TO STUDY THE VERTICAL TRANSMISSION OF HIV TYPE 1 IN TROPHOBLASTIC SWAN71 CELLS AND EVALUATION OF COMPOUNDS FOR THEIR ANTI-HIV ACTIVITY.

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE
REQUIREMENT FOR THE DEGREE OF

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THAPAR INSTITUTE OF ENGINEERING AND TECHNOLOGY,
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CERTIFICATE

This is to certify that the thesis entitled, **"To study the vertical transmission of human immunodeficiency virus type 1 in trophoblastic Swan71 cells and evaluation of compounds for their anti-HIV activity"** submitted by Ms. Divisha Bhatia, in partial fulfillment of the requirements for the award of degree of MASTER OF SCIENCE in BIOTECHNOLOGY of Thapar University, embodies the work done by the candidate under my guidance at the **National Institute of Immunology, New Delhi.** This work is original and has not been submitted in part or in full for any other degree or diploma of any university.

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CERTIFICATE

This is to certify that the thesis entitled **"TO STUDY THE VERTICAL TRANSMISSION OF HIV TYPE 1 IN TROPHOBLASTIC SWAN71 CELLS AND EVALUATION COMPOUNDS FOR THEIR ANTI-HIV ACTIVITY"** being submitted by **Ms. Divisha Bhatia (ROll No. 301101010)** in partial fulfillment of the requirement for the award of Master of Sciences in Biotechnology, Thapar University, Patiala, is a bonafide work carried out under the esteemed supervision of **Dr. Satish K. Gutpa, PhD, FNA, FASc, FNASc, FNAMS, Reproductive Cell Biology Laboratory, Deputy Director, National Institute of Immunology**

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Divisha Bhatia

DECLARATION

I hereby declare that the work being presented in the thesis entitled **"Vertical Transmission Of Hiv Type 1 In Trophoblastic Swan71 Cells And Evaluation Of Different Natural Compounds For Their Anti-Hiv Activity"** in partial fulfillment of the requirements for the award of degree of Masters in Microbiology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala is my own laboratory work **at Reproductive Cell Biology Laboratory** during the period of January 2013 to June 2013, under the conception and supervision of **Dr. Satish K. Gutpa, PhD, FNA, FASc, FNASc, FNAMS II, Deputy Director, National Institute of Immunology**. This dissertation has not been submitted elsewhere for the award of any degree or diploma by other universities.

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ABBREVIATIONS

AIDS	Acquired immunodeficiency Syndrome
ampho	amphotericin
AZT	Azidothymidine
BSA	bovine serum albumin
CTB	cytotrophoblasts
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	dimethyl sulphoxide
dNTPs	Deoxyribonucleotide triphosphates
EDTA	Ethlenediaminetetraacetic acid
EA	Ellagic acid
FBS	Fetal bovine serum
gp120	Glycoprotein 120
GA	Gallic acid
H	Hour
HIV	Human Immunodeficiency Virus
hMR	Human mannose receptor
IL-6	Interleukin-6
mg	milligram
ml	millilitre
mM	milli molar
mmol	milli mole
MTCT	mother-to-child transmission
M-tropic	Macrophage-tropic
MTT	(3-(4,5-Dimeethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
LTR	Long terminal repeat
RPMI	Roswell Park Memorial Institute
µg	microgram
µl	micro litre
N	normality
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram

nm	nanometre
nmol	nano mole
O.D	optical density
pH	potential hydrogen ion concentration
PCR	Polymerase Chain reaction
Pen	Penicillin
PBS	Phosphate buffer saline
SDS	sodium dodecyl sulphate
Strep	Streptomycin
Rnase	Ribonuclease A
ST	syncytiotrophoblast
TEER	Transepithelial electric resistance
T-tropic	T-cell tropic
TNF	Tumor necrosis factor

Acquired immunodeficiency Syndrome (AIDS) is an immunological disorder characterized by abnormalities of immunoregulations and opportunistic infections caused by Human Immunodeficiency Virus (HIV). HIV, responsible for rapidly growing fatality rate globally, was discovered in 1983-84 and has been shown to originate from Chimpanzees (Wain-Hobson, 1998). HIV, a lentivirus belonging to the Retroviridae family is subdivided in two type, HIV-1 and HIV-2. HIV-1 and HIV-2 were detected in the 1980s, causing infection in human populations in Africa (Eberle and Gürtler , 2012). The virus isolated from chimpanzees is known as HIV-1 and that transmitted from sooty mangabey monkeys is known as HIV-2. HIV-1 is more virulent and the main cause of AIDS pandemic while HIV-2 has shown a less virulent course in humans. HIV-1 is classified into two groups, M and O. Group M is further divided into ten genetically distinct subtypes, A-J, whereas group O contains another group of heterogeneous viruses (Simson *et al.,* 1999). HIV comprise of a nucleoprotein core surrounded by an outer coat. Each virion expressed 72 glycoprotein projections composed of gp120 and gp41. The gp41 molecule is a transmembrane molecule that crosses the lipid bilayer of the viral envelope. Gp120 is associated with gp41 and serves as the viral receptor for CD4 on host cells. The viral envelope derives from the host cells, contains some host-cell membrane proteins, including class I, and class II MHC molecules. Within the envelope is the viral core, or nucleocapsid, which includes a layer of a protein, called p17 and an inner layer of a protein p24. The HIV genome consist of two copies of single stranded RNA ,which are associated with two molecules of reverse transcriptase (p64) and nucleoid proteins p10 ,a protease, and p32 ,and integrase (Kindt *et al.,* 2006).

Vertical transmission of the HIV type 1 (HIV-1) is a serious global health problem. Currently, 2.1 million children <15 years of age are infected by HIV-1 worldwide and 90% of these infections are associated with mother-to-child transmission (MTCT) of this retrovirus (UNAIDS, 2002). The risk of vertical transmission lies within the range of 15–25% in industrialized countries and reaches 35% in developing countries (UNAIDS, 2002). MTCT may occur in utero, at birth during delivery, or through breastfeeding. Vertical transmission occurring through breastfeeding or at birth most likely involves ingestion of contaminated fluids (e.g., milk, vaginal secretion, and blood) by the infant. In contrast, *in utero* HIV-1 transmission requires a direct implication of the placenta.

Mathematical modeling has estimated that while 65% of the infections occur at birth, one third of the infants are considered to become infected *in utero* <2 month before delivery (Rouzioux, *et al*., 1995). This being said, *in utero* HIV-1 transmission has been documented by several groups (Magder *et al., 2005;* Biggar *et al*., 2003). In addition, vertical transmission can occur early during pregnancy because HIV-1 has been detected in aborted foetuses as early as after 8 week of gestation (Lewis *et al*., 1990) and in first and second trimester fetuses (Bhoopat *et al*., 2005; Soeiro *et al*., 2007; Mano and Chermann., 1991; Courgnaud *et al*., 1991). *In utero* transmission of HIV-1 is poorly understood, and the importance of studying such a process is related to the following facts. If we consider again the mathematical modeling cited above, it can be estimated that among the 2 million children currently living with HIV-1/AIDS, hundreds of thousands of these infections occurred *in utero*. Hence, although *in utero* transmission is unlikely the primary cause of infection in infants, given the scale of the pandemic, the resulting numbers are substantial. Antiretroviral therapies are known to significantly reduce the incidence of vertical transmission. However, according to recent statistics gathered in developing countries, only 10% of pregnant women received pre-birth counselling for preventing MTCT and only 7% of people had access to antiretroviral treatments in 2003 (UNAIDS, 2004 a). Consequently, initiatives to prevent vertical transmission of HIV-1 are now aimed at developing strategies that are readily available to women. In this context, it is well recognized that a better understanding of how and when vertical transmission occurs is crucial (UNAIDS, 2004 a). Despite the significant progress in reducing the incidence of HIV transmission from mother to fetus that has been achieved with the introduction of novel antiretroviral regimens, the pathogenesis, in particular regarding the passage of HIV across the placenta, is not fully understood. Therefore, there is a need to understand the mode of infection and entry of HIV in trophoblastic cells.

HIV-1 is an enveloped virus that gains entry into susceptible cells upon fusion of its outer membrane with the plasma membrane of a target cell. This process involves coordinated interactions between the viral envelope (Env) glycoproteins gp120 and gp41 and the primary cellular receptor like, CD4 and a coreceptor such as either CXCR4 or CCR5 and Mannose receptor. The possible mode of entry is through endocytic pathway, where the virus gets entrapped inside the endosome and leads to infection at later stage. Thereby, a deeper understanding for the entry of the virus is required. Cytokines such as TNF-α and IL-6 stimulate receptor mediated endocytosis and transcytosis of macromolecules across the epithelial barrier, and TNF-α upregulates transcription of HIV-1 promoters in trophoblast cells. The placenta produces a number of cytokines that may play a critical role in promoting or inhibiting vertical transmission of HIV-1, and inflammation of the placental villous membrane was associated with an increased rate of mother-to-child HIV-1 transmission in a large cohort of women (Zachar *et al.*, 2002; Patterson *et al*., 2001; Coulomb-L'Hermine *et al*., 2000; Wabwire-Mangen *et al*., 1999). Therefore, we hypothesized that TNF-α and IL-6 may promote transcytosis of HIV-1 across the placenta and anti-inflammatory agents may abrogate this effect.

Acquired immunodeficiency syndrome patients face great socio-economic difficulties in obtaining treatment. The probable inhibitors may target many stages in the virus life cycle: virus adsorption, virus-cell fusion, virus uncoating, HIV regulatory proteins and HIV enzymes (reverse transcriptase, integrase and protease). The problem of drug resistance in HIV infection is a consequence of the virus propensity to mutate (Flexner *et al*., 2007). The selective effect of these drugs, also favours emergence of mutations that can establish clinical drug resistance (O'Brien, 2000). Additionally, the dose-limiting side-effects and the necessity for long-term anti-HIV treatments are the limitations to standard HIV therapy (Hupfeld and Efferth, 2009). There is an urgent need for new, safe, and cheap anti-HIV agents. Traditional medicinal plants are a valuable source of novel anti-HIV agents and may offer alternatives to expensive medicines in future. Various medicinal plants or plant-derived natural products have shown strong anti-HIV activity and are under various stages of clinical development in different parts of the world.

In the present study, transmission of HIV-1 into Swan71 trophoblastic cells have been evaluated using various *in vitro* assays. This study also focuses upon the integration of HIV-1 trophoblastic genome and formation of productive infection. Further, the receptors and cytokines involved in entry of virus inside the cells have been elucidated. In this study, two compounds have been evaluated for anti-HIV activity along with their cytotoxicity using *in vitro* assays.

Acquired Immunodeficiency Syndrome (AIDS) is an immunological disorder characterized by the abnormalities of immunoregulations and opportunistic infections caused by Human Immunodeficiency Virus (HIV). HIV, responsible for rapidly growing fatality rate globally, was discovered in 1983-84 and has been shown to originate from Chimpanzees (Wain-Hobson, 1998). An understanding of the central events in the transmission of HIV-1 infection is critical to the development of effective strategies to prevent infection. HIV is transmitted by three main routes: sexual contact, exposure to infected body fluids or tissues, and from mother to child during pregnancy, delivery, or breastfeeding (known as vertical transmission).

Vertical transmission of the HIV type 1 (HIV-1) is a serious global health problem. Women are particularly vulnerable to heterosexual transmission of HIV-1 due to both socio-economical and biological reasons (UNAIDS, 2004 b). Therefore, blocking HIV-1 transmission in the female genital tract is key to prevent infection. Because barrier methods, such as the male and female condoms, are unfortunately not yet sufficiently accepted, efficacious vaccines and topical microbicides that interfere with viral transmission are high on the wish list (Cutler *et al*, 2008; Elias and Coggins, 1996). Currently, 2.1 million children < 15 years of age are infected by HIV-1 worldwide and 90% of these infections are associated with mother-to-child transmission of this retrovirus (UNAIDS, 2004 b), occurring *in utero*, at birth, or via breast-feeding (Rouzioux *et al.*,1995). HIV-1 has been detected in newborns at delivery (Laure *et al.*, 1998; Menu *et al.*, 1995; Zachar *et al.*, 1991) and in aborted foetuses. For an HIV+ woman not being treated for HIV, the chance of passing the virus to her child is about 25% during pregnancy, labor and delivery. If she breast feeds her infant, there is an additional 12% chance of transmission.

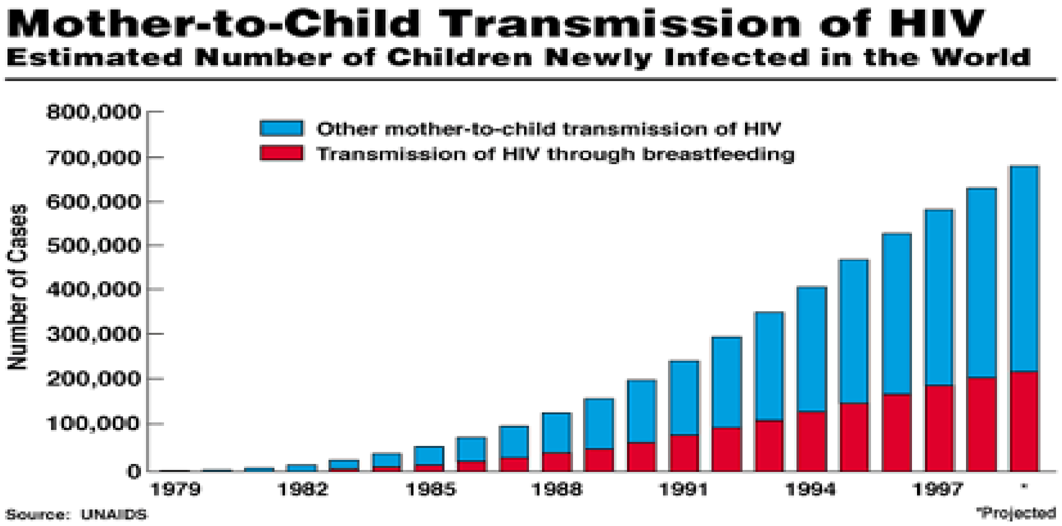


Figure1. Mother to child transmission of HIV by breast-feeding and other causes. *Source: WHO, 2002*

Anatomical considerations argue that *in utero* infection involves the passage of virus or virus-infected cells across the placenta into the fetal compartment (Aplin *et al*., 1991), although this remains to be established. The expression of viral gene products in the villi of placenta from HIV-positive women has been documented (Backe *et al*., 1994; Chandwani *et al*., 1991; Maury *et al*., 1989), although there is disagreement regarding the type and distribution of the placental cells involved.

In order to understand the mechanisms leading to placental and/or fetal infection, it is essential to understand the structure and physiology of the placenta. The placenta is made of maternal and fetal tissues. Humans have a hemomonochorial villous placenta. Maternal blood from spiral arteries in the decidua (uterine lining during pregnancy) flows into the intervillous space where it surrounds thousands of fetally derived floating placental villi. The entire villous surface is covered with a continuous layer of multinucleate syncytiotrophoblast, which is the major fetal surface in contact with maternal blood. The apical side of the syncytium consists of profuse, branched microvilli (Schiebler *et al*., 1991; Cantle *et al*., 1987) and provides abundant surface area for gas and nutrient exchange between mother and fetus. The syncytiotrophoblast (ST) is under girded by cytotrophoblasts (CTB) (Benirschke *et al*., 2006), which are separated from fetal capillaries in the villous stroma by a basement membrane. Some cytotrophoblasts leave the basement membrane and differentiate along the invasive pathway to form anchoring villi: columns of unpolarized cytotrophoblasts attach to and then penetrate the uterine wall where they give rise to extravillous cytotrophoblasts (Horse *et al.,* 2004). A subset of extravillous cytotrophoblasts breaches maternal spiral arteries in the decidua and differentiates into endovascular trophoblasts that replace the resident maternal endothelium to direct more blood into the intervillous space.

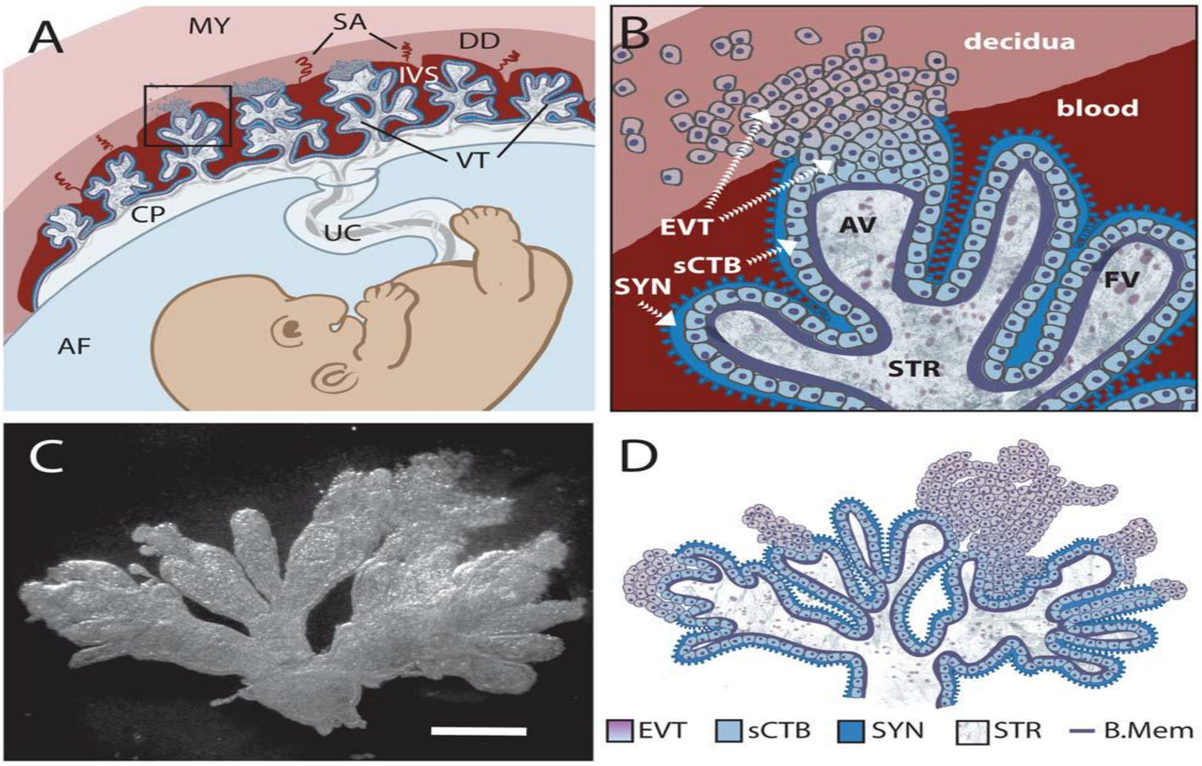


Figure 2. Comparison of in vivo placental structure to placenta explant model. (A) Structure and orientation of fetus and placenta in uterus at, 6 weeks of gestation. Fetal structures are represented in shades of blue and purple while maternal are in shades of red. Maternal structures: MY: myometrium, SA: spiral arteries, DD: decidua (uterine lining during pregnancy), IVS: intervillous space filled with maternal blood. Fetal structures: VT: villous tree, CP: chorionic plate, UC: umbilical cord, AF: amniotic fluid. (B) Enlargement of boxed area in panel A Maternal blood surrounds the villous tree composed of anchoring (AV) and floating (FV) villi, which are covered by a syncytiotrophoblast (SYN) that is underlaid by subsyncytial cytotrophoblasts (sCTB) and a basement membrane. The subsyncytial CTB layer grows increasingly discontinuous in later trimesters. Gas and nutrient exchange with the maternal blood occurs across the syncytiotrophoblast to supply fetal capillaries in the stroma (STR). At the uterine wall, extravillous cytotrophoblasts (EVT) anchor the villous tree in the decidua. Some invade the decidua and move away from the tip to remodel maternal spiral arteries, with altered gene expression patterns as they move (not shown). Notably, E-cadherin expression decreases as VE-cadherin expression rises in distal (relative to fetus) extravillous cytotrophoblasts. (C) A six-week placental explant anchored in Matrigel. Bar = 1 mm. (D) Cartoon representation of the relevant structures seen in panel C. S*ource :10.1371/journal.ppat.1000732.g001*

However, there is conflicting evidence regarding the susceptibility of the ST to HIV infection. HIV gene products have been both localized to the trophoblastic layer of the placenta by some authors and found only in placental macrophages of the villous stroma by others (Backe *et al*., 1994; Mattern *et al*., 1992). Thus, whether the ST is a barrier that prevents entry of free virus or actively promotes its dissemination into fetal tissues is not clear. Conclusively determining the contribution of ST infection by free virus is necessary for the evaluation of alternative routes (e.g., cell-mediated trophoblast infection) through the trophoblast barrier.

HIV, a lentivirus belonging to the Retroviridae family is subdivided in two type, HIV-1 and HIV-2. HIV-1 and HIV-2 were detected in the 1980s, causing infection in human populations in Africa (Eberle and Gürtler**,** 2012). The virus isolated from chimpanzees is known as HIV-1 and that transmitted from sooty mangabey monkeys is known as HIV-2. HIV-1 is more virulent and the main cause of AIDS pandemic while HIV-2 has shown a less virulent course in humans. HIV-1 is classified into two groups, M and O. Group M is further divided into ten genetically distinct subtypes, A-J, whereas group O contains another group of heterogeneous viruses (Simon *et al.*, 1998)

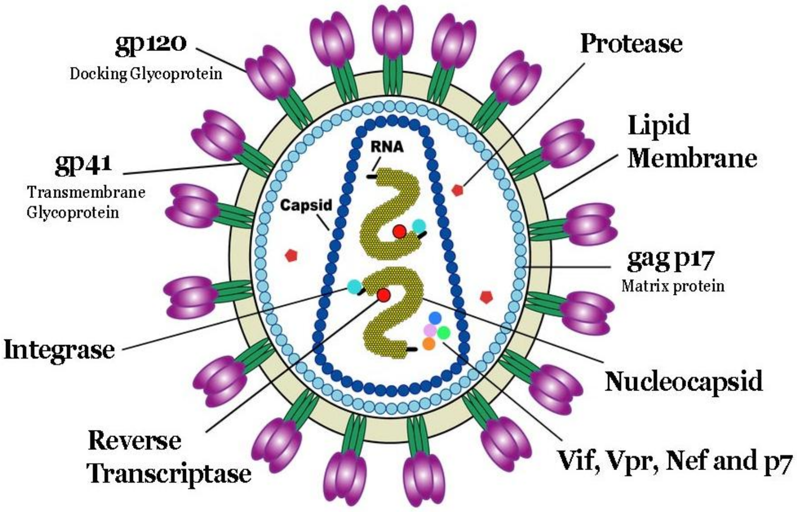


Figure 3. Diagrammatic representation of human immunodeficiency virus structure. *Source: http://www.eenzyme.com/hivresearchtools.aspx*

HIV comprise of a nucleoprotein core surrounded by an outer coat. The nucleoprotein core of the virion comprises of two copies of the viral genomic RNA coding for dozens of proteins with three major genes *gag*, *pol* and *env* (Kim *et al.*, 1989). The precursor gag protein encoded by *gag* gene is cleaved by protease enzyme into nucleocaspid (NC) protein and capsid matrix (CA) (Coffin, 1991). The *pol* gene codes for the viral enzymes reverse transcriptase, integrase and protease that are cleaved by GagPol precursor proteins (Deacon *et al.*, 1995). The *env* gene encodes for a 160 kDa precursor glycoprotein that is cleaved by an endopeptidase into two glycoproteins viz, gp120, and gp41. It is the high degree of variability in the envelope gene, which makes it problematic to prepare a vaccine that will protect against all strains of HIV-1 (Kaltz and Skalka, 1990). The nucleoprotein core is surrounded by an outer envelope of lipid bilayer containing surface gp120 that binds to the receptor present on the host cell surface and transmembrane (gp41) envelope glycoproteins, which anchors the gp120 onto the surface of the viral lipid bilayer.

Structure and expression of HIV-1 genome

The proviral DNA form of the HIV-1 genome consists of 8.5-9 kb of protein coding information, flanked on either side by a pair of identical long terminal repeats (LTRs). The coding portion encodes for at least 9 recognized genes (Baltimore, 1990). These are the virion structural genes (*gag*, *pol*, and *env*), the regulatory genes (*tat*, *rev*, and *nef*), and the accessory genes (*vpu*, *vpr*, and *vif*). Transcription of the provirus is controlled by a single promoter in the 5' LTR region and gives rise to a 9 kb primary transcript containing all nine genes. This primary transcript can either be packaged as such into virion particles to form the viral RNA genome or can be spliced into various mRNAs specifying the individual gene products (Mansky and Temin, 1995; Baltimore, 1990).

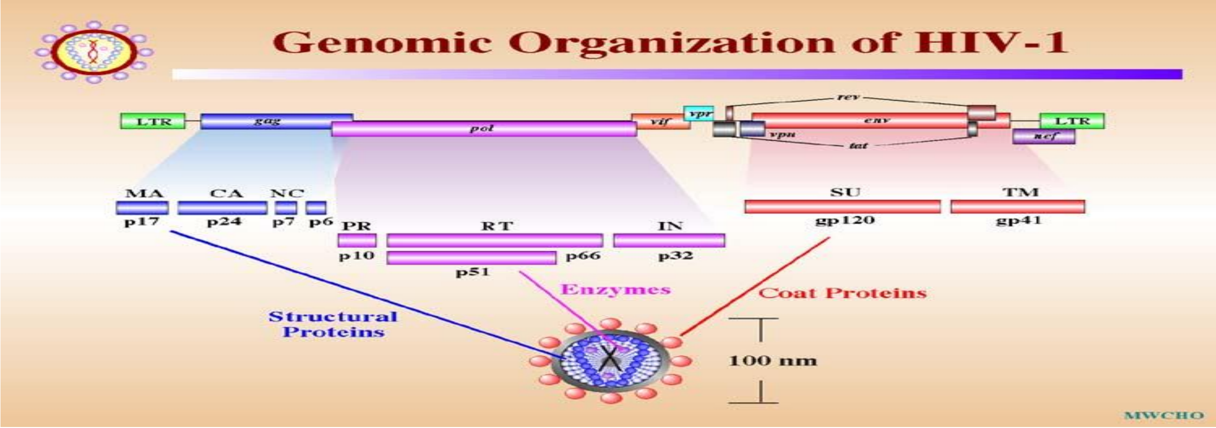


Figure 4. Genomic organisation of HIV-1.

Source*: http://www.stanford.edu/group/virus/retro/2005gongishmail/HIV.html*

During the early phase of HIV infection, only the fully spliced regulatory mRNAs can be detected in the cytoplasm of the infected cells. Cytoplasmic expression and subsequent translation of the structural mRNAs leading to the production of virions occurs only in the viral life cycle in response to the viral regulatory protein rev. The regulatory proteins tat, rev and nef together act to either up regulate or down regulate the rate of HIV viral replication in the infected host cell (Aiken *et al.*, 1994).

HIV Receptors and Co-receptors

For *in utero* HIV-1 transmission to occur, the virus must cross the selective placental barrier, which is composed of a double layer of polarized epithelial-type trophoblasts: cytotrophoblasts and syncytiotrophoblasts. HIV-1 has been shown to productively infect trophoblasts both *in vitro* (Vidricarie *et al.,* 2003; Lagaye *et al.,* 2001; Zachar *et al.,*1991) and *in vivo* (Dictor *et al.,* 2001; Menu *et al.,* 1999; Lee *et al.,* 1997). However, they exhibit a much lower susceptibility to productive HIV-1 infection than do CD4+ T cells (Vidricarie *et al.,* 2003; Zachar *et al.,*1991).

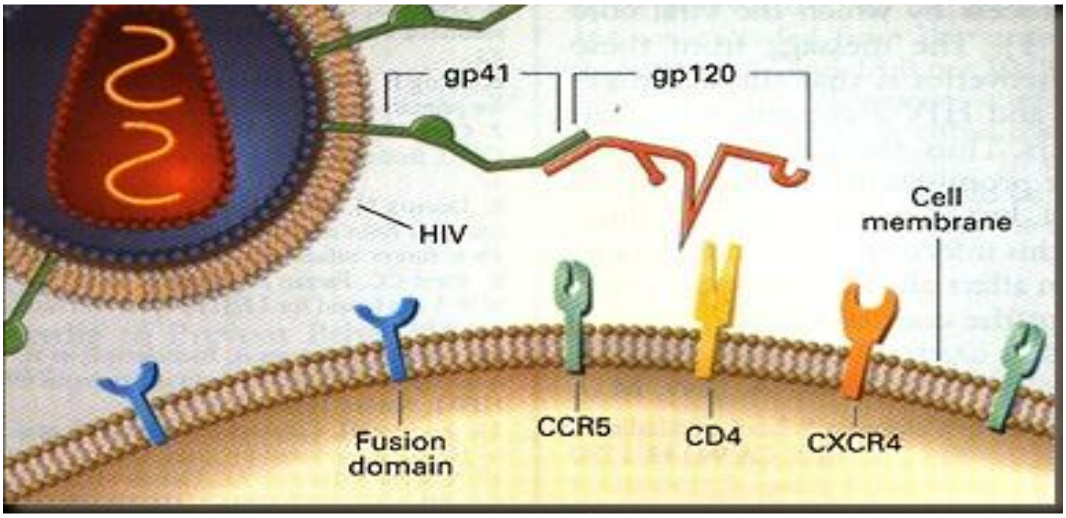


Figure 5. Diagrammatic representation of receptors involved in transmission of HIV across the cellular membrane. *Source: www.unaids.org/bangkok2004/photos/C-2876_768.jpg*

The precise structures of the placenta involved in fetal infection remains unknown, several possible mechanisms can currently be proposed: (i) the direct passage of infected maternal blood cells through the placental barrier; (ii) FcR mediated transport across the placenta of viral particles bound to maternal HIV-specific IgG antibody; and (iii) a direct viral binding to CD4 or CD4-like molecules, at the maternal-fetal interface (Poonia *et al.,* 2006; Sugaya *et al.,* 2004).

- ***CD4 RECEPTOR***

The primary cellular receptor for HIV entry is CD4. The HIV infection starts with the attachment of HIV by way of the gp120 protein to the CD4 receptor target cells. Virus entry into cells results on binding of virus env proteins to specific host-cell surface receptors. This process is believed to be driven initially by conformational changes in *env* following CD4 binding that expose fusion peptide virus domains located at the amino terminus of gp41 (Sakai *et al.,* 1999).

The CD4 receptors for HIV is composed of four extracellular immunoglobin (Ig)-like domains and is expressed at the surface of a subset of T lymphocytes and some macrophages. It assists the T cell receptor (TCR) in communicating with an antigen-presenting cell (Ansari *et al.,* 1996; Isobe *et al.*, 1988). Using its intracellular domain, CD4 amplifies the signal generated by the TCR by recruiting an enzyme, the tyrosine kinase Lck, which is essential for activating many molecular components of the signaling cascade of an activated T cell. CD4 also interacts directly with MHC class II molecules on the surface of the antigen-presenting cell using its extracellular domain. The extracellular domain adopts an immunoglobulin-like beta-sandwich with seven strands in two beta sheets, in a Greek key topology (Brady *et al.,* 1993)

However, expression of CD4 on a target cell is necessary but not sufficient for HIV entry and infection. Several chemokine receptors act as co-factors that allow HIV entry when co-expressed with CD4 on a cell surface.

- ***CXCR4***

CXCR4, or fusin, is expressed on T cells (Feng *et al.,* 1996). Co-expression of CXCR4 and CD4 on a cell allow T-tropic HIV isolates to fuse with and infect the cell. HIV gp120 interacts with both CD4 and CXCR4 to adhere to the cell and to effect conformational changes in the gp120/gp41 complex that allows membrane fusion by gp41. CXCR4 is expressed on many T cells, but usually not on macrophages and does not allow fusion by macrophage-tropic (M-tropic) HIV isolates (Feng *et al.,* 1996). It is interesting to note that stimulation with some bacterial cell wall products upregulates CXCR4 expression on macrophages and allows infection by T-tropic strains of HIV (Moriuchi *et al.,* 1998).

- ***CCR5***

  Shortly after the identification of CXCR4, another co-receptor was identified. CCR5, is expressed on macrophages and on some populations of T cells. It also function in concert with CD4 to allow HIV membrane fusion (Alkhatib *et al.,* 1996; Deng *et al.,* 1996; Dragic *et al.,* 1996). HIV gp120 binding to CCR5 is CD4-dependent, as antibody inhibition of CD4 can reduce binding to CCR5 by 87% (Trkola *et al.,* 1996). M-tropic HIV isolates appear to use CCR5 as their co-receptor for infection both of macrophages and of some T cells. Individuals with certain mutations in CCR5 are resistant to HIV infection (Alkhatib *et al.,* 1996; Dean *et al.,* 1996; Liu *et al.,* 1996; Samson *et al.*, 1996).

- **OTHER CO-RECEPTORS**

  CCR5 and CXCR4 appear to be the two major co-receptors for HIV entry into cells, but they are not the only such chemokine co-receptors. CCR3, a chemokine expressed on eosinophils and microglia, is used by some strains of HIV for infection of the microglia and resulting CNS pathology (He *et al.,* 1997). It is possible that other such chemokine receptors can also bind HIV gp120 and be used for HIV entry.

- **MANNOSE RECEPTOR**

  The mannose receptor (MR) is a C-type lectin carbohydrate binding protein primarily present on the surface of macrophages and dendritic cells. It also can be found on skin cells such as human dermal fibroblasts and keratinocytes (Sheik *et al.,* 2000). Many more roles have been ascribed to the MR including clearance of pathogens, capture of foreign antigens for presentation to MHC-II compartments (Engering *et al.,* 1997; Prigozy *et al.,* 1997), clearance of glycoprotein hormones (Simpson *et al.,* 1999), clearance of extracellular peroxidases (Lang *et al.,* 1994; Shepherd and Hoidal, 1990), endocytosis of lysosomal acid phosphatase (Imai and Yoshimura, 1994), and regulation of glycoprotein homeostasis (Lee *et al.,* 2002). Recent work has suggested that the MR may serve as an entry receptor for several important human pathogens (Vigerust *et al.,* 2005; Nguyen and Hildreth, 2003;

Reading *et al.,* 2000). In addition to a cysteine-rich domain and fibronectin type II repeat, the MR structurally contains eight carbohydrate recognition domains (CRD), of which 4, 5 and 7 are reported to be the most critical for binding and internalization of ligands with exposed oligosaccharides terminating in mannose, fucose or N-acetylglucosamine (Taylor *et al.,* 1992).

A characteristic feature of the MR (mannose receptor) and other members of this family is their rapid internalization from the plasma membrane via a clathrin-mediated mechanism that delivers the receptors to the endocytic pathway (Zvaritch *et al.,* 1996; Harding *et al.,* 1985). Several studies have shown that the MR binds and internalizes ligands via receptor-mediated endocytosis (Stahl *et al.,* 1980), and participates in phagocytosis of mannosylated particles and pathogens (Linehan *et al.,* 2001)

In humans, MR as a CD4 independent receptor plays a role in HIV transmission in different cell types including spermatozoa and vaginal epithelial cells (Fanibunda *et al.,* 2008; Trujillo *et al.,* 2007). In case of primary genital epithelial cells, HIV has also been reported to decrease the expression of tight junction proteins and increase the leakiness of the epithelial layer towards HIV (Kaushic, 2011; Nazli *et al.,* 2010). In human vaginal epithelial cells, HIV binds to human MR and activates MMPs, which in turn degrade the extracellular matrix proteins (López-Herrera *et al.,* 2005)

Characterisation of different viral strains

HIV-1 infects CD4+ T lymphocytes as well as monocytes/macrophages. Several subtypes of HIV-1 circulate in infected people worldwide, including subtype B in the United States and subtype C in Africa and India. The current understanding demonstrates that HIV-1 infectivity for macrophage-tropic (M-tropic) and T cell-tropic (T-tropic) viruses uses different cell surface co-receptors (Berger *et al.,* 1998). HIV-1 enters human cells in a process that comprises several steps, including the binding of the viral gp120 protein to the cellular receptor protein CD4 and a co-receptor protein, usually one of the two chemokine receptors CCR5 and CXCR4. The type of co-receptor used by the virus, the so-called co-receptor tropism, has a prognostic value, since patients with a CXCR4-tropic virus (T-tropic virus, also called as X4 virus) progress faster to Acquired Immunodeficiency Syndrome

(AIDS) compared to patients with a CCR5-tropic virus (M-tropic virus, also called as R5 virus) (D'Souza *et al*., 1996; Koot *et al*., 1993). In order to understand the mode of entry and infection of virus, there is an urgent need to get a better knowledge about the biological properties of HIV-1 subtypes, including cellular tropism, virus entry, replication efficiency, and cytopathic effects characterization. To comprehend better the viral dynamics various clones of HIV-1 have been constructed.

- ***PNL4.3***

  pNL4.3 is a T-tropic (sub-type B) recombinant proviral clone, that contacts the CXCR4 co-receptor (Rich *et al*., 2002). It contains DNA from HIV-1 NY5 and LAV strains in pUC18 vector (Abbas *et al*., 2007). Upon transfection, this clone directs the production of infectious virus particles in a wide variety of cells. The progeny, infectious virions, has been synthesized in several non-T cell lines, indicating the absence of any intracellular obstacle to viral RNA or protein production or assembly.

- ***PNLAD8-EGFP***

  pNLAD8 is an M-tropic (sub-type C) derivative of the T-tropic HIV-1 NL4.3 (Adachi *et a1*., 1986). The pNLAD8 is modified (by Eric Freed) by substituting a *Kpn* I to *Bsm* I fragement from M-tropic *env* for the counterpart *env* fragment in pNL4-3, making it fully infectious for cultured macrophages. Enhanced Green Fluorescent Protein (egfp) cDNA is placed into *nef* of pNLAD8, creating the pNLAD8-EGFP molecular clone.

Cytokines

The placental microenvironment abounds with cytokines that are key players in the regulation of normal growth and development of the placenta and fetus (Zachar *et al*., 2002). Enhanced production of inflammatory cytokines by trophoblast cells has been reported in HIV-1–infected pregnant women (Lee *et al*., 1997; Shearer *et al*., 1997), as has placental inflammation (Schwartz and Nahmias, 1991). Thus, cytokines involved in placental and fetal growth could also be important in HIV infection through placenta. Inflammatory cytokines may cause damage to the placental barrier, allowing transfer of the virus (Raghupathy, 1997). These cytokines such as tumor necrosis factor (TNF) could also stimulate viral replication (Bacsi *et al*., 2001) as they are potent inducers of nuclear factor-kB (NF-kB), which is required for HIV-1 long terminal

Table 1: Cytokines present during pregnancy and their involvement in HIV infection

Cytokines	**Location in human pregnancy**	**HIV production effect**	**References**
Interleukin (IL)-6	Produced by trophoblast in early pregnancy. Maximum expression in endometerium at implantation.	Elevated level of IL-6 increases the risk of infection; circulating IL-6 level correlates with residual HIV infection.	Bastard *et al.*, 2012; Ostrowski *et al.*, 2008
TNF- *α*	Decidua, trophoblast	Stimulates HIV infection by modulating transient expression from the HIV long terminal repeat (LTR).	Toder *et al.*, 2006; Zachar *et al.*, 2002
Epidermal growth factor (EGF)	Decidua, trophoblast	Leads to enhancement of HIV-1 expression. Maximum level of EGF has been obtained at high viral copy number.	Clare *et al.*, 1998; Mizrachi *et al.*, 1994
Granulocyte-macrophage colony-stimulating factor (GM-CSF),	Placenta, decidua	GM-CSF production may promote HIV-1 infection by facilitating sustained contact between viable neutrophils with bound HIV-1 and CD4 lymphocytes.	Fu *et al.*, 2011
Interleukin 1*β* (IL-1*β*)	Decidua, trophoblast	Enhances HIV-1 infection. HIV-infected cells produce increased amounts of IL-1*β* response to stimuli that could be present *in vivo*.	Molina *et al.*, 1989
Interferon *α* **(IFN-*α*)**	Placenta, decidua	Decreases HIV-1 viral levels. Delay disease progression by inhibiting viral replication. .	Manion *et al.*, 2012

repeat (LTR) transcription (Osborn *et al.,* 1989). Thereby, higher level of cytokines in placental barriers during pregnancy may readily effect the HIV infection.

- ***TNF-α***

Tumor necrosis factor-α is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. It is produced chiefly by activated macrophages, although it can be produced by many other cell types such as CD4+ lymphocytes, NK cells and neurons. Hallmarks of the inflammatory response, including elevated levels of tumour necrosis TNF-*α* in maternal serum, amniotic fluid and placental samples have been found consistently in association with spontaneous preterm labour and delivery (Gucer *et al.,* 2001; Maymon *et al.,* 1999; Steinborn *et al.,* 1996). TNF-*α* promotes HIV-1 replication in host cells through its interaction with the transcription factor NF-*κ*B and elevated amniotic fluid TNF-*α* levels have been associated with increased rates of maternal–fetal HIV-1 transmission (Anderson, 1997; Duh *et al.*, 1989). Thus, it promotes HIV-1 transmission across the placental barrier in pregnancies complicated by pro-inflammatory conditions, including co-infection with *Hepatitis C virus* or other sexually transmitted diseases, chorioamnionitis, premature rupture of the fetal membranes and spontaneous preterm delivery. TNF-*α* stimulate receptor-mediated endocytosis and transcytosis of macromolecules across the blood–brain barrier, and TNF-*α* upregulates transcription of HIV-1 promoters in trophoblast cells (Zachar *et al.,* 2002; Descamps *et al.,* 1997).

- ***IL-6***

IL-6 acts as both a pro-inflammatory and anti-inflammatory cytokine. It is secreted by T cells and macrophages to stimulate immune response to trauma, especially burns or other tissue damage leading to inflammation. IL-6 has been shown to play an important role in the immunopathology of various diseases; IL-6 is an autocrine growth factor for multiple myeloma (Kawano *et al.,* 1988). The unregulated, constitutive production of IL-6 may induce polyclonal B cell activation leading to hyper gammaglobulinemia and autoantibody production in certain tumor-bearing patients (Hirano *et al.,* 1990). It has been recently reported that HIV-1 infection leads to secretion and production of IL-6. Thus, it has been demonstrated that there are markedly elevated levels of plasma IL-6, IL-6 mRNA in PBMC, and IL-6 secretion by cells in the circulation of HIV-infected donors.

Medicinal plants as a source of anti-HIV agents

Acquired immunodeficiency syndrome patients face great socio-economic difficulties in obtaining treatment. The probable inhibitors may target many stages in the virus life cycle: virus adsorption, virus-cell fusion, virus uncoating, HIV regulatory proteins and HIV enzymes (reverse transcriptase, integrase and protease). The problem of drug resistance in HIV infection is a consequence of the virus propensity to mutate (Flexner *et al.*, 2007). Mutations arise because HIV's replication machinery lacks the proofreading mechanism and copies itself with low fidelity resulting in one mistake every time it replicates. The selective effect of these drugs, also favors emergence of mutations that can establish clinical drug resistance (O'Brien, 2000). Additionally, the dose-limiting side-effects and the necessity for long-term anti-HIV treatments are the limitations to standard HIV therapy (Hupfeld and Efferth, 2009). There is an urgent need for new, safe, and cheap anti-HIV agents. HIV reverse transcriptase inhibitors are important drugs for the treatment of AIDS and many natural products from plants belonging to a wide range of different structural classes, e.g., coumarins, flavonoids, tannins, alkaloids, lignans, terpenes, naphtho- and anthra-quinones, and polysaccharides have been shown to be active as RT inhibitors (Matthee *et al.*, 1999; el-Mekkawy *et al.*, 1995). Traditional medicinal plants are a valuable source of novel anti-HIV agents and may offer alternatives to expensive medicines in future. Various medicinal plants or plant-derived natural products have shown strong anti-HIV activity and are under various stages of clinical development in different parts of the world. Throughout the world, medicinal plants have been used, with the history that dates back to the origin of human civilization on earth. Approximately, ten thousand of the world's plants have been documented for their medicinal use. Traditional medicines has served as a source of alternative medicine, new pharmaceuticals and healthcare products. Considerable research on pharmcognosy, phytochemistry, pharmacology and clinical therapeutics has been carried out on potential Ayurvedic medicinal plants (Mann *et al.*, 2008). Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids and flavonoids, which have been found *in vitro* to have antimicrobial properties. These natural compounds may possess virucidal activity, restrict HIV entry and fusion in host cell or may inhibit the viral enzyme activity. Among various natural compounds screened for anti-HIV activity, gallic acid and ellagic acid have been selected for further inspecting the anti-HIV activity.

- ***GALLIC ACID***

Gallic acid is a trihydroxybenzoic acid, a type of phenolic acid, found in gallnuts, sumac, witch hazel, tea leaves, oak bark, and other plants (Reynolds and Wilson, 1991). Gallic acid is found both in free form and as part of hydrolyzable tannins. The synthetic n-alkyl esters of gallic acid (GA), also known as gallates, especially propyl, octyl and dodecyl gallates, are widely employed as antioxidants by the food and pharmaceutical industries (Kubo *et al*., 2002; van der Heijden *et al*. 1986,). Besides the antioxidant activity, other biological activities have been described for this group of molecules, mainly anticancer activity (Frey *et al*., 2006, Veluri *et al*., 2006; Kitagawa *et al*., 2005; Fiuza *et al*., 2004) as well as antibacterial and antifungal properties (Stapleton *et al*., 2004; Fujita and Kubo, 2002; Kubo *et al*., 2002). However, there are few reports about the antiviral activity of these compounds. In 1988, a study described the inhibition of HSV-1 and HSV-2 replication by methyl gallate (Kane *et al*., 1988). In 2002, as part of the screening of phenolic compounds against HIV-1 integrase, GA was found to be active (Nutan *et al*., 2013).

- ***ELLAGIC ACID***

Ellagic acid is a natural phenol antioxidant found in numerous fruits and vegetables. The antiproliferative and antioxidant properties of ellagic acid have spurred preliminary research into the potential health benefits of ellagic acid consumption. Ellagic acid is the dilactone of hexahydroxydiphenic acid. The highest levels of ellagic acid are found in blackberries, cranberries, pecans, pomegranates, raspberries, strawberries, walnuts, wolfberry ,and grapes (Vattem andShetty,2005). Ellagic acid has antiproliferative and antioxidant properties in a number of *in vitro* and small-animal models (Seeram *et al*., 2005; Narayanan *et al*., 1999). The antiproliferative properties of ellagic acid may be due to its ability to directly inhibit the DNA binding of certain carcinogens, including nitrosamines (Madal *et al*., 1988) and polycyclic aromatic hydrocarbons (Teel *et al*., 1986). As with other polyphenolic antioxidants, ellagic acid has a chemoprotective effect in cellular models by reducing oxidative stress (Vattem *et al*., 2005). Ellagic acid also posses anti-HIV activity which shows dose-dependent inhibition of HIV-1 protease enzyme (Nutan *et al*., 2013).

Cell line selection

To study the functional aspect of trophoblast cells, several *in vitro* cells have been used which closely resemble to the primary trophoblast cells in terms of the expression of trophoblastic specific markers. Choriocarcinoma cells like JEG-3 and JAR are trophoblastic tumor cell lines that are useful models to study placental infection. Further, HTR-8/Svneo trophoblastic cells was developed by transformation with SV40 large T antigen in the trophoblast cells isolated from the explant culture of first trimester trophoblast cells (Graham *et al.,* 1993). These cells also display many characteristics features of primary Extravillous trophoblast (EVT) cells and are in use as a model for primary EVT cells. Cell lines like ACH-3P and AC1m59 were developed by a hybridoma of JEG-3 and trophoblast cells isolated from first and third trimester of pregnancy but during transformation, they have acquired different ploidy level. So, to avoid this problem, Swan71 cell line was generated by transformation of first trimester trophoblast cells with human telomerase reverse transcriptase, the catalytic subunit of telomerase. These cells display the characteristics of first trimester trophoblast cells and have intact diploid status. So, using this cell line an attempt has been made to understand the mode of transmission of HIV-1 into trophoblastic cells.

Reagents

Chemicals

Azidothymidine (AZT), bovine serus albumin (BSA), dimethyl sulphoxide (DMSO), ethidium bromide, ethanol, IL-6 (Interleukin-6), mannan, methanol, polybrene, sodium bicarbonate ($NaHCO_3$), sodium phosphate dibasic (Na_2HPO_4), sodium azide, sodium dodecyl sulphate (SDS), sodium chloride (NaCl), tris, trypsin, TNF- α (Tumor necrosis factor- α) and xylene dye were procured from Sigma-Aldrich Inc., St. Louis, MO, USA. Agarose, ethylenediaminetetraacetic acid (EDTA), glycerol were purchased from BDH, Merck Limited, Worli, Mumbai, India. Bright-Glo^{TM} Luciferase assay susbtrate, MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole] and 5X Reporter lysis buffer were procured from Promega, Madison, WI, USA. Ellagic acid and gallic acid were procured from Sigma-Aldrich Inc. Anti-human CD4 conjugated with fluorescein isothiocyanate (FITC), anti-human CD195 (CCR5) conjugated with Phycoerythrin (PE), anti-human CD184 (CXCR4) conjugated with Allophycocyanin (APC) and anti-human mannose receptor conjugated with Phycoerythrin (PE) were purchased from eBiosciences, San Diego, California, USA. sCD4 inhibitor was obtained from NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA.

Medium and antibiotics

Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI)-1640 medium were obtained from Sigma-Aldrich Inc. Fetal bovine serum (FBS) and antibiotic-antimycotic solution [pen-strep-ampho sol; Penicillin (10000 units/ml), Streptomycin (10 mg/ml) and Amphotericin B (0.025 mg/ml)] were obtained from Biological Industries, Kibbutz BeitHaemek, Israel.

Primers, enzymes and molecular weight markers

Oligonucleotide primers were custom made by Sigma-Aldrich Inc. Deoxyribonucleotide triphosphates (dNTPs) were obtained from New England Biolabs (NEB), Beverly, MA, USA. Taq DNA polymerase was purchased from Genei, Banglore, India. DNAse I and

RNAseA were purchased from Ferments International Inc., Ontario, Canada. The 1 kb and 100 bp DNA ladders were purchased from NEB.

Viruses and Plasmids

Plasmids pNL4.3 obtained from NIH AIDS Research and Reference Reagent program, Division of AIDS, NIAID, NIH. Plasmid pNLAD8-GFP was a kind gift from Kuan-The Jeang, Chief, Molecular Virology Section, National Institute of Allergy and Infectious Diseases (NIAID), USA.

Cell culture materials and Kits

Serological pipettes, tissue culture flasks (T-25, T-75), tissue culture plates (12-, 24- and 96 –well) and transwell inserts (0.4 µm) were purchased from Greiner Bio-One, GmbH, Frickenhausen, Germany. p24 ELISA kit was procured from XpressBio, Life Science Products, MD, USA. Genomic DNA Mini Kit (Blood/Cultured Cell) was obtained from Geneaid Biotech ltd., Taipei, Taiwan, Japan.

Others

Filtration membranes of 0.22 and 0.45 µm pore size were procured from Millipore, Bedford, MA, USA. Black and white-optiplates (microplates for reading of fluorescence and luminescence, respectively) were procured from BMG LabTech, GmbH, Allmendgruen, Germany. Millicell ERS Volt-Ohm Meter was obtained from Millipore, Bedford, MA, USA.

METHODS

1. CELL CULTURE

1.1 Culture of trophoblastic Swan71 cells

Swan71 cells (kindly provided by Prof Gil Mor, Yale University, USA) were maintained in DMEM supplemented with 10% Fetal bovine serum (FBS) and an antibiotic antimycotic cocktail [Penicillin (100 µg/ml), Streptomycin (100 µg/ml) and Amphotericin B (0.25 µg/ml)] at 37°C in humified atmosphere containing 5% CO_2. Cells were sub-cultured at 70-75% confluency using the medium containing 0.5% trypsin and 0.2% EDTA to dislodge the cells.

1.2 Culture of TZM-bl cells

TZM-bl, previously designated JC53-bl (clone 13) is a HeLa cell line. The parental cell line (JC.53) stably expresses large amounts of CD4 and CCR5. The TZM-bl cell line was generated from JC.53 cells by introducing separate integrated copies of the luciferase and ß-galactosidase genes under control of the HIV-1 promoter. The TZM-bl cell line is highly sensitive to infection with diverse isolates of HIV-1. These cells (kindly provided by Dr. Debashish Mitra, National Centre for Cell Sciences, Pune) were maintained in DMEM supplemented with 10% FBS and an antibiotic antimycotic cocktail [as described for Swan71 cells] at 37°C in humified atmosphere containing 5% CO_2. Cells were sub-cultured at 70-75% confluency using the medium containing 0.5% trypsin and 0.2% EDTA to dislodge the cells.

2. TRANSWELL® DUAL-CHAMBER SYSTEM : EPITHELIAL CELL CULTURE

The apical chamber of a dual-chamber Transwell® system with a growth area of 0.3 cm^2 (pore size: 3.0 µm) was cultured with 1 x 10^5 Swan71 cells (in 100 µl). The trophoblast cells were fed by adding 700 µl of culture medium (DMEM medium plus 10% FBS, 20 mM glutamine, and antibiotics) to the basal chamber. The cells were cultured for 7 days in order to obtain a confluent monolayer. The medium in the basal chamber was replaced every 48 h. Transepithelial electric resistance (TEER) across each cell layer was measured every day with a Millicell ERS Volt-Ohm Meter (Millipore, Bedford, MA, USA). TEER values were corrected by subtracting the background TEER obtained from inserts containing the same volume of culture medium but in the absence of cells. The resulting TEER was corrected for membrane growth area and expressed in Ω×cm^2.

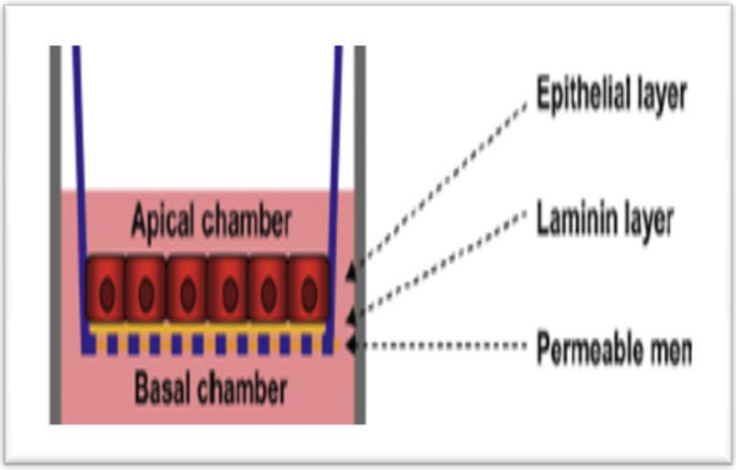


Figure 4. Schematic representation of the dual-chamber model. The model consists of an apical chamber (Transwell® insert) and a basal chamber. The permeable membrane of the apical chamber is coated with laminin, a component of the basal lamina. Swan71 cells are cultured in the apical chamber.

3. TRANSMISSION OF CELL-FREE HIV-1 IN THE DUAL-CHAMBER MODEL

Confluent Swan71 trophoblastic cells growing in dual-chamber Transwell were exposed to varying concentrations of either NL4.3 (MOI 0.05, 0.1 and 0.5) or pNLAD8-GFP (MOI 0.5 and 1). Once the Swan71 trophoblastic cells growing in dual-chamber transwell achieve confluency, the apical chamber was inserted in the flat bottom cup of a 12-well plate (basal chamber) containing DMEM medium supplemented with 10% FBS, 20 mM glutamine, and antibiotics. Immediately, preceding with the HIV infection, the cells were treated with polybrene (5 µl/ml) for an hour at 37°C. Cell-free HIV virus either NL4.3 or NLAD8-GFP strains were inoculated at varying concentrations onto the apical pole of trophoblastic barriers for 4 h at 37°C. Then, cell-free HIV was removed. For this purpose, the apical surface of Swan71 cell monolayers was carefully washed five times, using 1 ml of DMEM medium each time. Further, the Transwell® were transferred in new 12-well plate and 1.5 ml of medium was added in the basal chamber. The culture supernatants were harvested at two different time points (4 and 48 h), and virus production was measured using commercial ELISA kit. Each experiment was repeated four times.

4. HIV p24 ELISA

The p24 ELISA kit from Xpressbio, Life Science was used for carrying out p24 assay. All the reagents were allowed to reach room temperature (18-25°C). The positive control (200 pg/ml) at different dilutions was used to prepare standard curve and un-inoculated cell culture media was used as a negative control. To the antibody coated ELISA plates, 200 µl/wells of the test samples/standard were added. The test samples/control were treated with 20 µl of lysis buffer and incubated for 60 min at 37°C. The contents of the wells was aspirated and was washed 5 times with 1X wash buffer. Subsequent to washing, detector antibody (100 µl/well) was added. Following washing, Streptavidin HRP conjugate (100 µl /well) was added and incubated at room temperature (~25°C) for 30 min. The wells were further washed as described above. Without delay, 100 µl of substrate solution was added into each well and left at room temperature (~25°C) protected from direct light for 30 min. The reaction was stopped by adding 100 µl of stop solution to each well including the reagent blank. Reading was taken at OD_{450} using a microtitration plate reader blanked on the negative control well.
Concentration of p24 in the test samples was calculated by plotting their OD against the standard curve. The intensity of the color obtained is directly proportional to the amount of HIV-1 p24 captured.

5. ISOLATION OF GENOMIC DNA OF SWAN71 CELLS

The genomic DNA Mini Kit (Geneaids) was used as an efficient method for isolating genomic DNA from Swan71 cells. After infection of Swan71 cells with NL4.3 strain of HIV, the culture medium was removed, cells were washed with PBS and harvested by trypsinization. The cells were washed by centrifugation for 5 min at 300× g and the pellet was re-suspended in 150 µl of RBC lysis Buffer. Then, 200 µl of GB Buffer was added and incubated at 60°C for at least 10 min to ensure the cell lysate was clear. Following 60°C of incubation, 5 µl of RNAse A (10 mg/ml) was added to the clear lysate in order to obtain a RNA-free genomic DNA and was incubated at room temperature for 5 min. After RNAse treatment, 200 µl of absolute ethanol was added to the sample lysate and mixed immediately by shaking vigorously for 10 sec. GD Column was placed in 2 ml collection tube and transferred all the mixture to the GD Column. The column was centrifuged at 14,000 g for 30-60 sec and placed the GD column in new 2 ml collection tube. W1 Buffer (400 µl) was added to the GD Column and centrifuged at 14,000 g for 30-60 sec. The flow-through was discarded and 600 µl of Wash Buffer was added to the GD Column. The column was centrifuged at 14,000 g for 30-60 sec and discarded the flow-through. The Column was centrifuged again for 3 min at 14,000 g to dry the column matrix. The dried GD Column was transferred to a clean 1.5 ml microcentrifuge tube and added 100 µl of pre-heated Elution Buffer to the centre of the column matrix. The elution buffer was allowed to stand for 3 min and then the column was centrifuged at 14,000 g for 30-60 sec to elute the genomic DNA.

6. TO CHECK HIV INTEGRATION IN GENOMIC DNA USING PCR

The purified genomic DNA was quantified by nanodrop spectrophotometer and was subjected to polymerase chain reaction (PCR) with HIV-1 specific primers. The PCR was conducted in BioRad Thermocycle with four different primers amplifying the respective region of HIV-1 genome. The reaction mixture contained 2.5 U of Taq DNA polymerase in Taq reaction buffer with 25 mM $MgCl_2$, 10 mM of each deoxynucleoside triphosphate, 10 mM each of the primers, and 100 ng/µl of the genomic DNA. The reaction conditions were 45 s of denaturation at 95°C, 60 s of annealing at 57-60°C, and 60 s of extension at 72°C for 35 cycles followed by post extension for 10 min at 72°C. Amplified products were analysed on 1.5-2% agarose gels containing 2-3 µl ethidium bromide and photographed. The workability of PCR was verified by using pNL4.3 plasmid as positive control. Uninfected cells were used as negative control.

7. LUCIFERASE ASSAY

TZM-bl cells (4.0 x 10^4/well) were seeded in 24-well plate and cultured overnight. In separate transwells, Swan71 cells were infected with HIV-1 NL4.3 at a multiplicity of infection (MOI) of 0.05, 0.1, and 0.5 at two different time points (4 and 48 h). The supernatants were collected and subsequently added in duplicate to TZM-bl cells and cultured for 4 h. The cells were washed once with cold 50 mM PBS after incubation to remove the cell free virus followed by addition of fresh culture medium. Cells were further cultured for 48 h, washed twice with PBS, and lysed with 1X lysis buffer (Promega Corporation, Madison, WI, USA) by freeze-thaw. The supernatant was analysed for luciferase activity by adding luciferase substrate (Promega Corporation) using white optiplate in the Fluorimeter (BMG Labtech GmbH, Offenberg, Germany). The results are expressed as relative luciferase count, calculated by taking the luminescence in experimental group (i.e. in the presence of supernatant from infected Swan71 cells) with respect to the luminescence in uninfected cells.

8. FACS ANALYSIS FOR EXPRESSION OF CD4, CCR5, CXCR4 AND MANNOSE RECEPTOR ON SWAN71 CELLS

Swan71 cells were harvested from growing culture in 25 cm^2 culture flask and washed twice with 50 mM PBS, pH 7.4 containing 1% sodium azide. Subsequently, cells (1 ×10^5/group) were incubated with labelled antibodies against CD4, CCR5, CXCR4 and mannose receptor (0.25 µg/100 µl); eBiosciences for 30 min at 4°C in dark. The cells were washed 3 times by centrifugation at 400 g for 5 min and re-suspended in 500 µl of ice cold PBS. The fluorescence was measured using BD Flow Cytometer. Unstained Swan71 cells were taken as negative control.

9. CYTOTOXICITY ASSAY

Cytotoxicity of compounds (gallic acid and ellagic acid) was assessed using MTT [3-(4,5-dimethylthiazol-2-yl)- 2,5 diphenyltetrazolium bromide; Sigma-Aldrich Inc.] assay. Briefly, TZM-bl cells (6 x 10^3 /well/100 µl) were seeded in a 96-well culture plate (Greiner BioOne, GmbH, Frickenhausen, Germany) and grown overnight at 37°C in a humidified atmosphere of 5% CO_2. Next day, culture medium with increasing concentrations of gallic and ellagic acid were added in duplicate and further incubated for 48 h. The solvent used to prepare the compounds was included as negative control. After incubation, 20 µl of MTT reagent (5 mg/ml) was added per well and incubated at 37°C for 3 h followed by addition of MTT solvent (100 µl/well; 20% SDS and 50% dimethyl formamide in 50 mM PBS). The absorbance (OD) was read at 570 nm with reference filter at 690 nm. Cell viability was calculated using the equation,

% Viability = [(OD of compound treated cultures)/ (OD of untreated cell control cultures)] x 100

10. INHIBITION OF HIV INFECTION IN TZM-BL CELLS-BASED ASSAY

TZM-bl cells (4 x 10^4/well) were seeded in 24-well plate and cultured overnight. In separate vials, HIV-1 NL4.3 at a multiplicity of infection (MOI) of 0.05 were treated with compounds (gallic acid and ellagic acid) for 1 h at 37ºC. Subsequently, pre-treated viruses were added in duplicate to TZM-bl cells and cultured for 4 h. The cells were washed once with cold 50 mM PBS after incubation to remove the cell free viruses followed by addition of fresh culture medium with compounds. Azidothymidine (AZT; Sigma-Aldrich Inc) was used as positive control whereas negative control comprised cells with untreated HIV. Cells were further cultured for 48 h, washed twice with PBS, and lysed with 1X lysis buffer (Promega Corporation Madison, WI, USA) by freeze-thaw. The supernatant was analyzed for luciferase activity by adding substrate (Promega Corporation) using white optiplate in the fluorimeter (BMG Labtech GmbH, Offenberg, Germany). The results were expressed as percentage inhibition, calculated by taking the luminescence in experimental group (i.e. in presence of test compound/AZT) divided by the luminescence in infected cells in absence of test compound/AZT multiplied by hundred. Per cent inhibition was calculated by subtracting the above value from hundred.

Evaluation of monolayer integrity in the dual chamber

Transmembrane electrical resistance (TEER) is a measure of the formation of tight junctions (Balda *et al.,* 1996). Determining the transepithelial electrical resistance is a widely used method to analyze the dynamics of physiological barriers in cell culture models. Confluency of the Swan71 cell monolayer was established by measuring transmembrane electrical resistance. The resistance was measured using Millicell-ERS (Electrical Resistance System). After every 48 h, the medium was replaced to keep the cells in healthy condition. An increase in TEER detected with the electronic circuit of the Millicell ERS-2 meter and its electrode is an indication of cell monolayer health and confluency. A gradual increase was observed in TEER during the Swan71 cell culture and showed maximum value on day 7 of the culture, reflecting formation of tight intercellular junction (transmembrane electrical resistance 432 ± 108 ohms $\times cm^2$; Table 2). This finding confirmed the validity of the model as a confluent trophoblast cell monolayer.

Transcytosis of HIV-1 across the monolayer formed by Swan71 cells

Transmission of HIV isolates across the Swan71 cell monolayer was observed by determining the HIV-1 p24 concentration. Recombinant variants of both T-cell and macrophage-tropic HIV-1 isolates such as NL4.3 and NLAD8-GFP were used. Swan71 cells were cultured for 7 days till a monolayer was formed. The cells were inoculated with varying concentration (0.05, 0.1, 0.5 or 1.0 MOI) of HIV-1 virus. The cells were cultured for 4 or 48 h at 37°C in humified atmosphere of 5% CO_2. HIV-1 p24 levels in apical, basolateral medium and cell lysate were measured after 4 and 48 h of infection. A gradual increase in p24 concentration was obtained with both, enhancement of multiplicity of infection and time of incubation when infected by either of the viral strain. The typical results obtained with NL4.3 virus at 48 h after infecting Swan71 cells monolayer is shown in Figure 6. Maximum p24 concentration was detected with 0.5 MOI after 48 h of infection with NL4.3 in the basolateral medium indicating the release of HIV in the lower chamber after infection. Results with 0.5 MOI showed an approximately tenfold increase in production of HIV when compared to cells challenged with 0.05 multiplicity of infection (figure 6).

Kinetics of HIV-1 in acutely infected cells plays an important role in detection of productive infection and integration of HIV DNA in infected cells. It has been reported that full length linear DNA is first observed at approximately 3 to 4 h post-infection. The integrated HIV DNA, at the end of the first round of HIV replication (i.e., 48-50 h post-infection), is said to account for approximately 10% of the total HIV DNA synthesized at the peak of viral DNA accumulation (i.e., 4 h p.i.). Thus, two different times (4 h and 48 h) were taken to calculate the accumulation of viral DNA. Our infection model involved treating Swan71 at high multiplicity of infection (0.5). A significant enhanced HIV-p24 concentration in basolateral medium, apical medium and cell lysate was demonstrated in case of treatment with NL4.3 (Table 4). We observed approximately two fold increase of HIV-p24 level (in basolateral medium, apical medium and cell lysate) after 48 h of infection (p24 concentration in basolateral chamber 273.09 ± 5.46; apical chamber 169.05 ± 5.86; cell lysates 221.07 ± 8.98) compared to 4 h time point (p24 concentration in basolateral chamber 144.11 ± 3.94 pg/ml; apical medium 75.94 ± 8.51 pg/ml, and cell lysate 110.03 ± 3.96 pg/ml) when challenged with NL4.3 (Table 4).

However, when the infection was carried out with M-tropic NLAD8-GFP viral clone (0.5 and 1 MOI). The maximum concentration was observed at 1 MOI after 48 h of infection in apical medium and cell lysate (Table 5). However, the HIV-p24 level was insignificant in the basolateral medium indicating no release of virus in the lower chamber of the transmembrane. A very little increase in HIV-p24 antigen level was observed at an MOI of 1.0 in cell lysate from 4 h (p24 concentration, 38.11 ± 1.89 pg/ml) to 48 h (p24 concentration, 46.06 ± 7.26 pg/ml) of infection, which was not significant (Table 5). The formation of viral particles at 0.5 multiplicity of infection (p24 concentration, 32.77± 5.42 pg/ml) was also considerably low in comparison to the formation of viral particles when cells were treated with NL4.3 clone (p24 concentration, 221.068 ± 8.98 pg/ml).

Table 2: Confluency of Swan71 cell monolayers in dual chamber system

No. of Days	Transmembrane electrical resistance (ohms ×cm^2 ± SEM)*
No cells	0
Swan71 cells × day 1	162 ± 115
Swan71 cells × day 3	183 ± 137
Swan71 cells × day 4	233 ± 165
Swan71 cells × day 5	296 ± 120
Swan71 cells × day 7	432 ± 108
Swan71 cells × day 8	408 ± 167

*Each value reported above represents the mean value ± SEM of at least three transwell experiments.

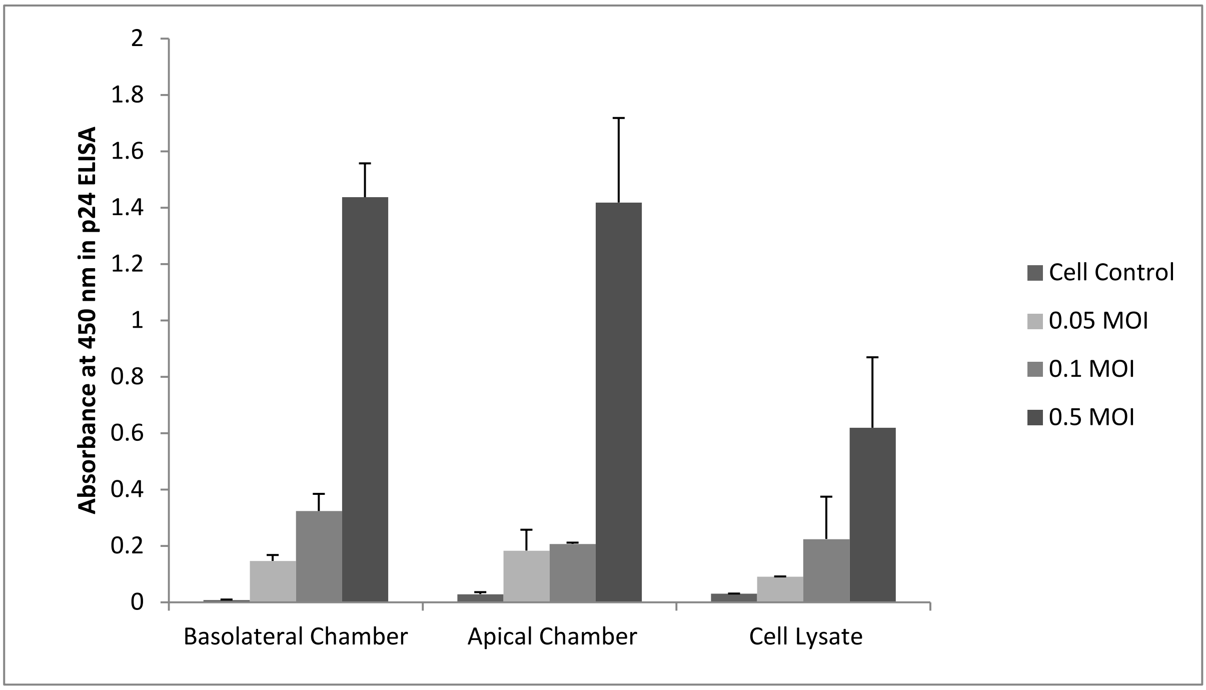


Figure 6. Infection of Swan 71 cells with varying concentration of NL4.3 Trophoblastic Swan 71 cells were grown in transwell for 7 days to establish monolayer. Subsequently cells were treated with NL4.3 at different MOI (viz. 0.05, 0.1 and 0.5) for 48h. After, incubation, culture medium collected from basolateral chamber and apical chamber as well as cell lysate were tested in HIV-1 p24 ELISA. Values are expressed as absorbance observed in p24 ELISA and represent means SE of 3 independent experiments performed in duplicate.

Table 4: Concentration of HIV-p24 level (pg/ml) indicating HIV production and release across Swan71 cell monolayer when infected with NL4.3

	Concentration of HIV-p24 (pg/ml)		
Experiments	Basolateral chamber	Apical chamber	Cell lysate
Control	49.44 ± 2.13	54.46 ± 7.35	51.95 ± 4.43
4 h	144.11 ± 3.94*	75.94 ± 8.51*	110.03 ± 3.96*
48 h	273.09 ± 5.46*	169.05 ± 5.86*	221.07 ± 8.98*

The result demonstrates that concentration of HIV particles significantly increases with respect to time in basolateral medium, apical medium and cell lysate. Cells were infected with NL4.3 at 0.5 MOI. p24 levels in culture supernatants were measured at two time points (4 h and 48 h) by ELISA. The concentrations of HIV-1 p24 antigen level are shown as mean ± SEM from at least three different experiments. *$p < 0.005$ as compared to Cell control.

Table 5: Concentration of HIV-p24 level (pg/ml) indicating HIV production and release across Swan71 cell monolayers when infected with NLAD8-GFP

	Concentration of HIV-p24 (pg/ml)		
Experiments	Basolateral chamber	Apical chamber	Cell lysate
Control	2.56 ± 0.14	1.77 ± 0.21	4.5 ± 0.07
4 h (0.5 MOI)	2.79 ± 0.98	18.22 ± 5.47*	23.11 ± 2.15*
48 h (0.5 MOI)	1.09 ± 0.2	44.59 ± 5.20*	32.77 ± 4.06*
4 h (1.0 MOI)	2.34 ± 0.53	25.72 ± 3.67*	38.11 ± 1.89*
48 h (1.0 MOI)	3.25 ± 1.02	61.63 ± 6.19*	46.06 ± 7.26*

The result demonstrates that concentration of HIV particles significantly increases with respect to time in apical medium and cell lysate. Cells were infected with NLAD8-GFP at 0.5 & 1.0 MOI. p24 levels in culture supernatants and all lysates were measured at two time points (4 h and 48 h). The concentrations of HIV-1 p24 antigen level are shown as mean ± SEM from at least three different experiments. *$p < 0.005$ as compared to Cell control.

Production of infectious mature HIV-1 virion particles after infection of trophoblast cells

The TZM-bl assay uses a stable cell line (TZM-bl, also called JC53bl-13) and a Tat-regulated firefly luciferase (Luc) reporter gene that is induced after infection with most strains of HIV-1 (Li *et al*., 2005; Wei *et al*., 2002: Platt *et al*., 1998). This cell line permits rapid, sensitive and reproducible measurements of infectious mature HIV-1 virion particles. Level of infection was measured as an enhancement in firefly luciferase (Luc) reporter gene expression in the presence of test sample compared to the absence of sample in TZM-bl cells. The supernatants collected after infection of Swan 7 cells with NL4.3 at different MOI (0.05, 0.1, and 0.5) for 48 h were used to infect TZM-bl cells. Transfection of TZM-bl cells with the supernatant collected from Swan71 cells infected with NL4.3 virus showed dose dependent increase in luciferase activity (Figure 7). The relative luciferase count observed at 0.1 MOI was significantly higher ($p< 0.01$) as compared to 0.05 MOI. Similarly, Swan71 cells infected with NL4.3 at 0.5 MOI also produced higher concentration of infectious HIV-1 virion particles as compared to when infected at MOI of 0.05 ($p< 0.005$). However, the difference in the production of infection virus particle by Swan71 cells was not significantly different when infected at MOI of 0.1 as compared to MOI 0.5. The results are expressed as percent Relative luciferase count (RLU) compared to virus control wells after subtraction of background RLU. Relative luciferase count was calculated by taking the luminescence in experimental group (i.e. cells treated by supernatant collect as above) minus the luminescence in uninfected cells divided by 4×10^4 cells.

To know if the production of infectious virus particle is also time dependent, another experiment was performed where Swan 7 cells were infected with NL4.3 at an MOI of 0.5 for either 4 h or 48 h. The culture supernatant thus collected were used to transfect TZM-bl cells grown in 24-well culture plate for 48 h. Higher luciferase was observed in TZM-bl cells which were infected with culture supernatant obtained at 48 h as compared to that collected at 4 h (Figure 8; $p< 0.05$).

These experiments conclusively proved that infection of trophoblastic Swan71 cells with NL4.3 not only lead to increase in the production of HIV-1 virus as analysed by p24 ELISA but also produce infectious virus particles as evident by an increase in luciferase activity when TZM-bl cells were incubated with culture supernatant from Swan71 cells.

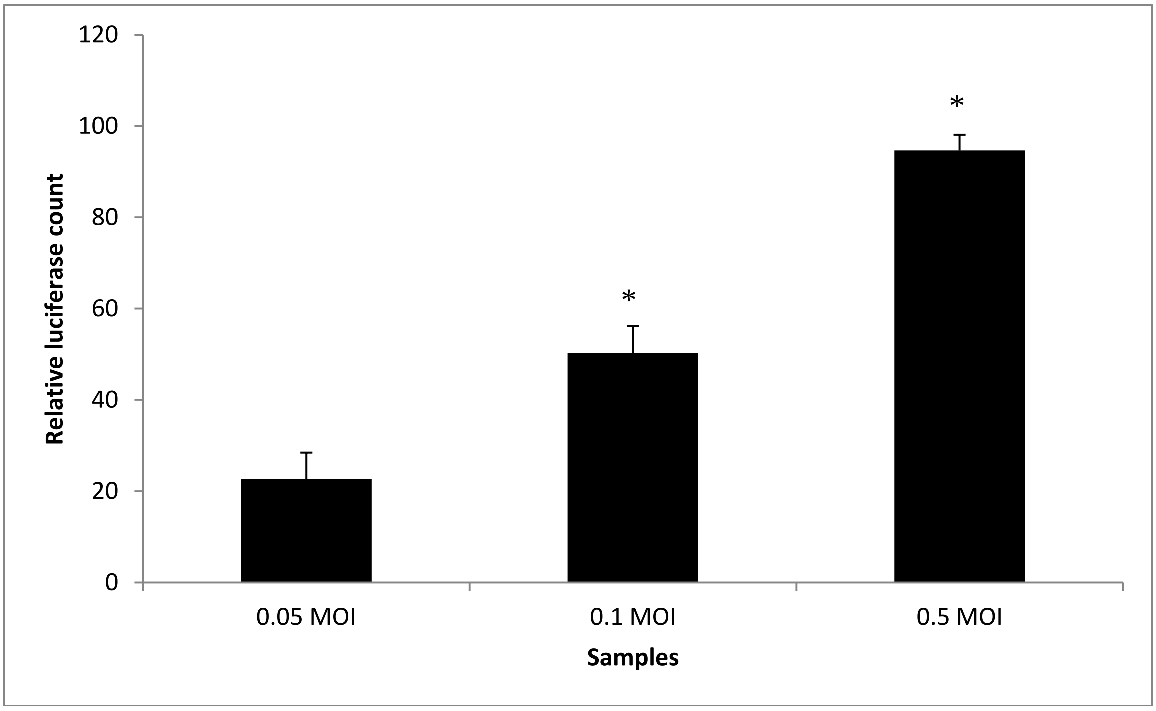


Figure 7. Analysis of the infectivity potential of HIV-1 virion particle produced after infection of Swan71 cells with NL4.3. Swan71 cells growing in transwell were infected with NL4.3 at an MOI of 0.05, 0.1, and 0.5 for 48 h. The above supernatants thus obtained from basolateral chamber (500 µl) were used to infect TZM-bl cells growing in 24-well culture plates for 48 h. After incubation, cells were washed with PBS and proceeded for calculation of luciferase activity as described in *Materials* and *Methods*. The values are represented as Percent Relative Luciferase Count (RLU). The values are referred as mean + SEM of 3 independent experiments performed in duplicate.
*p<0.05 as compared to cells infected with 0.05 MOI.

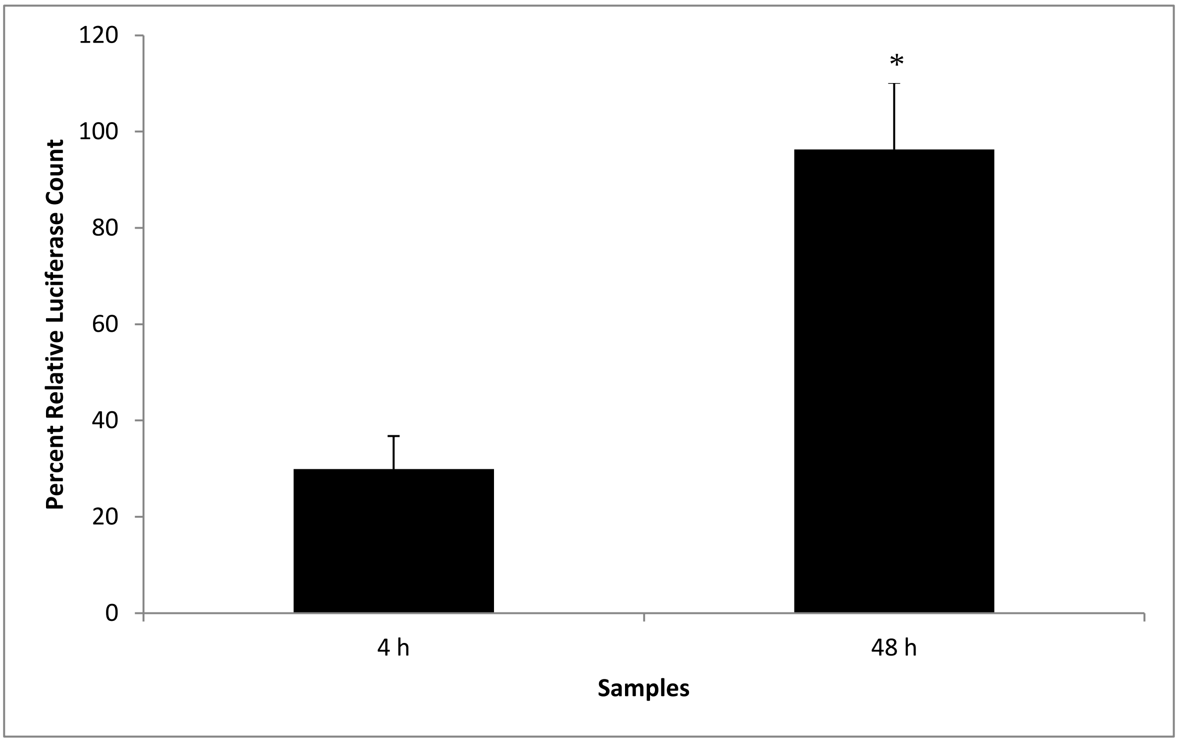


Figure 8. Increased production of infective HIV-1 virion particles in Swan71 cells as a function of time. Swan71 cells growing in transwell were infected with NL4.3 at an MOI of 0.5 for 4 or 48 h. The above supernatants thus obtained from basolateral chamber (500 µl) were used to infect TZM-bl cells growing in 24-well culture plates for 48 h. After incubation, cells were washed with PBS and proceeded for calculation of luciferase activity as described in *Materials* and *Methods*. The values are represented as Percent Relative Luciferase Count (RLU). The values are referred as mean + SEM of 3 independent experiments performed in duplicate.

*p<0.01 as compared to cells infected with 0.5 MOI for 4 h

Cytokines increases transcytosis of HIV-1 across Swan71 cell monolayer

Several inflammatory cytokines, including TNF-α and IL-6 play pivotal role during gestation and are essential for a successful pregnancy. These mediators produced locally by the placenta have been reported to augment HIV-1 infection and replication (Zachar *et al.,* 2002; Patterson *et al.,* 2001; Coulomb-L'Hermine *et al.,* 2000). Swan71 cells were treated with physiologically relevant concentrations of TNF-α (10, 50 and 100 ng/ml) and IL-6 (10 and 100 ng/ml) immediately preceding HIV-1 infection (Zachar *et al.,* 2002) to determine if TNF-α and/or IL-6 induce transcytosis. After an hour of treatment, cells were inoculated with HIV-1 NL4.3 (0.5 MOI) and incubated for 4 h. After 4 h incubation, cells were washed extensively (5 times) and transwells were transferred into new 12 well plates for 48 h incubation at 37°C in humified atmosphere of 5% CO_2. After incubation, p24 concentration was determined by ELISA as described in *Materials and Methods* in culture medium of basolateral chamber, apical chamber, and cell lysate. The results were compared with cells not treated with respective cytokines. Treatment with TNF-α and IL-6 resulted in significant increase in HIV-1 transcytosis when Swan71 cells were infected with NL4.3 (Figure 9). Prior treatment of Swan71 cells with 100 ng/ml of IL-6 led to a significant increase in the production of HIV-1 in basolateral chamber, apical chamber, as well as cell lysate. The increase in virus production was higher at 100 ng/ml of IL-6 as compared to 10 ng/ml of IL-6. A dose dependent increase in the production of HIV-1 virus was also observed in Swan71 cells treated with TNF-α. The extent of virus production was similar when Swan71 cells were treated with either 100 ng/ml of IL-6 or 100 ng/ml of TNF-α (Figure 9). Hence, TNF and IL-6 may be playing an important role during HIV-1 infection through placenta.

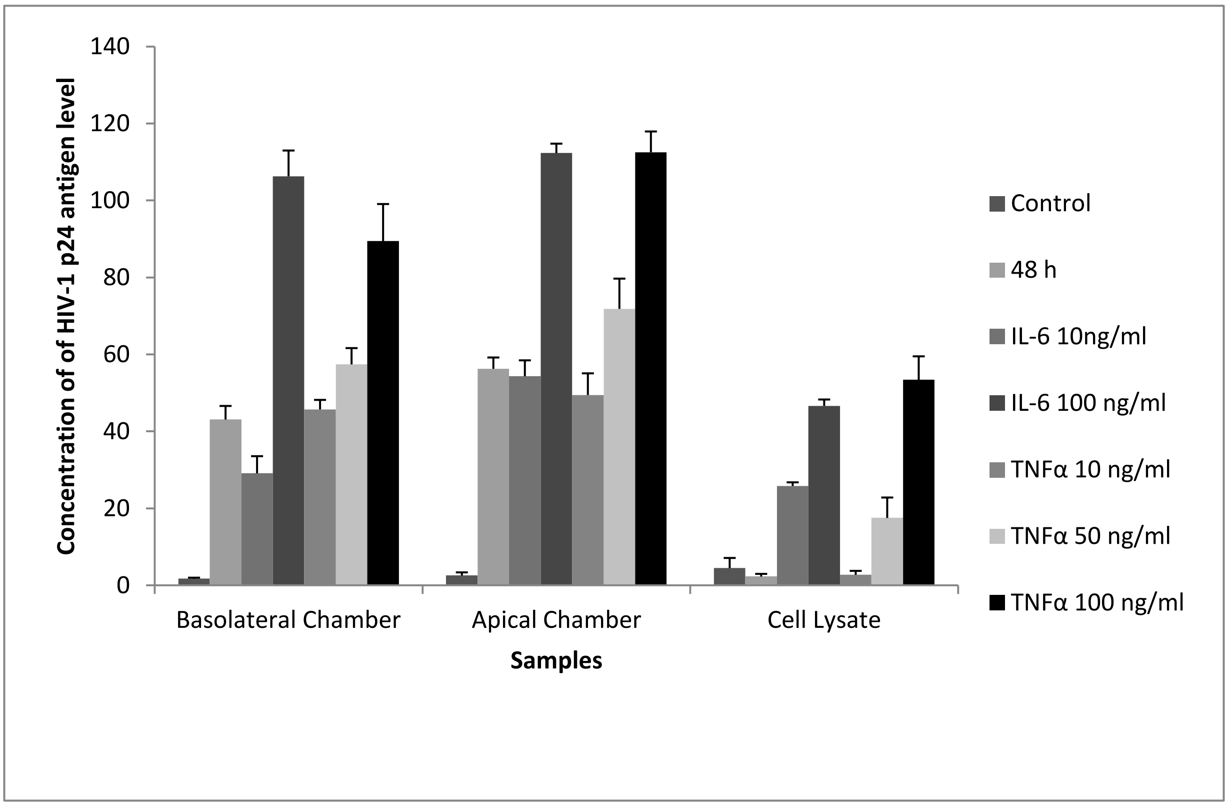


Figure 9: Effect of pre-treatment of Swan71 cells with either IL-6 or TNF-α on transcytosis of HIV-1. Swan71 cells growing in transwell were pre-treated with varying concentrations of IL-6 or TNF-α for 1 h followed by transfection with NL4.3 at 0.5 MOI. The cells were further processed and incubated for 48 h as described in Materials and Methods. After incubation, the culture medium collected from basolateral chamber and apical chamber as well as cell lysate was processed for p24 estimation by ELISA as described in *Materials* and *Methods*. Values are expressed as a mean + SEM of 3 independent experiments performed in duplicate.

Expression of CD4, CCR5, CXCR4, and mannose receptor by Swan71 trophoblastic cells.

Trophoblast cells express varied functional receptors including, CD4 receptors. The receptors play an important role in the entry of virus in trophoblastic cells. The Swan71 cells were incubated with anti-human CD4, anti-human CCR5, anti-human CXR4 and anti-human mannose receptor antibodies. The unstained Swan71 cells were taken as negative control. The flowcytometry results revealed that the Swan71 cells express mannose receptor on its surface (Figure 10). Analysis of the fluorescence revealed that 30% cells were stained with antibodies against mannose receptor. However, labelling of Swan71 cells with antibodies against CD4, CCR5 and CXCR4 did not show any specific cell population. These observations suggest that mannose receptor may be playing an important role in the transcytosis of HIV-1 into Swan71 cells.

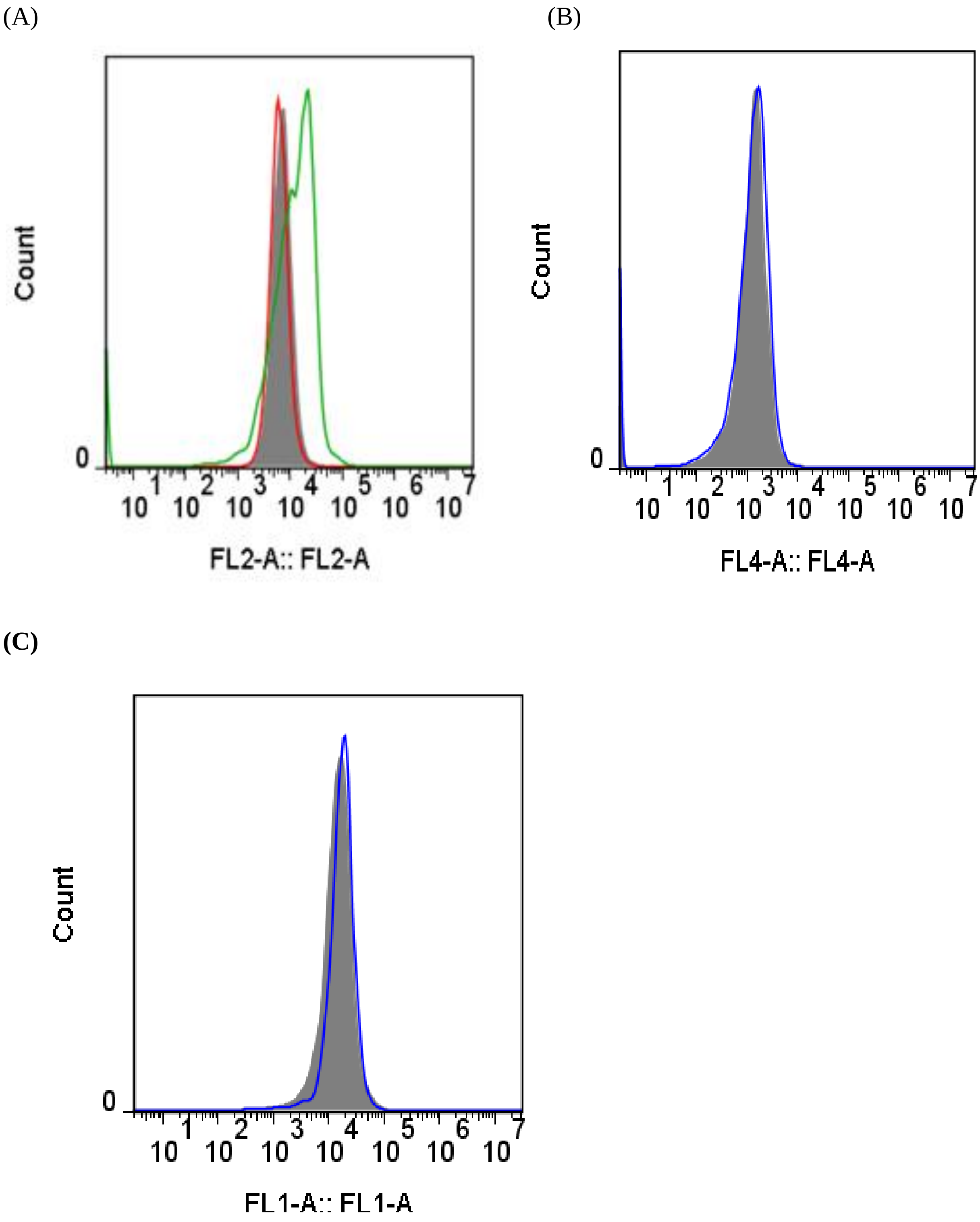


Figure 10: **Analysis of Swan71 cells by flowcytometry for the presence of CD4, CXCR4, CCR5 and mannose receptor**. Swan71 cells (0.1 x 10^6) were treated with labelled antibodies against human CD4, CXCR4, CCR5 and mannose receptor and cells processed for flowcytometric analysis as described in *Materials* and *Methods*. . The *y* axis indicates the cell number (count) detected in one channel; 10000 events were counted with each experiment. The *x* axis indicates the fluorescence intensity. A) The cells stained with activity against CCR5 (red color), mannose receptor (green color) whereas shaded area shows the profile of unstained cells; B) The cells stained with antibodies against CXCR4; and C) Cells stained with antibodies against CD4 receptor.

Role of mannose receptor in the transcytosis of HIV-1 virus in Swan71 cells

To delineate the relevance of mannose receptor in the transcytosis of HIV-1 virus, prior to infection with NL4.3, Swan71 cells were treated with Mannan (50 µg/ml) for an hour. In addition, cells were treated with SCD4 inhibitor C40 µg/ml for one hour. Further, the cells were inoculated with 0.5 MOI of pNL4.3. After 4 h incubation, cells were washed extensively (5 times) and transwells were transferred into new 12 well plates and incubated for 48 h at 37°C in humified atmosphere of 5% CO_2. After incubation, p24 concentration was determined by ELISA as described in *Materials and Methods* in culture medium of basolateral chamber, apical chamber, and cell lysate. The analysis of culture supernatant from both basolateral as well as apical chamber revealed a decrease in production of HIV-1 in presence of Mannan. However, analysis of p24 in cell lysate revealed an increase in p24 concentration in cells treated with Mannan. Treatment of Swan71 cells with sCD4 (CD4 inhibitor) did not led to any decrease in p24 levels as compared to untreated cells. However, a decrease in p24 levels were observed in cell lysate of Swan71 trophoblastic cells treated with sCD4. Due to the shortage of time, this experiment was performed only once and need to be confirmed. However, preliminary results suggest that mannose receptor may be playing an important role in transcytosis of HIV-1 in Swan71 cells.

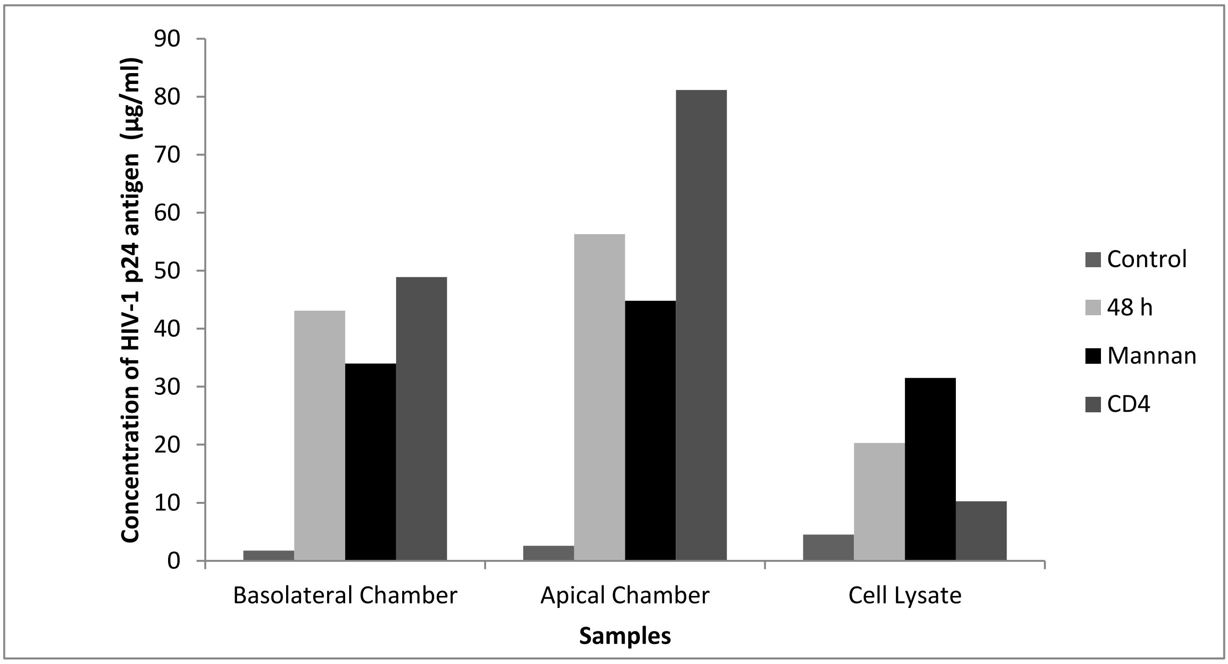


Figure 11: **Analysis of role of mannose receptor in transcytosis of HIV-1 into Swan71 cell**. Swan71 cells growing in transwell were treated with either Mannan or CD4 inhibitor for 1 h followed by transfection with NL4.3 at 0.5 MOI. The cells were further processed and incubated for 48 h as described in *Materials* and *Methods*. After incubation, the culture medium collected from basolateral chamber and apical chamber as well as cell lysate was processed for p24 estimation of ELISA as described in *Materials* and *Methods*. Values are expressed as mean of an experiment performed in duplicate.

Integration of HIV-1 in the host cell genomic DNA

Integration of newly synthesized viral DNA into the host cell chromosome is common to all retroviruses and is essential for a productive human immunodeficiency virus (HIV) infection. Polymerase chain reaction to detect HIV-1 provirus is widely accepted as a criterion of productive or latent infection since presence of provirus requires the initial stages of infection through reverse transcription. Upon reverse transcription of the viral genomic RNA, the resulting linear DNA molecule is actively transported to the nucleus within a complex of host and viral proteins known as the pre-integration complex, which is thought to be the immediate precursor to the integration reaction. Hence, PCR was carried out to detect the integration of HIV-1 in genomic DNA of trophoblastic Swan71 cells. To determine whether trophoblasts were latently infected, we extracted total DNA from HIV-1 challenged trophoblast cells cultured for 4 h and 48 h and carried out PCR analysis of provirus. Unchallenged trophoblasts containing no viral DNA were taken as negative control. After infection of trophoblastic cells with the T-tropic variant NL4.3 at an MOI of 0.5 at 4 and 48 h, DNA from the cells showed no viral DNA signal. PCR signals from DNA extracted from the cultured cells indicated no signals, predicting that the integration of HIV proviral did not occur in genomic DNA of host cell. These results show that all viral DNA in on the outside of Swan71 cells and none have been reverse transcribed from infected virus inside the cells. The results were compared with the signals provided by NL4.3 plasmid which acted as positive control. PCR was conducted with four different primers, specific to the genomic organisation of HIV-1.

Table 4: Primers used for PCR

Gene	**Primers**	**Annealing temperature**	**Product size (bp)**
Gag region	5-'AATTCGGTTAAGGCCAGGGG-3' 5'-TGGGGTGGCTCCTTCTGATA-3'	60°C	490
vif-vpu-vpr region	5'-TGGGGTCTGCATACAGGAGA-3' 5'-TCTGATGAGCTCTTCGTCGC-3'	60°C	764
Alu-LTR	5'-ACAAGCTAGTACCAGTTGAG-3' 5'-CTGCTAGAGAATTTTCCACACTGAC-3’	60°C	546
Alu-gag	5- GCCTCCAAAGTGCTGGGATTACAG -3’ 5′- GTTCCTGCTATGTCACTTCC -3′	60°C	1500

Cytotoxicity and anti-HIV activity of compounds using TZM-bl cells

Gallic acid and Ellagic acid were evaluated for their anti-HIV activity using TZM-bl cell based assay and were found to possess promising anti-HIV activity. The cytotoxicity of the above compounds by MTT assay was assessed on the reporter cell line used for determination of anti-HIV activity. TZM-bl cells were seeded in a 96-well culture plate and grown overnight at 37°C in a humidified atmosphere of 5% CO_2. Next day, culture medium with increasing concentrations of gallic and ellagic acid were added in duplicate and further incubated for 48 h and MTT assay was conducted as described in *Materials and Methods.* The CC_{50} values of gallic acid and ellagic acid were 47.35 and 38.65 μg/ml, respectively (Table 3). The anti-HIV activity evaluation of these compounds was performed at concentrations much below than that showing cytotoxicity.

In order to determine the inhibition activity of gallic and ellagic acid, TZM-bl cells were seeded in 24-well plate and cultured overnight. In separate vials, HIV-1 NL4.3 at a MOI of 0.05 were treated with compounds (gallic or ellagic acid) for 1 h at 37ºC. Subsequently, pre-treated viruses were added in duplicate to TZM-bl cells and cultured for 4 h. After incubation, the cells were washed to remove the cell free virus and further incubated for 48 h. After 48 h incubation, luciferase activity was determined as describe in *Materials and Methods.* The IC_{50} obtained by using non linear curve-fitting programme for gallic acid and ellagic acid were 0.51 μg/ml and 0.41 μg/ml respectively (Table 3). These compounds inhibited HIV infection in TZM-bl cells in a dose dependent fashion. Both of the compounds showed a dose dependent inhibition in HIV infection.

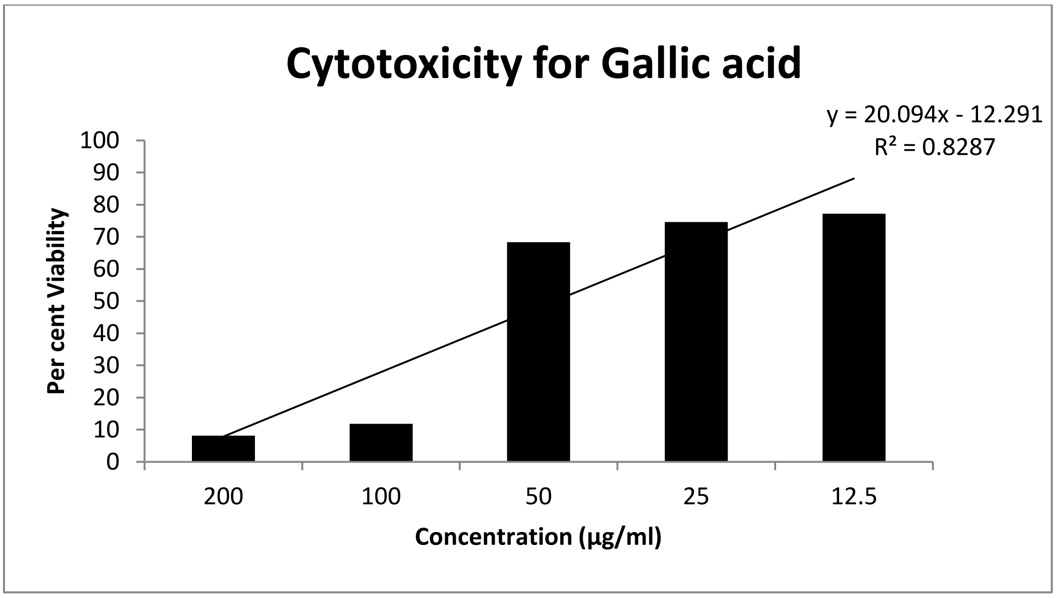
Table 3: *In vitro* cytoxicity and anti-HIV activity of the Gallic and Ellagic acid using TZM-bl cells

Compounds	**CC_{50} (µg/ml)**	**IC_{50} (µg/ml)**	**TI**
Gallic acid	47.35	0.512	92.48
Ellagic acid	38.65	0.41	93.69
AZT	176.03	0.091	>2021

CC_{50}: The cytotoxic concentration of the compounds that caused the reduction of viable cells by 50%.

IC_{50}: The concentration of the compounds that resulted in 50% inhibition in HIV infection.

TI, Therapeutic Index is CC_{50}/ $IC_{50;}$ AZT, azidothymidine



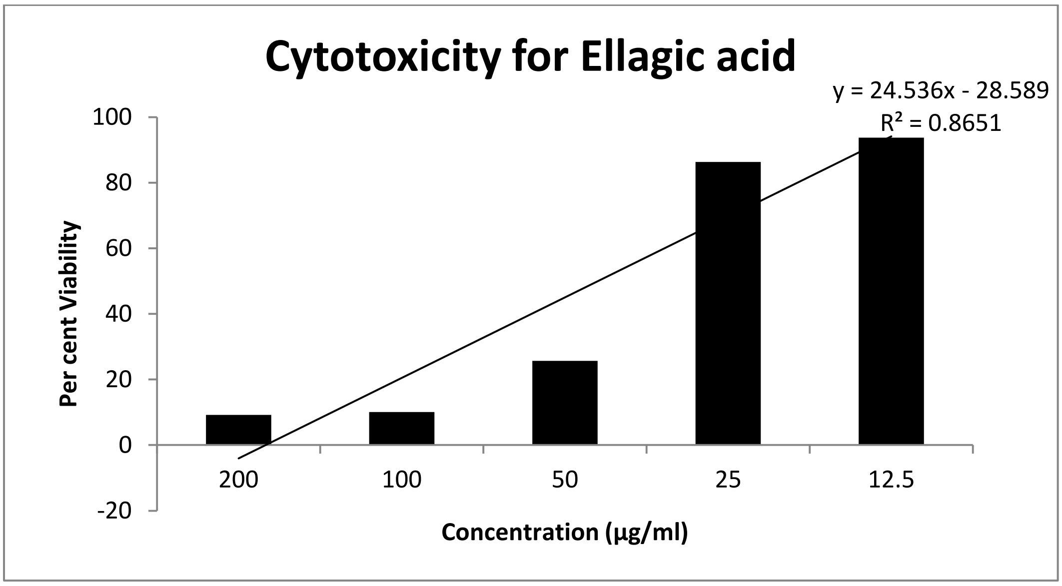
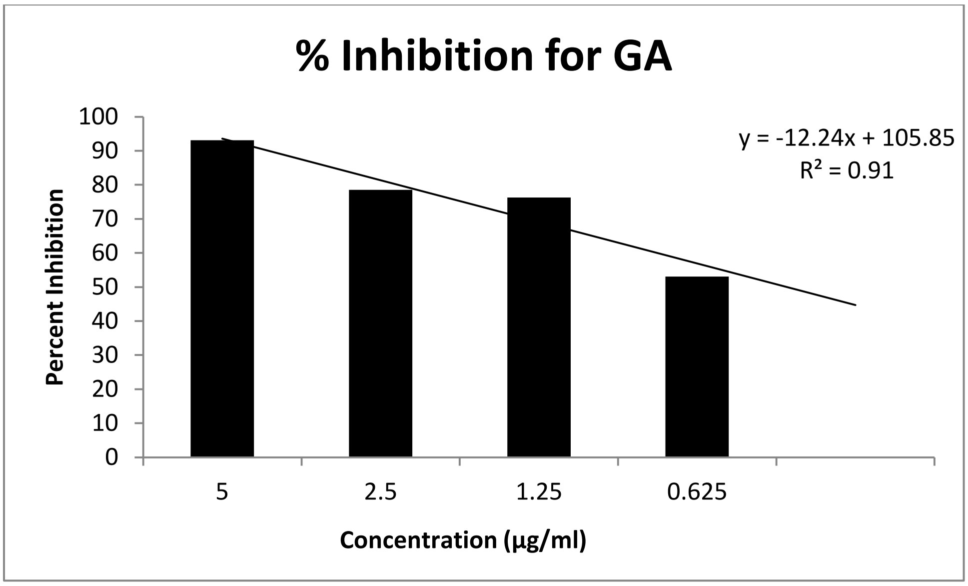


Figure 12 : **Cytotoxicity of gallic and ellagic acid in TZM-bl cells**. TZM-bl cells were seeded in a 96-well culture plate and grown overnight at 37°C in a humidified atmosphere of 5% CO_2. Next day, culture medium with increasing concentrations of gallic and ellagic acid were added in duplicate and further incubated for 48 h and MTT assay was conducted as described in *Materials and Methods.* Values shown as mean ± SEM of two independent experiments performed in duplicate. Both gallic acid and ellagic show maximum cytotoxic effect at 200µg/ml (8.10 and 9.19 µg/ml respectively)



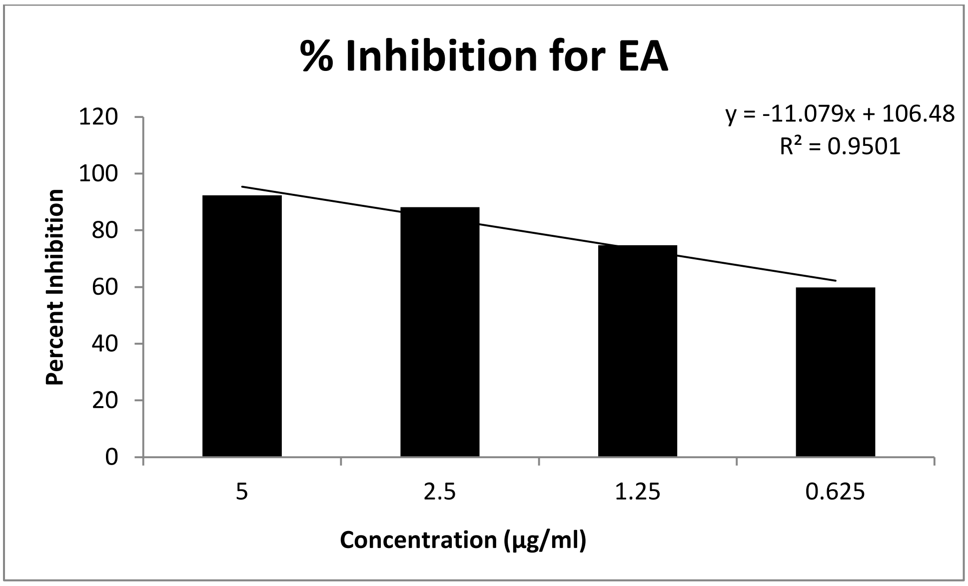


Figure 13: **Anti-HIV activity of gallic acid and ellagic acid using *in vitro* TZM-bl cells based assay.** TZM-bl cells were seeded in 24-well plate and cultured overnight. In separate vials, HIV-1 NL4.3 at a MOI of 0.05 were treated with compounds (gallic or ellagic acid) for 1 h at 37ºC. Subsequently, pre-treated viruses were added in duplicate to TZM-bl cells and cultured for 4 h. After incubation, the cells were washed to remove the cell free virus and further incubated for 48 h. After 48 h incubation, luciferase activity was determined as describe in *Materials and Methods.* Values shown as mean ± SEM of two independent experiments performed in duplicate. Both gallic acid and ellagic shows maximum inhibition at 5 µg/ml (93.08 and 92.31 µg/ml respectively)

Understanding mother-to-baby transmission of HIV-1 during gestation ultimately requires understanding how HIV enters fetal tissue. The development of microbicides could constitute a promising strategy to prevent sexual HIV transmission and consequently reduce HIV spread. Preclinical *in vitro* evaluation of new candidate microbicides is necessary before progressing to animal studies and clinical testing. As a potentially relevant model that mimics the structure of the female genital tract, a dual-chamber system with a female genital epithelial layer in the apical chamber is proposed. The primary trophoblastic cells were evaluated to form a confluent monolayer to carry out infection effectively and mimic the in vivo conditions. The developed models were used to investigate viral transmission parameters on one hand and to and to evaluate epithelial layer integrity on the other hand. TEER is a measure for epithelial layer integrity. It confirmed the formation of polarized monolayer with constant increase in resistance value.

Addition of cell-free virus on top of the epithelial layers resulted in infection and supported transmission across the transmembrane mainly at higher input titers. The relative role of HIV-infected cell transmigration through the genital epithelial barrier in heterosexual virus transmission has not yet been fully characterized. The observations made in the present study provide evidence supporting the hypothesis that HIV-infected mononuclear cells may cross the epithelial barrier. Entry requires transit of the virus cross the trophoblast, a fetal cell that separates all fetal from maternal tissue (Alpin *et al.*, 1991). We have shown in this study with sensitive methods of virus detection that pure populations of primary placental trophoblasts may productively infected with cell-free virus, however at a very low titre. The increase in viral concentration with time indicates that the virus do infects the primary trophoblastic cells but the productivity of virus is very low. Vertical transmission is associated with a selective transmission of marcrophage-tropic or T-cell-tropic HIV variants and therefore trophoblast-HIV interactions were analyzed in this context. The infection with T-tropic and M-tropic strain indicated that the cells readily are infected with pNL4.3; however, the infection with pNLAD8-GFP was considerably low. It predicts a picture that viral entry occurs effectively through co-receptor CXCR4 as compared to CCR5 co-receptor. The enhancement of luciferase activity in TZM-bl cells after treatment with the infected supernatant also revealed the viral entry and productive infection by HIV-1 particles in Swan71 cells. However, PCR results demonstrate that virus does not integrate inside the trophoblastic Swan71 genome. This throws light on the possibility that after internalization, the virus remains in vesicles and is rapidly transcytosed from the apical to the basolateral surface of the endothelium, from which infectious HIV-1 particles are released without being integrated inside the cell (Lagaye *et al.*, 2001; Bomsel, 1997) Thereby, trophoblastic cells appear not to play a role in HIV mother-to-child transmission since integration was not observed after trophoblast was challenged with two different isolates. This premise provides the information that trophoblast resist HIV infection by free virus at the stage of plasma membrane passage or shortly thereafter. These results highlight the importance of an intact syncitiotrophoblast. HIV infection, it if occurs, must proceed through other mechanisms, possibly involving the close apposition of infected maternal cells with the villous trophoblast or through endocytosis which may allow the occurrence of infection at a later stage

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Previous investigators have reported conflicting data regarding the expression of HIV-1 receptors and chemokine co-receptors on trophoblast cells (Douglas *et al.*, 2001; Ishii *et al.*, 2000; Moussa *et al.*, 1999). Most investigators agree that trophoblast cells do not express the CD4 antigen, to which HIV-1 envelope protein binds. Consequently, research efforts have focused on the expression of chemokine receptors, which may be involved in CD4-independent infection of trophoblast cells. The chemokine receptor CCR5 is a co-receptor for macrophage-tropic HIV-1 strains, which are usually non-syncytium inducing and are the most prevalent isolates in neonatal infections, while CXCR4 is a co-receptor for T-cell-tropic HIV-1 strains, which are usually syncytium inducing (Doms and Peiper**,** 1997). In some reports, these chemokine receptors were detected in first trimester trophoblast cells, but according to most reports, term trophoblast cells do not express the HIV-1 coreceptors CCR5 and CXCR4 (Douglas et al, 2001; Ishii *et al.*, 2000; Moussa *et al.*, 1999). We used sensitive techniques (i.e. flow cytometry) and were unable to detect expression of CD4, CCR5, and CXCR4, on primary trophoblast cells. Importantly, positive controls yielded expected results with flowcytomer. Our results are congruent with those reported for other endothelial cell, Jurkat cells, which express CD4, CCR5 and CXCR4 on their surface (Kanmogne *et al.*, 2000).

Therefore, we conclude from our findings that the absence of these receptors on trophoblast cells provides one mechanism by which the placenta resists transmission of HIV-1 from the maternal to the fetal circulation. The present study demonstrates that human vaginal epithelial cells interact with HIV gp120 via the hMR (Sashaina *et al.*, 2011).

Mannose receptors on trophoblast cells were found to be present and were inhibited by the mannose receptor antagonist mannan. In recent study (Horbul *et al.,* 2001), herpes simplex virus induced disruption of the surface epithelium allowed direst access of HIV to sub-mucosal CD4+ cells. Thus, the epithelial barrier integrity appears to be crucial determinant for HIV entry via the sexual root. However the trophoblastic cells do not contain CD4 receptors. This provides the evidence that the HIV entry occurs through other possible mean. This increases the prospect that hMR pathway would lead to mucosal transmission of HIV in villous trophoblasts. This argument was further supported by the decrease concentration of HIV obtained when treated with antagonistic mannan, though the decrease was minimal.

The villous syncytiotrophoblast appears to be susceptible to endocytosis of HIV-1 particles (Douglas *et al*., 2001; Bourinbaiar *et al*, 1993; Phillips and Tan, 1992). After endocytosis, the virus remains in vesicles and can be transported from the apical to the basolateral surface of the trophoblast layer by transcytosis, which is a rapid transcellular transport pathway that is specific to polarized epithelial cells (Lagaye *et al*., 2001; Bomsel, 1997). Cytokines such as TNF-α and IL-6 stimulate receptormediated endocytosis and transcytosis of macromolecules across the epithelial barrier, and TNF-α upregulates transcription of HIV-1 promoters in trophoblast cells (Zachar *et al*., 2002; Descamps *et al*., 1997). Therefore, we hypothesized that TNF-α and IL-6 may promote transcytosis of HIV-1 across the placenta and anti-inflammatory agents may abrogate this effect. After failing to detect the HIV internalization in primary trophoblast cells, we performed the infection in the presence of cytokines to establish an *in vivo* condition. Our findings also provide experimental evidence supporting the clinical observation that inflammatory mediators are associated with increased HIV-1 vertical transmission rates. The placenta produces a number of cytokines that may play a critical role in promoting or inhibiting vertical transmission of HIV-1, and inflammation of the placental villous membrane was associated with an increased rate of mother-to child HIV-1 transmission in a large cohort of women. We found that TNF-α and IL-6 significantly up-regulated transcytosis of HIV-1 isolates. Thus, we believe that local cytokine status may be correlated with trophoblast infection and the likelihood of prenatally acquired HIV-1 infection.

Women are particularly vulnerable to heterosexual transmission of HIV-1 due to both socio-economical and biological reasons (UNAIDS, 2002). Therefore, blocking HIV-1 transmission in the female genital tract is key to prevent infection. With a urgent need to reduce the HIV level of infection in mass, there’s a requirement for the establishment of novel HIV-treatment with least dose-limited side effects and long-term dependency. Therefore, it is essential to focus on isolation of anti-HIV therapeutics from natural compounds. Gallic acid and Ellagic acid are believed to have anti-HIV activity, which inhibits the activity of HIV-protease and integrase enzymes (Nutan *et al*., 2012). These phenolic extracts possess high antioxidant, anti-inflammatory and anti-viral activity. Because of their abundance, anti-neoplastic and anti-hyperlipidaemic properties, make them an eminent anti-HIV drug. Both the compounds inhibited HIV infection in dose dependent manner in TZM-bl cell assay. The supernatant from infected and compound treated cells showed maximum inhibition in virus loads. The therapeutic effect is the ratio between the toxic and the therapeutic dose and is used as a measure of its relative safety. A high TI suggests the compounds as an effective candidate for anti-HIV activity. The present results suggest that Gallic acid and Ellagic acid have a potential to be considered for the development of microbiocides for prevention of HIV infection.

Summary

Vertical transmission of HIV-1 is a serious public health issue. It is estimated that 1600 infants acquire HIV-1infection every day worldwide. The HIV-1 transmission rate is increasing in developing countries, ranging from10% to 40% in the absence of antiretroviral treatment. In order to understand the mechanism of transmission of HIV from mother to foetus, primary trophoblastic cells were infected with different strains of viral clone. Various mechanisms and receptors involved in entry of HIV into the cell were evaluated. The results claimed the absence of HIV-1 receptors; CD4 and its coreceptors CCR5 and CXCR4, while mannose receptor were present on the cell surface. The experimental set-up indicated that HIV genome does not get integrated into the genome of trophoblastic cells. This increased the possibility that of the fact that the viral genome gets endocytosed inside the endosome of the cell and later, get released through transcytosis across the trophoblastic monolayer. After failing to detect the HIV internalization in primary trophoblast cells, we performed the infection in the presence of cytokines to establish an *in vivo* condition. After failing to detect the HIV internalization in primary trophoblast cells, we performed the infection in the presence of cytokines to establish an *in vivo* condition. Our findings also provide experimental evidence supporting the clinical observation that inflammatory mediators are associated with increased HIV-1 vertical transmission rates.

With the advent of increase in HIV infection, blocking HIV-1 transmission in the female genital tract is key to prevent infection. Thus, natural drug compounds were used to evaluate the anti-HIV activity. Gallic acid and Ellagic acid are believed to have anti-HIV activity, which inhibits the activity of HIV-protease and integrase enzymes. The screening of this compounds suggest that both Gallic acid and Ellagic acid have a potential to be considered for the development of microbicides for prevention of HIV infection, with gallic acid being more effect drug in inhibiting HIV activity.

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