Thesis for the Degree of Master of Science

Inhibitory Effect of Sulfated Chitooligosaccharides on HIV-1 Infection

by

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August 2008

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Sulfated chitooligosaccharides 의 HIV-1 감염억제 효과

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Abstract

Human immunodeficiency virus type-1 (HIV-1) is identified as the causative agent of acquired immunodeficiency syndrome (AIDS) which is one of the most important diseases with about 33.2 million people infected worldwide. Recently, it has been crucial to identify compounds with less or no cytotoxicity and higher inhibitory activity against various HIV strains, including the ones which are resistant to drugs currently used for antiretroviral therapy. Despite the advances in rational drug design, natural products stand as the most important drug candidates for the inhibition of HIV infection. In the present study, *in vitro* anti-HIV properties of various molecular weight sulfated chitooligosaccharides (SCOSs) were investigated. According to the results obtained from the experiments, SCOSs inhibited the replication of HIV-1, as well as protected T-lymphoblasts from HIV-1 induced cytopathic effects and almost completely reduced cell culture HIV-1 p24 antigen level. The delayed addition analysis of SCOSs in co-culture studies revealed that SCOS's action was at the early stage of HIV-1 replication. SCOSs blocked not only cell-to-cell fusion process of HIV infected and uninfected cells, but also cell free virus attachment. SCOSs with different molecular weight also exhibited different inhibitory patterns. These results indicate that SCOSs are potentially safe HIV-1 inhibitors with high activity at non-cytotoxic concentrations.

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List of Abbreviation

AIDS	Acquired immunodeficiency syndrome
AZT	Azidothymidine
$CCID_{50}$	50% cell culture infectious dose
CD4	Cluster of differentiation number 4
DMSO	Dimethyl sulfoxide
DS	Dextran sulfate
EC_{50}	50% effective concentration
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
Gp	Glycoprotein
HIV-1	Human immunodeficiency virus type-1
ICTV	International Committee on the Taxonomy of Viruses
LTR	Long terminal repeat
MHC	Major histocompatibility complex
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PCP	*Pneumocystis carinii*
Rev	Regulator of virion
SCOS	Sulfated chitooligosaccharide
Tat	Transactivator of transcription
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U

Introduction

The History of AIDS and HIV

In 1981, *Pneumocystis carinii* pneumonia was noticed in homosexual male in New York and San Francisco. *Pneumocystis carinii* pneumonia is characterized as an infection occurred almost especially until then in senior people, organ recipients, those suffering from various types of cancer, or others whose immune systems had been severely weakened, or damaged by immunosuppressive drugs. These young male gays, however, had no obvious reasons for facing immune system failure. Furthermore, unusual clusters of Kaposi's sarcoma, which is a rare benign cancer usually occurs in elderly, was reported in homosexual men in the same area (Reeves et al., 2000; MMWR[1]). By the end of August 1981, the CDC (US Center for Disease Control) had received 107 reports of *Pneumocystis carinii* pneumonia, Kaposi's sarcoma, or both in 95 homosexual male, 6 heterosexual male, 5 male whose sexual orientation was not known and 1 female. Autopsies on the people who had died revealed to a serious collapse of the immune system in the disease. Those findings were clearly the indication of a new epidemic (MMWR[2]).

Initially CDC referred to the condition as "Kaposi's sarcoma and opportunistic infections in previously healthy persons", while the media referred to it as "GRID- Gay Related Immune Deficiency". Finally, in 1986, the nomenclature of the virus and syndrome was completed by the International Committee on the Taxonomy of Viruses (ICTV) which named the virus causing this disease as human immunodeficiency virus (HIV) and the disease caused by the virus as acquired immunodeficiency syndrome (AIDS).

The Structure of HIV-1

HIV is an enveloped retrovirus belongs to the lentivirus family (Figure 1). The structure of HIV is relatively complex with each virus expressing 160 kDa glycoproteins composed of gp120 and gp41. The gp41 molecule is a transmembrane glycoprotein that crosses the membrane of the viral envelope (Reeves et al., 2000). There is a non-covalent interaction between gp120 and gp41. The entry of HIV into the host cell requires the interaction of viral envelope glycoprotein, gp120, with the CD4 glycoprotein and a

chemokine receptor on the host cell surface (Kwong et al., 1998). The viral envelope is composed of the host cell membrane and naturally contains some host cell membrane proteins, including class I and class II major histocompatibility complex molecules which previously located on host-cell membrane (Reeves et al., 2000). Within the envelope, viral core (nucleocapsid) that includes a layer of protein called p17, and an inner layer of protein called p24 is located. The HIV genome is composed of two identical single-stranded RNA (+) and some proteins are attached to the genome such as two molecules of reverse transcriptase, a protease, and an integrase (Reeves et al., 2000).

The Viral Envelope

The envelope consists of a double layer lipid molecules derived from the plasma membrane of the host during budding which are called as lipid rafts (Aloia et al., 1993). The envelope can easily be damaged by detergents, disinfectants and drying which makes HIV rather fragile and less tolerant to harsh environmental conditions. The viral envelope contains a number of cellular proteins, such as MHC I and MHC II molecules besides major proteins gp120 and gp41 which are located on the membrane. These cellular proteins help the virus from the attacks of host's immune system (Gelderblom et al., 1987).

The gp160 glycoprotein is a precursor that yields the gp120 and gp41 glycoproteins when cleaved by the host-cell proteases (Figure 2). The gp120 molecule is a peripheral membrane glycoprotein that is attached the membrane of the viral envelope by the gp41 glycoprotein through non-covalent interactions. Infection of the host cell is initiated by interactions between gp120 and CD4 cell surface receptor. Gp120 binds to CD4 receptor and anchors the virus to the cell surface, and additional interactions with chemokine receptors trigger a conformational change that leads the viral and cellular membranes fuse (Klatzmann et al., 1984; McDougal et al., 1985; Bour et al., 1995). The gp120 molecule is recognized by the immune system, and a number of antibodies react to several different neutralizing sites on the gp120 molecule. If gp120 molecule neutralized by an antibody, it cannot interact with CD4 receptor, and hence the virus will not be able to anchor to the host-cell membrane. The most important target for neutralizing antibodies on the gp120 molecule is known as the V3 loop (Sierra et al., 2005; Nara et al., 1991).

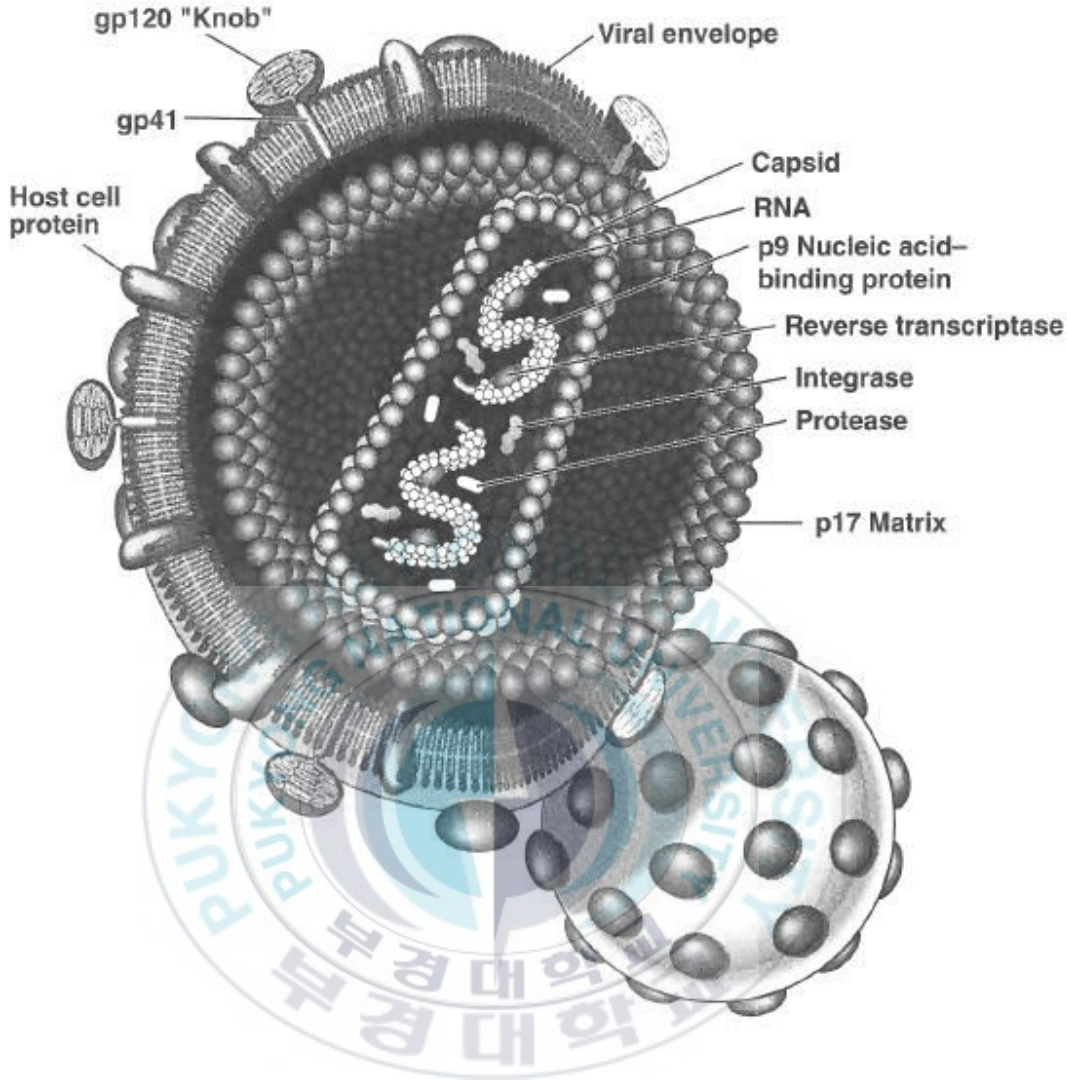

Figure 1: The structure of HIV-1 (Ward, 1999)

The V3 loop has an important function in the fusion of the viral envelope with the host-cell membrane. The V3 loop is a hypervariable region where genetic mutations can occur quite frequently, and is used by HIV as an advantage in order to escape from immune attack to avoid recognition by antibodies. The other important antibody-neutralization target on the gp120 molecule is the CD4 binding region (Back et al, 1990; Ho et al., 1991; Nara et al., 1991).

The Virion

The viral matrix sustains the structure of the HIV and helps the translocation of the cDNA copy of the viral RNA into the cell nucleus. The p17 matrix protein is linked to the viral envelope (Gelderblom et al., 1987). The capsid forms the main core of the virus and p24 core protein is the main building block for this structure (Gelderblom et al., 1987). Antibodies raised against p24 core protein form the main principle of HIV detection tests. Two identical strands of viral RNA are located in the capsid. Each strand of RNA contains a complete set of viral genes (Takasaki et al., 1997). Nucleic acid-binding protein (p9) is attached to each strand of RNA (Franke et al., 1994; Thali et al., 1994). HIV integrase (INT), protease (PR) and reverse transcriptase (RT) are present in the capsid. Table 1 show the proteins located in the virion.

Classification of HIV

Since describing the AIDS pandemic, several genetic mutations in HIV have been accumulated for distinct types and subtypes to arise. Eleven distinct types of HIV-1 have been identified up to day (Robertson et al., 2000).

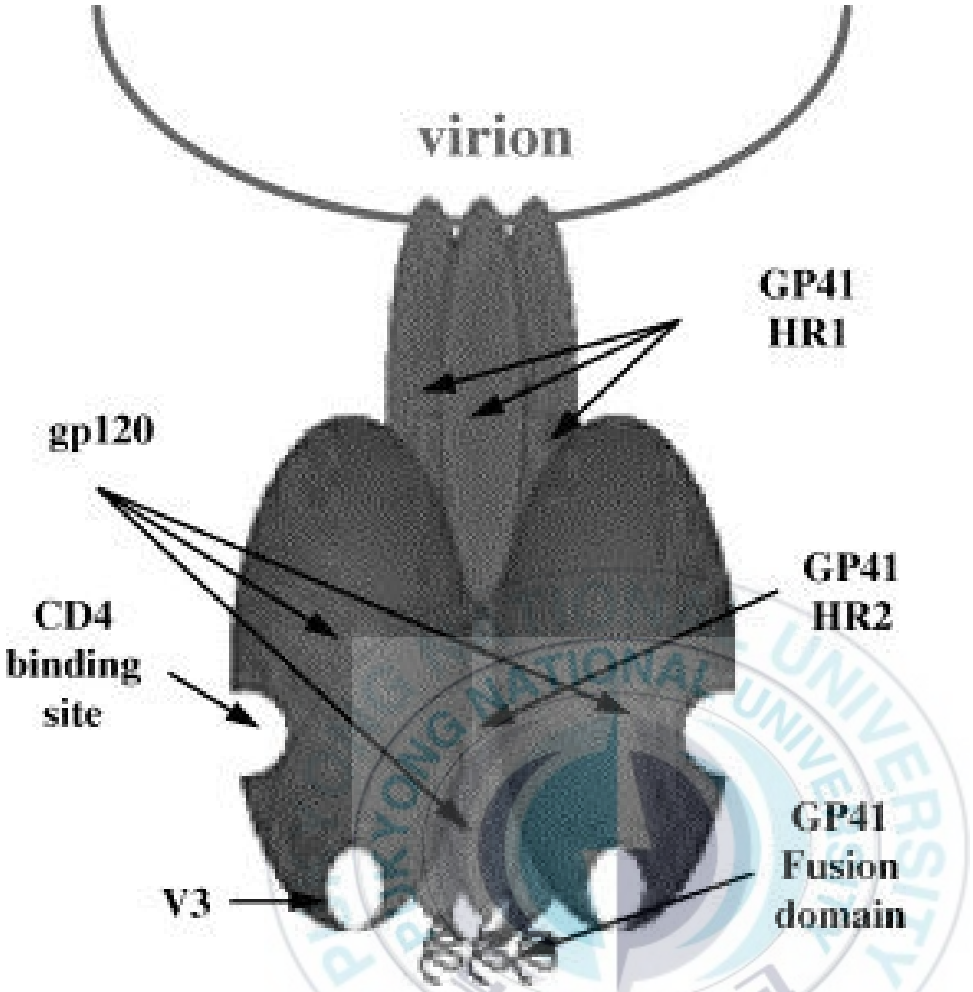

Figure 2: HIV envelope glycoprotein (Sierra et al., 2005). The Env glycoprotein is a heterodimer of the external gp120 non-covalently attached to the transmembrane domain gp41, organized in triangular symmetry.

HIV Genome

The HIV genome contains three major genes, which are *gag*, *pol* and *env* (Figure 3). Long terminal repeats (LTRs) flank the both 5'-end and 3'-end of the genome (Sierra et al., 2005). The promoter, enhancer and host cell DNA-binding protein domains reside in the 5'-end LTR. The *gag*, *pol* and *env* regions are located downstream from the 5'-end LTR. The *gag* gene encodes the structural proteins, the most importantly the p24 capsid protein. The *pol* gene serves as a template for the integrase, protease and reverse transcriptase which are located in the nucleocapsid. The *env* gene encodes for the viral envelope proteins, gp120 and gp41. The 3'-end LTR enables the viral genome to link with the 5'-end LTR, forming a circular plasmid-like structure.

The HIV genome also contains a number of genes encoding some accessory proteins which are crucial for viral replication (Turner and Summers, 1999). The "transactivator of transcription" (*tat*) gene encodes a potent transcriptional activator essential for HIV replication. The *rev* gene encodes a regulator of structural gene expression also essential for virus replication. Other viral genes include *vpr*, *vif*, *vpu* and *nef*.

Table 1: The genes of HIV-1 and the functions of their proteins (Goldsby et al., 2000)

Gene	Protein product	Function of encoded proteins
gag	**53-kDA precursor**	**Nucleocapsid proteins**
	p17	Forms outer core-protein layer
	p24	Forms inner core-protein layer
	p9	Is component of nucleoid core
	p7	Binds directly to genomic RNA
env	**160-kDA precursor**	**Envelope glycoproteins**
	gp41	Is transmembrane protein associated with gp120 and required for fusion
	gp120	Protrudes from envelope and binds CD4
pol	**Precursor**	**Enzymes**
	p66	Has reverse transcriptase and RNase activity
	p51	Has reverse transcriptase activity
	p10	Is protease that cleaves gag precursor
	p32	Is integrase
vif	**p23**	Promotes infectivity of viral particle
vpr	**p15**	Weakly activates transcription of proviral DNA
tat	**p14**	Strongly activates transcription of proviral DNA
rev	**p19**	Allows export of unspliced and singly spliced mRNAs from nucleus
nef	**p27**	Increases viral replication; down-regulates host-cell CD4
vpu	**p16**	Is required for efficient viral assembly and budding

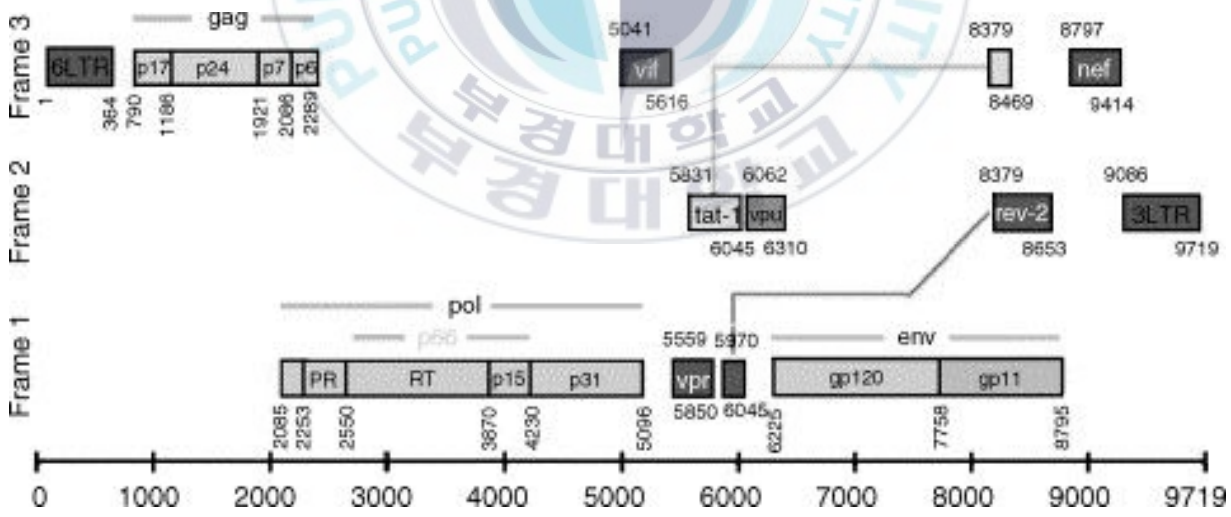

Figure 3: Organization of the HIV-1 proviral genome (Sierra et al., 2005).

HIV Types and Subtypes

HIV subtypes are categorized according to differences in DNA base sequences for the envelope glycoprotein gp120 and the gag protein. Amino acid sequences in the gp120 molecule differ by 20%-30% between subtypes, and by 15% in the gag protein. Classification of HIV-1 and HIV-2 is shown in figure 4. Phylogenetic analyses of known HIV-1 strains have identified three distinct groups of virus (M, N, and O), and nine genetic subtypes (A to D, F to H, J, and K) within major group (M). Group M (for Major) includes the vast majority of HIV-1 strains. Group O (for Outlier) is composed of highly variable, genetically-related strains which are not categorized in known clades. Group O infections can be detected in Central Africa (mainly Cameroon and some neighboring countries), but even in this area they form relatively a small portion of HIV-1 infections. Only a few cases of group N infections have been introduced to literature, and these were in patients from Cameroon.

Another type of HIV, known as HIV-2, was firstly identified in early 1986, which was recovered from females in Senegal (Clavel et al., 1986). This strain of HIV differed considerably from HIV-1: 55% of its proviral DNA base sequences were different from HIV-1. Furthermore, most antibodies raised against HIV-1 did not react with HIV-2. HIV-2 is mainly found in the West African countries. HIV type-2 causes an immune deficiency which is basically similar to AIDS, however, individuals infected with HIV-2 often sustain higher levels of CD4 cells for a longer period so that disease can be realized only much later on than for HIV-1 infected individuals. Other evidence also furnishes that HIV-2 is much less infectious than HIV-1, less likely to be transmitted from mother-to-child, and has a lower rate of replication (Markowitz, 1993; De Cock et al., 1993; Simon et al., 1993; Marlink, 1996).

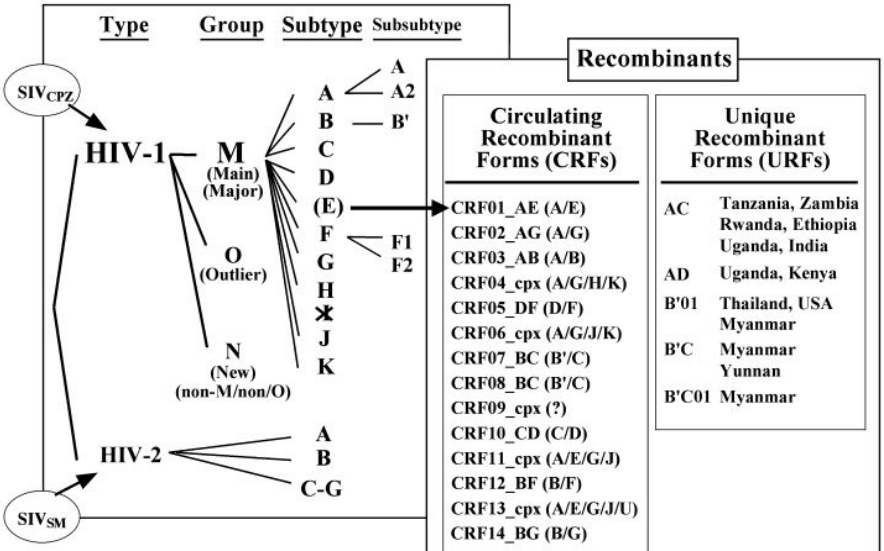

Figure 4: Classification of human immunodeficiency viruses (Takebe et al., 2004)

Current Status of HIV/AIDS in Korea

In comparison to other countries, Korea has a relatively low HIV/AIDS prevalence. Since the first reported case in 1985, total number of HIV infected people is 5323, 980 of whom have already died. However, since 1985, the number of new HIV cases has been increasing steadily (Figure 5). By gender, 4861 of HIV infected patients are male whereas 462 patients are female. Those aged 30-39 years represent the highest number of cases of infection. Sexual transmission is the main route of infection for AIDS patients and HIV infected people (UNAIDS, 2008).

The number of newly reported cases HIV cases in 2007 is 744 in Korea. Among 744, the number of HIV-positive cases is 701 in male and 43 in female, representing a ratio of 16:1. By age group, those in their 30s have the highest number of infection cases (Figure 6), followed by those in their 40s and those in their 20s. The main cause of transmission is sexual contact (UNAIDS, 2008).

HIV Replication

The knowledge of HIV replication is important for the understanding of its pathogenesis and the development of therapeutic agents.

Infection of Target Cell

Following entry of HIV into cells getting into contact with the appropriate receptors and co-receptors, formation of the viral double-stranded DNA genome is followed by the integration of proviral-DNA into the host cell genome, creating a provirus (Figure 7).

Attachment of the virus occurs upon the binding of the gp120 on the viral envelope to the CD4+ host cell. Before the virus can gain entry into the host cell, however,

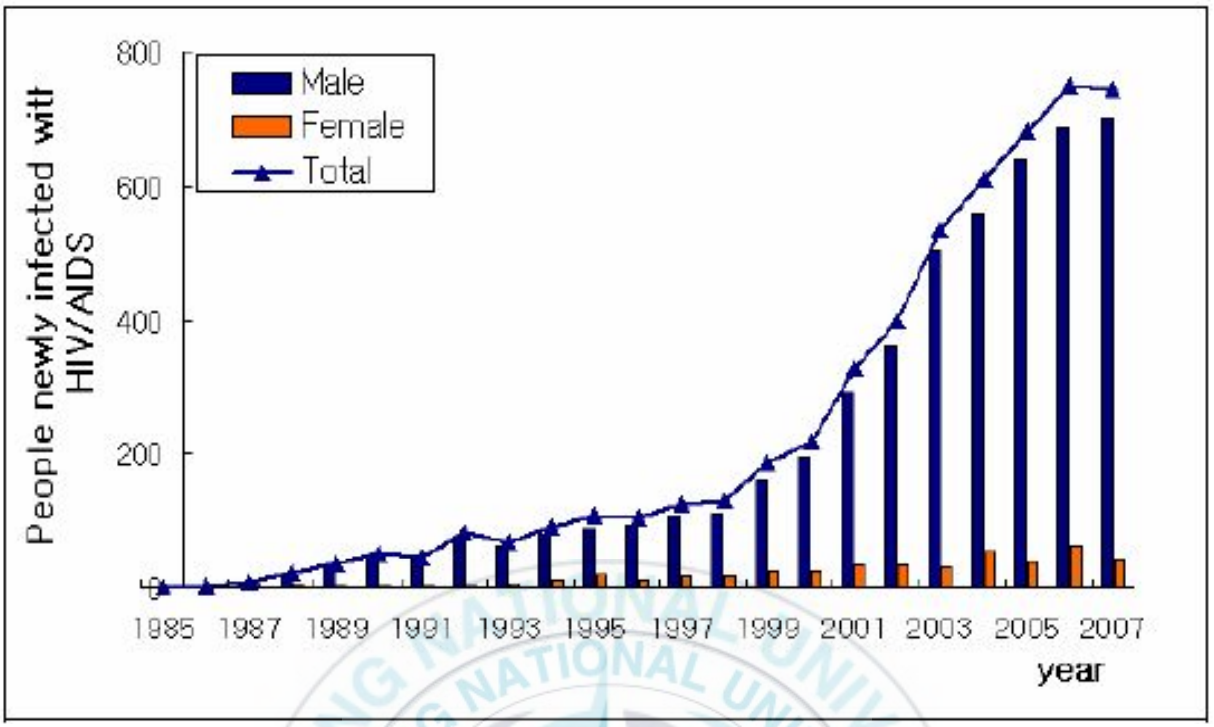

Figure 5: Reported Number of People Living with HIV/AIDS in Korea by Year (UNAIDS, 2008 Country Progress Report)

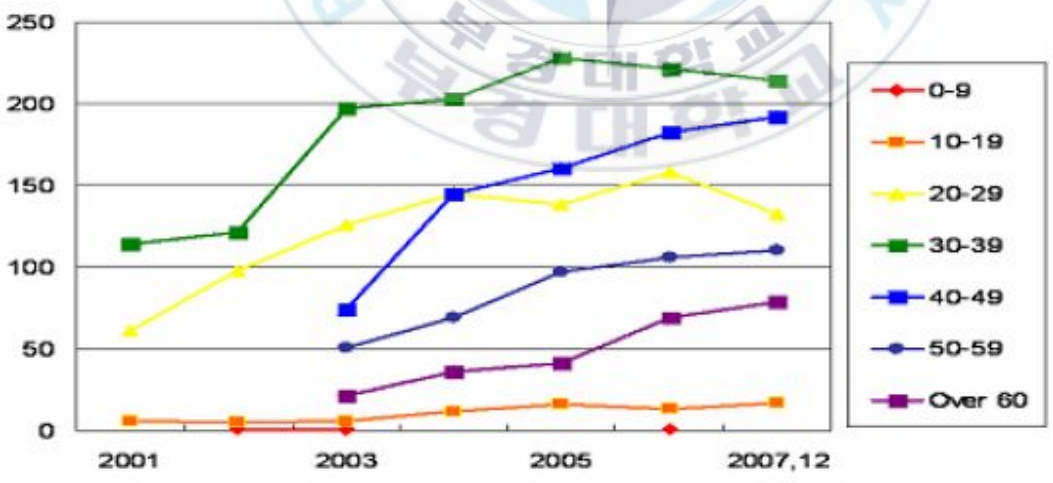

Figure 6: Annual Trend of HIV-Infected People in Korea by Age (UNAIDS, 2008 Country Progress Report)

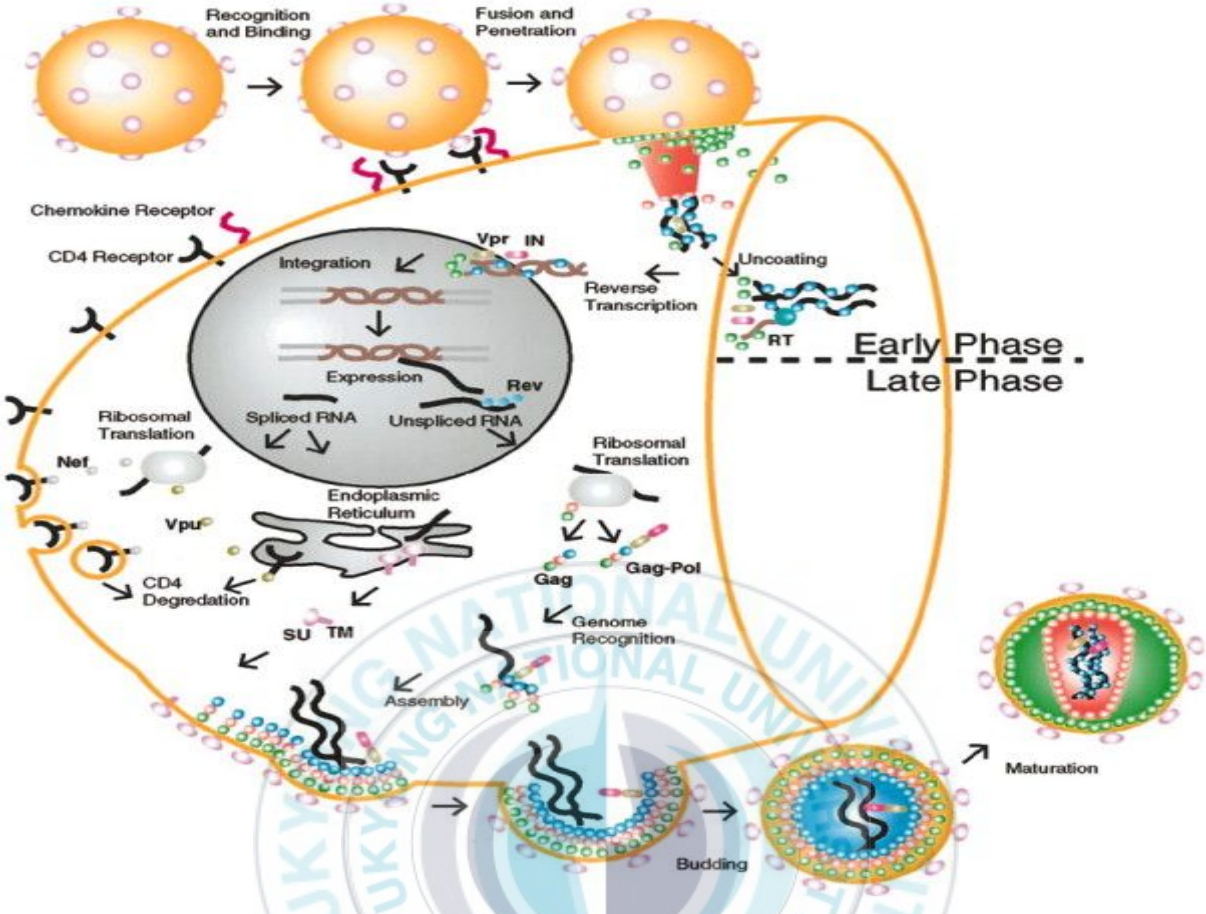

Figure 7: HIV life cycle (Turner et al., 1999)

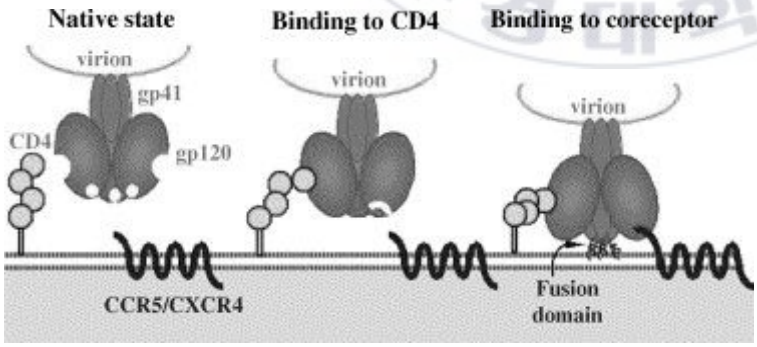

Figure 8: Co-receptors and model for virion attachment (Sierra et al., 2005)

attachment of a second receptor (co-receptor) to V3 loop of the gp120 glycoprotein must occur: CD4 molecule allow gp120 to bind to the second receptor, facilitating the attachment via bringing the virus and cell membranes into close proximity (Sattentau, 1998). The second receptor, or co-receptor, could be either one of the following major chemokine receptors (Figure 8), CXCR4 or CCR5. CXCR4 is found on naive T-cells whereas CCR5 is located on monocytes, macrophages, and activated or memory subset of T-cells (Alkhatib et al., 1996; Deng et al., 1996; Feng et al., 1996, Connor et al., 1997).

The use of one or the other of these chemokine receptors is important to categorize the HIV strain: CCR-5 is used by slow-growing, non-syncytium-inducing macrophage tropic strains of HIV-1 constituting the vast majority of virus present in newly infected individuals, while CXCR-4 is used by rapid-growing, syncytium-inducing T lymphocyte-tropic strains of HIV-1 which generally appear late in the course of infection (Schuitemaker et al., 1992; Zhu et al., 1993; Connor et al., 1997).

The gp41 molecule, which anchors the gp120 glycoprotein into the viral membrane, facilitates the fusion of the envelope with the cell membrane of the host cell (Ryser and Fluckiger, 2005). Gp41 goes through a conformational change after the binding of gp120 to CD4 and the co-receptor; the insertion of gp41 into the target cell membrane and consequent gp41 folding, brings the viral and target cell membranes into close proximity allowing membrane fusion and the transfer of the viral contents into the cell. Once in the cytoplasm, the viral RNA is converted to DNA by the action of a viral RNA-dependent DNA polymerase activity and a virus specified ribonuclease H activity found in the HIV-1 reverse transcriptase (RT) enzyme.

Reverse transcriptase is a heterodimer (Figure 9) consisting of two chains, p66 and p51, encoded by the same gene belonging to the *pol* open reading frame (ORF). p66 chain contains an additional RNase H domain; the rest of the sequence, except for the three-dimensional structure, is the same. The active sites for both primary activities of the enzyme (RNA- or DNA-dependent DNA polymerase, and RNase H) are contained in the p66 domain (Wlodawer, 2002).

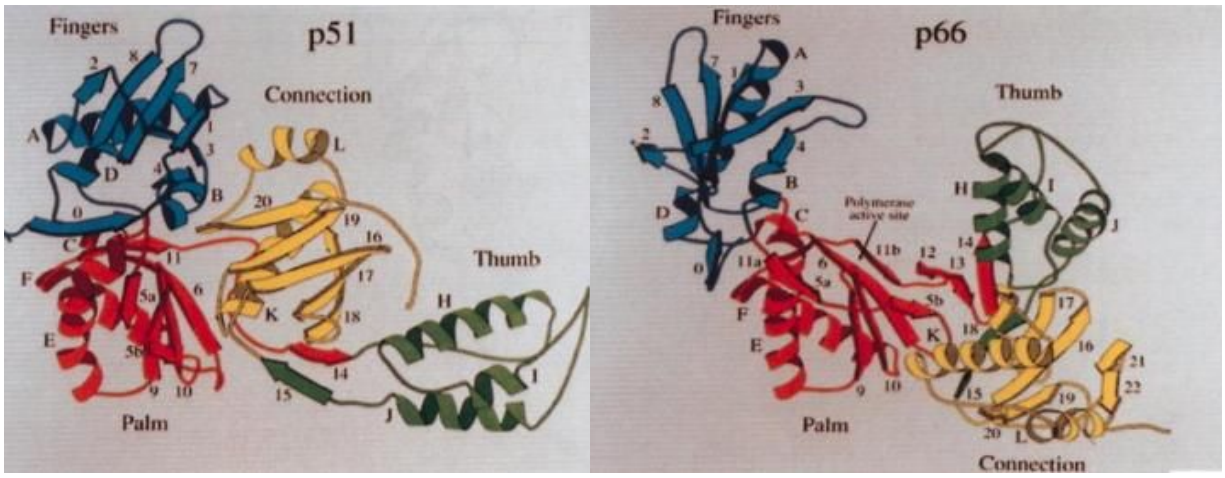

Figure 9: Ribbon drawings of the p51 and p66 that form the HIV-1 RT heterodimer (Turner and Summers, 1999)

There are two identical strands of the RNA genome in each HIV virus. In the cytoplasm, this single-stranded RNA genome is converted to a single double-stranded DNA copy by the simultaneous action of these two enzymes activities (Panganiban and Fiore, 1988): reverse transcriptase starts to copy a piece of one of the RNA genome strands, then it copies a piece of the second strand. It continues, "jumping" back and forth between the two strands as it assembles the DNA copy. The synthesis is carried out by using the nucleotide-building blocks provided by the host cell. A single-stranded DNA molecule is produced first. The reverse transcriptase also extends each end of the viral DNA molecule to form long-terminal repeats (LTRs). The LTRs enable the viral DNA to be integrated into one of the host cell's chromosomes, and play a major role in the regulation of viral protein production. After the synthesis of the viral DNA copy, the RNase H activity of the viral reverse transcriptase enzyme degrades the virus's original RNA genome. The second and matching DNA strand is synthesized through complementary base pairing to yield a double-stranded DNA copy of the virus genome.

After the reverse transcription of the viral RNA genome, the resulting viral DNA strands form a circular structure with the base-pairing of LTRs. A combination of protein with viral DNA , namely nucleoprotein

complex, containing the viral DNA, integrase and other necessary viral proteins, is then transported into the cell nucleus through connection with the cell nuclear import pathway and the aid of the Vpr accessory protein (Trono, 1995). The integration process also requires a host cell chromosomal protein (Farnet and Bushman, 1997). In the nucleus, the integrase protein has three activities: the enzyme cleaves the LTR ends of the double stranded proviral DNA for the recombination reaction; the integrase also cleaves the host cell DNA; and finally it splices the ends of the virus and host cell DNA (Bushman and Craigie, 1991). Host cell DNA ligase enzymes repair the site of insertion. The circular plasmid-like DNA is straightened and integrated into the host genome into regions of active transcription. The viral genome is now a double-stranded DNA provirus that resembles a cellular gene.

Activation of Provirus

Once is integrated, viral DNA resides permanently integrated into the host cell genome. The viral genome remains as part of the nuclear DNA as long as the cell is alive. As the virus occasionally kills the host cells, this feature of the viral life cycle ensures that, once infected, a person remains infected for life. If the host cell divides, the proviral DNA is duplicated and transmitted to the daughter cells, as is the cell's normal DNA. The consequence of this feature is, for the time being, that treatment for HIV-1 infection must remain life-long.

The provirus can remain latent in resting infected T-cells without a time limitation (Vermus, 1988). Once the infected cell is activated, however, the genome of the provirus is activated too. The host cell's enzymatic system processes the production of HIV. Since the host cell spend effort to produce viral proteins, this situation weakens the host cell itself.

HIV proteins can be categorized in three different kinds: structural proteins that are used to form the structure of the new virions, regulatory proteins that regulate the production of new virus particles, and additional proteins that serve other functions.

The full-length viral RNA (mRNA) is used as a template to make the structural proteins and the replication machinery enzymes (Haseltine, 1991); three genes in the HIV genome – *env*, *pol*, *gag* – encodes for these 10 structural proteins.

The *env* gene produces the gp160 glycoprotein that is cleaved by host proteases to form the gp120 and gp41 molecules. The *gag* gene serves as a template for a polyprotein (Schupbach et al., 1984; Ratner et al., 1985) that is processed by viral protease to produce three proteins: the capsid protein, the matrix protein, and the nucleic acid-binding protein. The *pol* gene encodes for a fusion protein with a capsid protein precursor (Jacks et al., 1988) that is trimmed by viral protease to produce the active reverse transcriptase (RT), RNase H, integrase, and protease.

Early in the viral replication cycle, newly transcribed mRNA is spliced multiply by the cellular splicing mechanism to produce the Tat, Rev, and Nef regulatory proteins. The Tat protein activates transcription (Arya et al., 1985; Cullen, 1986; Feng and Holland, 1988), the Rev protein regulates transport of HIV-1 transcripts from the nucleus to the cytoplasm (Cullen, 1992; Malim et al., 1989), and the Nef protein seem to have an impact on HIV-1 viral loads and the development of AIDS (Kestler et al., 1991; Wei et al., 2003), and the down regulation of major histocompatibility complex class I (MHC-I) antigens (Kerkau et al., 1989; Scheppler et al., 1989) and CD4 (Garcia and Miller, 1991). Two other regulatory proteins are viral protein U (vpu), that facilitates export of viral particles from the cell (Strebel et al., 1989), and viral protein R (vpr), that speeds viral replication (Ogawa et al., 1989).

In preparation for virus budding, the gp120 and gp41 molecules have to be displayed on the cell membrane. The mRNA transcribed from the *env* gene has to follow a similar pathway to cellular secretory proteins. While the mRNAs for most HIV proteins are translated to proteins by cytoplasmic ribosomes, the mRNA for the envelope gene has to be translated by ribosomes that are located on the membrane of the rough endoplasmic reticulum (RER). As the newly produced gp160 glycoprotein leaves the ribosome of the RER, it is anchored in the membrane of the RER. Here, the molecules move from the RER into the Golgi complex. The Golgi complex packages the glycoproteins into vesicles (Hallenberger et al., 1992). As it goes through the process, it is folded and glycosylated. The gp160 is trimmed by cellular proteases to

produce the gp120 and gp41 molecules. The gp120 and gp41 molecules combine with each other as meterotrimers to produce the gp120-gp41 complex that is anchored into the wall of the Golgi complex. After translocated from the Golgi complex in vesicles, they are then placed in position on the cell membrane.

All other proteins are produced during viral replication and accumulate in the host cell's cytoplasm (Fuller et al., 1997). Rev protein triggers the production of single stranded full-length RNA copies of the proviral DNA, forming the genomes of immature virions. In the cytoplasm, the *gag* gene proteins assemble themselves into the new capsid (Morikawa, 2003). As each capsid forms, it encapsulates the RNA genome, as well as the large polyprotein produced by the *pol* gene.

Only after complete placement of the single stranded RNA genome and proteins into the envelope, the fully organized progeny virion buds from the plasma membrane and released into the extracellular matrix (Gelderblom, 1991; Göttlinger et al., 1991). As the new virions bud off from the host cell, they take some part of the host membrane with. This part of cell membrane forms the envelope of the virus. It includes the gp120-gp41-heterotrimers deposited earlier on from the *env* gene product modifications in the Golgi apparatus. It also contains some of the host cell's proteins that were located on the membrane before viral release.

After budding off, the incorporated polyprotein product is trimmed by HIV protease inside the capsid. This process produces the functioning proteins such as reverse transcriptase, integrase, and protease enzymes. The newly released virions now have the capacity to infect new target cells, starting a new replication cycle as fully mature viruses.

Course of HIV Infection

The number of viruses increase and the number of CD4+ helper T-cells decrease in the plasma of infected individuals (Rosenberg and Faucci, 1991; Gougeon, 2003). T-cell damage can occur in a number of ways:

- Infected cells may simply lyse when tremendous amounts of virus bud from their lipid bilayer.

- Fusion of uninfected cells with infected cells during syncytium formation will lead to a significant damage in available T-cells since lysis and death of multinucleated cells occur within 48 hours of fusion.
- Since CD4+ T-cells capture, process, and display envelope gp120 glycoprotein on their membrane, uninfected T cells can be destroyed by HIV-1 specific cytotoxic T-cells.

HIV-infected T-cells may be stimulated for apoptosis when triggered by the presence of antigen: gp120-binding to CD4 provides one signal while gp120 presenting at a later stage may trigger an apoptotic response. Alternatively, antibody against gp120 may trigger apoptosis.

Currently, disease progression is monitored by CD4+ lymphocyte counts and viral load tests (Figure 10). The number of CD4+ lymphocytes per mm^3 of blood plays an important role in the management of HIV disease: taking multiple counts over time can serve as a measure for disease progression and an individual's immune-system function (Figure 10). Declining CD4+ counts closely relates to an increased risk of the development of certain opportunistic infections and cancers, and has been used to determine when to begin treatment to prevent certain opportunistic infections. Viral-load measures the amount of virions in blood plasma. Changes in viral load are earlier and more accurate indications of both disease progression and response to therapy than CD4+ counts, and are considered essential.

The Four Stages of HIV Disease

In most people, HIV infection progresses from the initial infection to AIDS in four stages: acute infection, asymptomatic HIV disease, early HIV disease (ARC), and advanced HIV disease (or AIDS).

Acute Infection Stage

This stage is also known as "primary infection" and begins after HIV entry into the host cell, ending approximately 2 – 6 weeks later. In the primary infection stage, there is an initial decrease in CD4 receptor counts in combination with seroconversion, and the return of CD4 counts to relatively normal numbers

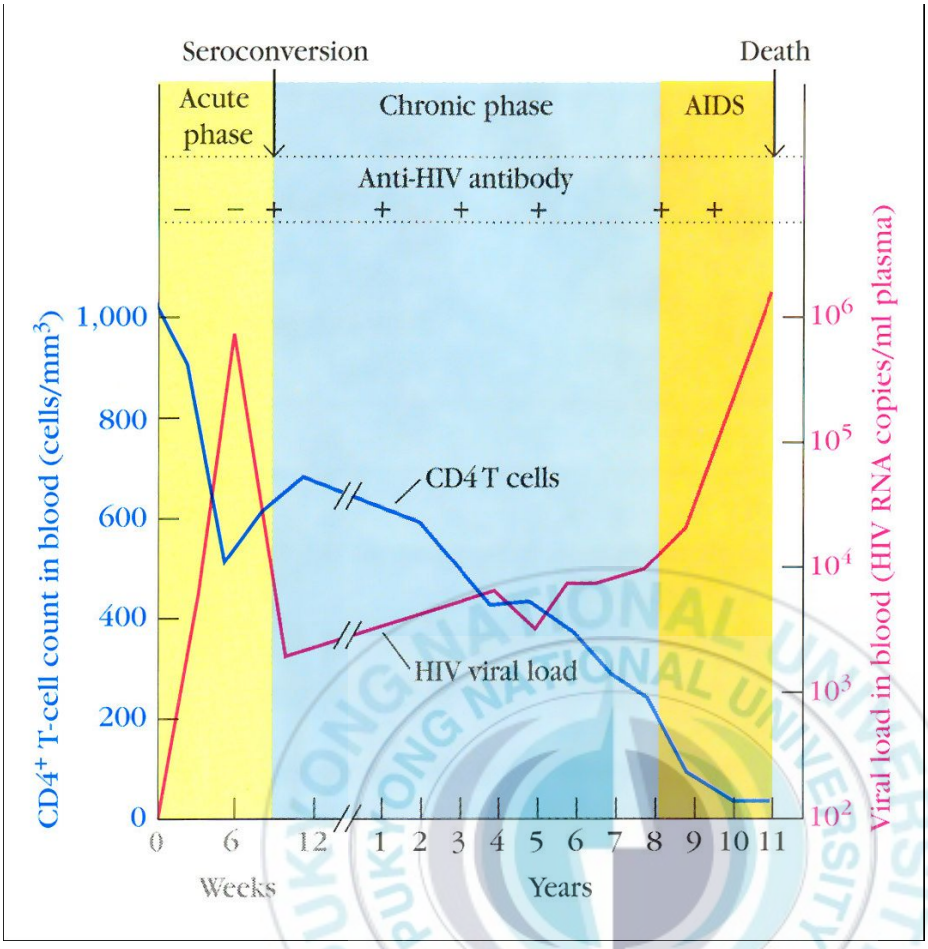

Figure 10: Serological Profile of HIV Infection (Goldsby et al., 2000)

Soon after infection, viral RNA is detectable in the serum. However, HIV infection is most commonly detected by the presence of anti-HIV antibodies after seroconversion, which normally occurs within a few months after infection. Clinical symptoms do not appear for at least 8 years after infection, but this interval is variable. The onset of clinical AIDS is usually signalled by a decrease in T-cell numbers and an increase in viral load.

(500-1200 cells/mm^3). Primary infection of host CD4+ cells mostly occurs in the lymph nodes draining the site of entry. The HIV strains present at this stage are likely to use the CCR5 co-receptor during the infection of CD4+ cells, have slower rates of virus production, and seem to be less lethal to CD4+ cells than the strains present during AIDS.

The immune system immediately responds to HIV infection with both cell-mediated and antibody-mediated immunity, and seems to win the first round. The viral load in the blood initially increases and then decreases shortly after the infection. Patients may experience primary disease symptoms including flu-like symptoms, pneumonitis, gastrointestinal and brain involvement. Symptoms of acute infection vanish and the acute phase of HIV infection ends.

Asymptomatic Stage

This is the longest stage of HIV infection, lasting for about 10 years as an average. Most patients exhibit few symptoms of HIV disease. The virus is, however, not latent: it is highly active in the lymphoid tissues. Patients may experience progressive generalized lymphodenopathy, which is indicative of an active immune response to the virus. The amount of viral load in the blood may fluctuate for a while, a strong CD8+ T-cell response is generated resulting in the clearance of HIV from the blood. A significant decrease in CD4+ cells in the blood is observed (>500 cells/mm^3) as well as a loss of cytokine regulation. The loss of cytokine regulation leads to an overproduction and/or underproduction of some cytokine, disrupting the signals needed by the immune system to accomplish effective immune responses. It also disrupts the ability of CD8+ cells to destroy HIV-infected cells and causes the spontaneous proliferation of B lymphocytes and overproduction of unnecessary antibodies. Approximately, 98% of HIV-infected cells are productively infected CD4+ lymphocytes, about 1% are latently infected T-lymphocytes and the remaining 1% are chronically infected macrophages, dendritic and glioma cells.

Early HIV Disease

The progression from the asymptomatic stage to early HIV disease often becomes obvious with the development of some certain opportunistic infections. This stage was originally named as "AIDS Related Complex (ARC)". The rate of HIV production in the lymph nodes and spleen remains high. The internal structure and function of the lymphoid-related organs (thymus, bone marrow, spleen, lymph nodes) gradually declines. Viral load in the blood rises. CD4 receptor counts fall to 200-500 cells/mm^3. Most patients at this stage of the progression are treated with the combination antiretroviral therapy, treatment for opportunistic infections as they arise, and treatment for *Pneumocystis carinii pneumonia* when T-cell counts reaches 200 cells/mm^3 and below.

Advanced HIV Disease

This stage is defined as AIDS. Aids-related illnesses included infection with a number of opportunistic diseases. CD4 counts fall below 200 cells per cubic millimeter and serious opportunistic infections and cancer gradually appear as the immune system starts to lose its function severely. During this late stage of disease, HIV virions are more virulent and more likely to use the CD4 molecule and CXCR4 co-receptor to gain entry and infect T-cells. These virions also promote the formation of syncytium. The levels of virus may be quite low in the blood (less than 400 copies of HIV RNA per milliliter of plasma). HIV levels in the blood then drastically increase as the lymph nodes are disrupted. Weight loss is a frequent complication and is indication of the death in individuals with HIV disease. Neurological disorders may arise from opportunistic infections, malignancies, or directly from HIV itself. Highly active antiretroviral therapy (HAART) is a must to slow the progression of the disease and protect immune system function. Resistance to antiretroviral drugs is characteristic to advanced disease. Treatments are also needed for opportunistic infections as they arise.

Polysaccharides as anti-HIV Agents

The antiviral activity of polysaccharides was firstly reported by Gerber et al. (1958). Mumps and influenza viruses were inhibited by algal polysaccharides. Ehresmann et al. (1977) reported the inhibition of herpes simplex virus and other viruses by polysaccharide fractions from the extracts of ten red algae. Sulfated polysaccharides from red alga *Schyzimenia pacifica* inhibited the HIV reverse transcriptase (RT) enzyme (Nakashima et al., 1987). Baba et al. (1988a, b) compared the HIV inhibitory effects of sulfated polysaccharides from different sources and found that most of them exhibited anti-HIV activity. Fucoidan, a complex sulfated polysaccharide from the alga *Fucus vesiculosus*, was proved to inhibit HIV *in vitro* (Sugawara et al., 1989).

Dextran is a high molecular weight polysaccharide produced from sucrose by several bacteria such as *Leuconostoc dextranicum*. Predominantly, α-(1→6) linked D-glucose molecules form the structure of its backbone. Different molecular weight fractions are obtained by partial hydrolysis of dextran. These fractions are sulfated and finally recovered as sodium salt of dextran sulfate (DS) (Witvrouw and De Clercq, 1997). Schols et al. (1992) demonstrated that DS showed effectively inhibited both HIV-1 and HIV-2 at very low concentrations without exhibiting cytotoxicity on MT-4 cells. However, DS exhibited different inhibition patterns at different molecular weights.

The semisynthetic sulfated polysaccharide, pentosan sulfate, is obtained by the sulfonation reaction of hemicellulose xylan. Pentosan sulfate also inhibited the cytopathic effects of HIV-1 and HIV-2 *in vitro*, especially HIV-2 with ah therapeutic index of round 10.000 (Witvrouw and De Clercq, 1997).

Galactan sulfate is a polysaccharide extracted from Venezuelan red alga *Agardhiella tenera*. When galactan sulfate is tested for its anti-HIV inhibitory activity, it also inhibited the HIV-1 and HIV-2 at a very low concentration and without any significant cytotoxicity (Witvrouw and De Clercq, 1997).

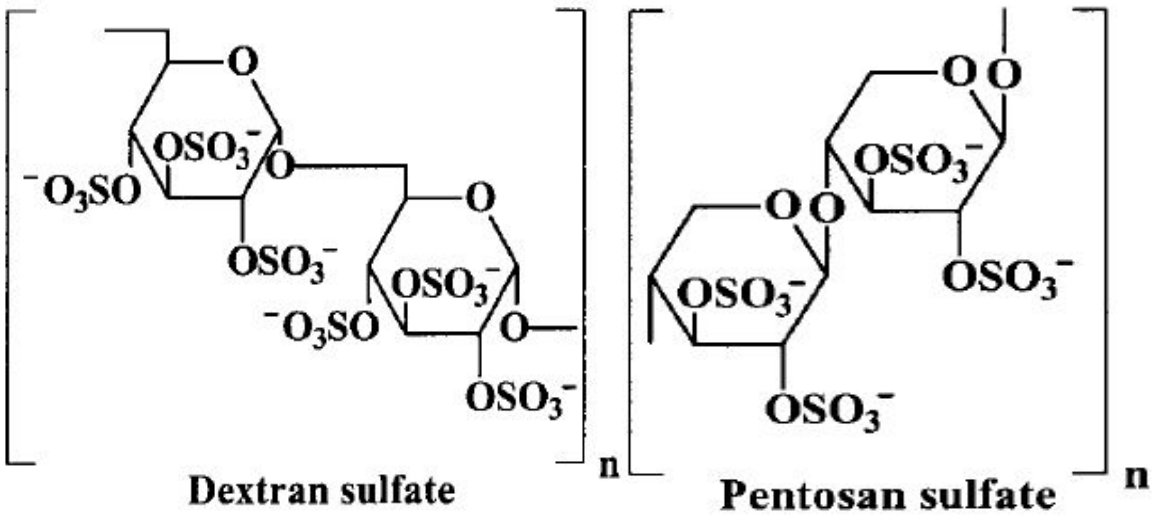

Figure 11: Sulfated polysaccharides: dextran sulfate (DS) and pentosan sulfate (PS) (De Clercq, 2000)

Chitosan and chitooligosaccharides (COS) as bioactive materials

Chitin is naturally abundant and simple β-(1→ 4) glycans composed of 2-acetoamido-2deoxy-D-glucopyranose units. It is the major constituent of shells of arthropods such as crabs, shrimps, lobsters, insects and it also is produced extracellularly by fungi and some brown alga. Chitin is a by-product or a waste from crab, shrimp and crawfish processing industries and a highly water-insoluble compound. Chitosan is a functional and basic linear polysaccharide prepared by *N*-deacetylation of chitin in the presence of alkaline. Generally, deacetylation cannot completely be achieved even under harsh treatment. The degree of deacetylation usually ranges from 70% to 95%, depending on the method used. Thus, chitosan is available with various molecular weights and deacetylation degrees. Chitosan is insoluble in water, alkali and organic solvents but is soluble in most solutions of organic acids when the pH of the solution is below 6. The industrial production and application fields of chitosan has been steadily increasing since 1970s. Early applications of chitosan were centered on the treatment of wastewater, heavy metal adsorption, food processing, immobilization of cells and ezymes, resin for chromatography, functional membrane in biotechnology, animal feed so on. The recent trend is toward producing high

valuable industrial products such as cosmetics, drug carriers and pharmaceuticals. Chitin and chitosan are known to exhibit antitumor, antibacterial, hypocholesterolemic and antihypertensive activity. The main motive for the development of new applications for chitosan lies in the fact that it is a very abundant polysaccharide, as well as nontoxic and biodegradable. Despite its functions and importance as a biomaterial, the applications of chitosan in food and biomedical industries are narrowed owing to its poor solubility, high molecular weight and viscosity. There are evidences about the no or poor absorption of chitin and chitosan in human intestine due to lack of enzymes to cleave the β-glucosidic linkage in chitosan. Since chitosan is a water insoluble large biopolymer, it is difficult to be absorbed by human body. In this respect, enzymatic hydrolysis of chitosan to obtain oligomers is of great interest recently (Kim and Rajapakse, 2005).

Chitosan oligosaccharides (COS) are hydrolyzed derivatives of chitosan composed of β-(1→ 4) D-glucosamine units. They have better properties such as lower viscosity, relatively smaller molecular size in comparison to chitosan and short chain length with free amino groups which makes COS highly soluble in aqueous solutions. COS are effective agents for lowering of blood cholesterol and pressure, controlling arthritis, enhancing antitumor properties.

Some sulfated chitosan derivatives are suggested to have antiviral effects. *N*-carboxymethylchitosan *N,O*-sulfate, which is derived from *N*-carboxymethyl chitosan by a random sulfation reaction, inhibited HIV-1 replication and binding to CD4 cell surface receptor (Jayakumar et al., 2007). Nishimura et al. (1998) suggested that selective sulfation of chitosan at *O*-2 and/or *O*-3 results with a potent anti-HIV agent showing a higher antiviral activity than 6-*O*-sulfated derivative. Since chitosan oligosaccharides are biodegradable, water-soluble and nontoxic compounds, they might be beneficial biomaterials for the inhibition of HIV and the treatment of AIDS patients. In this study, anti-HIV-1 properties of sulfated chitooligosaccharides (SCOS) and the effect of their molecular weight on the inhibition profile of HIV-1 was investigated.

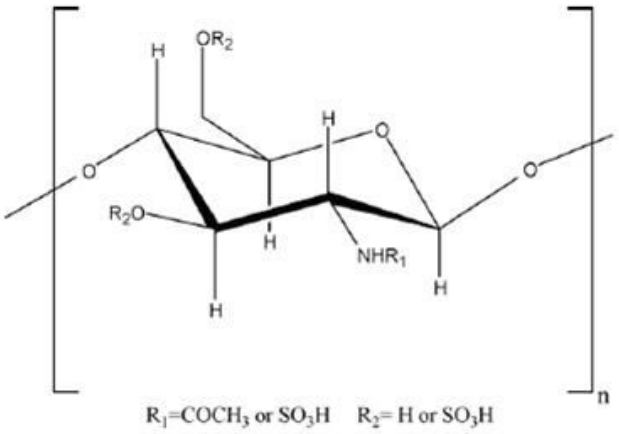

Figure 12: Structure of sulfated chitosan

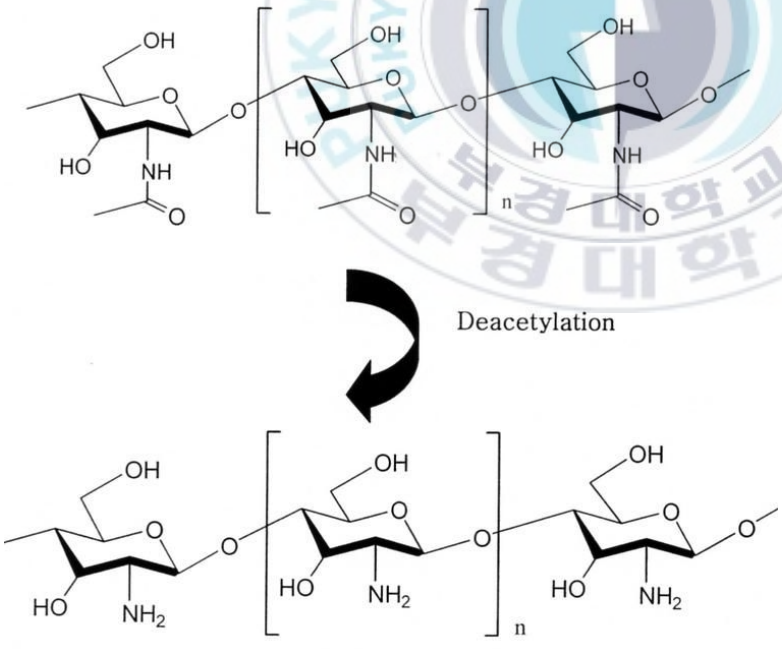

Figure 13: Structures of chitin and chitosan

Experimental Procedure

1. Materials

Different molecular weight of chitooligosaccharides (COS) were kindly donated by Kitto Life Co. (Seoul, Korea). HIV-1 p24 antigen capture ELISA was purchased from Perkin-Elmer (Boston, MA, USA). Reverse transcriptase activity assay kit was purchased from InvitroGen (CA, USA). Cell culture medium (RPMI 1640), penicillin/streptomycin, fetal bovine serum (FBS), and other cell culture materials were obtained from Gibco BRL, Life Technology (NY, USA) and Sigma Chemical Co. (St. Louis, MO, USA). MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), RNAse, proteinase K, dithiothreitol (DTT) and polyethylene glycol (Mw 8000 Da) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Primary and secondary antibodies used for western blot analysis were purchased from SantaCruz Biotechnology (CA, USA).

2. Preparation of chitosan oligosaccharides using an UF membrane bioreactor

Chitosan is mainly produced by deacetylation of chitin obtained from seashell materials, such as crab and shrimp shell, for the industrial-scale production of COS. Before starting the deacetylation reaction, chitin is chemically treated in order to remove the protein, inorganic salts and lipids, which are the main components of crustacean shells together with chitin. Crab shells are treated with 3~5% aqueous NaOH solution which is a crucial step for the removal of protein which is bound to crab shell in order to avoid the contamination of chitin products with proteins. Deproteinized shells are the neutralized and calcium is removed by treatment of 3~5% aqueous HCl solution resulting with a white or slightly pink precipitate of chitin. Chitosan is formed by *N*-deacetylation of chitin using 40-45% NaOH. The resulting crude sample is dissolved in 2% acetic acid and the supernatant is neutralized with aqueous NaOH solution to obtain chitosan as a white precipitate (Hirano, 1996). Generally, the reaction of deacetylating chitin in an alkaline solution cannot reach completion even under harsh treatment. Some of the amino groups remains acetylated distribute randomly along the polymer chain.

The preparation of chitosan oligosaccharides was carried out according to following method: chitosan was continuously hydrolyzed through a UF (ultra-filtration) membrane reactor system connected to an immobilized enzyme column reactor (Figure 14). Chitosanase from *Bacillus sp*. was adsorbed onto the chitin matrix as to immobilize the enzyme. Chitosan solution was prepared as 1% (w/v) in 1000 ml of water. Chitosan was dissolved in water, stirred, mixed with 400 ml of 1M lactic acid and the volume was completed to 15 L by adding water. The pH was adjusted to 5.5 with a saturated $NaHCO_3$ solution and the temperature was maintained at 60ºC. The chitosan was hydrolyzed by enzymatic reaction in the reactor system and fractioned through UF membrane of molecular weight cut-off 10 kDa, 5 kDa, 3 kDa and 1 kDa (Jeon and Kim, 2000).

3. Preparation of sulfated chitooligosaccharides

Chitooligosaccharides (10 g) was dispersed in 1 l of distilled water, treated with 2.2 g of sodium carbonate anhydrous and 4.5 g of trimethylamine-sulfur trioxide (Me_3N-SO_3). The mixture solution was heated at 65ºC for 12 hours. The resulting solution was cooled then dialyzed exhaustively against distilled water using an electrodialyzer (Micro Acilyzer G3, Asahi Chemical Industry Co., Tokyo, Japan), and lyophilized. Sulfated chitooligosaccharides were named as following: SCOS MW<1 KDa (SCOS I), MW 1-3 KDa (SCOS II), MW 3-5 KDa (SCOS III) and MW 5-10 KDa (SCOS IV).

4. Cell lines and virus

H9 and H9/HIV-1_{IIIB} cell lines were obtained through American Type of Culture Collection (Manassas, VA, USA). CEM-SS cell line from Dr. P. Nara and C8166 cell line from Dr. G. Farrar were provided by the EU Programme EVA Centre for AIDS Reagents, NIBSC, UK. H9 cell is a cutaneous T cell lymphoma derived from HUT 78 cell line. H9/HIV-1_{IIIB} cell is chronically infected with HIV-1_{IIIB} and gives high yield of virus. C8166 is a human T-lymphoblastoid cell which is reported to carry but not express HTLV-I genome. CEM-SS cell line is a human T4-lymphoblastoid cell line that has been cloned for both poly-L-

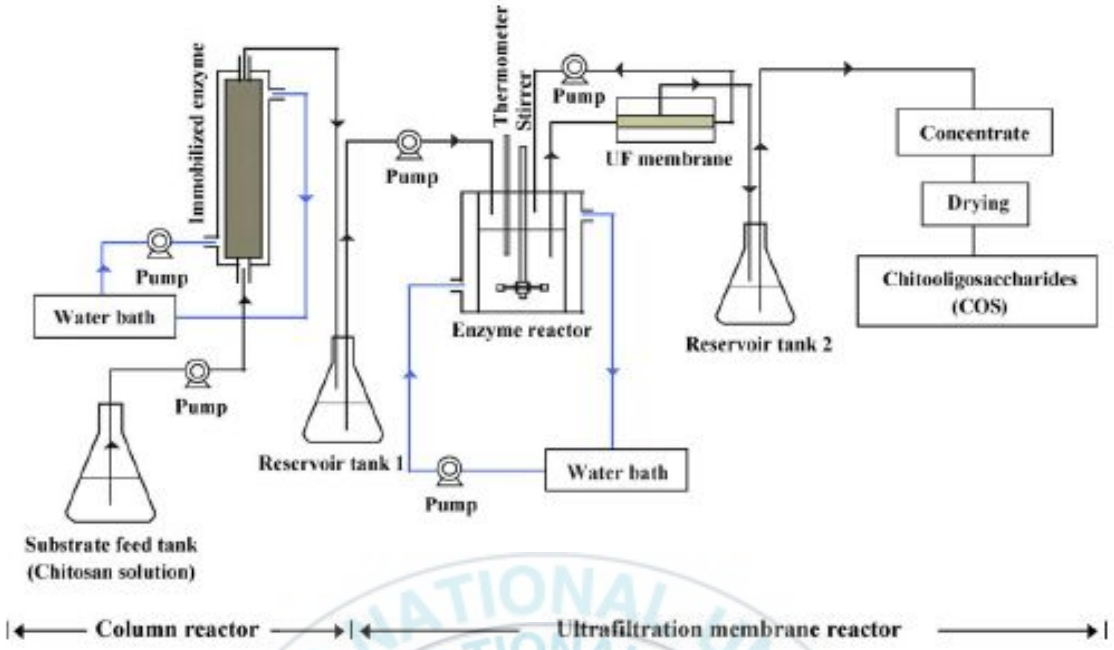

Figure 14: Schematic diagram of the dual reactor system developed for continuous production of chitooligosaccharide (COS). Adapted from Jeon and Kim (2000)

lysine induced adherence to microtitre plates and viral induced syncytial fusigenic sensitivity following infection with either cell-free or cell-associated HIV-1 and HIV-2. This cell line allows for infection with syncytium-inducing (SI) strains of HIV-1. These SI isolates are found in individuals with more advanced disease and are associated with accelerated loss of CD4+ lymphocytes. The CEM-SS cell line uses the CXCR4 co-receptor and X4 viruses show rapid replication kinetics in this T-cell line.

HIV-1_{IIIB} virus stock was obtained from the culture supernatant of chronically infected H9/ HIV-1_{IIIB} cells. Cell-free virus was harvested from the supernatants by centrifugation and filtration through 0.22 μm filter. The virus stocks were stored as small aliquots at -80 ºC until use.

5. Cell culture

H9, H9/HIV-$1_{IIIB,}$ CEM-SS and C8166 cell lines were propagated at 37ºC under 5% CO_2 in complete RPMI 1640 medium supplemented with 10% FBS, 100 μg of streptomycin per ml and 100 U of penicillin per ml. All cells were cultured in either T25 or T75 cell culture flasks. Cells were subcultured 2-3 times a week to yield a final concentration of 1 x 10^5 cells. Cells were routinely replaced every 2 months from frozen stocks.

6. Cell viability assay

The cytotoxic concentrations of 6,6'-bieckol were determined by MTT assay, a method based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Weislow et al., 1989). 400 μl of medium containing CEM-SS or H9 cells were cultured into a 48-well plate at a density of 10^5 cells/ml. The plate was incubated overnight and treated with 100 μl of RPMI 1640 medium containing different concentrations of sulfated chitosan oligosaccharides. After 72 hours of incubation, 100 μl of 500 μg/ml MTT solution was added to each well and the plate was incubated for another 4 hours at 37ºC. The blue formazan salt was dissolved in acidified propanol containing 50% DMSO and 4% triton X-100. Optical density was measured at 540 nm with a GENios microplate reader (Tecan, Austria GmbH,

Austria). The optical density of formazan formed by untreated cells was taken as 100% of viability.

$$\text{Percent MTT Reduction} = \frac{\text{Treated}^{A540nm}}{\text{Blank}^{A540\,nm}} \times 100$$

The data was expressed as a mean of three independent experiments and $P<0.05$ was considered as significant.

Figure 14: Molecular structure of MTT and its corresponding reaction product

Anti-HIV screening

1. Determination of syncytia formation

HIV-1 infection of CD4+ human T cell lines in culture results in significant cytopathic effects. Cell killing is characterized by initial formation of large, multinucleated giant cells and syncytia, followed later by destruction of single cells that swell and die.

1 x 10^5 C8166 or CEM-SS cells in aliquots of 300 μl were seeded in triplicate to a 48-well plate containing 100 μl of serial dilutions of compound in complete medium. After 2 hours of incubation, the cells were infected with 100 μl of stock supernatant of HIV-1_{IIIB} diluted in complete medium at 200 $CCID_{50}$. The plates were incubated at 37ºC for 72 hours and the number of syncytia was determined microscopically.

2. Determination of lytic effect of HIV-1

In order to determine the anti-HIV-1 activity of sulfated COS on acutely infected CEM-SS cells, an MTT-formazan-based assay was used. Cells in log-growth phase were washed and resuspended in complete medium, and a 300 μl aliquot containing 1 x 10^5 cells was added in triplicate to the wells of a 48-well plate containing the dilutions of compound in a volume of 100 μl of medium. Stock supernatants of HIV-1_{IIIB} were diluted in complete medium to yield sufficient cytopathicity (~90% cell kill in 7 days), and a 100 μl aliquot was added to the wells. Plates were incubated for 7 days at 37 ºC and at the end of 7 days, 100 μl of 500 μg/ml MTT solution was added to each well and the plate was incubated for another 4 hours at 37ºC. The blue formazan salt was dissolved in acidified propanol containing 50% DMSO and 4% triton X-100. Optical density was measured at 540 nm with a GENios microplate reader (Tecan, Austria GmbH, Austria). The optical density of formazan formed by untreated cells was taken as 100% of viability.

3. Co-culture assay

3 x 10^4 C8166 cells were pre-treated with various concentrations of sulfated COS or complete medium alone for 2 hours and co-cultured with 3 x 10^3 H9 cells chronically infected with HIV-1_{IIIB} at 37ºC in a humidified atmosphere of 5% CO_2. Dextran sulfate (DS) was used as positive control. After 24 hours of incubation, the number syncytia formed was counted using a microscope.

4. Delayed addition of sulfated COS to HIV-1_{IIIB} infected CEM-SS cells

Uninfected CEM-SS cells were cultured into individual wells of a 48-well microtiter plate at a density of 3 x 10^4 cells/well in 400 μl of medium. Diluted HIV-1_{IIIB} stock supernatants (100 μl) were added to appropriate wells to yield a final M.O.I of 1.0. At various times after the addition of virus, a 100 μl aliquot of COS sulfates at different concentrations was added to multiple wells. Another set of cells were only treated with SCOS and incubated without being infected with virus. After a total of 7 days incubation, cellular viability was assessed using the MTT assay as described above.

5. The p24 ELISA

The HIV-1 p24 antigen assay is an enzyme immunoassay (EIA) or ELISA that has been developed for the detection and quantification of the HIV-1 p24 core protein. Direct evidence for the presence of infectious virus in a specimen is routinely obtained by co-culturing specimens with target cells that are susceptible to infection by HIV-1, such as T-cells. It uses a mouse monoclonal antibody to HIV-1 p24 antigen coated onto microtitre strip wells.

HIV-1 consists of a number of polyproteins produced by the *gag, pol* and *env* genes. The *gag* polyprotein contains four proteins: the p17 matrix protein (MA); the major capsid protein, p24 (CA); the nucleic acid-binding protein, p6 (NC); and the p7 protein, which is important for virion assembly. The p24 core protein of HIV-1 was discovered early in the 1980's. Antibodies to this protein were raised shortly afterwards, which allowed for the development of an ELISA technique that could be used as a diagnostic tool in HIV research. The assay has since been enhanced and is now used as a highly

sensitive method to determine disease progression because of its close link to the viral load of an infected individual.

The detection of p24 was done on plasma samples to determine the initial viral infectivity of the sample using the Perkin-Elmer HIV-1 p24 antigen assay. This was also determined for co-culture supernatants from experiments to determine titre of the expanded sample as well as the infectivity of the supernatant after co-culture with various cells lines. Lastly, the p24 assay is also utilized to determine TCID50.

H9 cells (3 x 10^6 cells/ml) were incubated in the presence or absence of HIV-1_{IIIB} for 1 hour at 37ºC. Cells were washed to remove unbound viruses and resuspended at 3 x 10^5 cells/ml in culture medium. Aliquots of 1 ml were placed in a 24-well culture plate containing an equal volume of medium with/without sulfated COS. AZT was treated as positive control. In order to determine the amount of virus released to the medium, HIV-1 p24 antigen capture ELISA was carried out with a commercial kit (Perkin-Elmer Life Sciences, Boston, MA) according to the manufacturer's instructions.

6. Western blot analysis

1 x 10^6/ml H9 cells were cultured in 10 cm cell culture plates. Following sample treatment, plates were incubated for 2 hours and infected with HIV-1_{IIIB} at 200 $CCID_{50}$. After 96 hour of incubation at 37ºC, cells were pelleted at 1000 rpm for 10 minutes and supernatant was harvested. The cells were washed 3 times with PBS and lysed with 500 μl of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 0.4% Nonidet P-40, 120 mM NaCl, 1.5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 80 μg/ml leupeptin, 3 mM NaF and 1 mM DTT. 100 μg total protein was used for immunoblot analysis.

Culture supernatant containing virus was filtered through 0.22 μm filter, mixed with 30% polyethylene glycol (PEG) (50% v/v) in 0.4 M NaCl and virus particles were pelleted at 15000 rpm for 45 minutes (Lee et. al., 2005). The viral pellets were lysed, resuspended in SDS sample buffer and equal volume of viral lysates (20 μl) was loaded onto SDS gel.

The proteins were subjected to denaturating SDS PAGE in 25 mM Tris, 192 mM glycine, 0.1% SDS with a 4% stacking and 10% separating gel. Separated proteins were transferred onto a nitrocellulose

membrane, blocked in Tris buffered saline containing 0.1% (v/v) Tween-20 (TBS-T) and 5% skim milk powder. The membrane was probed with mouse anti-p24 monoclonal antibody (1:500, SantaCruz) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (1:5000, SantaCruz). The proteins were visualized by chemiluminescence (Fujifilm Life Science, Tokyo, Japan).

7. *In vitro* reverse transcriptase activity assay

The activity of HIV-1 reverse transcriptase, isolated from virus pellet of culture supernatant as described above, was evaluated using a fluorescence RT assay kit (InvitroGen) according to the manufacturer's protocol.

Firstly, 5 μl of poly(A) ribonucleotide template and 5 μl of oligo $d(T)_{16}$ primer were mixed and incubated at room temperature for 1 hour for annealing. The primer/template mixture was diluted 200 fold into polymerization buffer. 20 μl of reaction mixture containing a template/primer hybrid, poly(A)/$d(T)_{16}$, and dTTP as a triphosphate substrate, was added to the wells of a microtiter plate and mixed with 5 μl of viral lysate containing various concentrations of sulfated COS of different concentrations, which was diluted in 50 mM Tris-HCl, 20% glycerol, 2 mM DTT, pH 7.6. After incubation at 37ºC for 1 hour, the reaction was stopped by the addition of 2 μl of 200 mM EDTA to each reaction. Fluorescence intensity was measured at 480 nm (excitation) and 520 nm (emission) with a GENios® microplate reader (Tecan Austria GmbH, Austria) after the addition of 173 μl of fluorescent PicoGreen reagent prepared in TE buffer.

The fluorescent dsDNA quantitation reagent preferentially detects double strand DNA (dsDNA) or RNA-DNA heteroduplexes over single stranded nucleic acids or free nucleotides. In the assay, reverse transcriptase activity in the biological sample generates long RNA-DNA heteroduplexes from a mixture of a long poly(A) template, an oligo-dT primer and dTTP. The RNA-DNA heteroduplexes can be detected by the fluorescent reagent.

8. RNA extraction

CEM-SS cells were cultured with a concentration of 3 x 10^5 cells/ml in 10 cm^2 cell culture plates. After incubation overnight, the cells were treated with/without various concentrations of compounds and AZT as a positive control. Following a four-hour incubation period, the cells were infected with HIV-1 at 200 TCID50. Plates were incubated for 72 hours at 37ºC and cells were pelleted at 1000 rpm for 10 minutes. The pellet was treated with 1 ml of TRIzol® reagent and the lysate was passed through a pipette several times. The homogenized samples were incubated for 2 minutes at room temperature and transferred into eppendorf tubes. 200 µl of chloroform was added to each sample, tubes were vortexed and centrifuged at 12.000 rpm for 15 minutes at 4ºC. After centrifugation, the colorless, upper aqueous phase was transferred to a microtube without disturbing the lower protein phase. The RNA was precipitated by mixing with 2-propanol, incubation for 10 minutes at room temperature and centrifugation at 12000 rpm for 10 minutes at 4ºC. The supernatant is discarded; RNA pellet is resuspended in 1 ml of 75% ethanol and centrifuged at 12.000 rpm for 10 minutes at 4ºC. After removal of ethanol, RNA pellet is suspended in DEPC-treated water and incubated at 55ºC for 10 minutes. The purity of RNA is detected by taking absorbance at 260 nm and 280 nm using a microplate reader.

9. DNA extraction

1 x 10^5 CEM-SS cells were cultured, treated with compounds, incubated for 72 hours and pelleted by centrifugation at 1000 rpm for 10 minutes. Supernatant was discarded and pellet was resuspended in 1x PBS three times and centrifuged. Finally supernatant was discarded and pellet was treated with 350 µl of 0.2 M sodium acetate pH 7.2, 25 µl of 10% SDS, 25 µl of 0.5 mg/ml RNAse A, 10 µl of 10 mg/ml proteinase K and transferred into eppendorf tubes. Mixture was passed through the tip of a pipette several times, vortexed, incubated for 30 min. at 37ºC and 10 min. at 55ºC, respectively. 410 µl of phenol:chloroform:isoamyl alcohol/25:24:1 mixture was added to the tubes, vortexed and centrifuged at 12000 rpm for 5 min at 4ºC. After centrifugation, supernatant containing DNA was transferred into a new eppendorf tube, mixed with chilled absolute ethanol (1,5 volume of supernatant), vortexed and incubated

at -20ºC for 10 min. Then tube was centrifuged at 12000 rpm for 5 min. at 4ºC, supernatant was discarded. 50 µl of TE buffer pH 8.0 was added into tube, precipitate was dissolved and DNA amount was measured.

10. Polymerase Chain Reaction (PCR)

0,75 µg of total RNA was mixed with RNAse-free water to complete a total volume of 14 µl. 1 µl of oligo(dT)15 was added to the mixture, RNA was denaturated for 2 min at 70ºC and chilled on ice immediately for primer annealing. Later on, the mixture is added the RT-PCR reaction chemicals in table 1. After RT-PCR reaction, 80 µl of water was added to the mixture containing cDNA.

Table 2: Chemicals used for reverse transcription PCR

PCR Chemical	**Amount used**	**Stock**
MMLV reverse transcriptase	0.5 µl	200 U/ µl
dNTP mixture	1 µl	10 mM
DTT	1 µl	100 mM
5X reaction buffer	4 µl	
RNase inhibitor	0.5 µl	80 U/ µl

Table 3: RT-PCR conditions

Temperature	**Time**
42 ºC	1h 30 min.
95 ºC	5 min.
4 ºC	∞

4 µl of cDNA obtained from RT-PCR reaction was used for normal PCR reaction as a template. cDNA was mixed with the chemicals given in Table 3 and PCR reaction was carried out according to conditions mentioned in Table 4.

Table 4: Chemicals used for PCR reaction

PCR Chemical	**Amount used**	**Stock**
Taq polymerase	0.5 µl	5 U/ µl
dNTP	2 µl	2.5 mM
Reverse Primer	0.5 µl	50 pmole/ µl
Forward Primer	0.5 µl	50 pmole/ µl
10X reaction buffer	2 µl	
DW	10.5 µl	

Table 5: PCR conditions

Temperature	**Time**	**Cycle**
94 ºC	5 min.	1
94 ºC 61 ºC 72 ºC	45 sec. 45 sec. 1 min.	30
72 ºC	10 min.	1
4 ºC	∞	

Table 6: Primers use in PCR reactions

Amplified gene	Primer Sequence
HIV-1 Vif	Sense: 5'- GAGTCAACGGATTTGGTCGT- 3'
	Antisense: 5'- GACAAGCTTCCCGTTCTCAG- 3'
Human GAPDH	Sense: 5'- GGTACAGTGCAGGGAAAGA- 3'
	Antisense: 5'- CTTGCCACACAATCATCACC- 3'

11. Statistical analysis

All data were expressed as mean of at lest three independent experiments ± standard deviation (SD). For statistical analysis of significance, analysis of variance followed by student's *t*-test.

Results and Discussion

1. Effect of SCOS on viability of CEM-SS cells

MTT formazan assay was applied in order to investigate the cytotoxicity of sulfated chitooligosaccharides on the viability of CEM-SS T4-lymphoblastoid cell line. CEM-SS cells were treated with or without SCOSs in various concentrations. None of the SCOSs did exhibit any significant cytotoxicity as expected (Figure 16). CC_{50} (minimum concentration of compound to kill 50% of the cultured cell) values for all SCOS were higher than 1000 μg/ml (CC_{50}>1000 μg/ml). Results obtained from MTT assay revealed that SCOSs are safe compounds for *in vitro* cell culture experiments.

2. SCOSs inhibited HIV-1 induced syncytia formation on C8166 T-lymphoblast cells

The infection of human CD4+ T cell lines in culture with HIV-1 results in significant cytopathic effects. After infection of T cell lines with HIV-1, initial formation of large, multinucleated giant cells and syncytia are formed. Three days after the infection of cell culture with HIV-1, the number of syncytia was quantified using a microscope.

According to data obtained from quantification of syncytia induced by HIV-1 on C8166 cells, all SCOSs inhibited syncytia formation in a dose-dependent manner (Figure 17). SCOS III appeared to be the most effective compound to protect cells from HIV-1 induced syncytia formation. At the highest concentration treated (1000 μg/ml), COS III inhibited HIV-1 induced syncytia formation more than 90%. The least effective compound was SCOS IV, which exhibited milder HIV-1 inhibitory activity in comparison to other SCOSs. The order of the inhibitory effects of SCOSs on HIV-1 induced syncytia formation was as follows: SCOS III>SCOS II>SCOS IV>SCOS I. Data obtained from this experiment revealed that 3-5 kDa range is the most suitable molecular weight among the SCOSs tested. As the molecular weight increased above 5 kDa, the activity decreased. However, low molecular weight SCOS (MW<1 KDa) did not inhibit syncytia formation significantly. This data was consistent with the data explained by Witvrouw and Declercq (1997). They claimed that relatively low molecular weight (~1000 Da) polysaccharides

(namely dextran sulfate) did not inhibit HIV-1 induced syncytia formation despite their other HIV-1 inhibitory properties.

The importance of molecular weight of sulfated polysaccharides on the inhibition of HIV-1 induced cytopathic effects was emphasized by Witvrouw and De Clercq (1997) previously. They claim that a significant increase was observed in anti-HIV-1 activity of dextran sulfate when molecular weight increased from 1 KDa to 10 KDa. At higher concentrations (10-500 KDa), however, HIV-1 inhibitory activity of dextran sulfate tended to decrease steadily.

It was also claimed that most sulfated polysaccharides (pentosan sulfate, dextran sulfate, carrageenans, galactan sulfate) could inhibit HIV-1 induced syncytia formation. However, higher concentrations of compounds were crucial for the inhibition of giant cell formation (Witvrouw and De Clercq, 1997).

When the microscope images are examined, syncytia formation of untreated-virus infected cells can easily be realized (Figure 18F). The images were taken at the late-syncytia formation stage. Therefore, some cells are highly disrupted by HIV-1 release and some cells enter the lytic phase. In figure 18E, protective effect of dextran sulfate can be observed. SCOS III was the most protective agent of C8166 cells from syncytia formation at 1000 μg/ml concentration (Figure 18C) according to microscopic image when compared with other SCOSs.

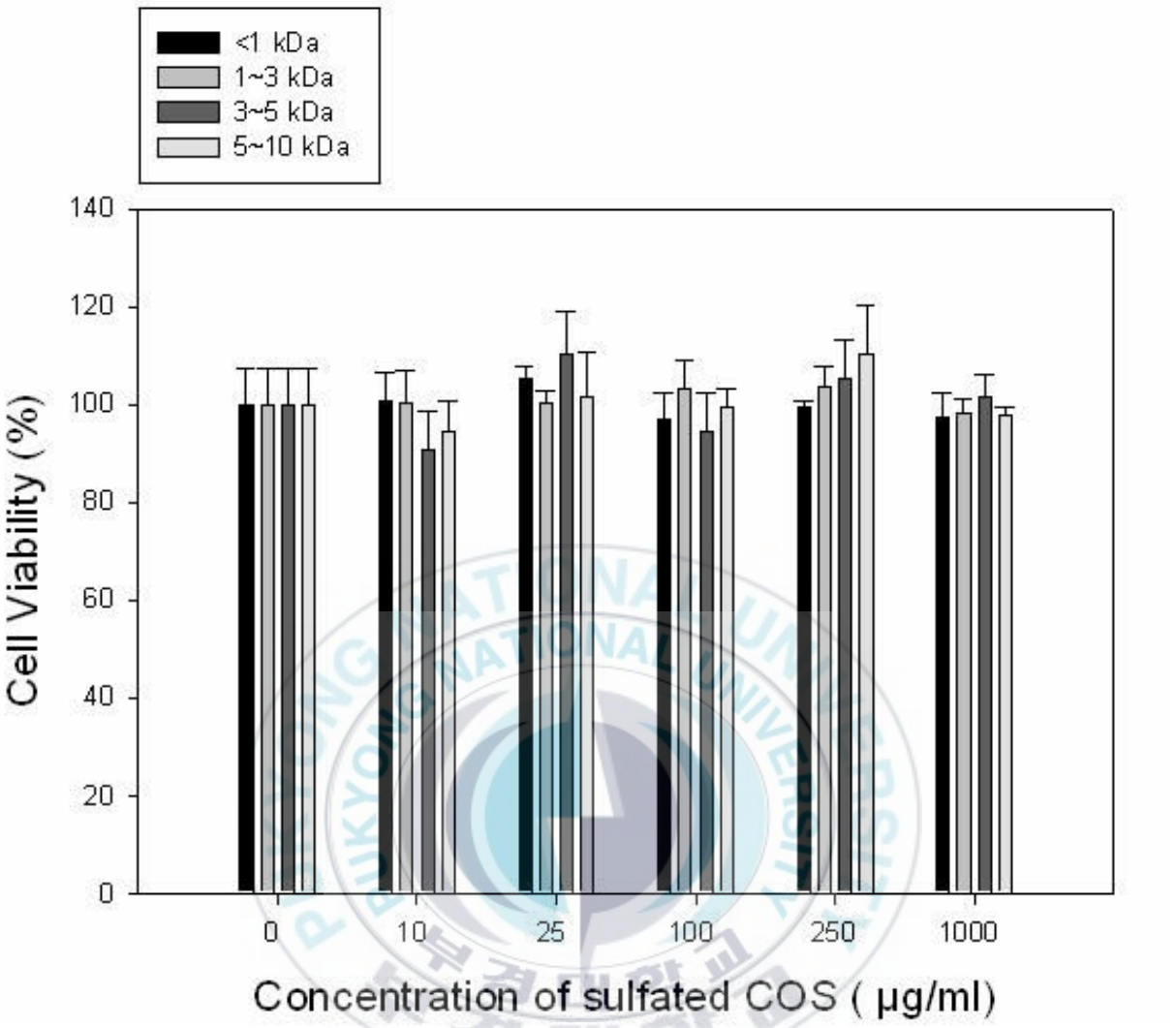

Figure 16: Effect of sulfated chitooligosaccharides on the viability of CEM-SS cells. Cells were treated with SCOS at the indicated concentrations. Cell viability was determined by MTT formazan assay after 72 hours of sample treatment. Values represent means ± SE (n = 3).

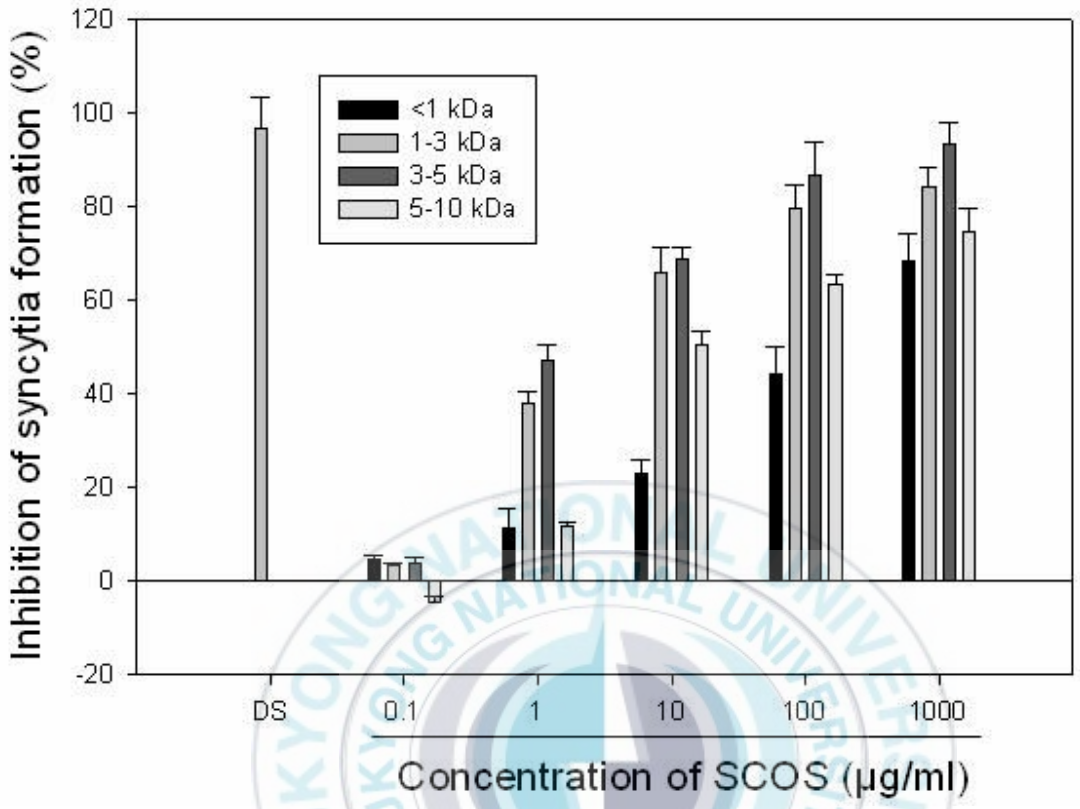

Figure 17: Inhibition effect of SCOS on syncytia formation on C8166 cell line infected with HIV-1_{IIIB}. Cells were treated with SCOS at the indicated concentrations 2 hours prior to infection. Dextran sulfate (100 μg/ml) was used as the positive control. Values represent means ± SE (n = 3).

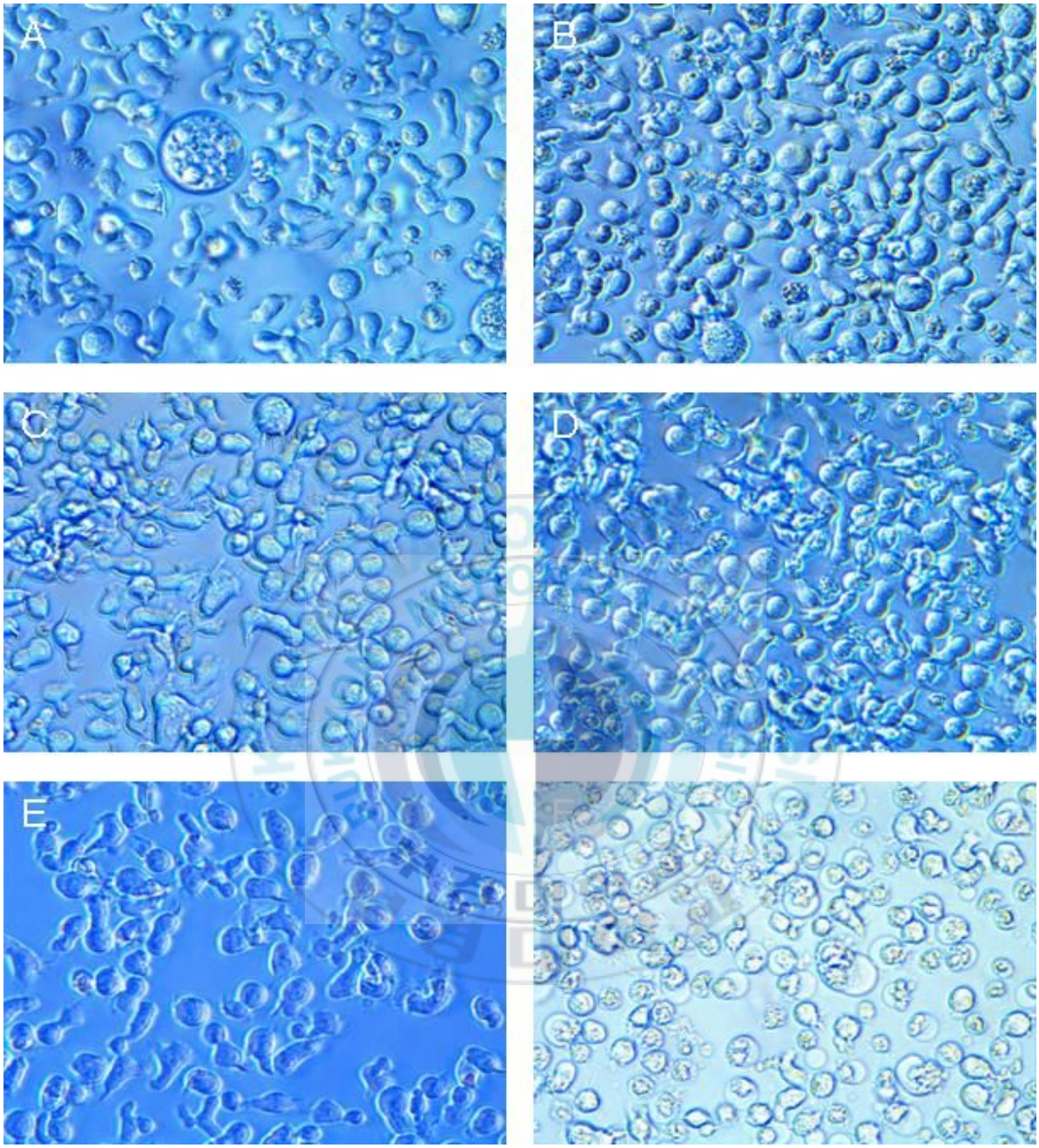

Figure 18: Microscopic image of syncytia formation analysis of C8166 cells infected with HIV-1_{IIIB}. C8166 cells were treated with 1000 µg/ml of COS I (A), COS II (B), COS III (C), COS IV (D) and the microscopic images were compared with 100 µg/ml dextran sulfate treated cells (E) and infected-untreated cells (F).

3. SCOSs protected CEM-SS cells from HIV-1 induced lytic effect

CEM-SS cell line, sensitive to the lytic effect of replicating HIV-1, was used for this experiment. The abilities of SCOSs on the suppression of HIV-1 replication were initially determined on the basis of the sensitivity of CEM-SS cell line to the lytic effect of HIV-1 infection. Cells were infected with sufficient amount of virus (~90% cell kill in 7 days) and the SCOS induced suppression of viral lytic effect was determined by generation of a soluble formazan (MTT) in surviving cells.

SCOS I, II and III protected the cells from lytic effect of HIV-1 at the highest concentration (1000 μg/ml) almost completely (Figure 19). The protective effect of SCOS IV at the highest concentration was about 80%.

SCOS III was the most effective compound to suppress the lytic effect of HIV-1. SCOSs protected cells from HIV-1 induced lytic effect at relatively lower concentrations when compared with the amount needed for the inhibition of syncytia formation which is consistent with the data explained before (Baba et al., 1990). This data indicates that SCOSs protect the cells from lysis once they are infected with HIV at some degree. However, the exact reason of this protection is not clear.

4. Co-culture of C8166 cells with H9/HIV-1_{IIIB} cells

This assay is based on the interaction between HIV-1_{IIIB} chronically infected cells and uninfected cells. The interaction of gp120 on infected cells with CD4 cell surface receptor on uninfected cells can induce the formation of multinucleated giant cells. An entry inhibitor is expected to inhibit the fusion process. C8166 cells were co-cultured with H9/ HIV-1_{IIIB} cells with a ratio of 1/10 after treatment with various concentrations of SCOSs and incubated for 24 hours. The number of syncytia was quantified using a microscope.

All SCOSs inhibited the fusion of infected cells with uninfected cells at some degree at the high concentrations (Figure 20). However, when the inhibition pattern is examined, it can be concluded that inhibition of syncytia formation was not as strong as the inhibition of cell-free virus. SCOS III exhibited

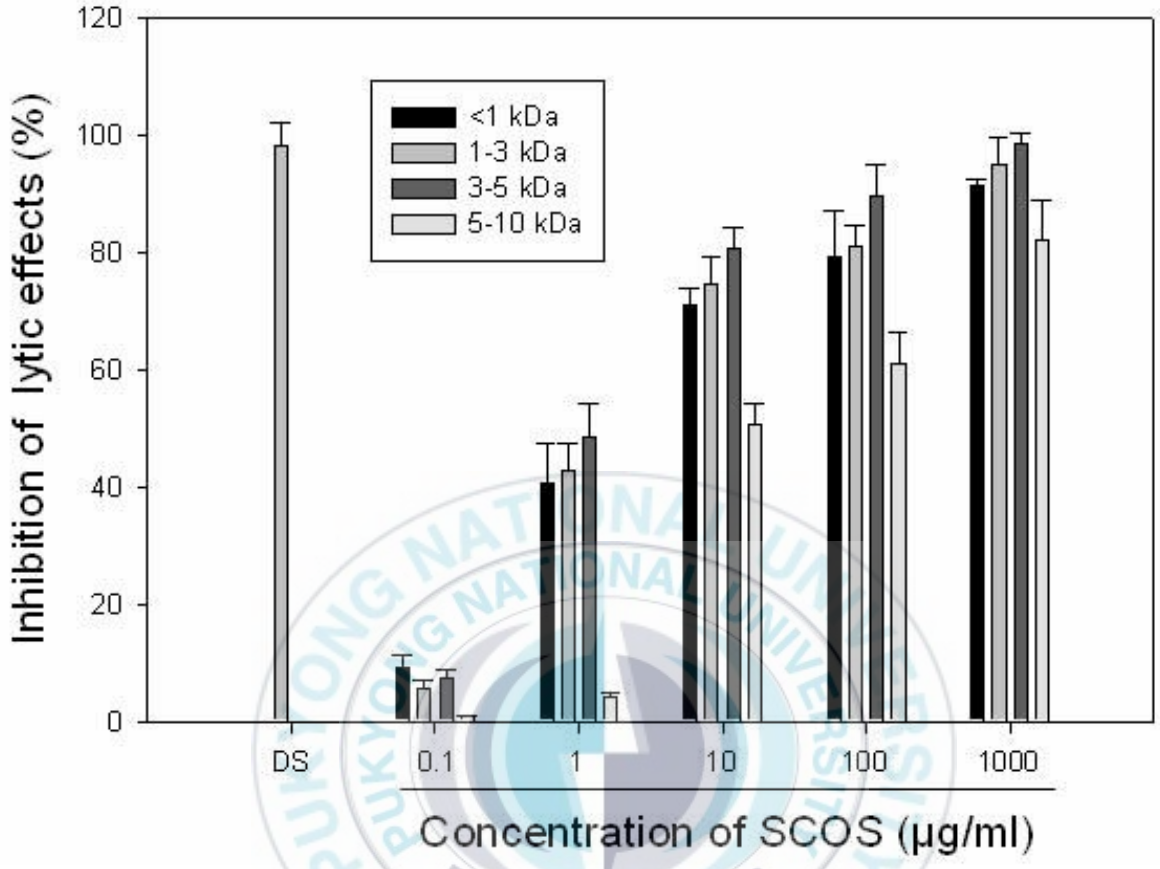

Figure 19: Inhibitory effect of SCOS on lysis of CEM-SS cell line infected with HIV-1_{IIIB}. Cells were treated with SCOS at the indicated concentrations, infected with HIV-1 and incubated for 7 days. Dextran sulfate (100 μg/ml) was used as the positive control. Values represent means ± SE (n = 3).

the strongest inhibition at 1000 μg/ml concentration about 80% inhibition of fusion. AZT, which is a reverse transcriptase inhibitor, failed to inhibit the fusion of infected and uninfected cells, as it was expected (Figure 20). As it was proved in previous experiments, SCOS I showed the weakest inhibitory pattern among SCOSs. The reason of this mild inhibition of SCOSs against fusion process is unknown. Above all, this experiment indicates that SCOSs should be treated against virus at the early stages of infection, possibly before the virus penetrates into the host cells.

5. Delayed addition of SCOSs to the cell culture

Delayed addition analysis of SCOSs to the cell culture was carried out in order to estimate the mode of action of SCOS. In other words, the experiment was carried out to determine at which step of infection SCOSs inhibited HIV-1. Initially SCOSs and HIV-1 were added to the cell culture simultaneously. Later on, the addition of SCOSs to the cell culture was delayed for 1, 2, 4 and 12 hours, respectively. In a parallel experiment, the same amount of cells was treated with only SCOS simultaneously with infected cells. AZT (HIV-1 reverse transcriptase inhibitor) and DS (HIV-1 entry inhibitor) treated-virus infected cells were used as the positive control. After a seven-day incubation period, the viability of HIV-1 infected-SCOS treated, uninfected-SCOS treated, AZT treated-infected and DS treated-infected cells were compared by measuring the optical density. The concentration of SCOSs treated was 1000 μg/ml in all sets of experiments.

When SCOS I and HIV-1 were treated at the same time, the viability of the cells after the incubation period was almost 90% compared with the uninfected cells, indicating that inhibition of HIV-1 replication correlates with this percentage (Figure 21). However, the percentage of cell viability decreases significantly when the cells are infected with HIV-1 one hour prior to SCOS I treatment. The cell survival decreases constantly up to treatment of virus 4 hours prior to SCOS I treatment. At and after this stage, almost none of the cells were viable which indicates that SCOS I is no more effective to inhibit the replication of HIV-1. This inhibition pattern is similar to that of positive control, dextran sulfate, which is an entry-inhibitor of HIV-1 infection. However, AZT-treated cells maintained their viability even after a

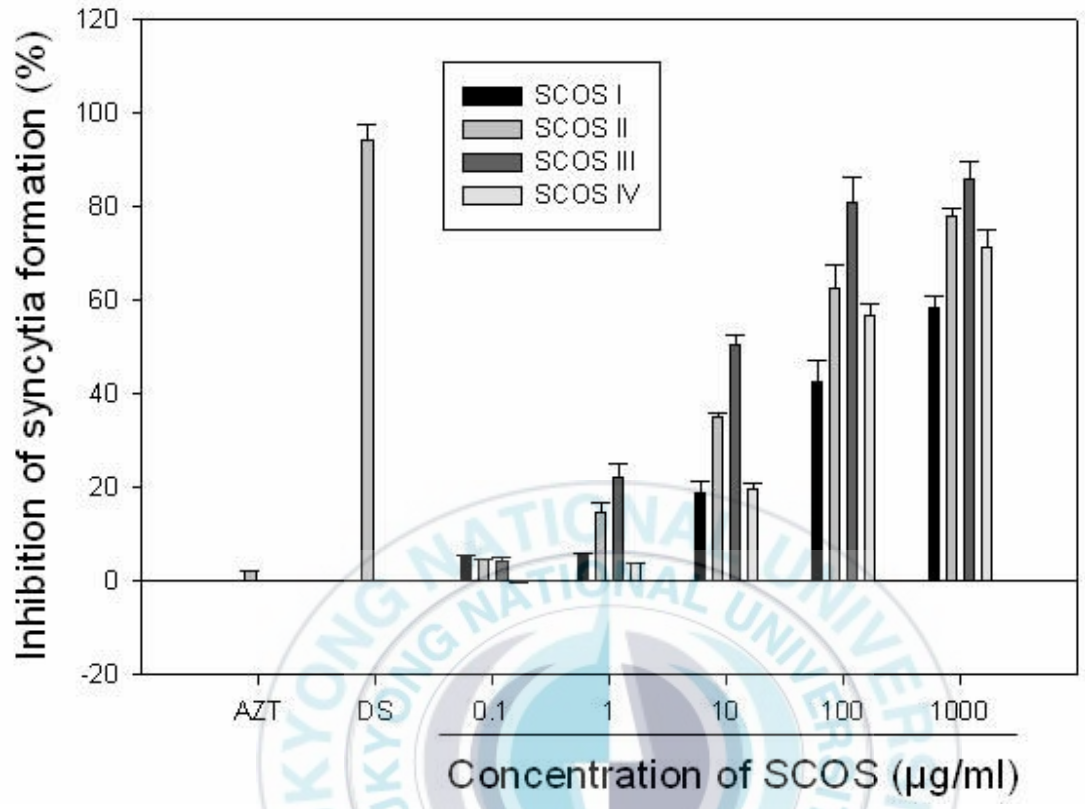

Figure 20: Co-culture study of C8166 cells with H9 cells chronically infected with HIV-1_{IIIB}. SCOS treated C8166 cells were co-cultured with H9/HIV-1IIIB cells at a ratio of 1:10 and incubated for 24 hours. Number of syncytia was quantified using a microscope. Azidothymidine (5 μM) and dextran sulfate (100 μg/ml) were used as drug controls. Values represent means ± SE (n = 3).

4-hour-delay of AZT treatment. This data indicates that AZT is an effective compound at later stages of HIV-1 infection, unlike SCOSs.

Data obtained from this experiment reveals that SCOS I is effective at the entry step of HIV-1. SCOS I inhibits the entry of HIV-1 into the cells probably by blocking the interaction of HIV-1 receptors with host cell surface receptors. Witvrouw and De Clercq (1997) suggested that sulfated polysaccharides inhibit HIV infection via blocking the attachment of the virus CD4 cell surface receptor subsequently inhibiting syncytia formation. They also furnished that sulfated polysaccharides interact with positively charged amino acids located in the V3 loop of glycosilated surface protein of HIV-1 (gp120). It also has been proven that dextran sulfate, which is a synthetic sulfated polysaccharide from marine origin, inhibits the binding of HIV-1 to the host cell via disrupting the interaction between gp120 and cell surface receptor CD4 (Callahan et al., 1991). Even though dextran sulfate has been shown to inhibit HIV-1 replication *in vitro*, orally administrated dextran sulfate and a number of sulfated polysaccharides were largely ineffective against HIV-1 *in vivo* (Hartman et al., 1990; Lorentsen et al., 1989). Therefore, it can be claimed that sulfated chitooligosaccharides potential HIV-1 inhibitors with superior properties such as high absorption rate in the intestines which is a crucial feature for a drug candidate (Kim and Rajapakse, 2005).

When the inhibitory activity of SCOSs is compared, SCOS III again seemed to be the most active inhibitor of HIV-1 infection whereas SCOS IV (Figure 24) was the least active compound among SCOSs as a result of delayed-addition of SCOS experiment. SCOS III probably blocked the interaction of CD4 cell surface receptor with V3 loop of viral gp120 molecule. Thus, SCOS III strongly inhibited the entry of HIV-1 into the host cell and protected the cells from HIV-1 induced syncytia (multinucleated giant cells) formation when it was added to the cell culture between 0 and 1 hour post-HIV infection. The protective effect of SCOS III dramatically decreased when it was treated at later time points. The same situation was observed with SCOSs of other molecular weights. The protective effect of SCOS II (Figure 22) was almost comparable to that of SCOS III.

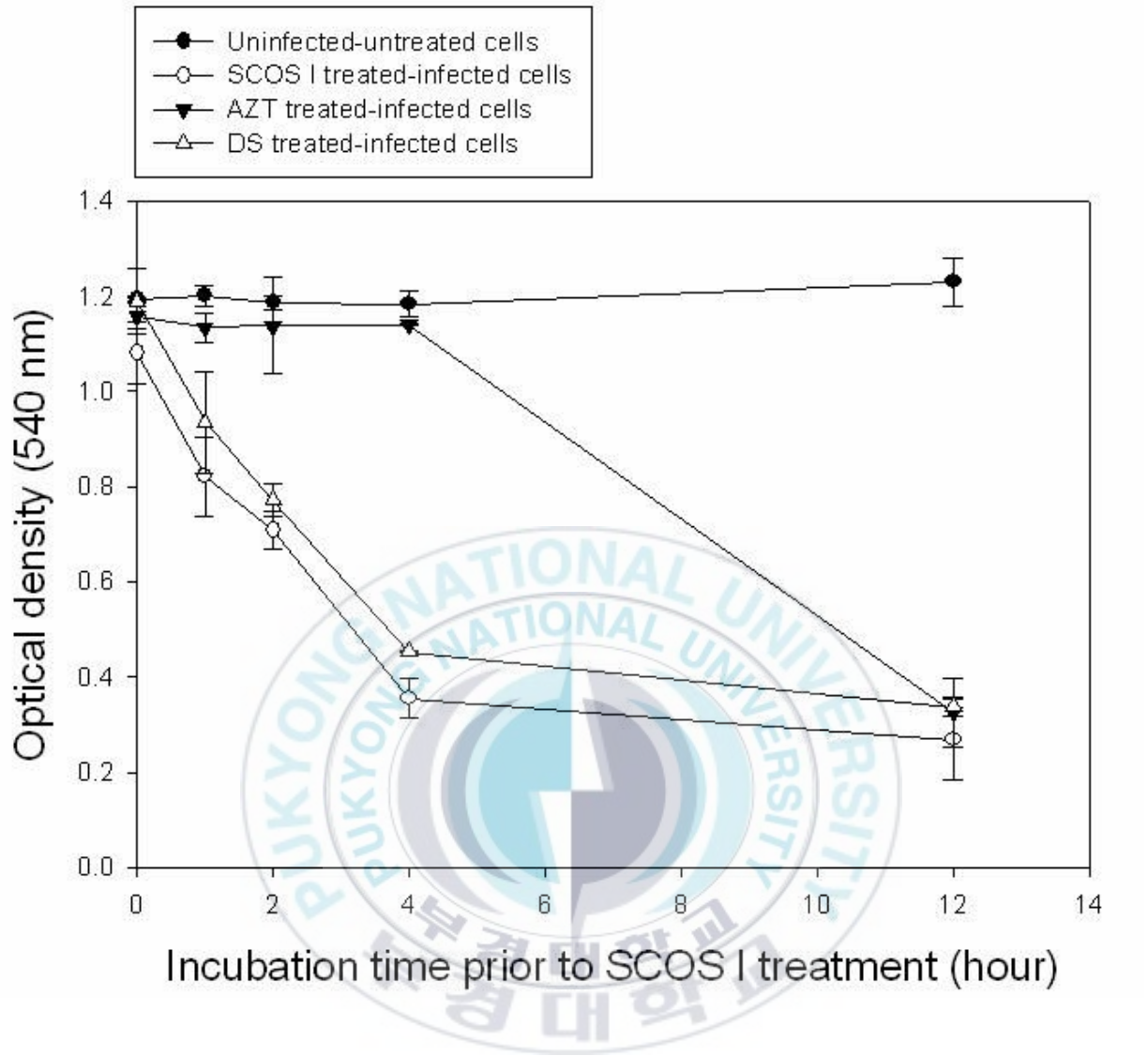

Figure 21: Effect of delayed addition of 1000 μg/ml of SCOS I on only HIV-1_{IIIB} infected, uninfected, AZT treated-infected and DS treated-infected CEM-SS cells analyzed after 7 days using the MTT assay. Azidothymidine (5 μM) and dextran sulfate (100 μg/ml) were used as drug controls. Values represent means ± SE (n = 3).

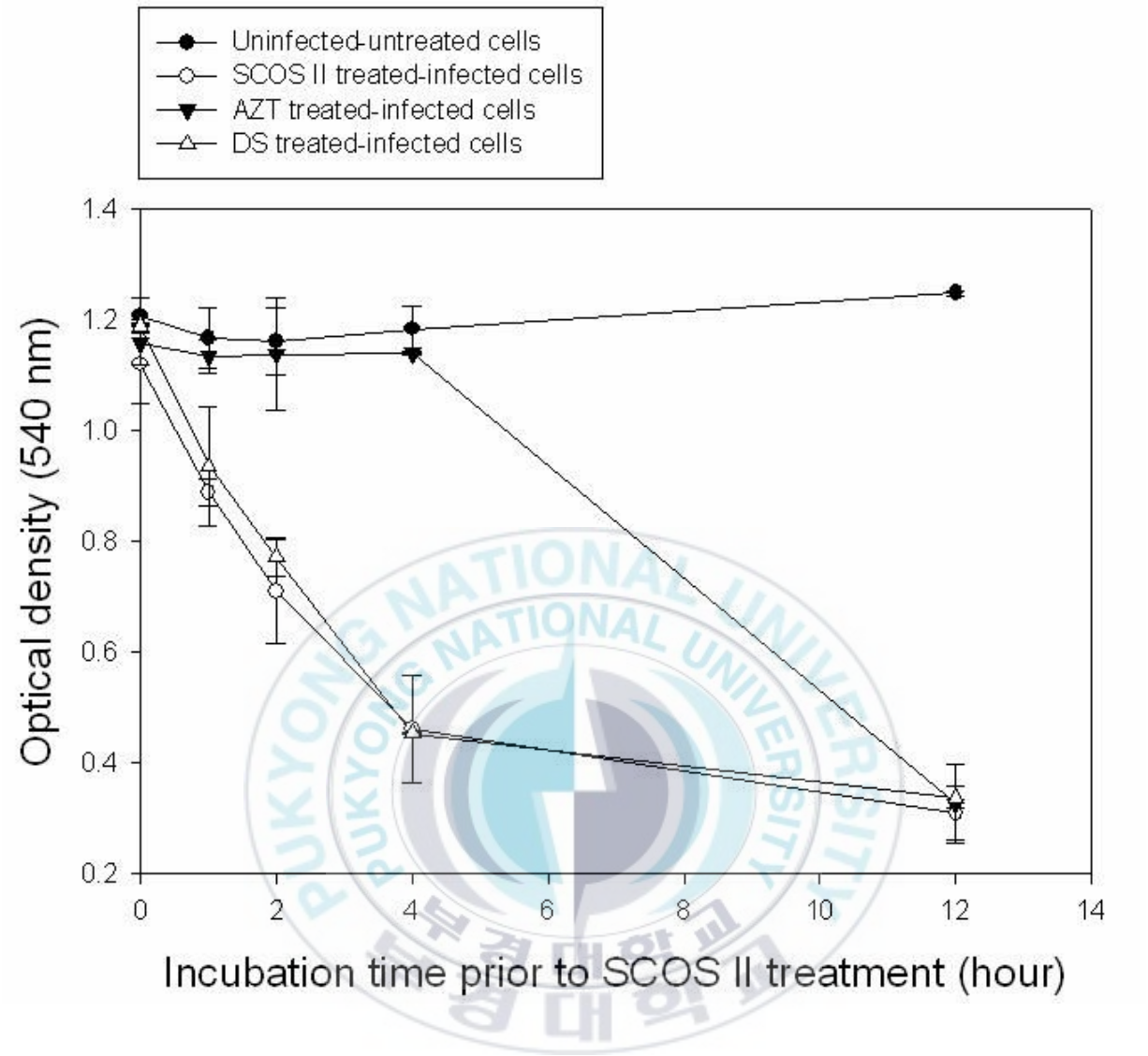

Figure 22: Effect of delayed addition of 1000 μg/ml of SCOS II on only HIV-1_{IIIB} infected, uninfected, AZT treated-infected and DS treated-infected CEM-SS cells analyzed after 7 days using the MTT assay. Azidothymidine (5 μM) and dextran sulfate (100 μg/ml) were used as drug controls. Values represent means ± SE (n = 3).

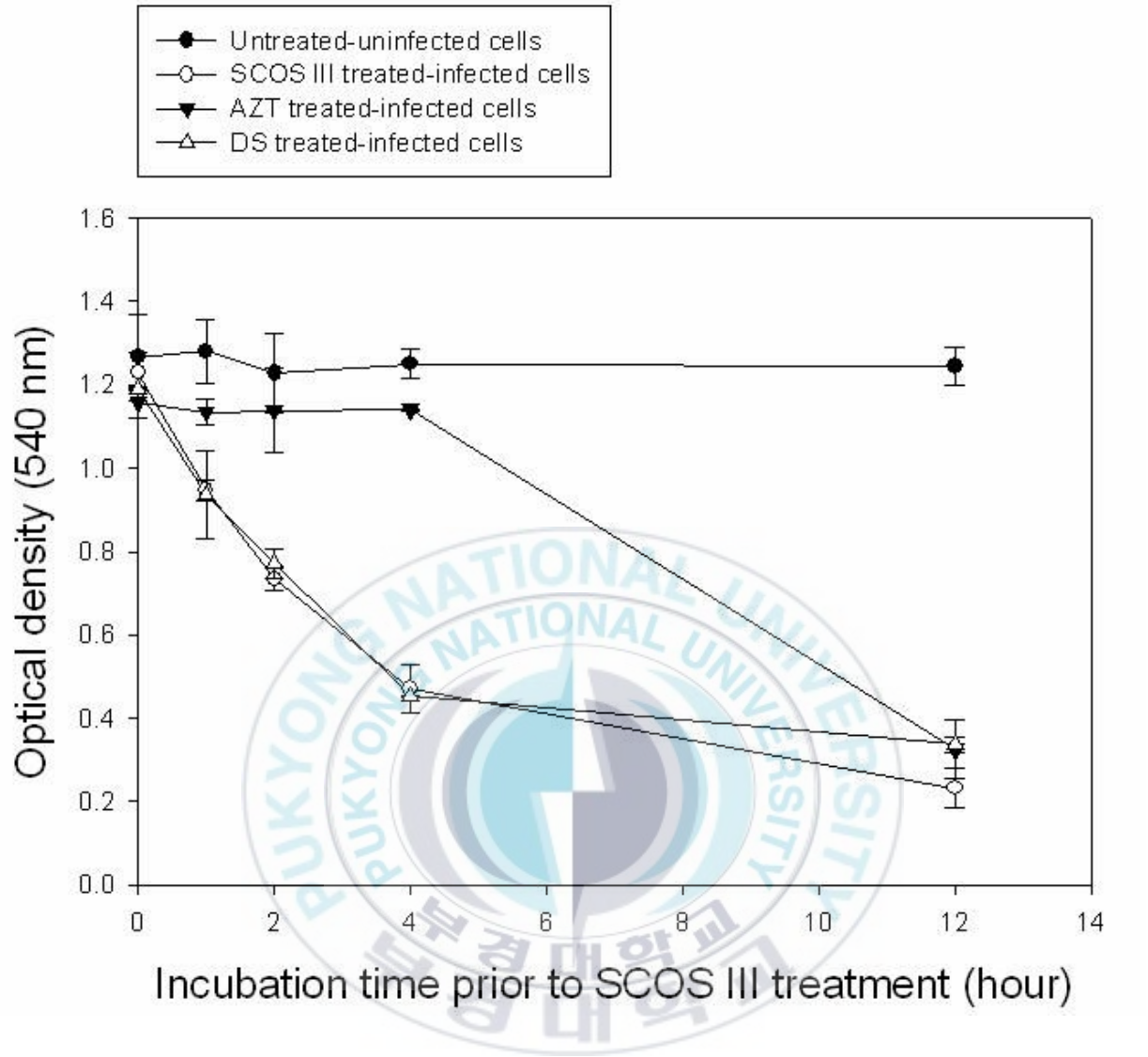

Figure 23: Effect of delayed addition of 1000 μg/ml of SCOS III on only HIV-1_{IIIB} infected, uninfected, AZT treated-infected and DS treated-infected CEM-SS cells analyzed after 7 days using the MTT assay. Azidothymidine (5 μM) and dextran sulfate (100 μg/ml) were used as drug controls. Values represent means ± SE (n = 3).

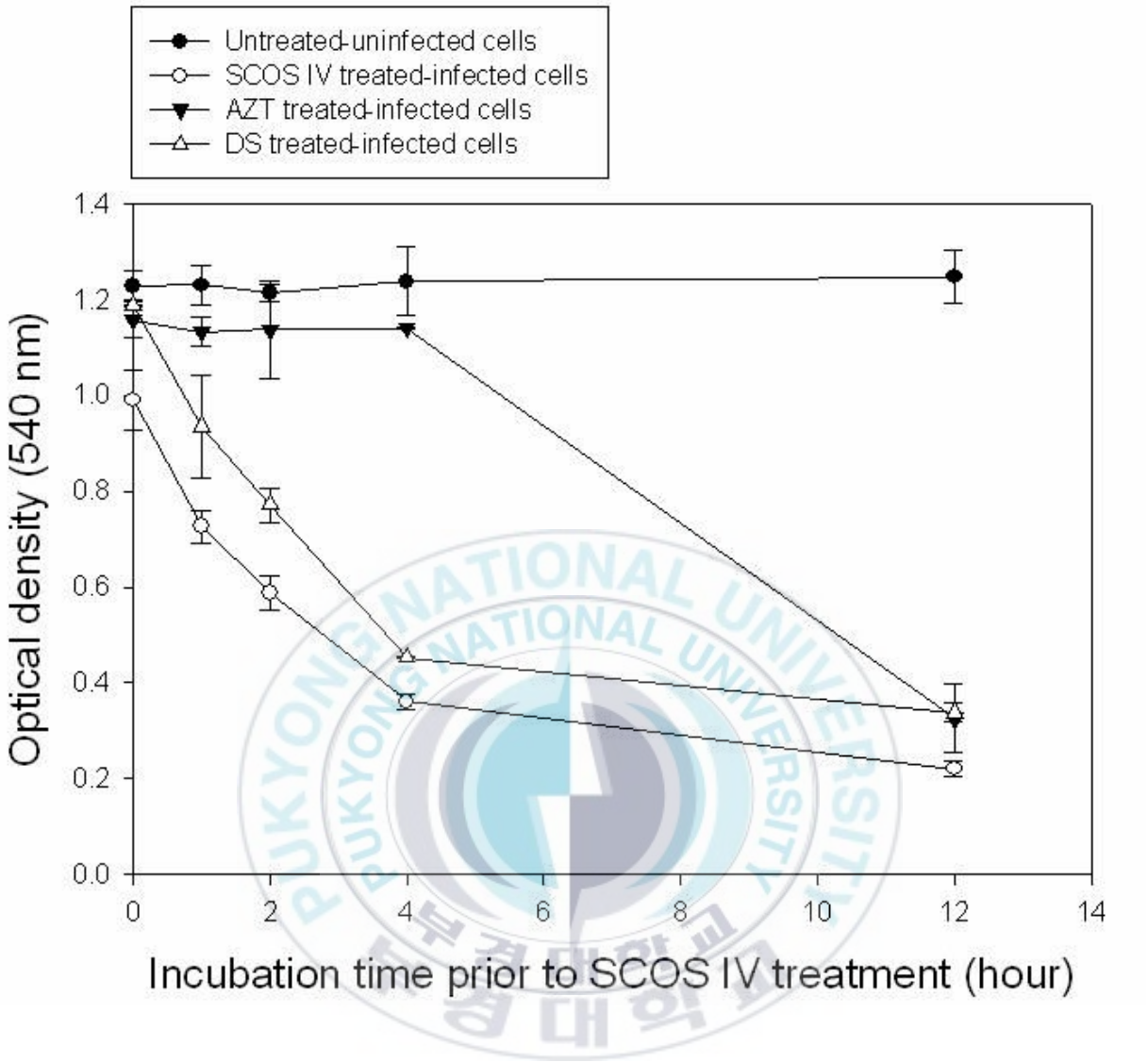

Figure 24: Effect of delayed addition of 1000 μg/ml of SCOS IV on only HIV-1_{IIIB} infected, uninfected, AZT treated-infected and DS treated-infected CEM-SS cells analyzed after 7 days using the MTT assay. Azidothymidine (5 μM) and dextran sulfate (100 μg/ml) were used as drug controls. Values represent means ± SE (n = 3).

6. Inhibition of p24 antigen production

ELISA technique is a highly sensitive method that could be used as a diagnostic tool in HIV research. The assay is used to determine disease progression because of its close link to the viral load of an infected individual. Since p24 core protein is very abundant in the cell or cell culture supernatant, it is very convenient to use p24 ELISA method to detect the severity of HIV-1 infection.

As a result of p24 ELISA experiment, SCOS III was the most effective compound to inhibit p24 antigen production (Figure 25). SCOS III inhibited p24 antigen production as comparable to that of positive control, dextran sulfate at the highest concentration (1000 μg/ml). The inhibition pattern of other SCOSs was similar to that of previous experiments.

7. Western blot analysis

To further characterize the anti-HIV-1 effect of sulfated chitooligosaccharides, immunoblot analysis was carried out on p24 protein from cell and cell culture supernatant of H9 cells treated with SCOSs. As expected, dextran sulfate treatment, as a positive entry inhibitor control, resulted in a decrease in p24 protein production. Treatment of cells with SCOSs also led to a dose-dependent decrease in p24 protein amount both in the cell and in the culture supernatant (Figure 26) comparable to that from p24 ELISA (Figure 25). According to western blot analysis, no or weak bands were detected at the locations of p55 gag related proteins (p55, p41 and p24) except for p24, which indicated that SCOSs did not inhibit HIV-1 protease (Davis et al., 2006). This reveals that gag proteins can be processed without facing any problem and SCOSs do not interfere with HIV-1 protease or the protein processing mechanism. The inhibitory mode of action of SCOSs is related to another mechanism.

Relatively, all SCOSs inhibited p24 protein production. However, when the cells are treated with 1000 μg/ml of SCOS III, the bands of p24 on the membrane can barely be detected, both in cell and culture supernatant p24, which is indicating that SCOS III is the most potent inhibitor of p24 production in comparison to other SCOSs.

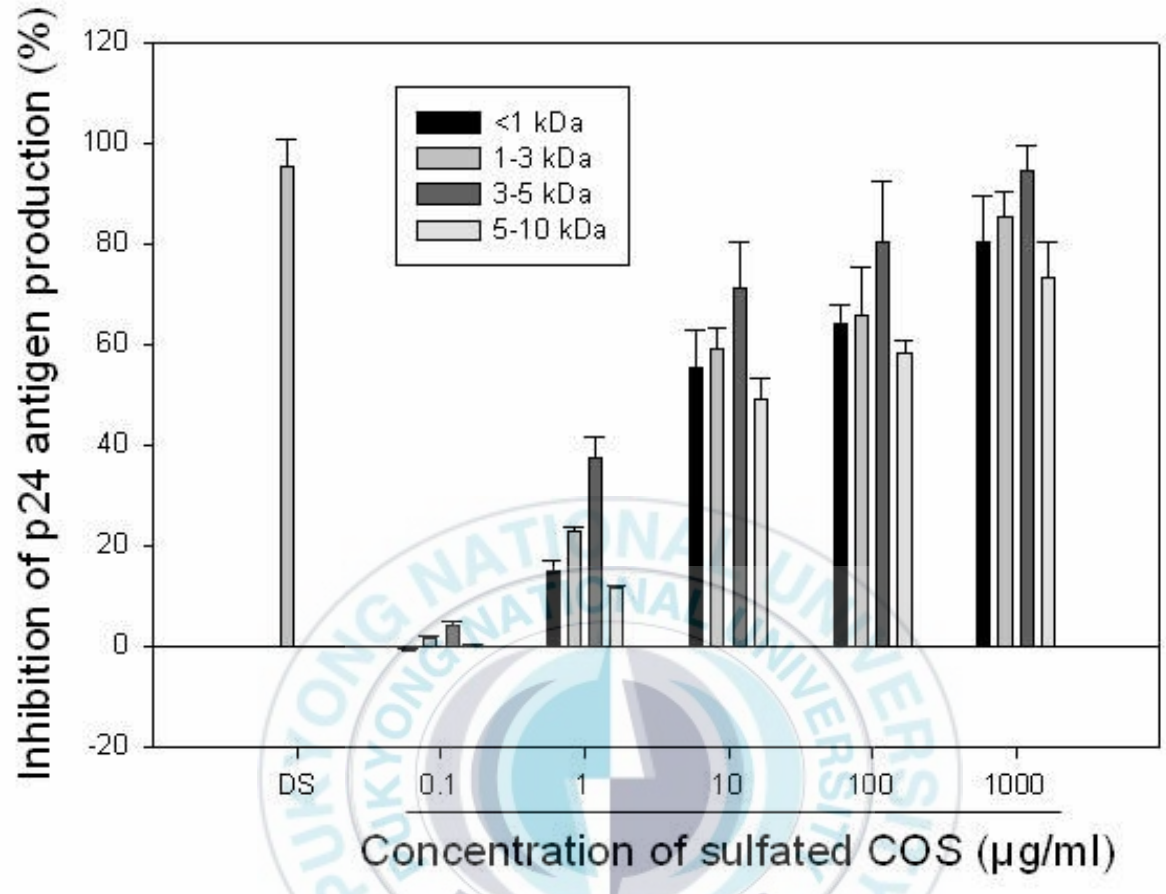

Figure 25: Inhibition of HIV-1_{IIIB} p24 antigen production in the cell culture supernatant. Cells were treated with the indicated concentrations of SCOS, infected with HIV-1_{IIIB} and incubated for 72 hours. Dextran sulfate was used as a positive control (100 μg/ml). Values represent means ± SE (n = 3).

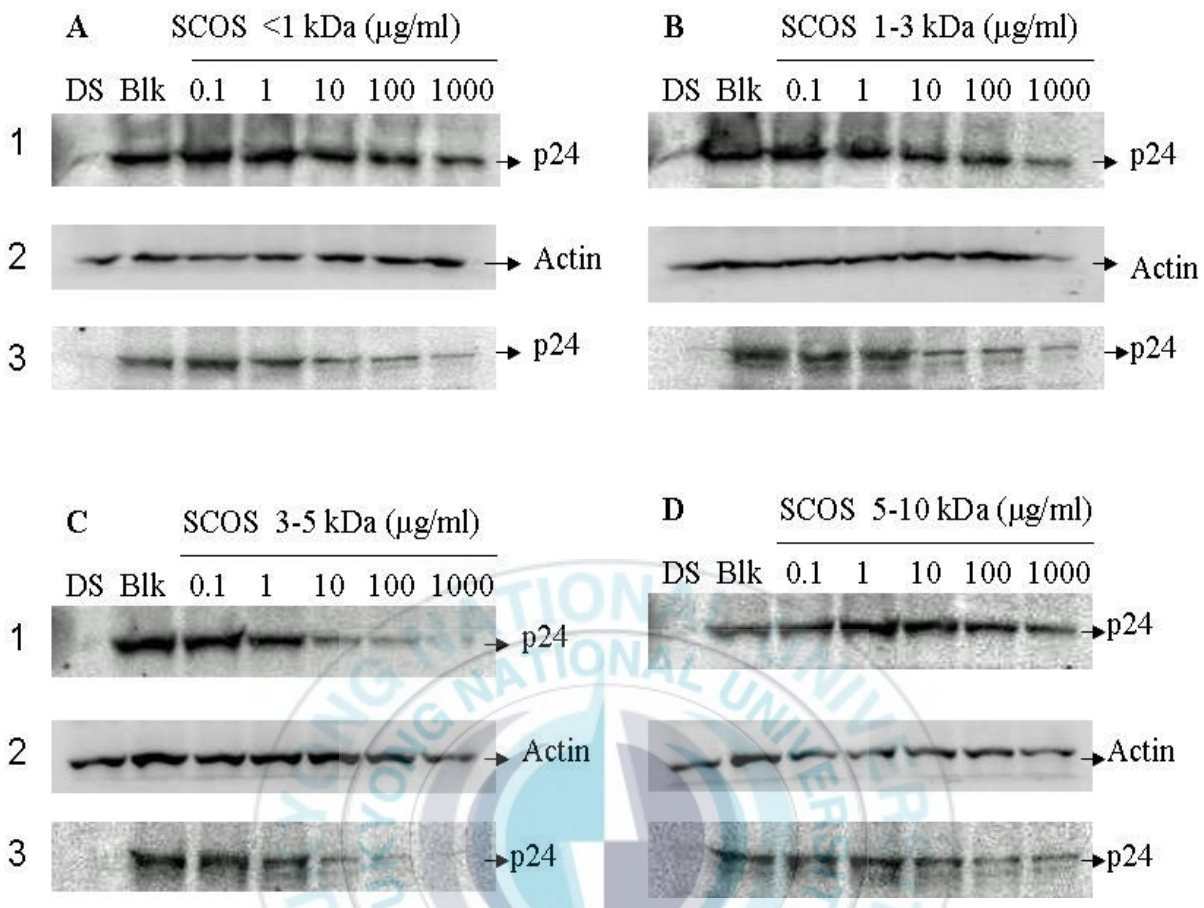

Figure 26: Western blot analysis of HIV-1_{IIIB} 24 kDa p24 protein. Cells were treated with indicated concentrations of SCOS <1 kDa (A), 1-3 kDa (B), 3-5 kDa (C) and 5-10 kDa (D). H9 cells were infected with HIV-1_{IIIB} and incubated for 96 hours. HIV-1 p24 core protein in the cell lysate (Lane 1), β-actin (Lane 2) and HIV-1 p24 core protein in culture supernatant (Lane 3) was detected using specific antibodies raised against p24 and β-actin. 100 μg/ml of dextran sulfate (DS) was used as a positive control.

8. Effect of SCOSs on HIV-1 reverse transcriptase enzyme activity

Reverse transcription is a crucial step in viral infection and it was the most important target for first generation HIV inhibitors. AZT was introduced as the first HIV inhibitor in 1989. After gaining entry into the host cell, HIV-1 RNA is reverse transcribed into proviral DNA by viral RT enzyme. CEM-SS cells were infected with HIV-1, incubated for 4 days and cell culture supernatant was collected to obtain viral lysate including reverse transcriptase. The same amount viral lysate was incubated with various concentrations of SCOSs and the inhibitory activity of SCOSs on HIV-1 reverse transcriptase in the viral lysate was evaluated using a fluorescence RT assay kit.

None of the SCOSs inhibited the activity of HIV-1 while AZT, a reverse transcriptase inhibitor, inhibited RT activity almost completely.

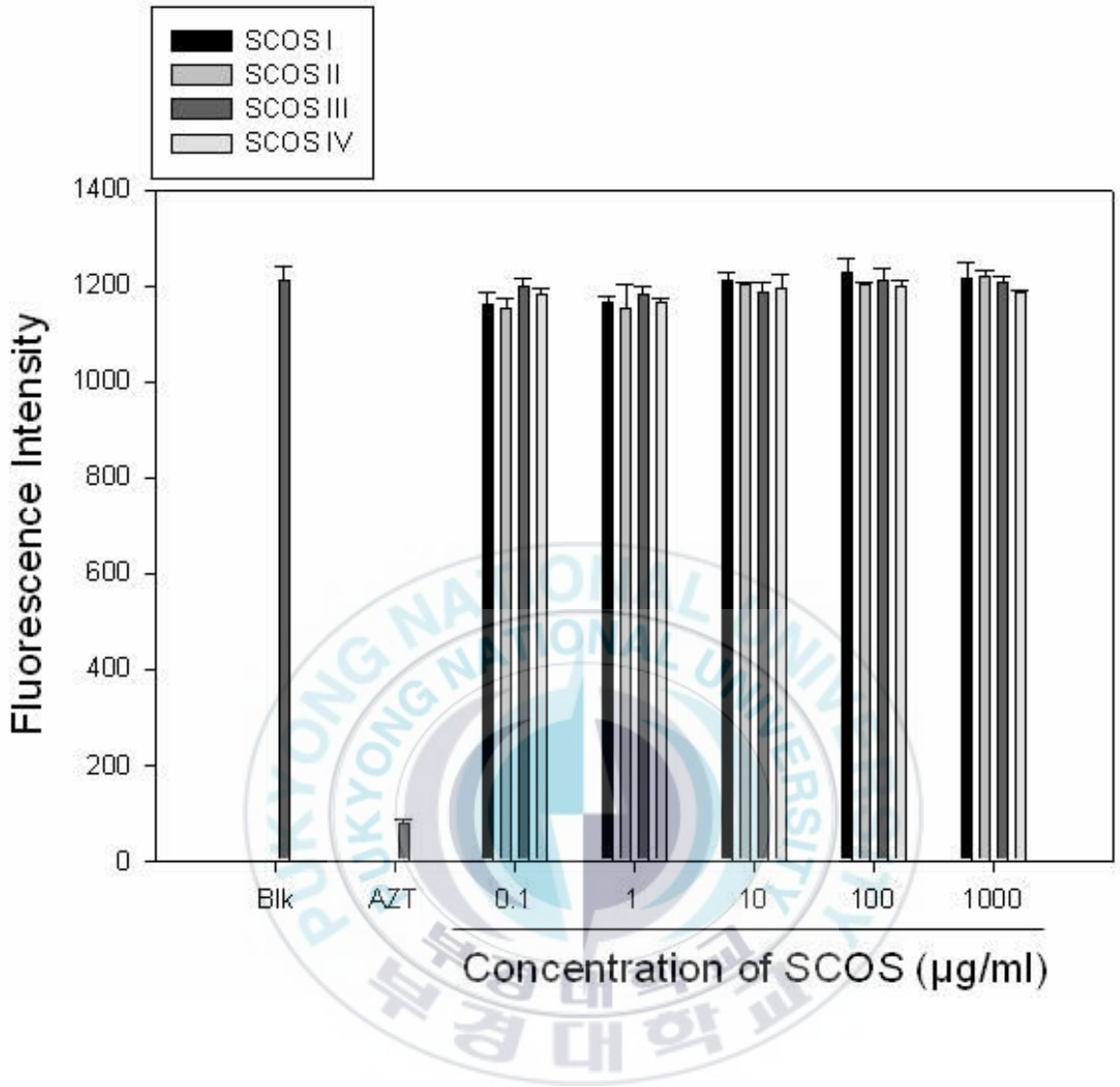

Figure 27: Effect of sulfated chitooligosaccharides on the inhibition of HIV-1 reverse transcriptase (RT) activity. The effect of SCOSs on HIV-1 RT activity inhibition was determined by fluorescent RT activity assay. HIV-1 reverse transcriptase enzyme was recovered from cell culture supernatant. Viral lysate including RT enzyme was incubated with various concentrations of SCOS and RT activity was measured as fluorescence intensity at 480 nm (excitation) and 520 nm (emission). Azidothymidine (5 μM) was used as drug control. Values represent means ± SE (n = 3).

9. Effect of sulfated chitooligosaccharides on HIV-1 gene expression

To determine the effect of SCOSs on the gene expression of HIV-1 genome, the expression of HIV-1 Vif gene was investigated. Total RNA was recovered from CEM-SS cells and reverse-transcribed into cDNA and it was used as a template for PCR to amplify a 271 bp region of HIV-1 Vif using primers corresponding HIV-1 Vif gene.

According to data, SCOSs of all molecular weight strongly inhibited the expression of HIV-1 genome (Figure 28). However, using this data, it cannot be deduced whether SCOSs directly inhibited HIV-1 gene expression or the inhibition occurred prior to or during the integration of proviral DNA into the host cell genome. Therefore, HIV-1 genome proviral DNA integration was investigated in order to illustrate the mode of action of sulfated chitooligosaccharides.

10. Effect of SCOSs on HIV-1 proviral DNA integration

Total DNA was extracted from CEM-SS cells, after treatment with SCOSs and infection with HIV-1. Total genomic DNA or intracellular circular plasmid-like proviral DNA was used as a template for PCR reaction to determine the presence of proviral DNA in the host cells. A 271 bp region of HIV-1 Vif using primers corresponding HIV-1 Vif gene was obtained as PCR product and electrophorosed on 2% gel. The bands, stained with EtBr, clearly indicated that the only a minor amount of cells were infected with HIV-1 and integration of proviral DNA was inhibited strongly (Figure 29). This data also revealed that SCOSs did not interfere with the expression of HIV-1 genome.

Data obtained from proviral integration study pointed that HIV-1 SCOSs inhibited HIV-1 prior to integration of proviral DNA into the host cell genome.

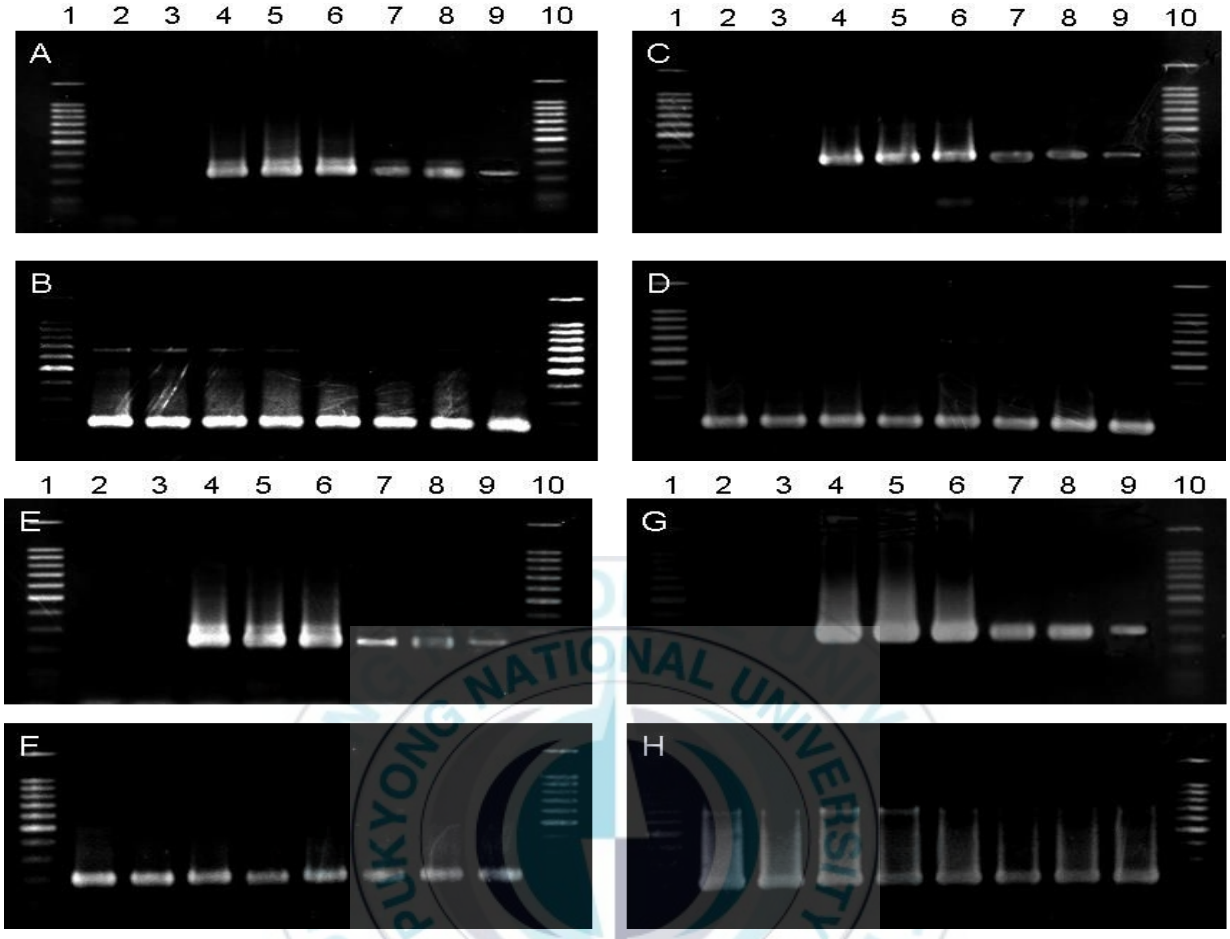

Figure 28: Gene expression analysis of HIV-1 genome. Total RNA from cell lysate was recovered and reverse transcribed into cDNA. 271 bp region of the HIV-1 vif (viral infectivity factor) gene was amplified using primers specific for vif gene. A 185 bp region of human GAPDH gene was amplified as a control. SCOS I Vif (A), SCOS I GAPDH (B), SCOS II Vif (C), SCOS II GAPDH (D), SCOS III Vif (E), SCOS III GAPDH (F), SCOS IV Vif (G) and SCOS IV GAPDH (H) were treated at different concentrations. 100 bp DNA marker (1), DNA of uninfected cells (2), Dextran sulfate as positive control (3), Blank (4), SCOS 0.1 µg/ml (5), 1 µg/ml (6), 10 µg/ml (7), 100 µg/ml (8), 1000 µg/ml (9) and 100 bp DNA marker (10).

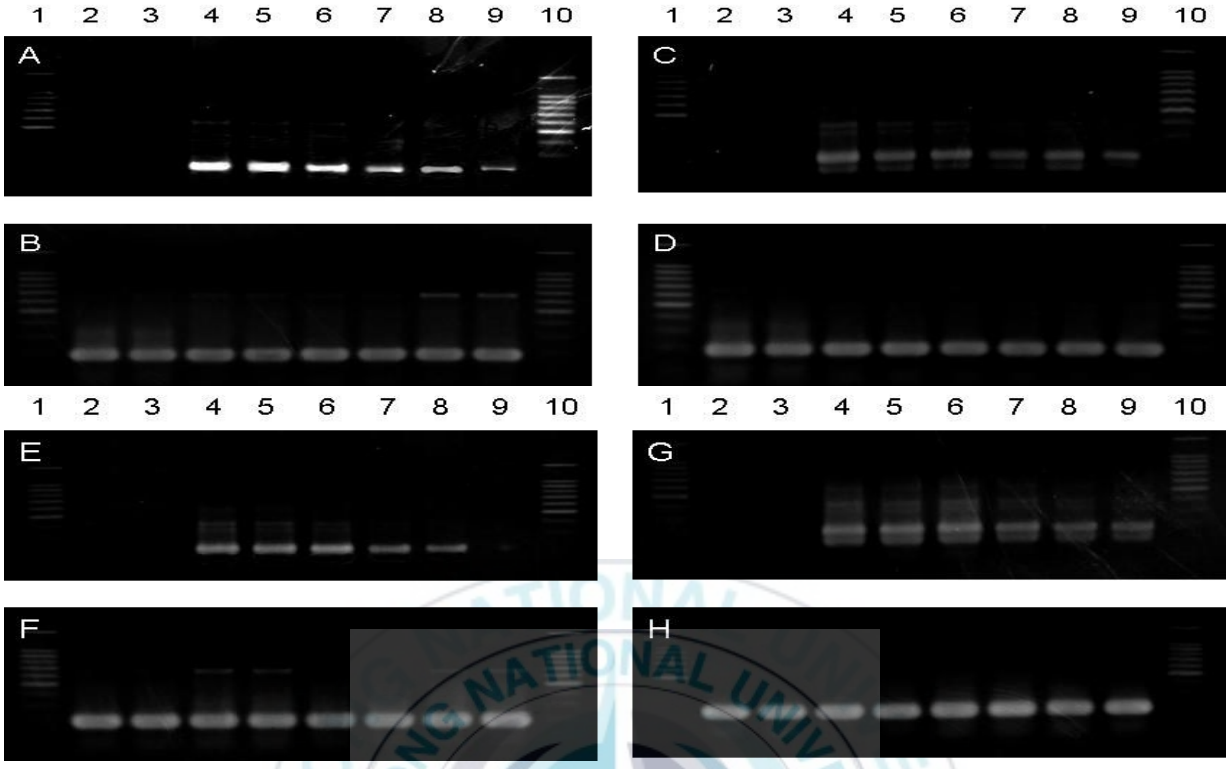

Figure 29: Host cell genome integration analysis of HIV-1 genome. Total DNA from cell lysate was recovered using phenol:chloroform:isoamyl alcohol. 271 bp region of the HIV-1 vif (viral infectivity factor) gene was amplified using primers specific for vif gene and 185 bp region of human GAPDH gene as a control. SCOS I Vif (A), SCOS I GAPDH (B), SCOS II Vif (C), SCOS II GAPDH (D), SCOS III Vif (E), SCOS III GAPDH (F), SCOS IV Vif (G) and SCOS IV GAPDH (H) were treated at different concentrations. 100 bp DNA marker (1), DNA of uninfected cells (2), Dextran sulfate as positive control (3), Blank (4), SCOS 0.1 µg/ml (5), 1 µg/ml (6), 10 µg/ml (7), 100 µg/ml (8), 1000 µg/ml (9) and 100 bp DNA marker (10).

11. Synthesis of chitooligosaccharide sulfates

Sulfated chitooligosaccharides were identified by FT-IR spectroscopy. Chitooligosaccharide sulfates were obtained in over 90% yields as white, fluffy, and water-soluble materials. The synthetic mechanism was substituted by sulfate at the C-2, C-3 and C-6 positions. Characteristic absorptions derived from the sulfo groups in the IR spectrum at 800, 1,240 and 1,350 cm^{-1} were assigned to C-O-S, S=O and S-N, respectively (Figure 30).

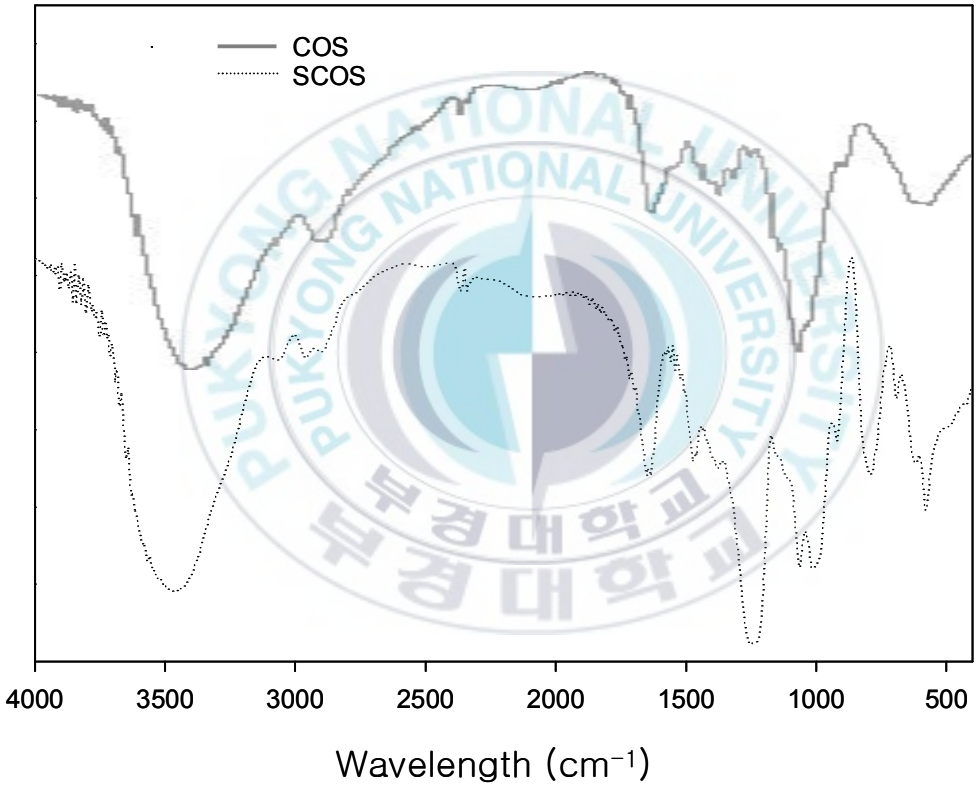

Figure 30: FT-IR spectra of 90% deacetylated chitosan and chitosan sulfate

Conclusion

Human immunodeficiency virus type-1 (HIV-1) is identified as the causative agent of acquired immunodeficiency syndrome (AIDS) which is one of the most important epidemics in the world. HIV is assumed to infect the immune system cells and cause the collapse of immune system by disrupting the cells, leaving the organism defenseless.

In the present study, chitooligosaccharides of various molecular weights were sulfated and their HIV-1 inhibitory activities were investigated. Results obtained from experiments revealed that all SCOSs inhibited HIV-1 replication at some degree. However, 3-5 KDa SCOS was proved to be the most effective compound against HIV-1 infection. 3-5 KDa SCOS inhibited HIV-1 induced syncytia formation, p24 antigen production and protected T-lymphoblasts from lytic effect of HIV-1. The inhibitory effect of all SCOSs was dose-dependent. Especially at the highest concentration treated, HIV-1 replication was almost completely inhibited by 3-5 KDa SCOS supported by p24 ELISA, western blot and syncytia formation analysis data.

Several experiments were carried out to illustrate the possible inhibitory mechanism of SCOSs on HIV-1 replication. p24 ELISA data revealed that SCOSs strongly inhibited p24 antigen production in cell culture supernatant. Western blot data was consistent with p24 ELISA data, however, any additional bands from gag-related proteins could not be observed, which meant that SCOSs did not interfere with HIV-1 protease and protein processing mechanism. HIV-1 reverse transcriptase enzyme activity was investigated and it was concluded that SCOSs did not inhibit HIV-1 RT enzyme. HIV-1 genome expression and proviral DNA integration was investigated. The data obtained from these experiments revealed that HIV-1 was inhibited prior to integration of proviral DNA into the host cell genome.

SCOSs inhibited HIV-1 induced syncytia formation as well as cell-to-cell fusion, indicating that SCOSs can inhibit not only cell-free virus, but also the fusion of HIV-1 infected and uninfected cells. In the delayed-addition studies of SCOSs, the inhibitory mechanism of SCOSs was compared with other known HIV-1 inhibitors. The inhibition pattern of SCOSs was similar to that of dextran sulfate, an entry inhibitor

of HIV-1. All the experiments pointed that SCOSs might have inhibited HIV-1 replication via blocking the interaction between HIV-1 glycoprotein gp120 and cell surface receptor CD4. This data was consistent with the data obtained from previous research which claimed that sulfated polysaccharides of different kinds can interact with V3 loop of viral gp120 glycoprotein.

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Sulfated chitooligosaccharides 의 HIV-1 감염 저해 효과

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초록

인체면역결핍바이러스 1 형 (HIV-1)은 전세계적으로 약 3 천만명 이상이 감염된 후천성면역결핍증 (AIDS)의 감염인자로 알려져 있다. 최근 항레트로바이러스치료제에 대한 내성을 갖는 다양한 HIV 종이 발견됨에 따라, 이들에 대한 높은 저해 활성을 가지는 무독성의 화합물을 발견하는 것이 매우 중요시 되고 있다. 그러나, 현재까지 의약디자인 기술의 진보에도 불구하고 획기적인 HIV 감염저해제가 개발 되지않아 천연물등에서의 HIV 감염저해제에 대한 후보물질 탐색 및 개발이 요구되고 있는 실정이다. 본 연구에서는 다양한 분자량의 sulfated chitooligosaccharides (SCOSs)의 *in vitro* 항에이즈 활성을 검토한 결과, SCOS 는 HIV-1 의 복제를 저해하였고, 세포변성효과를 유도한 HIV-1 으로부터 T-lymphoblast 를 보호하였으며, HIV-1 p24 항원의 level 을 감소시켰다. 또한, 세포실험을 통해 확인한 결과, SCOSs 의 항에이즈활성은 HIV-1 의 복제 초기단계에서 일어나는 것으로 확인 되었으며, HIV 감염세포와 비감염 세포간의 세포융합과정을 저해할 뿐만 아니라, HIV 가 세포에 부착하는것을 저해하였다. 이러한 SCOSs 의 항에이즈활성은 분자량에 따라 서로 다른 저해활성을 나타내었다. 이상의 결과로부터 HIV-1 저해제로서 SCOSs 는 높은 활성 및 안전성을 가지고 있음을 확인할 수 있었다.

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