Design and Development of Epap-1 derived Anti-HIV-1 peptides

A thesis submitted for the degree of

Doctor of Philosophy

By

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DECLARATION

I hereby declare that the work presented in my thesis is entirely original and was carried out by me in the Department of Biochemistry, School of Lifesciences, University of Hyderabad, under the supervision of Prof.Anand K. Kondapi. I further declare that this has not been submitted before for the award of degree or diploma from any Institute or University.

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CERTIFICATE

This is to certify that thesis entitled "**Design and Development of Epap-1 derived anti-HIV-1 peptides"** submitted to the University of Hyderabad by **Mr. C. BHASKAR** for the degree of Doctor of Philosophy, is based on the studies carried out by him under my supervision. This work has not been submitted before for the award of degree or diploma from any University or Institution.

Prof. Anand K. Kondapi
Supervisor

Head, Department of Biochemistry

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Date:

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ABBREVIATIONS

AIDS	: Acquired Immunodéficiency Syndrome
bp	: Base pairs
BSA	: Bovine serum albumin
Bis-acrylamide	: N,N'- methylene - bis - acrylamide
CCR5	: C-C chemokine receptor type 5
CD4	: cluster of differentiation 4
cDNA	: Complementary DNA
CO_2	: Carbon dioxide
CXCR4	: C-X-C chemokine receptor type 4
DMEM	: Dulbecco's Minimum essential media
DNA	: Deoxy ribonucleic acid
dNTPs	: Deoxynucleotidetriphosphates
dsDNA	: Double-stranded DNA
ELISA	: Enzyme linked immunosorbent assay
Epap-1	: Early pregnancy associated protein
FBS	: Fetal Bovine Serum
FCS	: Fetal calf Serum
Fig	: Figure
gp120	: Envelope glycoprotein GP120
HIV	: Human immunodeficiency Virus

IC-50	: half maximal inhibitory concentration
IFN-γ	: Interferon-gamma
IgG	: Immunoglobulin G
KDa	: Kilo daltons
LIF	: Leukemia inhibitory factor
Mab	: Monoclonal antibody
MALDI-TOF	: Matrix-assisted laser desorption/ionization
mg	: Milligram
min	: minutes
mM	: Millimolar
mRNA	: Messenger RNA
MTP	: Medical Termination of Pregnancy
FACS	: Fluorescence-activated cell sorting
MTT	: 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide
PAGE	: Polyacrylamide gel electrophoresis
PBMC	: peripheral blood mononuclear cell
PBS	: Phosphate buffer saline
PHA	: Phytohaemagglutenin
recEpap-1	: recombinant Epap-1
RITC	: rhodamine isothiocyanide
SDS	: sodium dodecyl sulphate

TEMED	: N’,N’,N’,N’- tetra methyl ethylene diamine
TRIS	: Tris (hydroxy methyl) amino methane
µg	: Microgram
µl	: Microlitre
µM	: Micromolar

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Chapter 1

Introduction

Human immunodeficiency virus (**HIV**) is a lentivirus (a member of the retrovirus family) that causes *acquired immunodeficiency syndrome* (AIDS) (Weiss 1993;Douek et al.,2009), a condition in humans in which the immune system fails, finally leading to life-threatening opportunistic infections was discovered in 1983 (Barré-Sinoussi, 1983; Broder 1984; Gallo,1984). Infection of the target cells by HIV-1 is dependent on the surface representation of cluster determinant 4 (CD4), which serves as a specific virus receptor (Klatzmann et al, 1984; Maddon et al, 1986). The high-affinity interactions between CD4 and the surface (SU) envelope glycoprotein of HIV-1 (gp120) are critical to initiate infection and viral entry (Klatzmann et al, 1984; McDougal et al, 1986). The HIV infection leads to progressive loss of CD4-expressing cells, including $CD4^+$ T lymphocytes (Barre-Sinoussi et al, 1983) that are the regulators of immune responses, and the host becomes susceptible to opportunistic infections, ultimately develops AIDS (Gupta 1993; Vento 1993; Fauci, 1988). In addition to peripheral blood lymphocytes, HIV-1 targets $CD4^+$ cells in the thymus, which is the primary site for $CD4^+$ T-cell selection (Bonyhadi et al, 1993; Stanley et al, 1993), and the lymph nodes, which are major HIV reservoirs in asymptomatic infected individuals (Pantaleo et al, 1993). The HIV infection causes cytopathic effects, including syncytium formation and cell death (Lifson et al, 1986). HIV persistently infects $CD4^+$ cells both *in vivo* and *in vitro* (Hoxie et al, 1985; Psallidopoulos et al, 1989; Schnittman et al, 1989), which result in immunological dysregulation and loss of immune competence in infected individuals (Fauci, 1993).

HIV has been found in saliva, tears, nervous system tissue and spinal fluid, blood, semen (including pre-seminal fluid, which is the liquid that comes out before ejaculation), vaginal fluid, and breast milk. However, only blood, semen, vaginal secretions, and breast milk generally transmit infection.

The initial infection with HIV occurs when the body fluids from an infected person is transferred to an uninfected one. The first stage of infection/ the primary/ or acute infection, is characterized by rapid viral replication (Mellors 1995) and in the absence of any detectable adaptive immune response, the viral titers reaches the levels >100 million copies of HIV-1 RNA/ml. This initial cycle of viral replication is important for the development of pathogenic processes i.e., the infection of range of tissue reservoirs and the destruction of HIV-1 specific $CD4^+$ T lymphocytes. The high levels of HIV-1 viremia persist for a short phase and decline by several orders of magnitude before reaching a viral setpoint. The host then generates an HIV-1-specific humoral and cellular immune responses to control initial viral replication (Mellors, 1995).

Several factors influence the viral replication during acute infection. These include the fitness of the infecting virus, host genetic factors and host immune responses. The reports of pantaleo (1993) show that there is clonal expansion of $CD8^+$ T cell (HIV- 1-specific) responses, which is indirectly proportional to the initial decline of viremia (Koup et al., 1995). These $CD8^+$ T cells eliminate HIV-1-infected cells directly by MHC class I-restricted cytolysis or indirectly by producing cytokines, chemokines or other soluble factors and restrict the generation of new viral progeny (Yang 1999). Host genetic factors play an

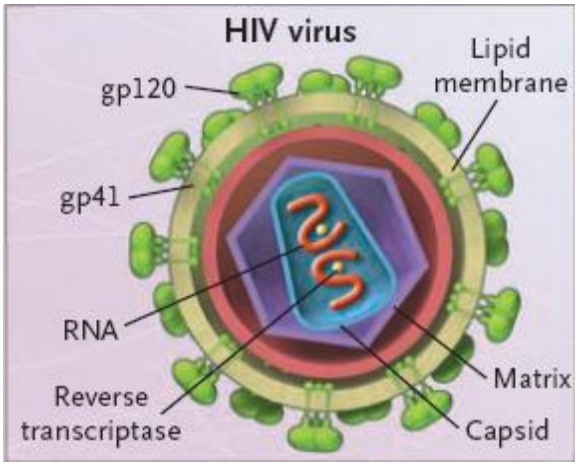
important role in both susceptibility and resistance to HIV-1 infection. One of these is the well characterized CCR5delta32, a major coreceptor for the HIV-1 entry (Samson 1996). Homozygotes for this 32 base pair deletion (CCR5delta32) do not express the receptor at cell surface and are not susceptible for infection with R5 tropic viruses (Samson 1996). HLA class I alleles have also been reported to be associated with both, lower viral set points and slower disease progression, including HLA-B27 and -B57 (Brien 2001, Kaslow 1996). Individuals expressing HLA-B57 exhibited a better control of viral replication following an acute infection (Altfeld 2003). Therefore host immune system and genetic factors influence the clinical manifestations of acute HIV-1 infection and the speed of disease progression.

Treatment

There is no cure for AIDS at this time. However, a variety of treatments are available that can help keep symptoms at bay and improve the quality of life for those who have already developed symptoms. Antiretroviral therapy suppresses the replication of the HIV virus in the body. A combination of several antiretroviral drugs, called highly active antiretroviral therapy (HAART), has been very effective in reducing the number of HIV particles in the bloodstream. This is measured by the viral load (how much virus is found in the blood). Preventing the virus from replicating can improve T-cell counts and help the immune system recover from the HIV infection. HAART is not a cure for HIV, but it has been very effective for the past 12 years. People on HAART with

suppressed levels of HIV can still transmit the virus to others through sex or by sharing needles. There is good evidence that if the levels of HIV remain suppressed and the CD4 count remains high (above 200 cells/mm^3), life can be significantly prolonged and improved. Treatment with HAART has complications. HAART is a collection of different medications, each with its own side effects.

HIV Structure



Obtained from Johnston and Fauci, NEJM, 2007

Fig 1.1

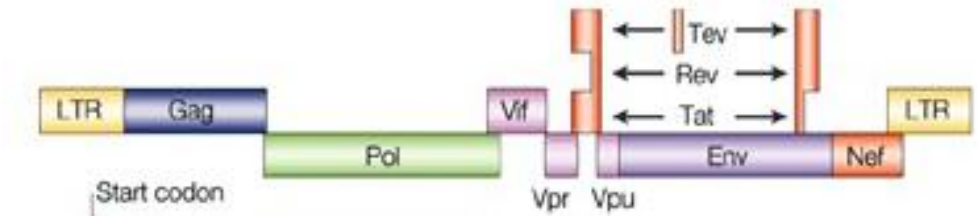
HIV is different in structure from other retroviruses. It is around 120 nm in diameter (around 60 times smaller than a red blood cell) and roughly spherical.

HIV-1 is composed of two copies of single-stranded RNA enclosed by a conical capsid comprising the viral protein p24, typical of lentiviruses (Figure 1). The RNA component is 9749 nucleotides long (Ratner et al., 1985). This is in turn surrounded by a plasma membrane of host-cell origin. The single-strand RNA is

tightly bound to the nucleocapsid proteins, p6, p7 and enzymes that are indispensable for the development of the virion, such as reverse transcriptase and integrase. The nucleocapsid (p7 and p6) associates with the genomic RNA (one molecule per hexamer) and protects the RNA from digestion by nucleases. A matrix composed of an association of the viral protein p17 surrounds the capsid, ensuring the integrity of the virion particle. Also enclosed within the virion particle are Vif, Vpr, Nef, p7 and viral Protease. The envelope is formed when the capsid buds from the host cell, taking some of the host-cell membrane with it. The envelope includes the glycoproteins gp120 and gp41.

As a result of its role in virus-cell attachment, the structure of the virus envelope spike, consisting of gp120 and gp41, is of particular importance. It is hoped that determining the envelope spike's structure would contribute to scientific understanding of the virus and its replication cycle, and help in the creation of a cure. The first model of its structure was compiled in 2006 using cryo-electron microscopy and suggested that three copies of gp120-gp41 heterodimers are thought to form a trimer as the envelope spike (Zhu et al., 2006). However, published shortly after was evidence for a single-stalk "mushroom" model, with a head consisting of a trimer gp120s and a gp41 stem, which appears as a compact structure with no obvious separation between the three monomers, anchoring it to the envelope (Zanetti et al., 2006). There are various possibilities as to the source of this difference, as it is unlikely that the viruses imaged by the two groups were structurally different (Subramaniam, 2006). More recently, further evidence backing up the heterodimer trimer-based model has been found (Zhu et al., 2008).

Genome Organization



Obtained from Peterlin and Trono, Nat. rev Immunology,2003

Fig 1.2

HIV has several major genes coding for structural proteins that are found in all retroviruses, and several nonstructural ("accessory") genes that are unique to HIV. The *gag* gene provides the basic physical infrastructure of the virus, and *pol* provides the basic mechanism by which retroviruses reproduce, while the others help HIV to enter the host cell and enhance its reproduction. Though they may be altered by mutation, all of these genes except *tev* exist in all known variants of HIV

- *gag* (group-specific antigen): codes for the Gag polyprotein, which is processed during maturation to MA (matrix protein, p17); CA (capsid protein, p24); SP1 (spacer peptide 1, p2); NC (nucleocapsid protein, p7); SP2 (spacer peptide 2, p1) and p6.

- *pol*: codes for viral enzymes reverse transcriptase, integrase, and HIV protease.

- *env* (for "envelope"): codes for gp160, the precursor to gp120 and gp41, proteins embedded in the viral envelope which enable the virus to attach to and fuse with target cells.

- Transactivators: *tat*, *rev*, *vpr*

- Other regulators: *vif*, *nef*, *vpu*

- *tev*: This gene is only present in a few HIV-1 isolates. It is a fusion of parts of the *tat*, *env*, and *rev* genes, and codes for a protein with some of the properties of tat, but little or none of the properties of rev.

RNA secondary structure

Several conserved secondary structure elements have been identified within the HIV RNA genome. These include the trans-activating responsive (TAR) element located within the 5' end of the genome and the HIV Rev response element (RRE) within the env gene (Berkhout,1992; Paillart et al., 2002). RNA secondary structures have been proposed to affect the HIV life cycle by altering the function of HIV protease and reverse transcriptase, although not all elements identified have been assigned a function.

An RNA secondary structure determined by 2' hydroxyl acetylation and primer extension (SHAPE) analysis has shown to contain three stem loops and is located between the HIV protease and reverse transcriptase genes. This *cis* regulatory RNA has been shown to be conserved throughout the HIV family and is thought

to influence the viral life cycle (Wang et al., 2008). The complete structure of an HIV-1 genome, extracted from infectious virions, has been solved to single-nucleotide resolution (Watts et al., 2009).

HIV Tropism

HIV Tropism refers to the cell type that the human immunodeficiency virus (HIV) infects and replicates in. HIV tropism of a patient's virus is measured by the Trofile assay. HIV can infect a variety of cells such as CD4+ helper T-cells and macrophages that express the CD4 molecule on their surface. HIV-1 entry to macrophages and T helper cells is mediated not only through interaction of the virion envelope glycoproteins (gp120) with the CD4 molecule on the target cells but also with its chemokine coreceptors.

Macrophage (M-tropic) strains of HIV-1, or non-syncitia-inducing strains (NSI) use the beta-chemokine receptor CCR5 for entry and are thus able to replicate in macrophages and CD4+ T-cells (Barré-Sinoussi et al., 1983). The normal ligands for this receptor, RANTES, macrophage inflammatory protein (MIP)-1-beta and MIP-1-alpha, are able to suppress HIV-1 infection *in vitro*. This CCR5 coreceptor is used by almost all primary HIV-1 isolates regardless of viral genetic subtype.

T-tropic isolates, or syncitia-inducing (SI) strains replicate in primary CD4+ T-cells as well as in macrophages and use the alpha-chemokine receptor, CXCR4, for entry (Barré-Sinoussi et al., 1983). The alpha-chemokine, SDF-1, a ligand for

CXCR4, suppresses replication of T-tropic HIV-1 isolates. It does this by down regulating the expression of CXCR4 on the surface of these cells.

Viruses that use only the CCR5 receptor are termed R5, those that only use CXCR4 are termed X4, and those that use both, X4R5. However, the use of coreceptor alone does not explain viral tropism, as not all R5 viruses are able to use CCR5 on macrophages for a productive infection (Barré-Sinoussi et al., 1983). HIV can also infect a subtype of dendritic cells (Gallo, 1983), MDC-1, which probably constitute a major reservoir that maintains infection when T helper cell numbers have declined to extremely low levels.

Life Cycle

Entry

HIV can only replicate (make new copies of itself) inside human cells. The process typically begins when a virus particle bumps into a cell that carries on its surface a special protein called CD4. The envelope spikes gp-120 on the surface of the virus particle interacts with its high affinity receptor CD4 on the target cell via a conserved site on it. Then the V3-loop of gp-120 binds to the core receptor CD26 on the target cell. Proteolyic cleavage of the V3 loop by CD24 induces a conformational change in gp120 & gp41 exposing the fusogenic domain in gp-41. This fusogenic domain then mediated fusion between the viral envelop and target cell plasma membranes.The contents of the HIV particle are then released into the cell, leaving the envelope behind.

Reverse Transcription and Integration

Following the nucleocapsid into the host cell cytoplasm, the core proteins are removed, releasing single stranded RNA and RT which copies the single stranded RNA forming RNA-DNA hybrid. After the original RNA template is partially degraded by ribonucleaseH, the synthesis of the second DNA strand proceeds. The double stranded DNA is then translocated to the nucleus and integrated into the host chromosomal DNA by the viral integrase enzyme which is packaged together with the RT in the virion. Once integrated the viral DNA is permanently associated with the host cell DNA. In this form, the virus can remain latent for years together without expressing its genes. Once integrated, the HIV DNA is known as provirus.

Transcription and Translation

HIV provirus may lie dormant within a cell for a long time. But when the cell becomes activated, it treats HIV genes in much the same way as human genes. First it converts them into messenger RNA (using human enzymes). Then the messenger RNA is transported outside the nucleus, and is used as a blueprint for producing new HIV proteins and enzymes.

Assembly, Budding and Maturation

Among the strands of messenger RNA produced by the cell are complete copies of HIV genetic material. These gather together with newly made HIV proteins and enzymes to form new viral particles, which then begin to assemble within the

host cell, begin to assemble within the host cell, the host cell plasma membrane is modified by insertion of gp-41 & associated gp-120. The viral RNA and core proteins then assemble beneath the modified membrane, acquiring the modified membrane as its envelope during budding. The enzyme protease plays a vital role at this stage of the HIV life cycle by chopping up long strands of protein into smaller pieces, which are used to construct mature viral cores.

The newly matured HIV particles are ready to infect another cell and begin the replication process all over again. In this way the virus quickly spreads through the human body. And once a person is infected, they can pass HIV on to others in their bodily fluids.

Structure of envelope

The HIV envelope spike is formed as a complex between gp120 and gp41 (Wyatt and Sodroski, 1998). The gp120 unit mediates attachment of the virus to the target cell, whereas gp41 is required for the fusion of virus and target cell membranes. During HIV infection, the viral envelope spike is first synthesized as a single polypeptide precursor (Wyatt and Sodroski, 1998). In the Golgi complex, the protein subsequently oligomerizes and undergoes extensive glycosylation. The glycosylation process, which is required for proper folding and conformational stability of the envelope glycoprotein (Fenouillet et al., 1994), mainly involves the attachment of N-linked highmannose- type oligosaccharides to the protein backbone. As the glycoprotein is transported through the Golgi, accessible glycan moieties are trimmed and modified by various cellular enzymes (Wyatt and

Sodroski, 1998). These modifications generate complex-type oligosaccharides; glycans that are relatively inaccessible to modifying enzymes remain as high-mannose type glycans (Leonard et al., 1990). The resulting glycoprotein, which has a molecular mass of ~160 kDa, is cleaved in the trans-Golgi network by furin or equivalent endoproteases into gp120 and gp41 (Wyatt and Sodroski, 1998). The gp120-gp41 complexes, which remain associated through weak non-covalent interactions, are initially expressed at the surface of infected cells. During the HIV budding process, the gp120-gp41 complexes are then incorporated into the virus envelope and displayed on its surface as viral spikes (Wyatt and Sodroski, 1998).

Organization of gp120 on the Viral Surface

Experimental evidence suggests that the functional unit of the envelope spike is a heterodimeric trimer complex of gp120 and gp41. Electron tomography study revealed structures on the surface of negatively stained virions of SIV and HIV-1 appear to be tri-lobed envelope glycoproteins (Zhu et al.,2003). The HIV core matrix that interacts with gp41 is organized in a trimeric configuration (Hill et al., 1996), and the crystal structures of HIV-1 gp41 cores resemble the transmembrane proteins of other viruses that have been shown to display trimeric envelope spikes (Chan et al., 1997; Tan et al., 1997; Weissenhorn et al, 1997).There are evidences that other envelope species may be present on the surface of HIV-1. Atomic force microscopy analyses have failed to reveal any uniform trimeric envelope species on the surface of virions (Kuznetsov et al., 2003). It was shown that viruses can be captured onto ELISA plate wells using

antibodies that are unable to neutralize viral particles in solution (Poignard et al., 2003; Herrera et al., 2005). Though trimers may likely represent the functional envelope spike, both functional and nonfunctional forms of the envelope may be present on the virion surface. These nonfunctional envelope entities may be monomers, dimers, or tetramers and could possibly arise as the result of (*a*) the dissociation of functional gp120-gp41 complexes, which could perhaps cause gp120 to be shed from the viral surface, or (*b*) inefficient trimerization of the spike in the Golgi (Wyatt and Sodroski, 1998; Parren et al., 1997; Burton and Montefiori, 1997).

Topology of gp120

Based on comparative sequence analyses, gp120 is divided into five conserved (C1– C5) and five variable (V1–V5) segments (Modrow et al, 1987; Willey et al., 1986). Before obtaining the gp120 core crystal structures, many topological features of monomeric and oligomeric gp120 were deduced from antibody binding experiments and mutagenic studies (Helseth et al., 1991; Kowalski et al., 1987; Moore et al., 1994(a); Pollard et al., 1992; Moore and Sodroski ,1996;Olshevsky et al., 1990; Moore et al., 1994(b)). The C1 and C5 regions were thought to be the main areas on gp120 for contact with gp41, as these regions are accessible to antibody on monomeric gp120 but not on gp120- gp41 complexes (Helseth et al., 1991; Moore et al., 1994(a); Moore et al., 1994(b)). Major segments of the C2, C3, and C4 regions were thought to form a hydrophobic core buried within the gp120 molecule (Moore et al., 1994(a); Pollard et al., 1992). It was proposed that this gp120 core harbors several discontinuous neutralizing

antibody epitopes that overlap the binding sites for CD4 and the coreceptor (Moore et al., 1994(a); Pollard et al., 1992; Moore and Sodroski ,1996;Olshevsky et al., 1990). In contrast to the conserved regions, the variable regions (mainly V1, V2, and V3) were well exposed on the surface of monomeric gp120 (Moore et al., 1994(a)). Deletion of V1/V2 and V3 generally increases the binding affinity of antibodies to epitopes that overlap the binding sites for CD4 and the coreceptor, which suggests that these variable regions may cover conserved epitopes from antibody recognition (Sullivan et al., 1998; Wyatt et al, 1995; Wyatt et al., 1993; Cao et al., 1997). Deletion of the V4 region has been shown to disrupt gp160 folding (Pollard et al., 1992; Wyatt et al., 1993), V4 also seems to tolerate insertion of foreign antibody epitopes (Ren et al., 2005). Determination of the structures of gp120 molecules from HIV and SIV has supported many of the interpretations made from these earlier observations.

Molecular Structure of gp120

Four crystal structures of HIV-1 gp120 and one of SIV gp120 have been reported (Chen et al., 2005; Kwong et al., 2000; Kwong et al., 1998; Huang et al., 2005). All five structures are of the gp120 core; i.e., the structures lack the V1/V2 and V3 variable regions, and the N and C termini are truncated. The HIV-1 gp120 structures were determined in complex with the D1D2 fragment of CD4, whereas the SIV gp120 structure was solved unliganded. Because all crystal structures determined so far are of monomeric gp120, they may not adequately represent the structure of oligomeric gp120 on the virus. Despite these and other caveats the structures do provide insight into the conformational flexibility of monomeric

gp120 as well as the locations of receptor-binding sites and putative antibody epitopes on gp120.

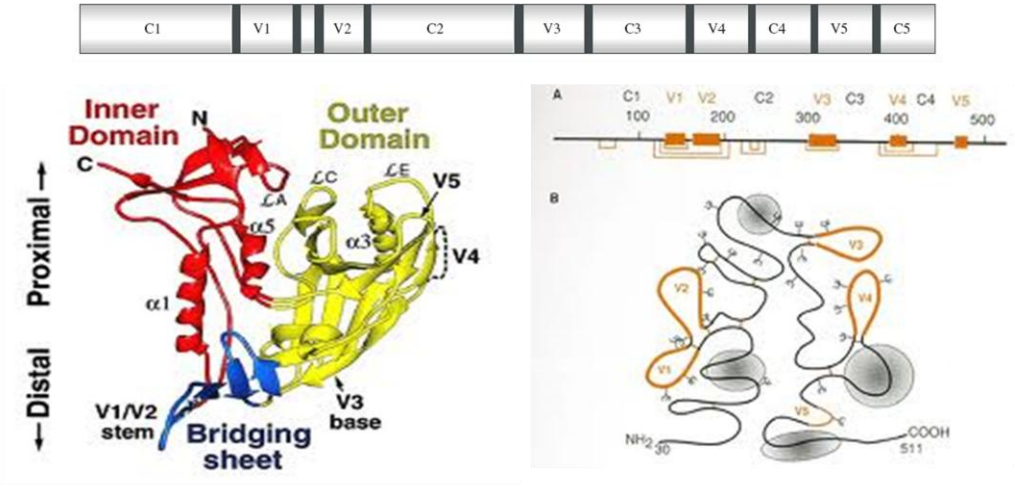
Crystal Structure of HIV-1 gp120 Core in Complex with CD4

The structure of the gp120 core from the laboratory-adapted virus HXB2 was the first determined in complex with CD4 (**Figure 1.4**) (Kwong et al., 1998). HXB2 is highly sensitive to antibody neutralization. The second crystal structure was that of CD4 complexed with the gp120 core of the primary virus YU-2 (Kwong et al., 2000(a)), which exhibits a marked resistance to antibody neutralization. The two gp120 core structures are virtually superimposable (Kwong et al., 2000(a)), which is consistent with earlier predictions that the ability of HIV to resist antibody neutralization may manifest mainly in the context of the gp120 quaternary structure on the viral surface rather than in the monomeric gp120 form (Moore et al., 1995). Based on these CD4-bound structures, gp120 is organized into three general areas (**Figure 1.5*a***): (*a*) the inner domain,(*b*) the outer domain, and (*c*) the bridging sheet (Wyatt et al., 1998).

Inner domain, outer domain, and bridging sheet

The inner domain is formed mainly by the C1 and C5 regions and is devoid of glycans (Kwong et al., 1998; Wyatt et al., 1998), which implies that these regions function as the major contact interface with the gp41 transmembrane unit. The outer domain, in contrast, is largely covered by glycans (Kwong et al., 1998; Wyatt et al., 1998). Modeling of the gp120 oligomer suggests that these glycans cover large sections of the outer surface of the spike to lower its overall

immunogenicity (Wyatt et al., 1998; Kwong et al., 2000(b)). Comparison of the liganded gp120 structure of HIV-1 (**Figure 1.5*a***) and the unliganded gp120 structure of SIV (**Figure 1.5*b***) shows that the respective outer domains are highly similar (Chen et al., 2005). The



HIV gp120 glycoprotein with five constant (C1–C5) and five variable (V1–V5) regions

Inner domain - C1 – C5 regions – no glycans- binds to gp41
Outer domain - V1 – V5 regions – glycosylated – binds to CD4
Bridging sheet – 4 β strands – binds to CCR5

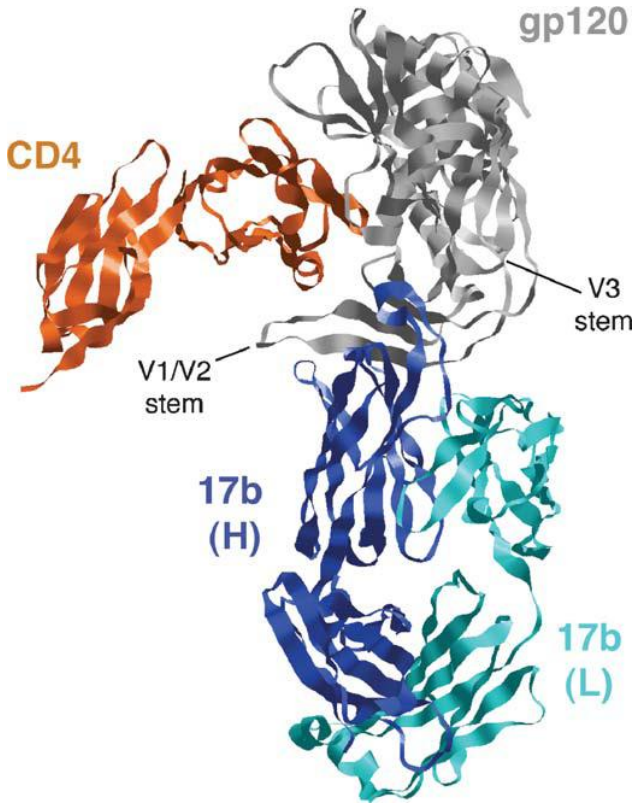
Adapted from Wyatt et al., Nature, 1998

Fig 1.3

conformation of the inner domain in the unliganded structure deviates significantly from the conformation in the liganded structures. This observation suggests that the inner domain may have significant conformational flexibility in the absence of CD4. Comparison of the inner domain substructures in the unliganded and liganded core structures suggests that, upon CD4 binding, these substructures are repositioned somewhat independently of each other, rather than a shift of the inner domain as a single unit (Chen et al., 2005). The large structural

rearrangements associated with the repositioning of inner domain substructures correclates with the large negative entropy and enthalpy changes measured by isothermal titration microcalorimetry (Myszka et al., 2000). It is noteworthy that the majority of gp120 conformational shifts resulting from CD4 binding are in the portion of gp120 that interacts with gp41. Thus, these conformational changes may be necessary to lock the coreceptor-binding site into a fixed conformation and also trigger gp41 into initiating the fusion process (Wyatt and Sodroski, 1998). The conformational changes that occur within the inner domain also affect the formation of the bridging sheet, which links the inner and outer domains. In the CD4- liganded gp120 conformation, the bridging sheet is folded into a compact antiparallel, four-stranded β-sheet (β2-β3 and β20-β21)(Kwong et al., 1998; Wyatt et al., 1998). However, in the unliganded structure, the β-strands that constitute the bridging sheet lie in separate pairs at a distance of approximately 20 ˚A (Chen et al., 2005); the two β-strands (β2-β3) that constitute the V1/V2 stem are located in the vicinity of the inner domain, whereas the other two strands (β20-β21) are situated near the outer domain in approximately the same location as they are on the liganded structure. Conformational changes that occur within the inner domain upon CD4 binding would result in a 40 ˚A shift of the V1/V2 stem to form the bridging sheet (Chen et al., 2005). However, molecular modeling of the liganded and unliganded gp120 structures suggests that the unliganded structure may be one of many conformations that gp120 may adopt in the absence of CD4 (Pan et al., 2005). In fact, the models suggest that the β2-β3 strands may oscillate from the conformation observed in the unliganded structure

to a conformation resembling the CD4-bound structure via a series of intermediate conformers (Pan et al., 2005).



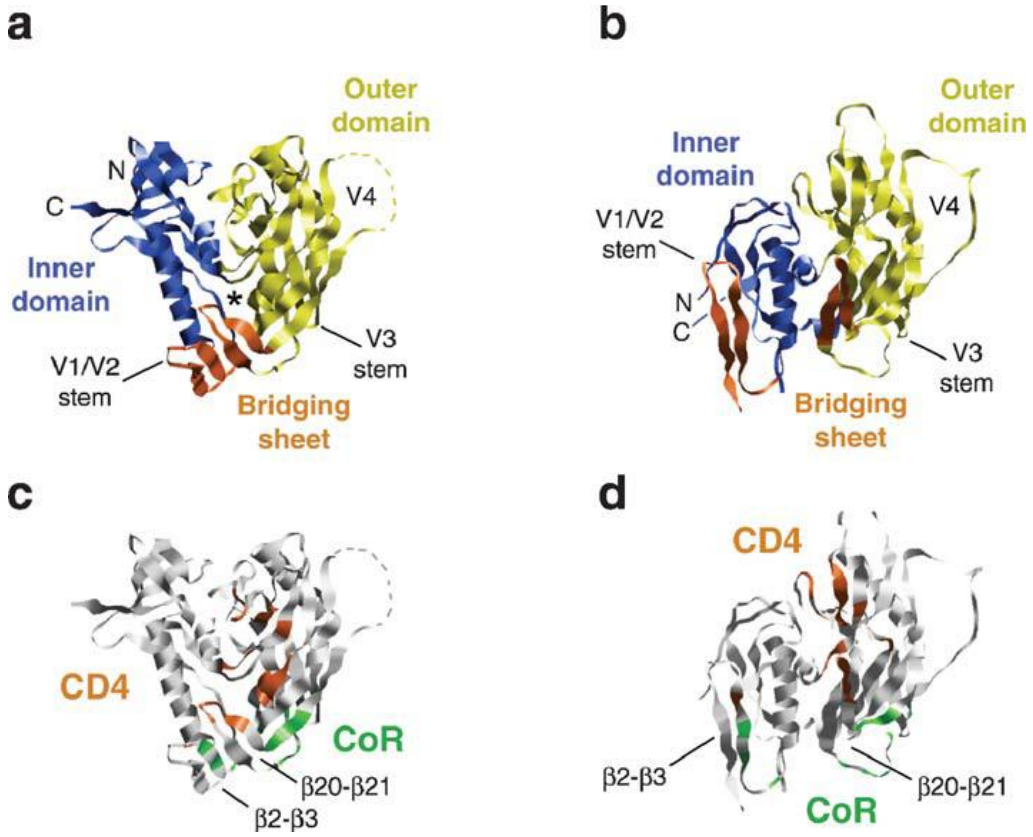
Adapted from Kwong et al., Nature 393:648–59

Fig 1.4: Crystal structure of HIV-1 gp120 complexed to CD4 and an antibody antigen-binding fragment (Fab). The gp120 core of HXB2 (Protein Data Bank ID 1G9M) (*gray*), CD4 (*orange*), the antibody heavy chain (H) (*blue*) and light chain (L) (*green*) are shown.

Epitope-mapping studies of antibodies to the CD4-binding site (CD4bs) also suggest that gp120 can adopt a conformation resembling the CD4-bound form relative to the conformation of the unliganded structure.

The CD4-binding site

The binding site for CD4 on the liganded gp120 structure is formed by the interface between the inner domain, bridging sheet, and outer domain (Kwong et al., 1998; Wyatt et al., 1998). At the center of this interface lies a hydrophobic cavity that has been dubbed the Phe43 cavity (**Figure 1.5*a***) (Kwong et al., 1998). However, most of the CD4 contact residues are located on the outer domain of the liganded HIV-1 gp120 structures and form a contiguous binding region (**Figure 1.5*c***). On the unliganded SIV gp120 structure no such region is discernible (**Figure 1.5*d***) (Chen et al., 2005), assuming that equivalent residues in SIV and HIV-1 contact CD4. On the unliganded SIV gp120 core, many of the residues that are presumed to contact CD4 upon complexation with gp120 are located near or within a long cavity that is formed primarily by portions of the inner and outer domains and the β20-β21 segment of the bridging sheet. The location of these conserved residues minimizes their immediate recognition by antibodies, while preserving the ability to contact CD4. In this regard, the curved structure of the D1D2 fragment of CD4 is particularly noteworthy (**Figure 1.4**); it permits CD4 to curl over the outer domain, so residues located near or within the cavity formed by the inner and outer domains can be reached. Given that the CD4bs is not coherently present on the unliganded structure, it indeed seems likely that gp120 transiently forms conformations that are reflective of the liganded structure; upon interaction with CD4, the gp120 structure is locked in the bound conformation.



Obtained from PDB ID 2BF1, HXB2.

Fig 1.5: Comparison of the crystal structures of HIV-1 and SIV gp120 core. (*a*) Structure of the CD4-liganded HIV-1 gp120 core (HXB2), viewed from the perspective of CD4. The gp120 inner domain (*blue*), outer domain (*yellow*), and bridging sheet (*orange*) are shown. The locations of various gp120 regions are also denoted. (*b*) Structure of the unliganded SIV gp120 core (Protein Data Bank ID 2BF1), viewed from the perspective of CD4 as in (*a*). (*c*) HIV core gp120 in same orientation as (*a*), depicting CD4 contact residues (*orange*) and residues that influence coreceptor binding (*green*). (*d*) SIV gp120 core in the same orientation as in (*b*), colored according to the scheme for HIV gp120 in (*c*).

The coreceptor-binding site

The region that is important for the interaction with the β-chemokine receptor CCR5 has been mapped to residues in the bridging sheet and near the V3 stem (Rizzuto et al., 1998; Rizzuto and sodroski, 2000). These residues lie close together on the liganded HIV-1 gp120 structure, but the equivalent residues on the unliganded SIV gp120 structure are separated into two areas (**Figure 1.5*c*,*d***) (Chen et al., 2005). These differences are consistent with the notion that CD4 binding is required to lock these areas into a contiguous binding site. The fact that the coreceptor site is not presented until after CD4 binding suggests that the site may be susceptible to antibody recognition. Several studies have shown that HIV strains that do not require CD4 for entry are highly sensitive to antibody neutralization (Hoffman et al., 1999; Edwards et al., 2001; Kolchinsky et al., 2001; Decker et al., 2005). Due to neutralizing antibody–driven selection pressure in vivo, the prevalence of such viruses during infection is likely low. However, in the absence of circulating neutralizing antibodies, e.g., in the central nervous system, such viruses may occur more frequently (Decker et al., 2005; Martin et al., 2001). We note here that the unliganded SIV gp120 structure is derived from strain SIVmac32H. This simian virus is able to infect CD4-negative target cells in vitro with medium efficiency (Reeves et al., 1999). The gp120 from this strain may thus harbor certain structural features that are not observed in the gp120 of HIV-1 or SIV strains that require CD4 for entry.

Antibody epitopes on HIV-1 gp120

The b12 epitope : This is the most broadly cross neutralizing epitope present on the CD4 binding site. The possible aminoacids that interfere with gp120-CD4 binding are Asp 457 , Pro 470 .

The 2G12 epitope : This present on the silent face of gp120. The aminoacids that aid in the neutralization are Asn 295, Thr 297, Ser 334, Asn 386, Asn 392, Asn 397. The mechanism of virus neutralization is unknown.

The 447–52D epitope: This is another neutralization restricted epitope on the V3 loop region which is often masked by V1 and V2 regions in various viral clades. The aminoacids responsible for the activity are Glu 370 , Tyr 384.

There are other CD4 – induced epitopes against which two antibodies 17b and 48d are produced that will interfere with coreceptor binding. The aminoacids involved here are Asn 88, Lys 117, Lys 121 ,Lys 207, Ser 256, Thr 257, Asn 262, DV3, Glu 370, Glu 381, Phe 382, Arg 419, Ile 420, Lys 421, Gln 422, Ile 423, Trp 427, Tyr 435, Pro 438, Met 475.

Mechanism of fusion

Infection of target cells by HIV is a complex, multi-stage process involving attachment to host cells and CD4 binding, coreceptor binding, and membrane fusion. The initial interaction between HIV and a target cell may be facilitated by nonspecific interactions between positively charged domains on the gp120 protein and negatively charged proteoglycans on the cellular membrane (Mondor et al., 1998; Moulard et al., 2000) or by specific interactions with cell surface lectin

binding proteins such as DCSIGN. Such attachment factors can enhance the efficiency of virus infection (Geijtenbeek et al., 2000). HIV interaction with CD4 occurs at a structurally conserved, recessed surface on gp120 that is formed by epitopes that are discontinuous in the primary protein sequence (Kwong et al., 1998). Upon binding to CD4, gp120 undergoes a conformational shift that has several important consequences. First, two sets of β-sheets that are spatially separated in unbound gp120 are brought together by CD4 binding into a four-stranded β-sheet minidomain called the bridging sheet (Chen et al., 2005; Kwong et al., 1998). Second, CD4 binding results in movement and exposure of the V1/V2 and V3 loop structures. Third, binding of CD4 changes the orientation of gp120 such that the bridging sheet and the V3 loop are directed towards the host cell membrane, where they can subsequently interact with coreceptor (Huang et al., 2005; Trkola et al., 1996;Wu et al., 1996).Thus, CD4 binding is a prerequisite to the formation and exposure of the coreceptor binding site of gp120. In humans, the primary coreceptors for HIV-1 are the chemokine receptors CCR5 and CXCR4, members of the seven-transmembrane G protein-coupled receptor family (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996; Oberlin et al., 1996; Zhang et al., 1998). These proteins are integral membrane proteins with seven transmembrane helices, an extracellular N-terminus and three extracellular loops (ECLs) that form a small pocket. The N- terminus of chemokine receptors contains sulfated tyrosine residues and elements within and around ECL2 that are critical for gp120 binding (Atchison et al., 1996; Edinger et al., 1997; Rucker et al., 1996). These spatially

separated domains of CCR5 interact with distinct regions of gp120: the N-terminus with the bridging sheet and the base of the V3 loop, and ECL2 with the tip of the V3 loop (Basmaciogullari et al.,2002; Cormier and Dragic, 2002; Cormier et al., 2001; Hoffman et al, 1999; Huang et al., 2007). The relative dependence on the N-terminus compared with the ECL2 loop appears to vary for different R5 viruses. The N- terminal interaction has now been investigated using a crystal structure between gp120 and an unusual sulfated antibody, 412d, that mimics the N-terminus of CCR5 (Huang et al., 2007). These studies suggest that prior to coreceptor binding, the V3 loop of gp120 is flexible and located close to the target cell membrane. Engagement of the N - terminus by gp120 requires the formation of a conserved sulfotyrosine binding pocket and converts the V3 stem from a flexible structure into a rigid β-hairpin. In contrast, the interaction between the tip of the V3 loop and ECL2 is less well defined, but contact between these regions is particularly important for HIV entry (Lee et al., 1999; Platt et al., 2001; Samson et al., 1997;Wu et al., 1997). These data are consistent with a crystal structure of gp120 in which the V3 loop is found to extend nearly 30Å from its base towards the cellular membrane, where it could presumably make contact with the Extracellular loops (ECLs) of the coreceptor (Huang et al., 2005). Binding of gp120 to CXCR4 appears to occur in a similar fashion (Basmaciogullari et al., 2002; Chabot et al., 1999; Doranz et al., 1999; Lin et al., 2003a), although the V3 loop of viruses that utilize CXCR4 tend to be more positively charged, particularly at positions 11, 24, and 25 of V3 (De Jong et al., 1992; Fouchier et al.,1992; Milich et al., 1993; Xiao et al., 1998). Binding of

gp120 to coreceptor triggers further conformational changes in the envelope trimer that result in the exposure of the hydrophobic fusion peptide of gp41 and its insertion into the host cell plasma membrane. After the insertion of the fusion peptide, the heptad repeat regions HR1 and HR2 of gp41 undergo a highly energetically favorable rearrangement in which they fold back on each other. In a functional trimer spike, this forms a six-helix bundle structure where the three HR1 domains form a central coiled-coil and the three HR2 domains wrap around in an anti-parallel direction around the central coil (Ch an et al., 1997; Weissenhorn et al., 1997). This structural rearrangement brings the transmembrane region of gp41, which is embedded in the viral membrane, into close proximity to the fusion peptide, which is inserted into the host cell membrane. This juxtaposition results in the formation of the fusion pore, allowing the viral capsid to enter the cell. The entry process of HIV has traditionally been thought to occur at the plasmamembrane of the cell, but recent evidence suggests that endocytosis of viral particles may be required for complete fusion (Miyauchi et al., 2009).

Entry inhibitors

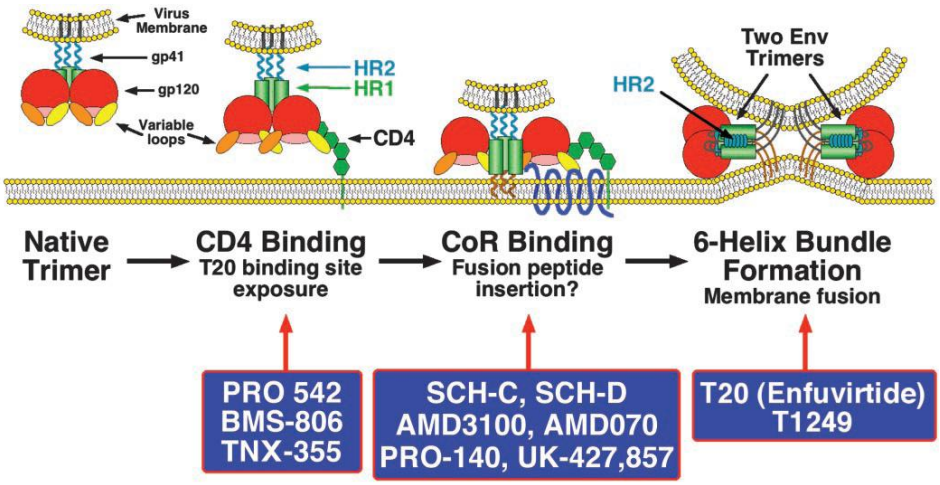
Antiretroviral (ARV) drugs are broadly classified by the phase of the retrovirus life-cycle that the drug inhibits. They include nucleoside and nucleotide reverse transcriptase inhibitors (NRTI), Non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors, integrase inhibitors, entry inhibitors and maturation

inhibitors. In turn entry inhibitors are classified into 3 types depending upon their mode of action namely,

a. Inhibitors blocking the gp120–CD4 interaction

b. Inhibitors blocking the gp120–coreceptor interaction

c. Inhibitors blocking gp41-mediated membrane fusion



Adapted from Moore and Doms , PNAS, 2003

Fig 1.6

a. Inhibitors blocking the gp120–CD4 interaction

In contrast to cell associated CD4, which is required for HIV entry, soluble CD4 (sCD4) was found to inhibit HIV entry at high doses *in vitro*. But clinical administration of this protein did not reduce viral loads in HIV-infected patients, and detailed analysis revealed that the levels of sCD4 achieved in patients were not sufficient to inhibit primary HIV isolates (Daar et al., 1990). Still, class of sCD4 derivatives and CD4 mimics, including the PRO-542 CD-IgG2 tetrameric fusion protein and the NBD-556 and NBD-557 compounds (Allaway et al., 1995;

Arthos et al., 2002; Martin et al.,2003; Schon et al., 2006; Trkola et al., 1995) (Fig. 3) were developed. These compounds appear to work by inducing a short-lived activated state of gp120 that spontaneously and irreversibly converts into a nonfunctional conformation. In contrast, the activated intermediate of gp120 generated by cell-surface CD4 is far more stable (Haim et al., 2009). Other small-molecule inhibitors of the CD4-gp120 binding interaction are the BMS-378806 and BMS-488043 compounds. These agents also target the conserved CD4-binding site of gp120, but their precise mechanism of action is unclear. Some studies suggest that these compounds compete with sCD4 for binding to gp120 (Hoet al., 2006; Lin et al., 2003b), while others indicate that they do not block sCD4 binding (Schon et al., 2006) and may exert their antiviral effects by preventing conformational changes in gp120 upon CD4 engagement (Si et al., 2004). The clinical utility of BMS-806 is limited by a low genetic barrier to resistance, as 1–2 amino acid changes of gp120 result in 40–500-fold resistance to drug (Lin et al., 2003b). Amino acids that confer resistance to BMS-806, including Trp 112, Thr 257, Ser 375, Phe 382, and Met 426, line the 'phenylalanine 43 cavity' on gp120 that is involved in stabilization of the CD4-bound conformation of gp120 (Madani et al., 2004). Although BMS-806 was discontinued in phase II clinical development, is has shown success in an animal model as a potential topical microbicide (Veazey et al., 2005). The humanized antibody ibalizumab (TNX-355) binds to the D2 domain of CD4 and blocks CD4-induced conformational changes in gp120 (Moore et al., 1992). This agent has been demonstrated to reduce viral loads and increase CD4 T cell counts in

combination with optimized background therapy (Kuritzkes et al., 2004), but is not orally bioavailable.

b. Inhibitors blocking the gp120–coreceptor interaction

The discovery of CCR5 and CXCR4 as the critical coreceptors for HIV entry (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996; Oberlin et al., 1996; Zhang et al., 1998) was rapidly followed by the identification of a subset of individuals that were homozygous for an inactivating deletion for CCR5,Δ32-*ccr5*, which conferred high-level resistance to HIV-1 infection without significant immunological consequences (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996). Coupled with the observation that patients heterozygous for Δ32-*ccr5* had delayed rates of disease progression (Dean et al., 1996; Huang et al., 1996; Michael et al., 1997; Rappaport et al., 1997; Samson et al., 1996), these findings indicated that pharmacological blockade of the gp120–CCR5 interaction could be an effective strategy for inhibiting HIV infection. A number of naturally occurring ligands for the CCR5 receptor block HIV infection, including CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) (Cocchi et al., 1995). These chemokines exert their antiviral effects by blocking Env binding to CCR5 and by inducing the internalization of CCR5 from the cell surface (Alkhatib et al., 1997), but have potentially undesirable agonist activity on CCR5. Several RANTES derivatives, including AOP-RANTES, NNY-RANTES, and PSC-RANTES, have been developed in an effort to maintain anti- HIV activity while reducing or

eliminating the agonistic effects on CCR5 (Mosier et al., 1999; Simmons et al., 1997). PSC-RANTES is in development as a potential microbicide for HIV (Cerini et al.,2008; Lederman et al., 2004). These agents compete with gp120 for binding to coreceptor and therefore are competitive antagonists of HIV infection. Another strategy for inhibiting gp120–coreceptor interactions is binding of small compounds to a hydrophobic pocket in the transmembrane helices of CCR5 and are believed to exert their antiviral effects by altering the conformation of the extracellular loops that HIV interacts with during coreceptor binding. These agents do not bind to the same binding site as gp120, making them allosteric rather than competitive inhibitors. Many small-molecule antagonists have demonstrated efficacy against HIV replication *in vitro*, and three of these agents have been tested extensively in humans. Aplaviroc (GW873140) was tested in phase IIb studies before reports of idiosyncratic hepatotoxicity halted its development in 2005(Nichols et al., 2008). Vicriviroc (SCH-D, SCH-417690) is currently in phase III clinical trials, and is a second-generation compound based on ancriviroc (SCH-C), which was discontinued after being associated with an elongatedQTcardiac interval in clinical trials (Strizki et al., 2005; Tagat et al., 2004). Maraviroc (UK-427857) was approved in 2007 by the FDA for the treatment of HIV-infected patients with viral replication and HIV strains resistant to multiple antiretroviral agents. Maraviroc is the result of medicinal chemistry optimization of the compound UK-107543 and is effective against CCR5-using (R5-tropic) HIV strains in the low nanomolar range (Dorr et al.,2005). It is administered orally twice daily, with dosages that vary depending on the presence

of strong CYP3A inducers or inhibitors in the antiviral regimen. Antibodies that block the CCR5 receptor and prevent HIV infection have also been developed. PRO-140 is a humanized mouse anti-CCR5 antibody that prevents gp120 from engaging CCR5 but does not block CCR5 ligand activity. It has demonstrated potent efficacy against CCR5-tropic HIV strains both *in vitro* and in HIV infected adults (Jacobson et al., 2008; Trkola et al., 2001). It is currently in phase II clinical trials. Unlike CCR5, CXCR4 is essential for multiple physiological processes. In mice, knockout of the CXCR4 gene or its ligand, CXCL12 (SDF-1), is embryonic lethal due to defects in vascularization, hematopoiesis, cardiogenesis, and abnormal cerebellar development (Tachibana et al., 1998; Zou et al., 1998). In humans, heterozygous truncating mutations in the cytoplasmic tail of CXCR4 have been associated with WHIM syndrome, an immunodeficiency syndrome characterized bywarts, hypogammaglobulinemia, infection, and myelokathexis (Hernandez et al., 2003). Several polypeptide mimics of the natural ligand for CXCR4, CXCL-12, have been developed. These compounds, including T-22, T-134, T-140, and ALX40-4C, act by binding specifically to the CXCR4 receptor and preventing gp120 binding (Arakaki et al., 1999; Doranz et al., 2001; Tamamura et al., 1994, 1998). ALX40-4C was tested in humans prior to the identification of CXCR4 as a coreceptor for HIV, and despite being well-tolerated did not have a significant effect on reduction of HIV viral loads. However, the majority of patients in this study were later found to have CCR5-tropic strains of HIV (Doranz et al., 2001). Small-molecule antagonists and partial agonists of CXCR4 are under development. The bicyclam analog AMD3100

demonstrates potent activity against CXCR4-using (X4-tropic) strains of HIV *in vitro*, but possessses cardiac abnormalities and no significant viral load reduction was observed (Hendrix et al., 2004). Interestingly, patients treated withAMD3100were found to have increased mobilization of CD34+ stem cells into the peripheral circulation (Liles et al., 2005), and the drug was approved by the FDA as a hematopoietic stem cell mobilizer for transplantation under the trade names Plerixafor and Mozobil. The compound AMD070 is a third-generation small molecule CXCR4 antagonist that is orally bioavailable and inhibits X4-tropic strains of HIV with similar potency to AMD3100 (Stone et al., 2007), but development has been halted due to liver histological changes in preclinical toxicity studies. Although no CXCR4 antagonists are in active clinical trials for the treatment of HIV-1 infection, several are in development and have demonstrated potent inhibitory effects on X4-tropic strains (Iwasaki et al., 2009; Murakami et al., 2009).

c. Inhibitors blocking gp41-mediated membrane fusion

Pharmacological agents that disrupt gp41-mediated membrane fusion, collectively called fusion inhibitors, were the first entry inhibitors to be approved for the treatment of HIV infection. Synthetic peptides corresponding to the HR1 and HR2 domains of gp41 were found to have potent antiviral effects (Jiang et al., 1993; Wild et al., 1992). These agents were initially analyzed during epitope-mapping experiments designed to identify targets for vaccine development (Matthews et al., 2004). However, biochemical and crystallization studies subsequently

revealed their true mechanism of action: prevention of the formation of the six-helix bundle by competing for binding to the HR1 and HR2 domains on gp41(Chan et al., 1997;Weissenhorn et al., 1997;Wild et al., 1994).The fusion inhibitor enfuvirtide (T-20)was approved by the FDA for treatment-experienced, HIV-infected patients in 2003. Enfuvirtide is a linear, 36 amino acid synthetic peptide with a sequence identical to part of the HR2 region of gp41 (Wild et al., 1993),and competes for binding to HR1. It has demonstrated potency against HIV in clinical trials (Kilby et al., 2002; Lalezari et al., 2003), although there is considerable variability in the enfuvirtide sensitivity of primary virus strains (Derdeyn et al., 2000; Melby et al.,2006; Reeves et al., 2002). A number of other next-generation peptidic fusion inhibitors are under investigation, several ofwhich have improved pharmacodynamics and efficacy compared with enfuvirtide (Dwyer et al., 2007; Lalezari et al., 2005b). Additionally, certain agents are active against some enfuvirtide-resistant strains of HIV (Dwyer et al., 2007; He et al., 2008), and fusion inhibitors that bind to different functional domains of gp41 can have synergistic effects (Pan et al., 2009). However, since peptidic fusion inhibitors are not orally bioavailable and must be administered via injection, the development of small-molecule inhibitors of gp41-mediated fusion remains a goal in drug development .An alternative approach that has shown considerable potential is the generation of d-peptide pocket-specific inhibitors of entry (PIEs) that bind to gp41 and which, unlike natural l-peptides, are not digested by proteases and have the potential for oral bioavailability (Welch et al.,2007).

Vertical transmission of HIV

The present work has its origins in the protection offered by the maternal placental environment during pregnancy. The vertical (mother-to-infant) transmission of HIV-1 is the major cause of AIDS in children and accounts for more than 10-39% (Domachowske, 1996; Zachar et.al., 1999, Ahmad, 2005). The viral load, which is related to clinical and immunological status in mother, is the main contributing factor for HIV-1 vertical transmission (Bongertz, 2001) along with numerous maternal parameters like low CD4+ve lymphocyte counts, disease progression rate and immune competence during various stages of pregnancy (Ahmad, 2005; Elizabeth et al., 2003). Low frequency of HIV transmission is due to the presence of various innate immune factors expressed in women during pregnancy. Also, the viral load in seropositive women reported to decrease during pregnancy (Cameron, 2009). Placenta is not only the major nutrient supplier but also a barrier to various pathogens that attack the developing foetus (Burton and Watson, 1997). So protective environment around this is highly important in control of viremia by the maternal immune system. Such innate immune factors include 55KDa placental factor (Tiensiwakul et al., 2004), LIF (Patterson et al., 2001), IFN γ (Druckmann et al., 2005), Killer specific secretory protein (Hayano, 2002), Indoleamine-2, 3-deoxygenase (Sedlmyr et al., 2002), collectins (Ohtani et.al., 2001) and hormonal factors (Hunt et al., 2000) to improve protection of the foetus from harmful pathogens.

Epap-1 and its genesis

Earlier studies identified and characterized a 90 KDa Epap-1, which is isolated from the first trimester placental tissue (Kondapi et. al., 2002; Rani et. al., 2006). Epap-1 was also found to be present in blood and urine of pregnant women during first trimester. Epap-1 inhibits HIV-1 entry and is found to have affinity to HIV-1 gp120. Molecular analysis of Epap-1 and gp120 interaction showed that it possesses recognition sites that can interact with V3 and C5 regions of gp120 (Rani et. al., 2006). Further, Epap-1 expressed in bacterial as well as baculoviral system was shown to be biologically active as the native form. Anti-HIV-1 activities of native and recombinant forms are conserved and specific to V3 and C5 regions. In addition, Epap-1 inhibits the replication of both X4 and R5 viral strains.

Rationale

Epap-1 was shown to block the recognition of V3 and C5 regions of gp120, identification of the region of Epap-1 interacting with V3 loop region would help in understanding the molecular action of Epap-1 in blocking gp120 fusion. Some proteins like Cyanovirin (a protein of 30KDa) suffered in preclinical and clinical studies. So the larger molecular weight of Epap-1(90 KDa) may have various limitations in stability, immunogenicity and production. Thus a peptide may be more stable and can evade unwanted immune reactions if any. Hence the identification of gp120 targeted HIV-1 neutralizing peptides derived from Epap-1 will form new lead in the development of entry inhibitors.

Objectives

- Analysis of anti-HIV potential of proteolysed extract of Epap-1
- Modeling of Epap-1 and gp120 interactions to identify potential gp120 binding regions in Epap-1
- Analysis of cytotoxicity and anti-HIV activity of Epap-1 derived peptides
- Characterization of molecular action of potent peptides
- Design of small peptides with enhanced affinity and activity

Chapter 2

Materials and Methods

Materials: AZT, 3'-azido-3'-deoxythymidine(Sigma chemicals),T-20(Virchow labs) Calcein AM and Calcein blue AM (Molecular probes, USA), RPMI-1640(Invirogen),DMEM(Invirogen), FBS (Invitrogen, Carlsbad, CA), C-18 Reverse phase column (Restek), SNA matrix (EY laboratories), RITC (Himedia), Histopaque (Sigma)

Cells Lines: The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA: Chinese hamster ovary (CHO) JRL, clone A 9 (J. Arthos); HL 2/3 cells (Dr. B. Felbar and G. Pavlakis) expressing IIIB HIV-1 Env, CD4+ SupT1 cells (Dr. J. Hoxie), TZM-BL cells

Antibodies: The various epitope specific gp120 Monoclonal antibodies were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA:

gp120 epitope specific monoclonal antibodies	Contibutors
V2 specific: 697-30D, V3 epitope: 257-DIV (cf 257D) C5 region: 670-30D	Dr. S. Zolla-Pazner
V3 loop: V3-21, SVEINCTRPNNNTRKSI, 298-315	Dr. J. Laman
V3 loop: F425 B 4a.1 (cf F425) and gp41: F240	Dr. M. Posner and Dr. L. Cavacini
CD4 Mab: SIM4	Dr. J. E. K Hildreth
C1 reactive: B2-FNMW, 94-97; C2: B13-TQLLLN, 257-262; C3: B32-FFY, 382-384 and V4 domain : B15	Dr.G.W.Lewis (Personal request)

Viruses: The following HIV-1 strain is procured form NIH-AIDS Research and reference reagent program, USA:

HIV-$1_{93IN101}$ is of biotype-NSI (X5) (Dr. R. Bollinger), isolated from a seropositive individual in India.

HIV-1 $_{93RW024-}$ The UNAIDS Network for HIV Isolation and Characterization, and the DAIDS, NIAID.

HIV-1 $_{94UG103-}$ The UNAIDS Network for HIV Isolation and Characterization, and the DAIDS, NIAID.

Methods

Isolation of native Epap-1

Placental tissue (MTP-Medically Terminated Pregnancy) of Human I Trimester is washed with PBS pH-7.3 (Sterile and Chilled) to completely remove the blood. Processing is done to remove the connective tissue with clean and autoclaved foreceps and scissors. Homogenization was done in PBS. Centrifuge at 16000 x g for 20 min at 4^0C. The protein in the supernatant is precipitated at 0-60% Ammonium Sulphate saturation. Centrifuge at 14000 x g for 15min. Protein in the supernatent was reprecipitated with 60-80% Ammonium Sulphate, pellet was collected at 800 x g for 20min at 4^0C .The pellet is suspended in minimum volume of 1 X PBS. Dialyse extensively against 1X PBS. Dialysate is Centrifuged at 16000 x g for 20min at 4^0C. Supernatent containing protein was eluted and estimated by Bradford method.

Purification of Protein by SNA Affinity Chromatography

10mg of Protein was loaded onto SNA column (Sambrucus nigra Agarose Lectin). Flow through was adjusted to 1ml/5min and flow through was stored at -20^0C. Unbound protein was washed with 10 times the bed volume, with 1X PBS-pH-7.3 until the A_{280} is < 0.05. Bound protein was eluted with buffer containing 100mM Galactose in 1X PBS pH-7.3. 1ml Aliquots of protein were collected in 1.5ml eppendorfs and stored at -80^0C.

Isolation of rec Epap-1

E. coli BL21 cells were grown in LB medium containing 100 mg/ml ampicillin until OD_{595nm} of 0.6 is reached. Protein expression was induced using 1 mM IPTG for 4 hours. Bacterial pellet was lysed in presence of lysozyme followed by sonication. recEpap-1 (Recombinant Epap-1) was purified using Ni-NTA column (Roda Rani et al., 2006) and the protein eluted with 200 mM imadazole was estimated using Bradford method (Bradford, 1976). Protein is stored at -80^oC until use.

Partial Tryptic digestion of Epap-1

Epap-1 was digested by Trypsin (1:50 ratio) by using the following protocol. 70µl of Epap-1 (0.75mg/ml) was mixed with 1 µl Trypsin (1mg/ml) in 50 mM pH 7.0 Tris-HCl buffer. The reaction was carried out by incubation for at 37^{o}C for 1 hour.

Purification of digested peptides by HPLC

The digested fragments of Epap-1 were purified by HPLC (Waters e2695) using c-18 (reverse phase) column through the following gradient program. Here, 2 solvents A, B were used. Solvent A is water + 0.1% TFA and the solvent B is Acetonitrile + 0.1% TFA. The Flow rate was adjusted to 1ml/min and the pressure was adjusted to 5000 psi. Initially washing was done with Buffer B for 10min followed by equilibration with Buffer A for 10min. HPLC gradient program: Step 1 - A – 100% , B – 0% (upto 3 min); Step 2 - A - 0% , B - 100% (upto 55min); step 3 - A – 100%, B - 0% (upto 60 min). Finally washing was done with 100% Acetonitrile after reversing the column for 10min. Two major peaks were obtained at the retention time 22 min (F-1) and 35 min (F-2) along with the unbound peak. These were collected and concentrated by lyophilisation. Now these three fractions were kept for antiviral assay.

SDS- PAGE

Polyacrylamide gel electrophoresis was performed according to the method of Lammelli (1970) in 0.1% SDS on a vertical slab gel system. The gels contained 10% acrylamide with 30:1 ratio of acrylamide to N, N, N', N'- methylene-bis-acrylamide. Samples were boiled at 100^{o}C for 10 min in the presence of loading dye to dissociate proteins into their individual polypeptide chains. The loading dye contained: SDS (1% w/v), 2-mercaptoethanol (5% v/v) in 0.063 M Tris.HCl, pH 6.8. The protein mixture was subjected to electrophoresis on 4.5% (w/v) polyacrylamide stacking gel in 0.125 M Tris-HCl, pH 6.8 and 10% (w/v)

resolving gel in 0.375M Tris. HCl, pH 8.8 at 100 volts till the dye front reaches the end of the gel. The electrode buffer (pH 8.5) contained 0.025M Tris buffer, 0.192M glycine and 0.1% SDS.

Validation of Epap-1 model

Structural modeling of Epap-1 was done earlier in our lab using Threading technique by WURST server and the energy minimization was done by GROMACS. This model was further validated by L-ALIGN and EMBOSS to proceed for docking.

Molecular docking

Molecular docking was carried out between Epap-1 and gp120 trimer (obtained from PDB) using HEX 5.6 software (Ritchie, 2008 ; Ritchie et al., 2010). Based on the docking conformation, potential gp120 interacting peptides of Epap-1 were selected for analysis of their interaction with gp120.

Analysis of Cytotoxicity of peptides

Reduction of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) by active mitochondria was chosen as a cell viability measurement optimal endpoint. SupT-1 cells ($0.2x10^6$) in RPMI 1640, 10 %FCS were seeded in 96 well plates. Increasing concentrations of compounds were added to the cells and incubated at 37^0C for 14 hrs in a CO_2 Incubator with 5% CO_2. The media was replaced with a fresh growth medium along with 20µl of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma). After

incubation for 4 hours in a humidified atmosphere, the media was removed and 200 ul of 0.1 N acidic isopropyl alcohol was added to the wells to dissolve the MTT-formazan crystals. The absorbance was recorded at 570 nm immediately after the development of purple colour. Each experiment was conducted in triplicate and the data was represented as an average with standard deviation.

Isolation of PBMCs from human blood

Ten milliliters of blood was collected form healthy volunteers and diluted it to 1:1 ratio with saline. Diluted 20ml blood was layered on 10 ml of histopaque in a 50 ml round bottomed centrifuge tube. The sample was subjected to centrifugation at 400 x g for 20 minutes in a bucket centrifuge (Heareus) without applying brakes. The clear white band of cells is seen at the histopaque gradient. The cells were carefully pipetted out without disturbing the contents in the tube and resuspended in saline (50ml). This was again centrifuged at 150 x g for 10 minutes. The supernatant is discarded and the pellet was washed 3 times in saline. The cells were seeded at density of $1x\ 10^6$ cells /ml in RPMI 1640 containing 10% FCS and 2µg of PHA. The cells were cultured at 37^0C and 5% CO_2.

Anti-viral activity in Sup-T1 cells

One million of SupT1 cells with 100% viability were seeded with RPMI 1640, 0.1% FBS in 12-well plates. Increasing concentration of peptides (50, 100, 150 µg/ml) were added to the cells and they were infected with HIV-$1_{93IN101}$ at a final concentration of virus equivalent to 2 ng of p24 per ml. The infected cells were incubated at 37 ^{0}C in 5% CO_2 incubator for 2 h. After 2 h, the cells were pelleted

at 350x g for 10 min, supernatant was discarded and cells were washed with RPMI 1640 containing 10% FBS. The cells were resuspended in the same medium and incubated for 96 h. The supernate were collected after 96 h and analyzed using p24 antigen capture assay kit (ABL, USA).The infection in the absence of Epap-1 was considered to be 0% inhibition. Azidothymidine (AZT) and T20 were taken as positive controls. The result given was an average of three independent experiments.

Conjugation of Peptides with RITC and binding to gp120 expressing HL2/3 cells

Rodmine isothiocynate (30µl, 1mg/ml PBS) was added to 50 µg of Peptide and kept for overnight incubation at 4 ^{o}C. The conjugated peptide was dialysed for 1hr against PBS. The RITC conjugated peptides were incubated with HL 2/3 cells for 1hr. The cells were washed with PBS and monitored for their Peptide binding using Flow Cytometer (Partec) and confocal microscope (Leica)

Dye transfer assay to examine cell fusion

Calcein AM labeling (ex/em 496/517): HL2/3 cells expressing HIV-1IIIB gp120 on surface were incubated with 0.5 µM of calcein AM for 1 h at 37 ^{0}C, washed, incubated in fresh medium for 30 min at 37 ^{0}C, washed and then resuspended in complete medium at 1 million cells/ml.

Calcein blue loading (ex/em 354/469): SupT1 cells were loaded with 20 µM of calcein blue for 1 h at 37 ^{0}C, washed, incubated in fresh medium for 30 min at 37 ^{0}C, washed and then resuspended in complete medium at 1 million cells/ml.

Fluorescently labeled gp120–41 expressing (HL2/3) cells and CD4+, CXCR4+ (SupT1) cells were co-cultured at 1:1 ratio for 2 h at 37 ^{0}C. Cell fusion was monitored using a dye redistribution assay. The fusion inhibition was monitored in the presence of peptides. The cell fusion in the absence of peptide was considered to be control cell fusion.

Reporter based assay in TZM-BL cells

Action of Peptides on virus infection is further confirmed by using a reporter cell line, TZM-Bl which upon HIV infection leads to Tat induced LTR –associated β-gal expression. In the control HIV infection, the cells express β-gal which reduces the substrate, X-gal when added. The reduced X-gal fluoresces in the presence of Azo dye (fast red violet). 1 million of TZM-BL cells with 100% viability were seeded with DMEM, 0.1% FBS in 12-well plates. Increasing concentrations of peptides were added with T20. The cells were incubated at 37 ^{0}C in 5% CO_2 incubator for 48 h. After this X-gal with azo dye was added to count for the number of fluorescent cells. No fluorescence was observed in the presence of T20 which was used as positive control.

Peptide interaction with gp160 in the presence of receptor and coreceptor

Mouse monoclonal anti-human gp160 antibodies spanning different regions of HIV-1 gp160 were added into wells of 96-well RIA plate at 10 ng per well in PBS, the plates were incubated overnight. Following day the wells were blocked with 3% BSA for 2 h at 37^{0}C, binary complexes containing gp160–peptides were formed by incubation of gp160 in PBS with increasing concentrations of peptides

at 37 ^{0}C for 1 h. Binary complexes were captured with gp160 monoclonal antibody pre-coated wells and incubated for 1 h at 37 ^{0}C. The unbound complexes were removed by washing thrice with wash buffer. Captured binary complexes were probed for the peptides using 10 ng of affinity purified rabbit polyclonal anti-human Epap-1 antibody by incubating for 1 h at 37 ^{0}C and wells were washed thrice with wash buffer. Bound rabbit polyclonal was probed with 1:2000 dilution of goat–anti-rabbit IgG-peroxidase antibody by incubating at 37 ^{0}C for 30 min, the wells were washed thrice with wash buffer and developed with TMB substrate system. The reaction was stopped after 30 min with 1N HCl and plates were read at 450 nm. Each experiment was done in triplicates and average, standard deviations were calculated. The same experiment was repeated in the presence of receptor and coreceptor.

Peptide interaction with virus surface gp120

HIV-1 virus was incubated in the presence of different concentrations of peptides. Bound complex of HIV-1 and peptide were captured by mouse monoclonal gp160 antibodies spanning different regions of gp160 as mentioned in the previous protocol. The captured complex was estimated for HIV-1 bound in terms of p24 released with 1% of Triton X-100 using ABL kit.

Chapter 3

Anti-HIV potential of proteolysed Epap-1

Introduction

Epap-1 is a 90 KDa protein expressed in placental tissue with significant affinity to gp120 (Kondapi et al, 2002). The bacterial expressed Epap-1 showed significant affinity to gp120 and anti-HIV activity but stoichiometrically the recEpap-1 binds to Epap-1 at 1:1 ratio while the native Epap-1 binds to gp120 at 1:2 ratio. It would be interesting to analyze the molecular region of Epap-1 associated with high affinity to gp120. Hence rec Epap-1 was partially digested with Trypsin. The cleaved fragments were separated on HPLC to analyze if the anti-HIV-1 activity resides in the smaller fragments of Epap-1.

Results

Native and recEpap-1 possess similar surface peptide backbone

Native Epap-1 was purified from MTP placental tissue using Sambucus *nigra* agarose matrix. Bound protein was eluted with galactose. Rec Epap-1 was expressed in BL21 cells and protein production was induced by IPTG. Expressed protein was isolated using Ni-NTA column. Partial Tryptic digestion was carried out by incubating Epap-1 with Trypsin(1mg/ml) in Tris-Hcl, pH 7.4 for 1 hour **(Fig 3.1)**. Peptide profile is analyzed by MALDI-TOFF. The MALDI analysis revealed an identical digestion pattern for the both native and recEpap-1 suggested similar surface organization of Epap-1 polypeptide chain that is accessible to Trypsin **(Fig 3.2)**.

Protease digest of Epap-1 retain anti-HIV-1 activity

The digested Epap-1 yielded proteins ranging from 72KDa to small peptides. The protease digest was tested for anti-HIV activity in Sup-T1 cells. In this assay, 1 million cells were infected with virus equivalent to 2ng of p24 per ml in the presence and absence of rec Epap-1 and digested pool of Epap-1. AZT was used as positive control. After 96h, the supernatants were analyzed for the amount of HIV-1 p24 protein equivalence of the virus replicated. Both Epap-1 and its proteolytic digest showed significant inhibition of HIV-1 replication with 60% inhibition at 1µg protein concentration **(Fig 3.3)**. This suggested that the entire tertiary structure of Epap-1 is not necessary to exert its anti-viral activity. Thus peptides in the digest were separated by HPLC using C-18 reverse phase column and two major fractions at 22min (Fraction-1) and 35min (Fraction-2) respectively **(Fig 3.4)** were obtained. These fractions were tested for their anti-HIV activity. Fraction 1 results showed 40% inhibition of HIV replication whereas the fraction 2 possesses only 20% inhibition of HIV-1 replication while small peptides could not be isolated **(Fig 3.5)**. The binding affinity of peptides in these two fractions to gp120 expressed on HL2/3 cells and their inhibitory activity in cell fusion assay was analyzed. In the former assay, the peptides in fractions were coupled with RITC and incubated with HL2/3 cells **(Fig 3.6)**. The cells bound with RITC-peptide(s) bound were counted to monitor whether the peptides possess affinity to gp120. Peptides in fraction 1 did bound to cells whereas the peptides in fraction 2 do not possess affinity to gp120. This was confirmed by the inhibitory action of peptides of fraction 1 on the fusion mediated dye transfer

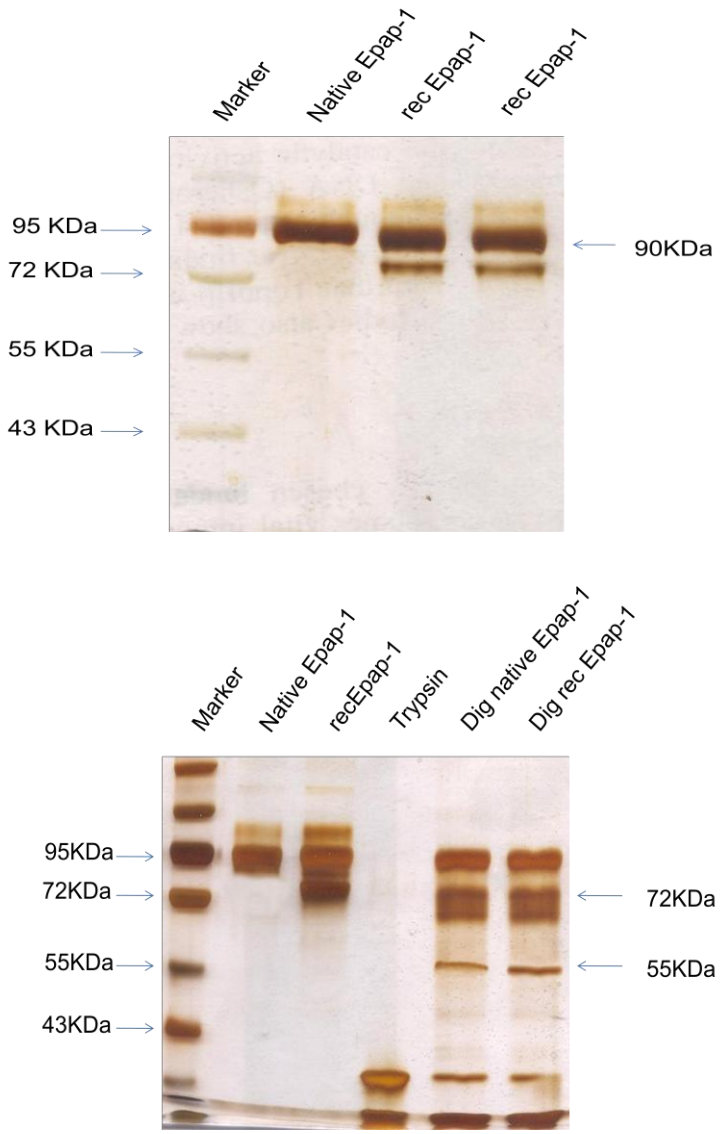
between calcein Blue loaded SupT1 and calcein AM loaded HL 2/3 cells where in peptides of the fraction 2 do not possess any activity while peptide(s) in fraction -1 blocked cell fusion **(Fig 3.7)**.

Discussion

The results of these studies showed that the entire tertiary structure is not essential for the anti-HIV activity of Epap-1. Since the Polypeptide component of Epap-1 showed partial activity, identification of the important residues that were involved in binding to gp120 remains a challenging task. It is difficult to analyze the residues which were anti-HIV because the random digestion of peptides results not only in the loss of crucial tertiary conformations but also in the loss of small peptides. Isolation and characterization of small peptides which contribute to the residual anti-HIV is complex because their separation by HPLC becomes increasingly difficult in the selection of an ideal column which can separate the protein fragments ranging from several Kilo Daltons to small peptides. Even though the peptides in the fractions could show affinity to gp120 on HL 2/3 cells and inhibited dye transfer between Sup-T1 and HL 2/3 cells at the present isolation of small individual peptides was not possible. Thus, prediction of the structure of Epap-1 and interaction contributed by the peptide backbone with gp120 would help in identification of exact regions of Epap-1 that can exhibit potential interaction with gp120.

Fig 3.1

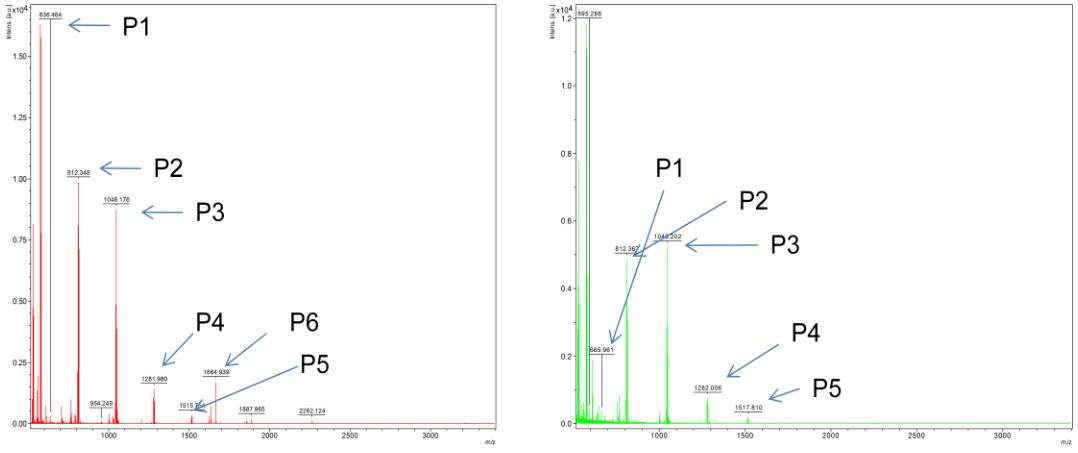
SDS-PAGE analysis of native, rec and digested Epap-1



Native, recombinant Epap-1 and the digested Epap-1 were separated by SDS-PAGE and stained by silver staining according to the mentioned protocol. Both forms were found to be of 90KDa molecular weight whereas the digested forms resulted in the fragments ranging from 72KDa to smaller peptides.

Fig 3.2

MALDI Analysis of rec and native Epap-1



Rec Epap-1 Native Epap-1

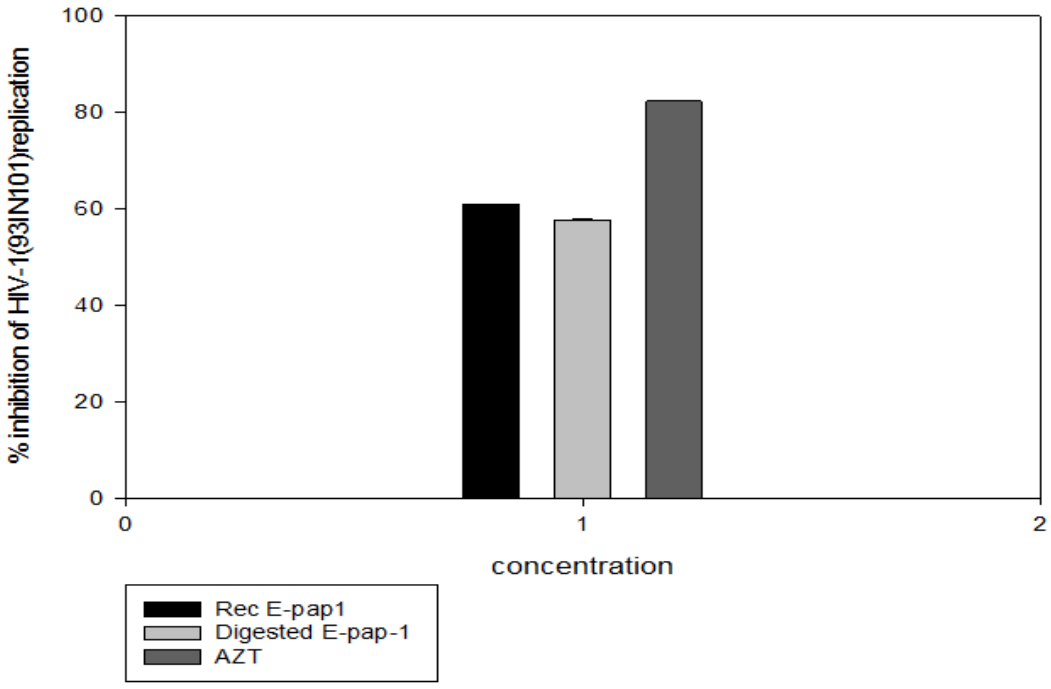
Major identical peaks in both proteins

Peak 1 - 636.464	Peak 4 -1281.980
Peak 2 - 812.348	Peak 5 - 1515.754
Peak 3 - 1046.202	Peak 6 - 1664.939

Both native and recombinant proteins were subjected to extensive digestion of Trypsin as per protocol and given for MALDI analysis. MALDI analysis reveals identical digestion pattern of both native and recEpap-1. There are six major identical peaks in both proteins. This confirms the similar folding of both the proteins.

Fig 3.3

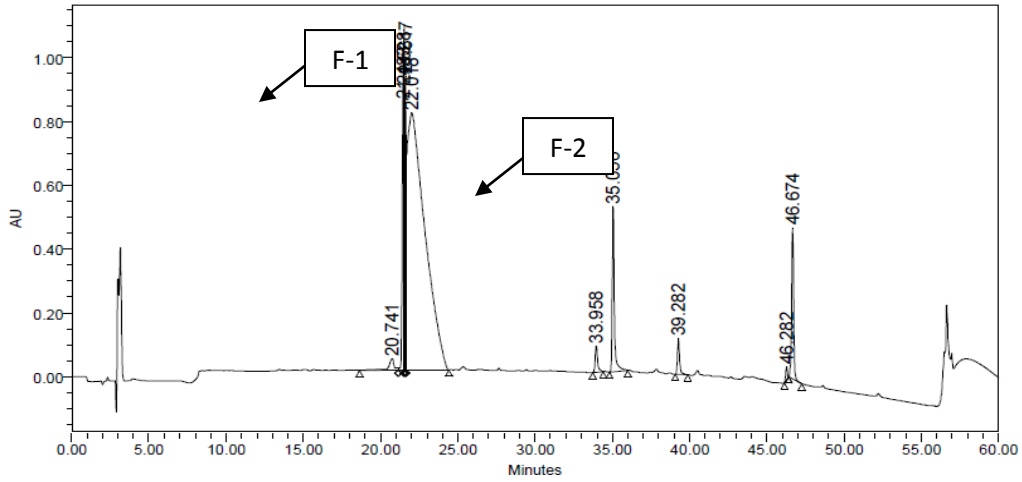
Anti-HIV-1 activity of digested Epap-1 in Sup-T1 cells



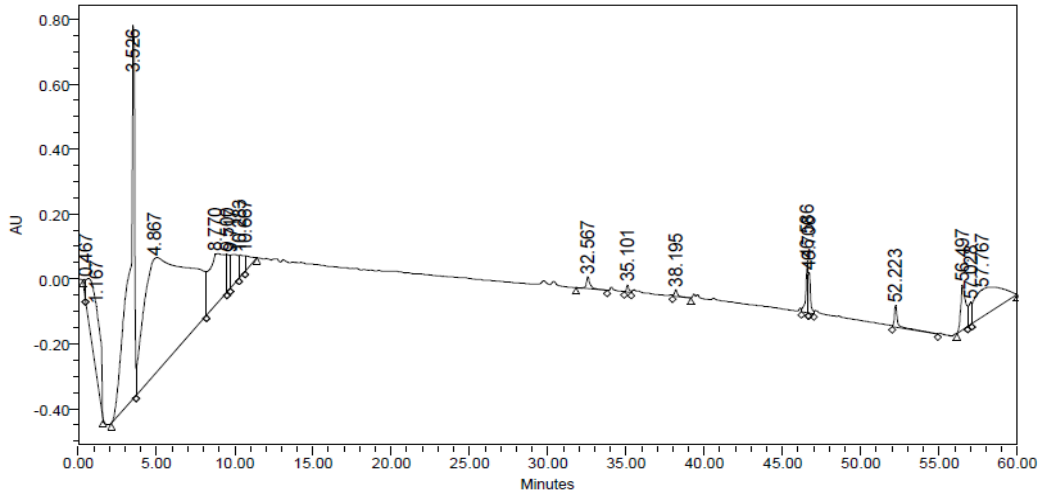
Sup T-1 cells were challenged with HIV-1 $_{93IN101}$ in the presence and absence of Peptides for 2 hours. Cells were washed, re-cultured in fresh medium and amount of virus replicated at day 4 was estimated. Inhibition is calculated with reference to the control infection in the absence of virus, whose p24 was 8 ng/ml at day 4. 10 µg/ml of nativeEpap-1 and recEpap-1 were used. 1 µM AZT is used as control.

Fig 3.4

HPLC profile of Digested Epap-1 and Trypsin



Digested Epap-1

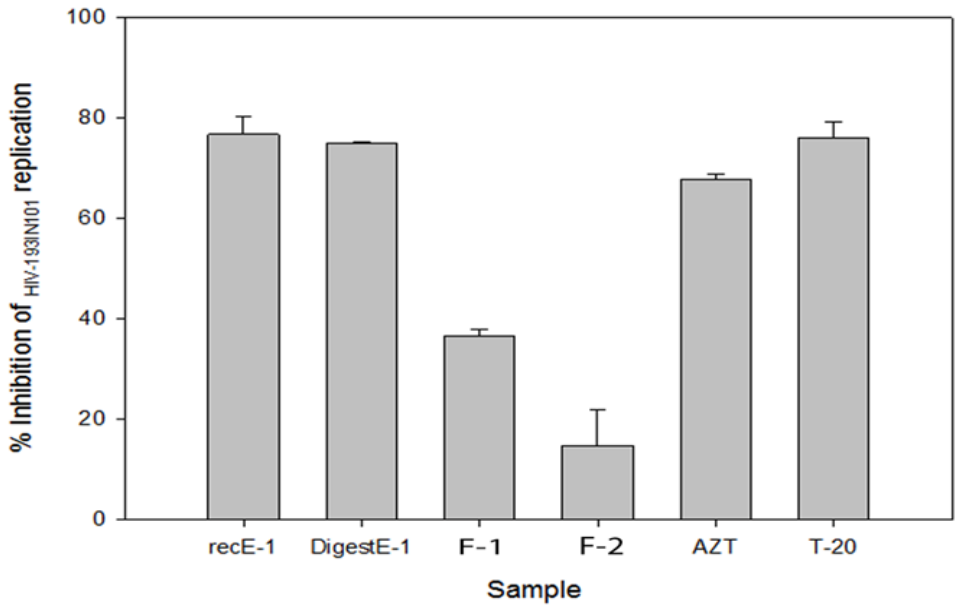


Trypsin alone

HPLC profile of recEpap-1 tryptic digest: Trypic digested protein was loaded on C-18 column and washed with water containing 0.1% TFA. Bound peptides were eluted with a gradient of Water-acetonitrile.

Fig 3.5

Anti-viral activity of separated fractions



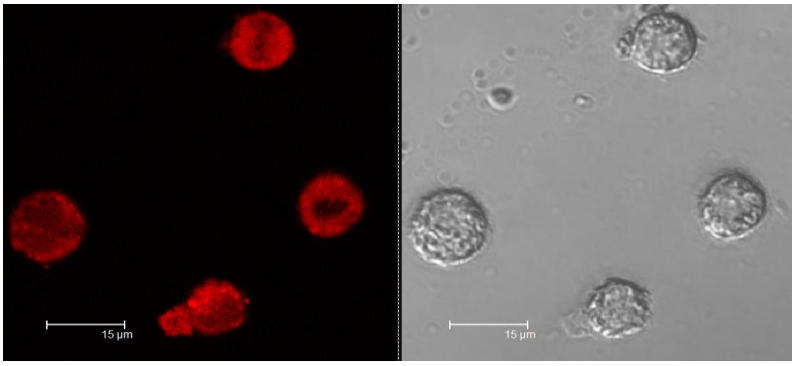
F-1: Fraction 1
F-2: Fraction 2

Sup T-1 cells were challenged with HIV-1 93IN101 in the presence and absence of Peptides for 2 hours. Cells were washed, re-cultured in fresh medium and amount of virus replicated at day 4 was estimated. Inhibition is calculated with reference to the control infection in the absence of virus, whose p24 was 8 ng/ml at day 4. 20 µg/ml of Epap-1 was used for recEpap-1 (recE-1), Tryptic digested recEpap-1 (DigestE-1), 60 µg/ml of Fraction-1 (F-1) and Fraction-2 (F-2) were used. 1 µM AZT and 2 µg/ml of T-20 were used as controls.

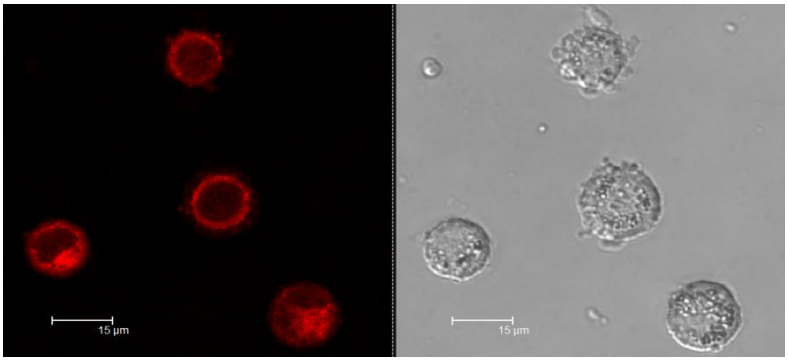
Fig 3.6

Binding of Fraction 1-RITC to HL2/3 cells

Epap-1 RITC



Fraction-1-RITC

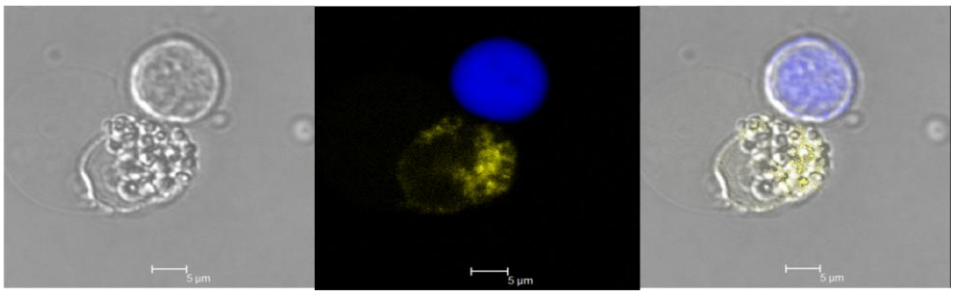


HL 2/3 cells were incubated with Fraction-1 conjugated with RITC for 2 hours at 37^{o}C. Cells were washed extensively and visualized using Confocal microscope. Red colour on the surface of the cells shows the binding of peptide – RITC conjugate to the cells. Here Epap-1 – RITC conjugate is used as a positive control for the assay.

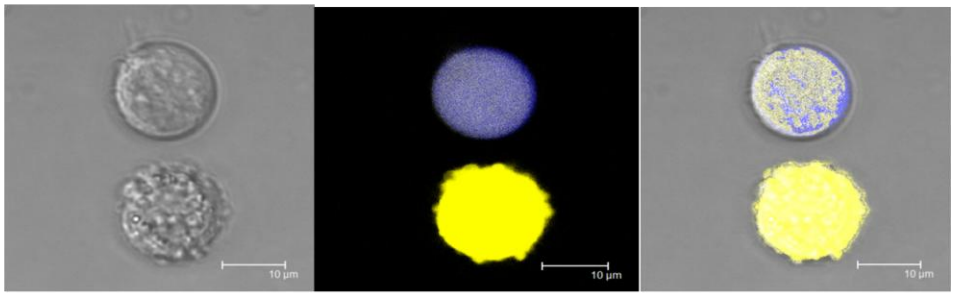
Fig 3.7

Action of Fraction -1 on entry

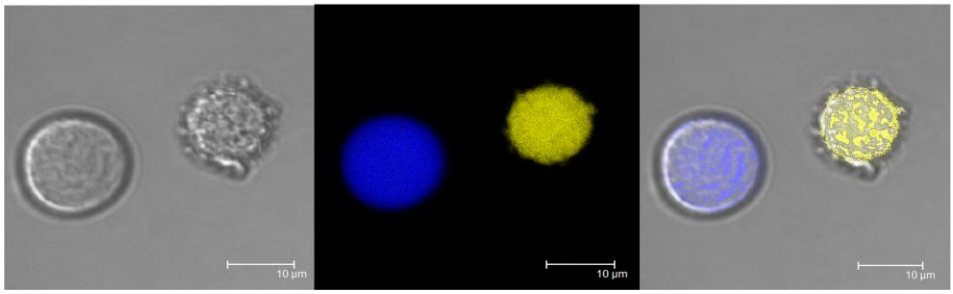
A : Control fusion



B: In the presence of 2 μg/ml of Fraction-1



C: In the presence of 1μg/ml of Epap-1



HL 2/3 cells were incubated with Fraction-1 for 2 hours at 37°C. The Sup T-1 cells were added and incubation continued 2 more hours. Cells were visualized using Confocal microscope. Transfer of dye takes place in the absence of any inhibitor. Panel A: Control fusion in the absence of drug or peptides, Panel B: Fusion inhibition in the presence of Fraction-1.Panel C: Fusion inhibition in the presence of Epap-1

Chapter 4

Design and evaluation of Epap-1 peptides

Introduction

Identification of the exact domain in the Epap-1 which interacts with the gp120 remains the next major step towards the development of an efficient therapeutics. To achieve this docking between Epap-1 and gp120 was considered as a potential approach. In silico structure prediction is generally done by Homology modeling. Since Epap-1 is a novel protein required sequence homology was not found between Epap-1 and the proteins in existing structure database, PDB. To achieve this, threading procedure was implemented to predict the structure of Epap-1 previously in our lab. Optimization of the structure was carried out to obtain a optimized structure of Epap-1 for docking with gp120. Since the interacting gp120 in the HIV virus particle is a trimer, the putative trimeric structure of gp120 (Wyatt et al.,1998) was considered as an appropriate model for study **(fig 4.2)**. The V3 loop residues exposed were one of the prime binding sites for Epap-1 situated adjacent to the CD4 binding pocket and the chemokine receptor binding regions.

Results

Modeling of Epap-1 and gp120 protein interaction to identify potential gp120 binding regions

Epap-1 amino acid sequence was derived from the cDNA sequence based on degeneracy code (Rani et.al., 2006). Homology of Epap-1 sequence using BLAST was <35% and hence its structure was derived by fold recognition using threading

on WURST server. The structure was optimized by the energy minimization using GROMACS software. Optimized structure was further validated with L ALIGN and EMBOSSES softwares which have given 78.4% and 78.8% homology **(fig 4.1)**.

Molecular docking was carried out between Epap-1 and gp120 trimer (obtained from PDB) using HEX 5.6 software (Ritchie, 2008 ; Ritchie et al., 2010). Based on the docking conformations, four potential gp120 interacting peptides of Epap-1 were selected for analysis of their interaction with gp120 (indicated in parenthesis).

P1. H_2N-CNPSLVPPSILISFAATRTKRMAYTPFTSNIC-COOH (114-147) (GLU246 of gp120)

P2. H_2N-RSTCALTAATAKAYATRSLEHRVVYRILHDC-COOH (383-412) (VAL270-GLN352 of gp120)

P3.H_2N-VYNLDCLFRSICLHPWWWWMGVYNLVRDFITLH-COOH(614-646) (GLU268-LYS357 of gp120)

P4. H_2N-SIMCLVLVISSKNKTPGPLTIVQVPAVATCFML-COOH(685-718) (GLN267 of gp120)

These peptides possess molecular interactions with gp120 in terms of 1, 8, 7, 1 number of interactions for peptides P1, P2, P3 and P4 respectively **(fig 4.4 - 4.7)**. These were synthesized commercially and analyzed for their affinity to gp120 and anti-HIV activity.

Binding affinity of peptides

The affinity of the RITC- conjugated peptides to gp120 expressed on the surface of HL2/3 cells was monitored by confocal microscope. The results show that P2, P3, P4 possess higher affinity to HL2/3 cells, whereas P1 did not exhibit any affinity. These results were confirmed by peptide bound fluorescently labeled cells using FACS analysis. None of the peptides could bind to SupT1 cells which are devoid of gp120 expression **(Fig 4.8).**

Analysis of cytotoxicity and anti-HIV activity of designed peptides

Cytotoxity of all the 4 peptides was analyzed in Sup-T1, Colo 205, SK-N-SH and Cos-7 cells at 50, 100 and 150 µg/ml concentrations by using MTT dye based cellular proliferation assay. All the four peptides were considerably less toxic (<15%)**(Fig 4.9 – 4.12)**. Analysis of anti-HIV activity of the peptides in both Sup-T1 and PBMC's revealed that P2 and P3 could exhibit 80% and 50% inhibition at 100 µg concentration respectively whereas P1 and P4 were inactive **(Fig 4.13 & Fig 4.14)**. This confirmed that the peptides P2 and P3 have affinity to gp120 and anti-HIV active. Further analysis was taken up to analyze its molecular action.

Discussion

Epap-1 molecular model was developed and docked with trimeric gp120 resulted in four interacting regions of Epap-1 with V3 loop region of gp120. The number of residues in the peptide-1 are 32 and it comprises of three helical moieties linked by two small loops. Even though all of the residues lie in the allowed regions of Ramachandran's plot only one residue of it is interacting with Glu 246

of gp120. The peptide-2 has got 31 residues which lie completely in the allowed regions of Ramachandran's plot. It consists of two helices linked by a loop and shows eight interactions with gp120. The peptide-3 consists of 33 residues which completely fall in the conformationally allowed regions of Ramachandran's plot. It consists of three helices and interacts with seven residues of gp120. The peptide-4 also consists of 33 residues that fall in the allowed regions of Ramamchandran's plot. It consists of two small helices linked by loop and finally assumes circular shape and exhibits only one interaction with gp120. Out of these four regions, peptides, P-2 and P-3 possess a fair number of interactions with V3 region and P-2 exhibited a considerable anti-HIV-1 activity at 100µg/mL concentration. One of the constraint observed in this modeling is the domain that interacts with C5 region was not found. Other is the number of residues interacting with gp120 are more in the region surrounding the V3 loop ie., from val 270 to glutamine 352 whereas the V3 region spans from aa 296 to 325. This might be one of the reasons for a higher IC-50 value of the potent peptide. Since the multiple domains in Epap-1 that interact with gp120 in many regions, the efficient inhibition of virus entry with a lesser IC-50 value might be possible with the synergisitic action of few peptides which exhibit strong binding to gp120.

Method of finding the interactions of Epap-1 and gp120

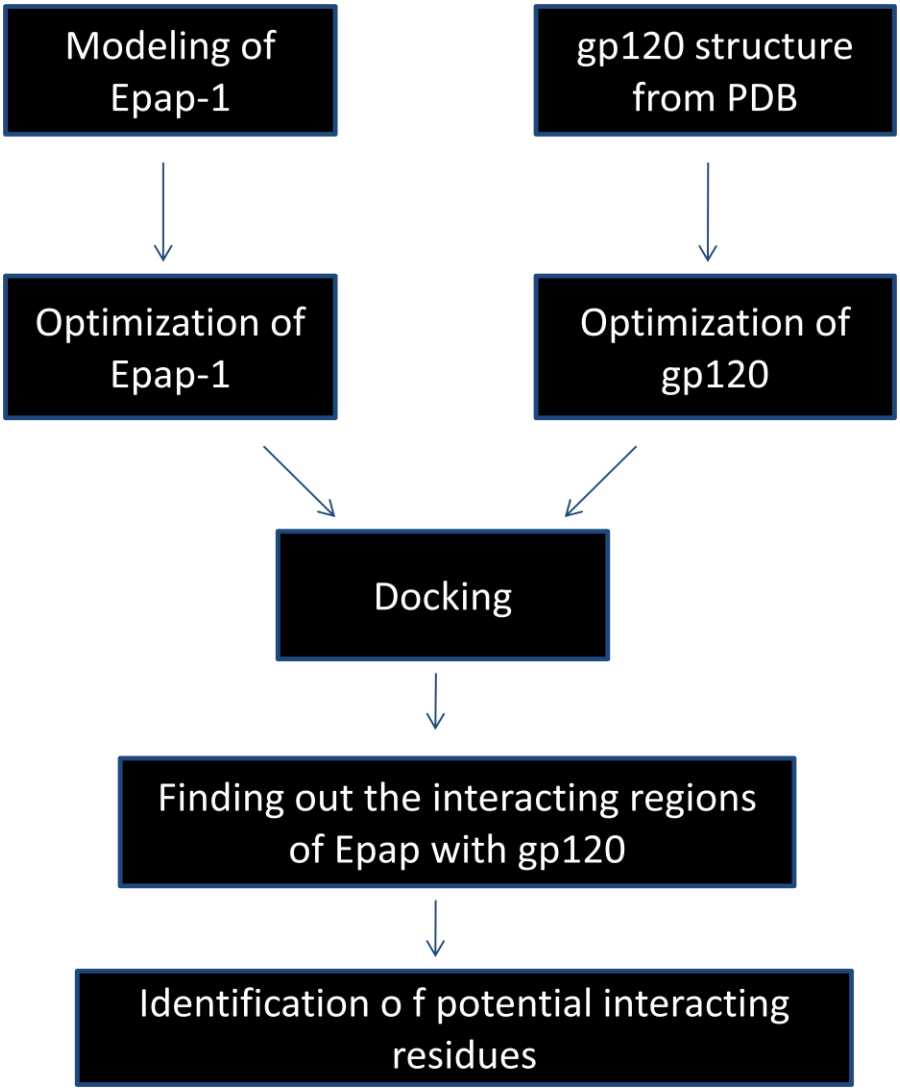


Fig 4.1

Box-1: lalign output for 2p04A vs. epap-1

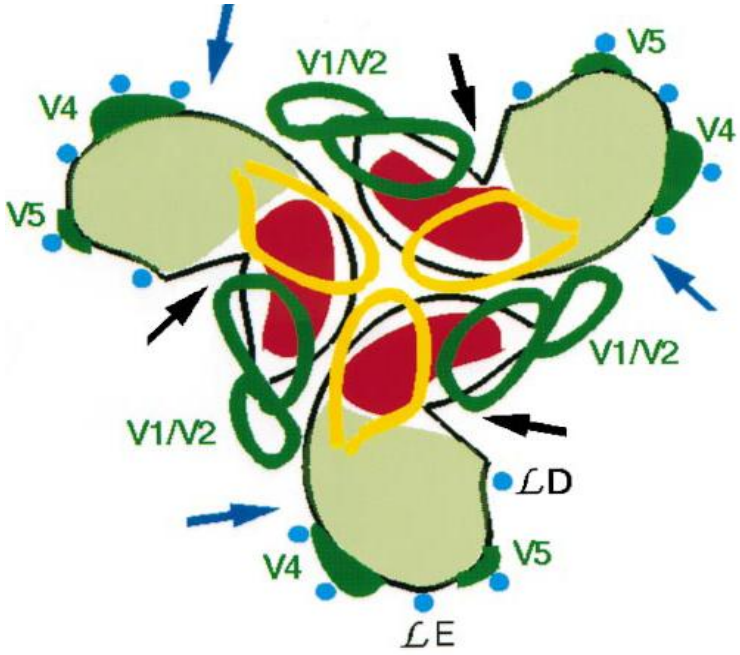
```
Comparison of:
(A) ./wwwtmp/.9989.1.seq 2p04A   - 609 aa
(B) ./wwwtmp/.9989.2.seq epap-1 - 733 aa
 using matrix file: BL50 (15/-5), gap-open/ext: -14/-4 E(limit)   0.05
  78.4% identity in 733 aa overlap (1-609:1-733); score: 2690 E(10000):
4.2e-263
```

Box-2: EMBOSS Align Results for 2p04A vs. epap-1

```
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 733
# Identity:     595/733 (78.8%)
# Similarity:   597/733(79.1%)
# Gaps:         151/733 (20.0%)
# Score: 2556.5
```

Results of the Final validation with L-ALIGN and EMBOSS of Epap-1 structure generated after Threading and energy minimization by Gromacs. Both the softwares have generated 78.4% and 78.8% homology respectively that were acceptable to proceed further with docking studies.

Fig 4.2

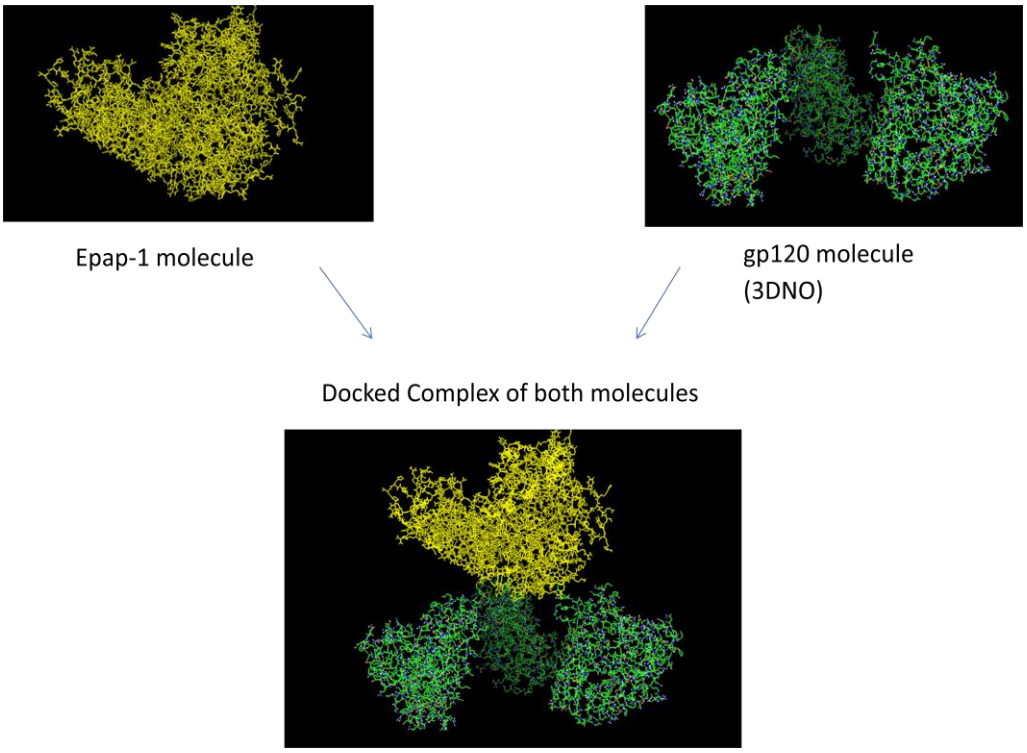


Adapted from Wyatt et al., Nature, 1998

A likely arrangement of the HIV-1 gp120 glycoproteins in a trimeric complex. The CD4-binding pockets are indicated by black arrows, and the conserved chemokine-receptor-binding regions are in red.Areas shaded in light green indicate the more variable, glycosylated surfaces of the gp120 cores. The approximate locations of the 2G12 epitopes are indicated by blue arrows; those of the V3 loops (yellow) and V4 regions (green) are indicated. The positions of the V5 regions (green) and some complex-carbohydrate addition sites (asparagines 276, 463, 356, 397 and 406) (blue dots) are shown. The approximate locations of the large V1/V2 loops, centred on the known positions of the V1/V2 stems, are indicated (green). On one of the gp120 subunits, the positions of the LD and LE loops are indicated. The distance of each of the gp120 monomers from the 3-fold symmetry axis is arbitrary.

Fig 4.3

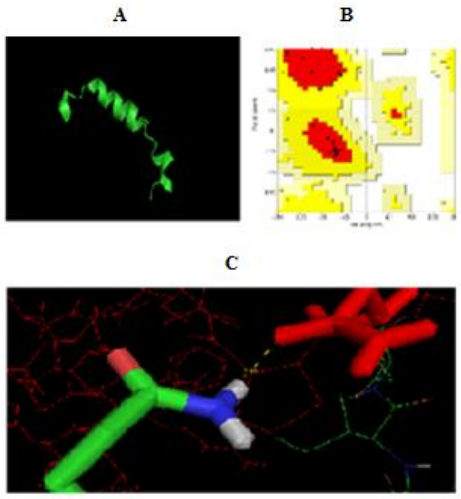
Molecular structure of Epap-1 and Epap-1 and gp120 docked conformation



The complex of Epap-1 and trimeric gp120 obtained after docking using HEX 5.6 software. The interacting regions between the two molecules are visualized by pymol. From this four major regions of Epap-1 which were interacting with V3 loop of gp120 were selected for further analysis.

Fig 4.4

Structure and Molecular interactions of peptide, P-1



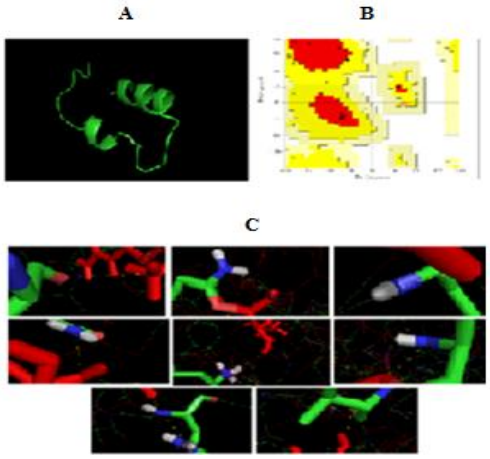
Plot Statistics

Residues in most favored regions [A,B,L]	26	100%
Residues in additional allowed regions [a,b,l,p]	0	.0%
Residues in generously allowed regions [~a,~b,~l,~p]	0	.0%
Residues in disallowed regions	0	.0%
Number of non-glycine and non-proline residues	26	100%
Number of end residues(excl. Gly and Pro)	2	
Number of Glycine residues (shown as triangles)	0	
Number of proline residues	4	
Total number of residues	32	

Panel A shows the structure of the peptide,P-1 and the ramachandran plot of the same in panel B whereas the lower panel C indicates the number of interactions showed by peptide with gp120. Here peptide P1 exhibited only one interaction that is with Glu246 of gp120.

Fig 4.5

Structure and Molecular interactions of peptide, P-2



Plot Statistics

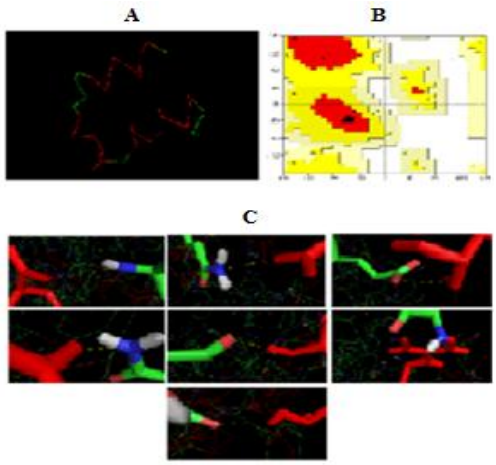
Residues in most favored regions [A,B,L]	22	75.9%
Residues in additional allowed regions [a,b,l,p]	6	20.7%
Residues in generously allowed regions [~a,~b,~l,~p]	1	3.4%
Residues in disallowed regions	0	.0%
Number of non-glycine and non-proline residues	29	100%
Number of end residues(excl. Gly and Pro)	2	
Number of Glycine residues (shown as triangles)	0	
Number of proline residues	0	
Total number of residues	31	

Panel A shows the structure of peptide, P-2 and the ramachandran plot of the same in panel B whereas the lower panel C indicates the number of interactions showed by peptide with gp120.Here peptide P-2 exhibited eight interactions with gp120 that were shown in the below table.

Sl. No	Interacting Molecules	Bond Distances in A
1	ALA346(O) - (N) ARG1	3.24
2	GLN352 (N) - (O)ARG1	3.23
3	GLN344 (NE2) – (0) ALA13	2.59
4	GLN352(NE2) – (OG1) THR3	2.77
5	THR290 (N) – (O) ALA11	2.20
6	VAL270(O) –(N) CYS31	3.42
7	LYS348 (NC) –(O) ARG26	3.36
8	GLN352(NE2) -(O)ARG1	2.77

Fig 4.6

Structure and Molecular interactions of peptide, P-3



Plot Statistics

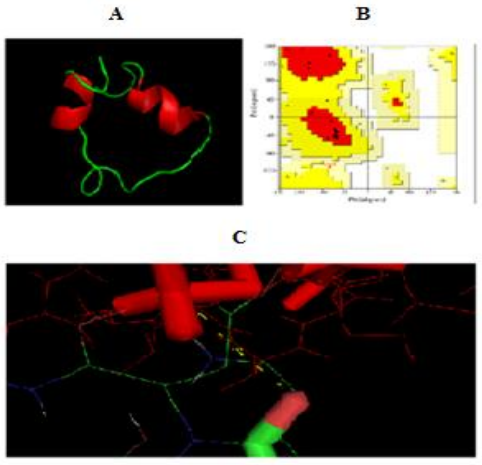
Residues in most favored regions [A,B,L]	28	96.6%
Residues in additional allowed regions [a,b,l,p]	1	3.4%
Residues in generously allowed regions [~a,~b,~l,~p]	0	.0%
Residues in disallowed regions	0	.0%
Number of non-glycine and non-proline residues	29	100%
Number of end residues(excl. Gly and Pro)	2	
Number of Glycine residues (shown as triangles)	1	
Number of proline residues	1	
Total number of residues	33	

Panel A shows the structure of peptide, P-3 and the ramachandran plot of the same in panel B whereas the lower panel C indicates the number of interactions showed by peptide with gp120.Here peptide P3 exhibited seven interactions with gp120 that were shown in the below table.

Interacting Residues of gp120 with epitope	Bond Distances in A
GLU268(OE2) – (N)TYR2	3.20
GLN344(NE2) – (OD1)ASP5	2.67
SER347(0) – (O)VAL26	2.38
GLN352(NE2) – (OD2)ASP28	2.74
ALA346(N) – (O)HIS33	3.44
LEU342(O) – (O)THR31	3.05
LYS357(O) – (NH2)ARG21	2.53

Fig 4.7

Structure and Molecular interactions of peptide, P-4



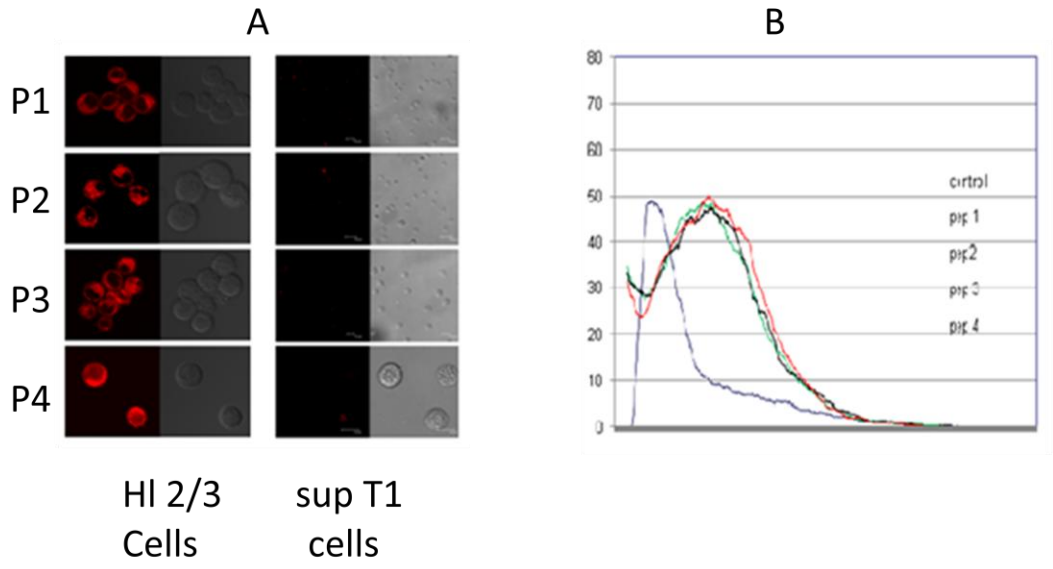
Plot Statistics

Residues in most favored regions [A,B,L]	24	88.9%
Residues in additional allowed regions [a,b,l,p]	2	7.4%
Residues in generously allowed regions [~a,~b,~l,~p]	1	3.7%
Residues in disallowed regions	0	.0%
Number of non-glycine and non-proline residues	27	100%
Number of end residues(excl. Gly and Pro)	2	
Number of Glycine residues (shown as triangles)	1	
Number of proline residues	3	
Total number of residues	33	

Panel A shows the structure of peptide, P-4 and the ramachandran plot of the same in the panel B whereas the lower panel C indicates the number of interactions showed by peptide with gp120.Here peptide P4 exhibited only one interaction with Gln267 of gp120.

Fig 4.8

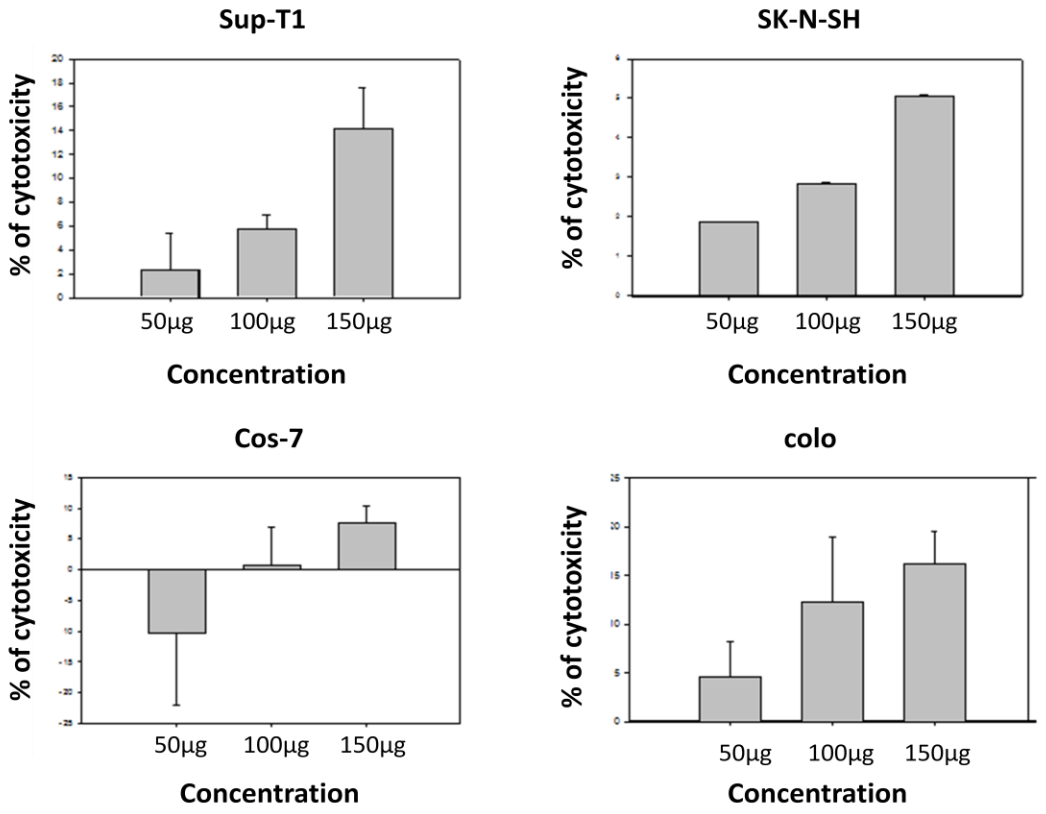
Binding affinity of peptides



Peptides were conjugated with RITC according to the mentioned protocol. The RITC conjugated peptides were incubated with HL 2/3 and Sup-T1 cells to test their affinity towards gp120. Panel A shows the confocal images of binding affinity of all the peptides to HL2/3 cells. Here peptides P-2, P-3, P-4 bound to the gp120 expressing HL2/3 cells more efficiently when compared to peptide P-1 whereas none of the peptides bound to SupT1 cells (control) that possess CD4 on its surface. The same results are shown by the FACS analysis shown in panel B.

Fig 4.9

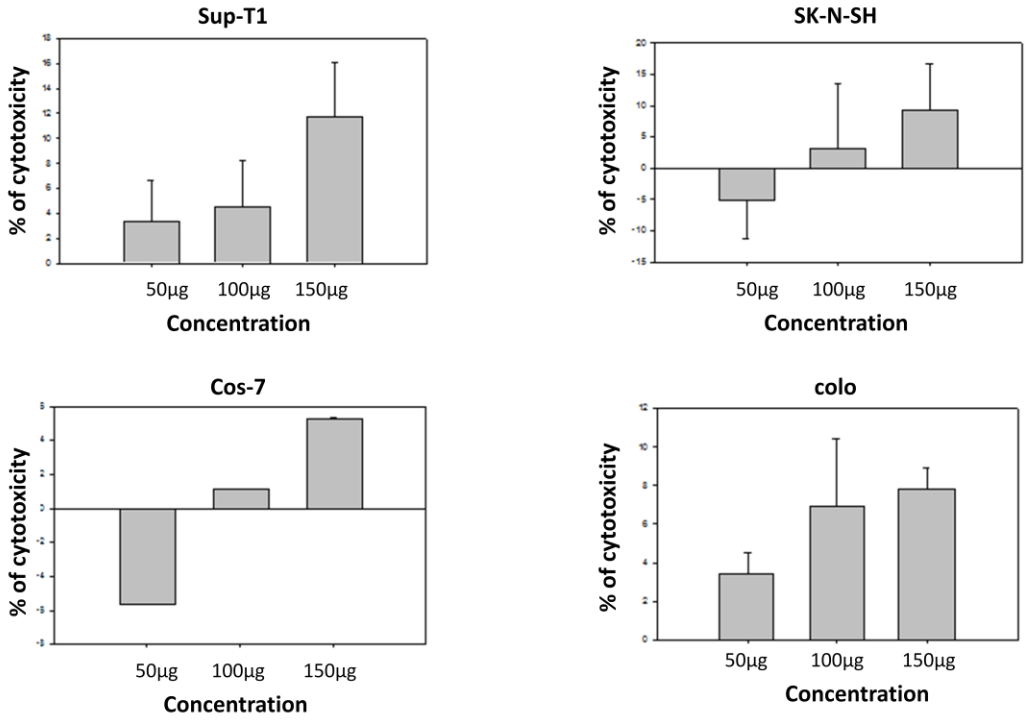
Cytotoxic profile of peptide, P-1



Cytotoxicity of peptide, P-1 was tested using MTT cell proliferation assay as per mentioned protocol for 16 hours in four different cell lines. Absorbance was taken at 570 nm to quantitatively measure the purple colour which indicates the percentage of live cells. All the 4 panels represent the cytotoxic profile of P-1 in different cell lines viz., colo 205, cos 7, SK-N-SH and Sup T1 cell lines. Even at 150 µg concentration only upto 20% toxicity was shown.

Fig 4.10

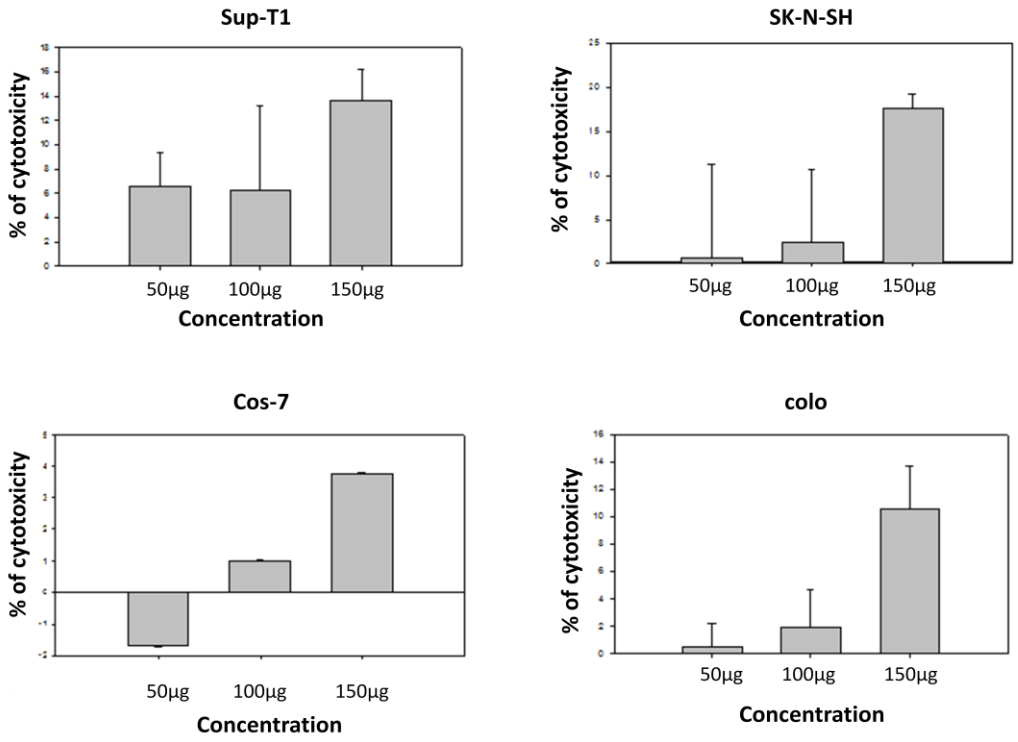
Cytotoxic profile of peptide, P-2



Cytotoxicity of peptide, P-2 was tested using MTT cell proliferation assay as per mentioned protocol for 16 hours in four different cell lines. Absorbance was taken at 570 nm to quantitatively measure the purple colour which indicates the percentage of live cells. All the 4 panels represent the cytotoxic profile of P-2 in different cell lines viz., colo 205, cos 7, SK-N-SH and Sup T1 cell lines. Even at 150 µg concentration only upto 15% toxicity was shown.

Fig 4.11

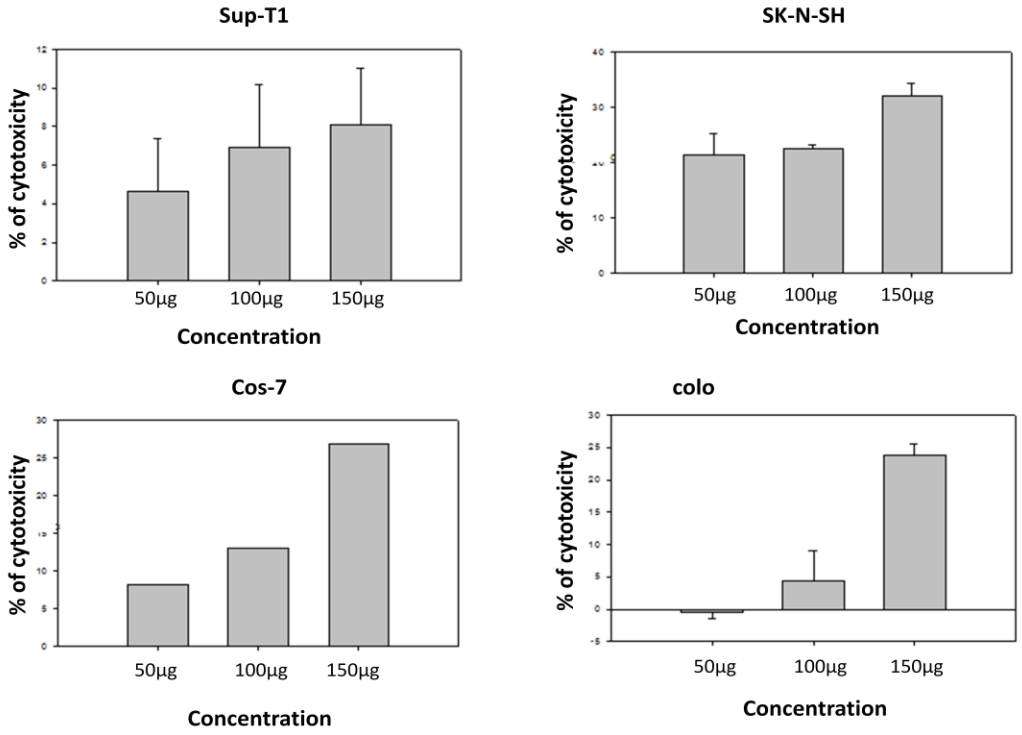
Cytotoxic profile of peptide, P-3



Cytotoxicity of peptide, P-3 was tested using MTT cell proliferation assay as per mentioned protocol for 16 hours in four different cell lines. Absorbance was taken at 570 nm to quantitatively measure the purple colour which indicates the percentage of live cells. All the 4 panels represent the cytotoxic profile of P-3 in different cell lines viz., colo 205, cos 7, SK-N-SH and Sup T1 cell lines. Even at 150 µg concentration only upto 15% toxicity was shown.

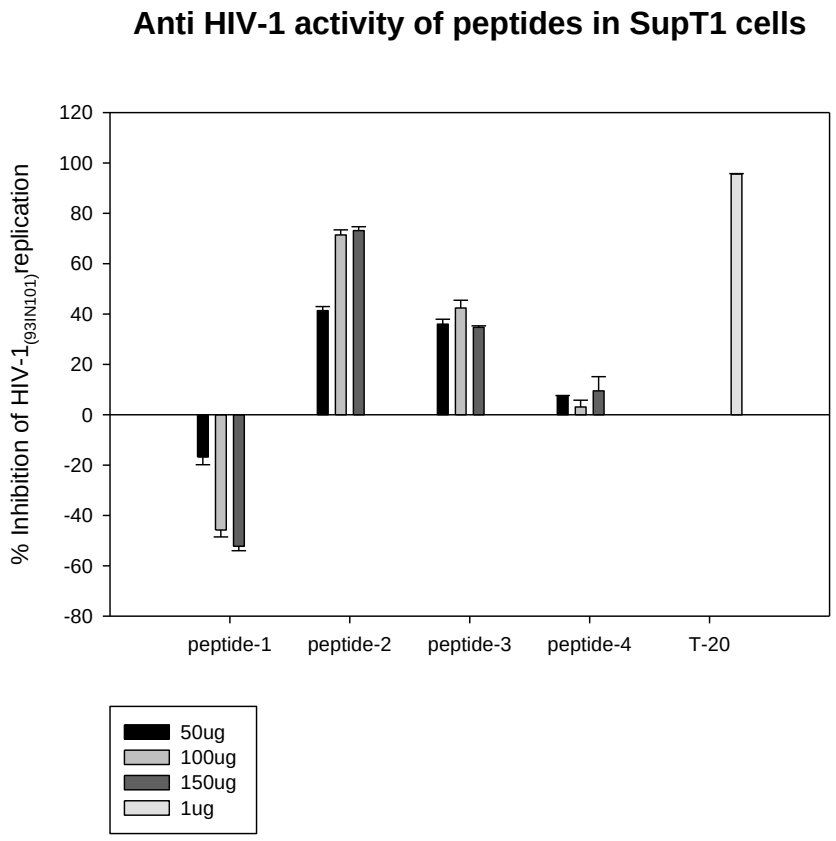
Fig 4.12

Cytotoxic profile of peptide, P-4



Cytotoxicity of peptide, P-4 was tested using MTT cell proliferation assay as per mentioned protocol for 16 hours in four different cell lines. Absorbance was taken at 570 nm to quantitatively measure the purple color which indicates the percentage of live cells. All the 4 panels represent the cytotoxic profile of P-4 in different cell lines viz., colo 205, cos 7, SK-N-SH and Sup T1 cell lines. Even at 150 µg concentration only upto 25% toxicity was shown.

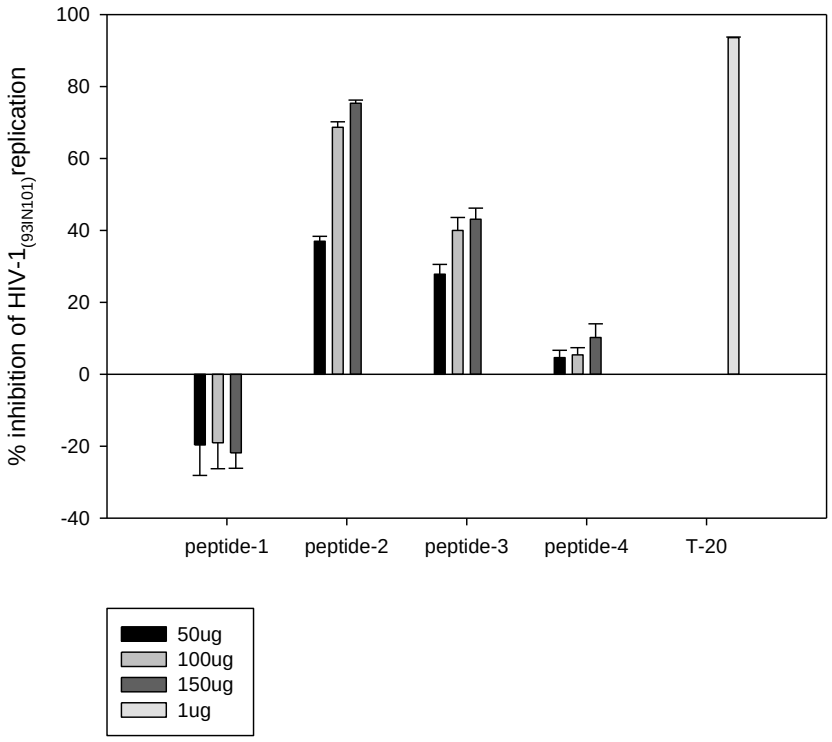
Fig 4.13



Sup T-1 cells were challenged with HIV-1 93IN101 in the presence and absence of Peptides for 2 hours. Cells were washed, re-cultured in fresh medium and amount of virus replicated at day 4 was estimated. Inhibition is calculated with reference to the control infection in the absence of virus, whose p24 was 8 ng/ml at day 4. Three concentrations 50, 100, 150 µg/ml of all four peptides were used. 1 µg/ml of T-20 was used as control. Out of all peptides P2 has shown 80% antiviral activity and P3 has shown 40% activity.

Fig 4.14

Anti HIV-1 activity of peptides in PBMC's



PBMC's were challenged with HIV-1 93IN101 in the presence and absence of Peptides for 2 hours. Cells were washed, re-cultured in fresh medium and amount of virus replicated at day 4 was estimated. Inhibition is calculated with reference to the control infection in the absence of virus, whose p24 was 8 ng/ml at day 4. Three concentrations 50, 100, 150 µg/ml of all four peptides were used. 1 µg/ml of T-20 was used as control. Out of all peptides P2 has shown 80% antiviral activity and P3 has shown 40% activity.

Chapter 5

Molecular action of potent peptides

Introduction

Since peptide, P-2 exhibited considerable anti-viral activity and binding to HL2/3 cells, it would be important to characterize the binding interaction of peptide against various epitopes of gp120 using monoclonal antibodies against those regions to know the specificity of the interaction. The amino-acid sequence of human immunodeficiency virus gp120 glycoproteins consists of five variable regions (V1–V5) interposed among more conserved regions (C1-C5). Variable regions V1–V4 form exposed loops anchored at their bases by disulphide bonds. Neutralizing antibodies recognize both variable and conserved gp120 structures. The V2 and V3 loops contain epitopes for strain-restricted neutralizing antibodies. More broadly neutralizing antibodies recognize discontinuous, conserved epitopes in three regions of the gp120 glycoprotein viz., CD4 binding site, CD4- induced epitopes (CD4i) and coreceptor binding site.

Development of potent and specific inhibitors and vaccines against Human immunodeficiency virus-1 (HIV-1) infection is current challenge in prevention and control of Acquired immunodeficiency syndrome (AIDS). Mutagensis and antigenic variability by the virus prompted a closer understanding of the molecular events of virus entry that would help in identification novel conserved dynamic epitope(s) of gp120 from various HIV clades. It needs to known whether such dynamic conformational changes of gp120 during virus invasion are comprised of single or multiple epitopes that are potential target for virus neutralization.

The peptide interacting epitopes of HIV-1/gp120 has been mapped using the various epitope specific gp120 monoclonal antibodies V2 specific: 697-30D, V3 epitope: 257-DIV (cf 257D) and C5 region: 670-30D (Dr. S. Zolla-Pazner); V3 loop: V3-21, SVEINCTRPNNNTRKSI, 298-315 (Dr. J. Laman); V3 loop: F425 B 4a.1 (cf F425) and gp41: F240 (Dr. M. Posner and Dr. L. Cavacini); CD4 Mab: SIM4 (Dr. J. E. K Hildreth); C1 reactive: B2-FNMW, 94-97; C2: B13-TQLLLN, 257-262; C3: B32-FFY, 382-384 V4 domain : B15 (Dr.G.W.Lewis).

Results

P-2 interacts with variable loop epitopes of $gp160_{JR-FL}$

Since peptide, P-2 showed significant anti-HIV activity, we have taken-up to analyse the molecular action of P-2 that interacts with gp120. To monitor the involvement of all the envelope epitopes in P-2 interaction, $gp160_{JR-FL}$ was used in all these studies. To understand molecular interaction of P-2 with gp160, the $gp160_{JR-FL}$ was incubated with increasing concentrations of P-2 and gp160-P-2 complexes were captured onto various anti-gp160 monoclonal antibody coated wells. The binary complexes were detected with affinity purified Epap-1 polyclonal antibody. The results shown in **Fig 5.1** suggest P-2 forms a binary complex with gp160 through an interaction with variable regions of the gp160, with a strong interaction at V3 loop regions. The interaction at V3-F425 and V3-257D is enhanced with increasing concentration of P-2 suggesting its strong binding to gp120 in V3 loop regions. P-2 shows a week interaction with V4 loop regions and exhibits negligible interaction with the constant regions of soluble monomeric gp160.

P-2 can interact with gp160 in presence of CD4

The ability of P-2 interaction with CD4-gp120 binary complex was analyzed by incubation of soluble gp160 and CD4 with increasing concentrations of P-2. The results in **Fig 5.2** show that P-2 interaction with gp160-CD4 would block the exposure of variable (F425, 257-DIV) regions of gp160 suggesting higher specificity of its binding in this region.

P-2 interacts with gp160 in presence of coreceptor and CD4

To examine the molecular epitope(s) of gp160 that may be involved in the interaction of P-2 to form a quaternary complex, we have incubated CD4-gp160-CCR5 in the presence of increasing concentrations of P-2. The complexes containing gp160 were captured by the wells coated with anti-gp160 monoclonal antibodies and the captured quaternary complexes were detected with affinity purified Epap-1 polyclonal antibody. The results show that P-2 can interact with gp160 even in the presence of CD4 and CCR5 (**Fig 5.3**). Further, the P-2 binding with V3 loop regions is remained unaltered in the quaternary complexes of P-2-gp160-CD4-CCR5 suggesting the strong interaction of P-2 with V3 loop regions (F425, 257DIV).

P-2 shows strong interaction with virus surface gp120

Since the gp120 epitopes exposed in soluble gp160 may be different from that of the epitopes of gp120 exposed on the surface of the virus, studying the interaction of peptide, P-2 with virus surface gp120 would be functionally relevant. The virus

was captured using monoclonal antibodies spanning gp120 and gp41 regions in the presence of P-2. The exposure of various epitopes of gp120 in the presence of P-2 was monitored in terms of the amount of virus binding to corresponding antibody. The amount of bound virus was estimated by quantifying in terms of viral p24. The results of these experiments show that P-2 binding to gp120 could block specifically at V3-F425 and 257DIV epitopic regions (**Fig 5.4**). Hence, P-2 interacts with monomeric soluble gp160 and virus surface gp120 in a similar mode, where in, it interacts with epitopes V3-425.

Analysis of P-2 interaction with HIV-1-CD4 complex

Virus surface gp120 interaction with P-2 and CD4 was investigated in two ways.

In the first, HIV-1 was pre-incubated with P-2 and the HIV-1-P-2 binary complexes were incubated with CD4 to form ternary complexes comprising P-2-HIV-1-CD4. The epitopes of gp120 present in the ternary complexes were then analyzed. The results show that epitopes V3-F425 and 257-DIV masked by P-2, while the remaining epitopes were unaltered with increasing concentration of P-2 (**Fig 5.5**). This suggests that P-2 binding can specifically block V3 loop epitopes, V3-F425, 257-DIV.

To examine whether P-2 can interact with the virus alone and/or also with CD4 bound conformation of virus, we have analyzed P-2 binding ability with binary complex of CD4 and virus. Virus was pre-incubated with soluble CD4 to form binary complexes; these binary complexes were further incubated with increasing concentrations of P-2. The ternary complexes containing gp120-CD4-P-2 were

captured using monoclonal antibodies spanning different regions of gp120. The results of analysis show that P-2 can block epitopes, V3-F425 and 257-DIV. These results confirm that P-2 can interact with the conformation of gp120 in virus as well as virus complexed with CD4 (**Fig 5.6**). The conserved epitopes that is blocked by P-2 in both states were primary isolates neutralizing epitope of gp120 V3 loop, V3 F425 and 257-DIV. This suggests that the P-2 interacts selectively and induces partial conformational changes in various epitopes of gp120 without completely interfering with its CD4 interaction.

Action of P2 on gp120 mediated cell fusion

Cell fusion bioassay was carried out by using CD4 positive Sup T-1 cells and gp120 expressing HL 2/3 cells. HL 2/3 cells and sup T-1 cells were incubated in the presence and absence of P2. Then the cells were incubated for 2 hours. The results in **Fig 5.7** shows that the P2 indeed bound to HL 2/3 cells, and inhibited the fusion of Sup-1 cells and HL 2/3 cells, while cells are fused in the absence of P2.

Action of P2 on Reporter based TZM-BL cells

Anti-HIV-1 activity of peptide-2 was reconfirmed by reporter based assay. TZM-BL cells were challenged with HIV-1 in the presence and absence of peptides. Expression of β-gal occured during HIV infection to the untreated TZM-BL cells and the reduction of X-gal took place, whereas the presence of T20 and P2 inhibited the viral infection and the subsequent expression of β-gal (**Fig 5.8,**

Panel A). The results of FACS analysis presented in **Fig 5.8 Panel B**, confirm that Peptides possess affinity to gp120 and prevented HIV-1 infection.

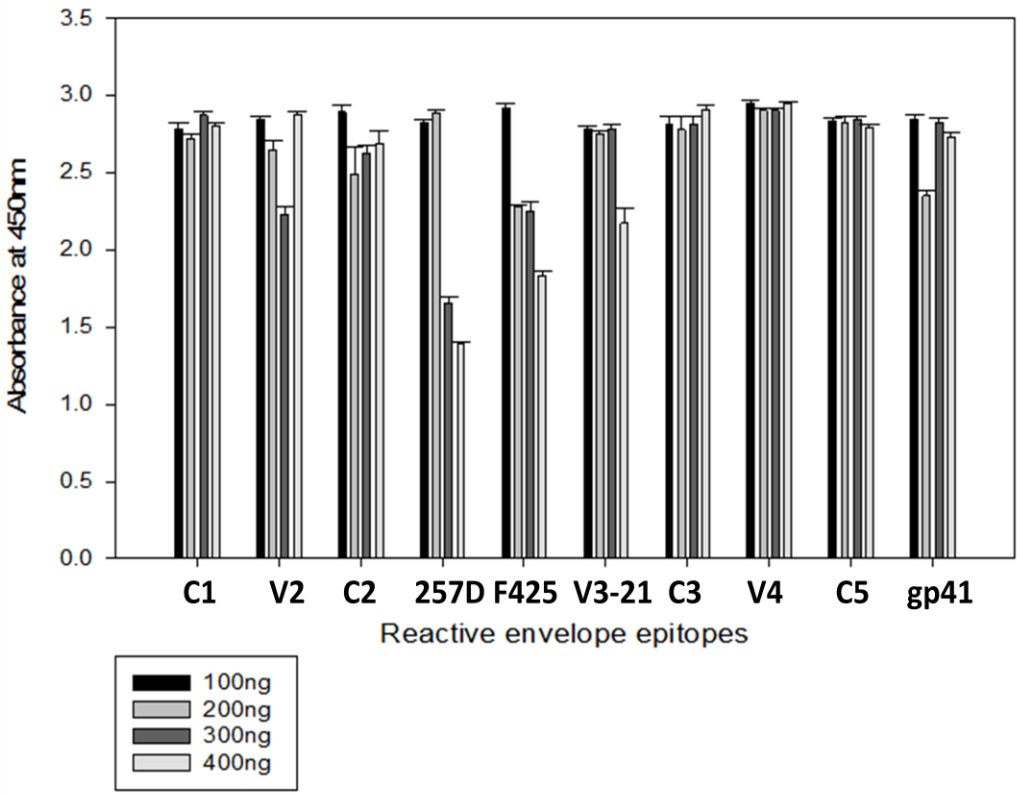
Discussion

The interaction studies done between the peptide and the gp120 antibodies with both soluble gp160 and virus bound gp120 showed that it specifically interacts with the regions recognized by 257D and F425 antibodies by ELISA. The inhibition of viral entry was validated by cell fusion assay and β-gal reporter assay. Even though the interaction of peptide with gp120 is specific, it is not possible to assess the energetic contribution of a given residue side chain to binding affinity from structural results alone (Cunningham and Wells, 1993;Dall'Acqua et al., 1998; Seligman et al., 1996) and results from antibody binding assays with peptides as solid-phase antigens are not always good predictors of antibody binding to V3 regions in the context of gp120 (Gorny et al., 2002; Moore, 1993; Moore et al., 1994). That might be another reason of the higher IC-50 value of P-2 .The V3 region of HIV-1 gp120 is arguably a potential target for HIV vaccine design (Zolla-Pazner, 2005). It is often presumed that the inability of anti-V3 antibodies to neutralize broadly is largely due to limited accessibility of the V3 region to antibody when V3 is in the context of the functional envelope spike on the viral surface. Indeed, recent studies have shown that V3 may be shielded – at least in part and on certain viruses – by the V1/V2 region (Krachmarov et al., 2006; Pinter et al., 2004), thus supporting the results from earlier investigations into the functional properties of V1/V2 (Cao et al., 1997; Wyatt et al., 1993). However, it should also be noted that sequence

variability in the regions flanking the conserved β-turn in the V3 crown (GPGR sequence) may alter the stability of the turn or alter its surface accessibility (Catasti et al., 1995). The influence of V1/V2 and sequence variation in V3 on the binding interaction of mAb F425-B4e8 with V3 will be subject to further investigation.

Fig 5.1

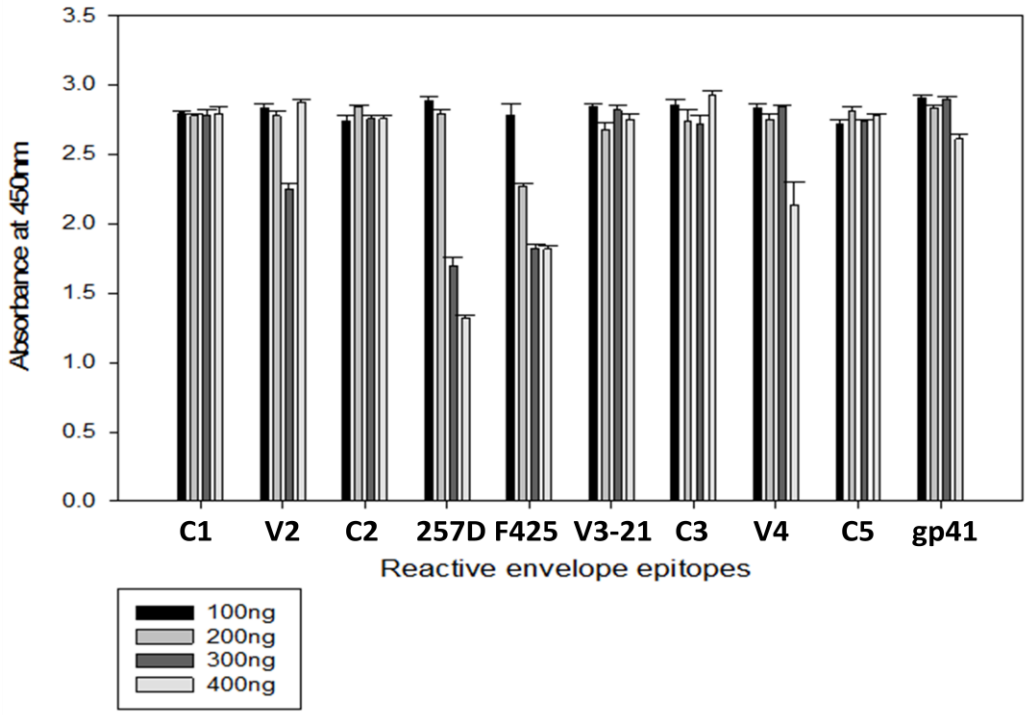
Binding of P-2 at V3 regions of gp160



HIV-1 gp160 was incubated with increasing concentrations of P-2. P-2-gp160 complexes were captured by gp160 monoclonal - coated wells as indicated on X-axis. The P-2 in gp60 - P-2 complexes was probed using polyclonal antibody against Epap-1. Absorbance was taken at 450 nm. Lesser O.D value in the antibody well indicates the potential binding region of peptide against that particular antibody since the peptide blocks the binding of antibody to the gp160 at that region.

Fig 5.2

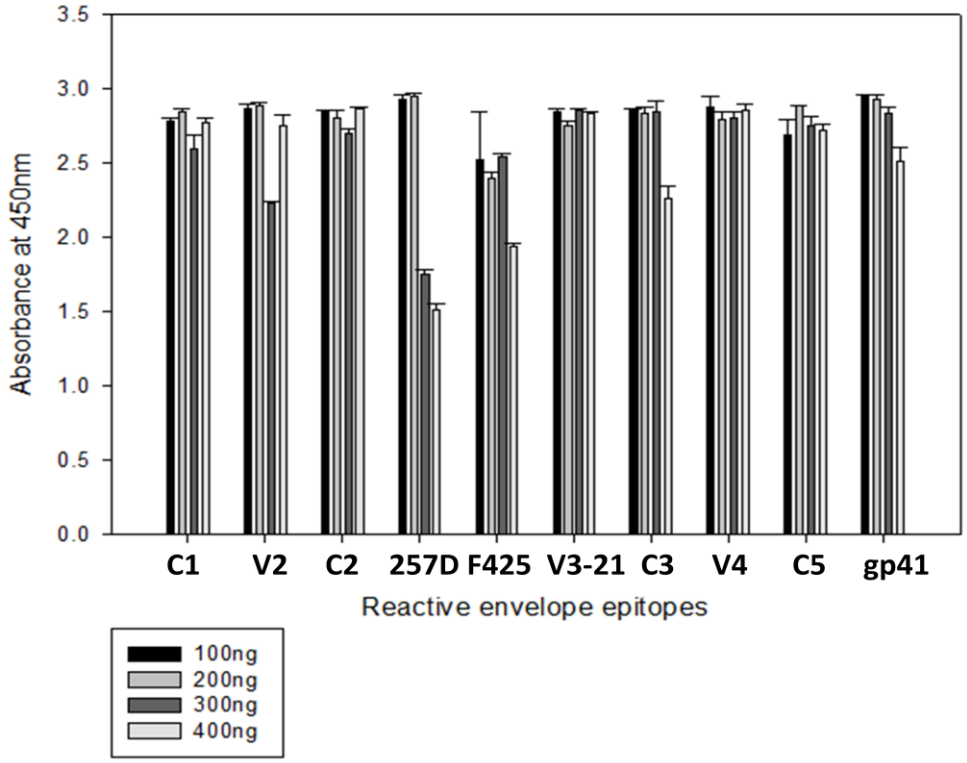
Binding of P2 with gp120 in the presence of CD4



HIV-1 gp160 and CD4 were incubated with increasing concentrations of P-2. P-2-gp160 - CD4 complexes were captured by gp160 monoclonal- coated wells as indicated on X-axis. The P-2 in gp60 – CD4 - P-2 ternary complexes was probed using polyclonal antibody against Epap-1. Absorbance was taken at 450 nm. Lesser O.D value in the antibody well indicates the potential binding region of peptide against that particular antibody since the peptide blocks the binding of antibody to the gp160 at that region.

Fig 5.3

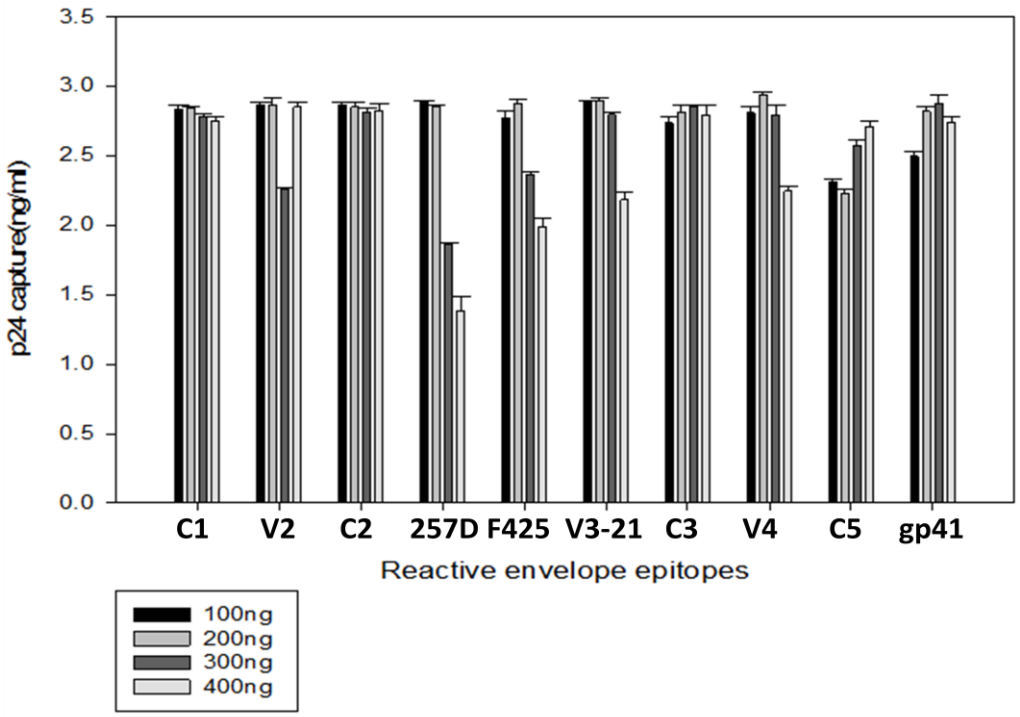
Binding of P-2 with gp120 in the presence of Cd4 and CCR5



HIV-1 gp160, CD4 and CCR5 were incubated with increasing concentrations of P-2. P-2 - gp160 - CD4 – CCR5 complexes were captured by gp160 monoclonal- coated wells as indicated on X-axis. The P-2 in the complexes was probed using polyclonal antibody against Epap-1. Absorbance was taken at 450 nm. Lesser O.D value in the antibody well indicates the potential binding region of peptide against that particular antibody since the peptide blocks the binding of antibody to the gp160 at that region.

Fig 5.4

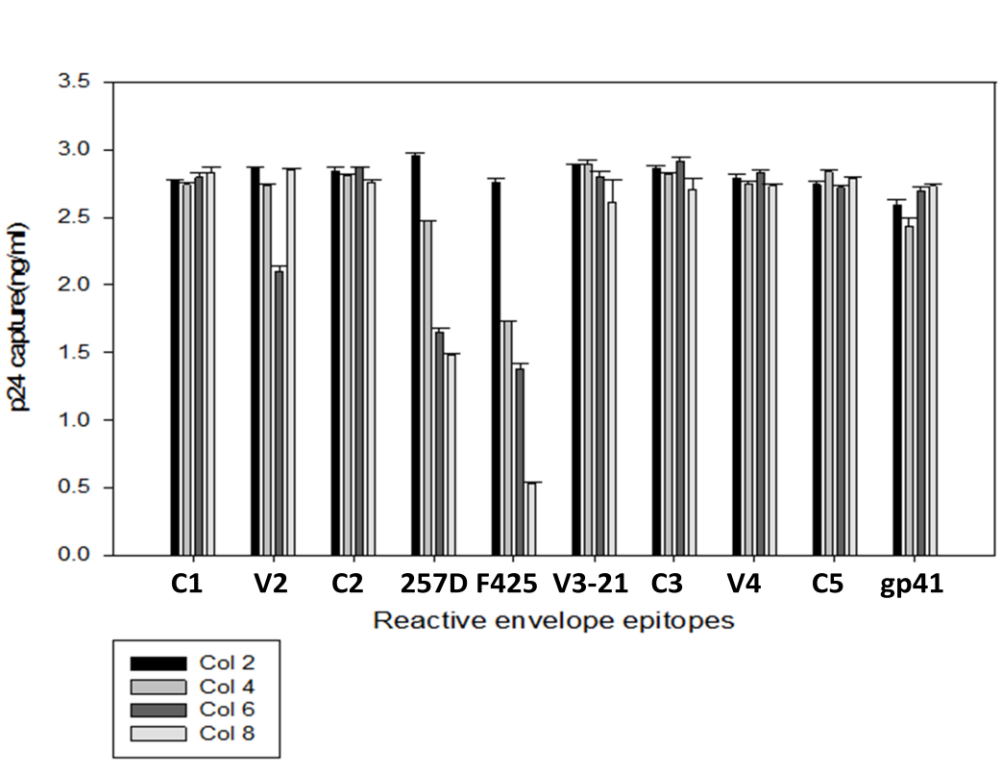
Interaction of P-2 with virus surface gp120



Virus was incubated with increasing concentrations of P-2, virus was captured with gp120 monoclonal antibody coated wells as indicated on X-axis. The bound virus was lysed and p24 was estimated and plotted on Y-axis. Decreased p24 value in the experiment conducted with a particular antibody well indicates the potential binding region of peptide against that particular antibody since the peptide blocks the binding of antibody to the gp160 at that region.

Fig 5.5

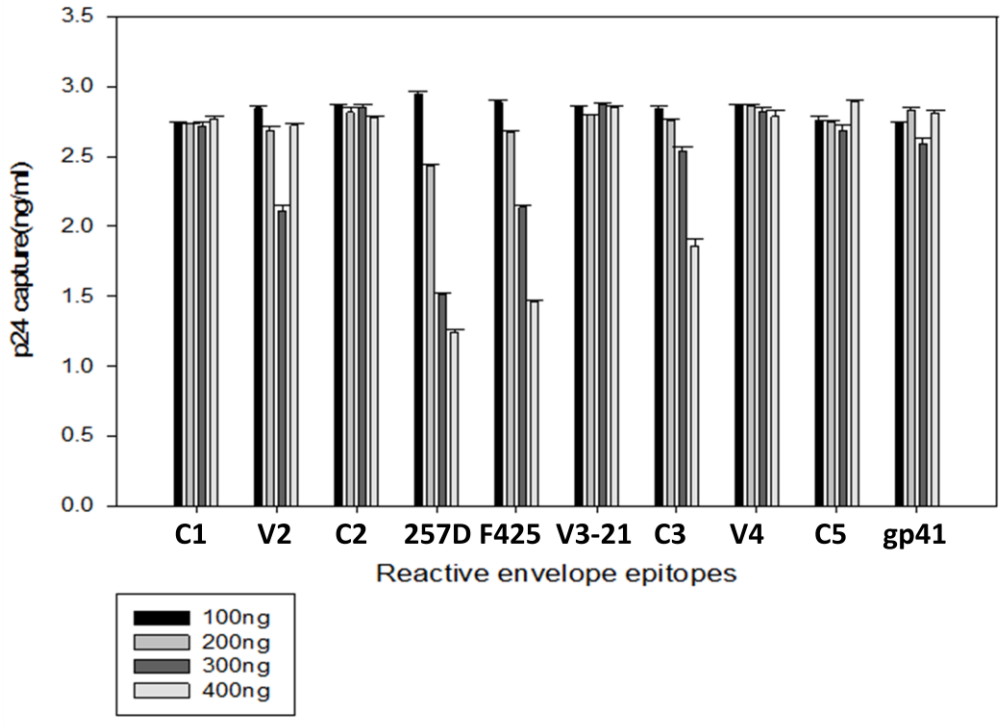
Interaction of virus surface gp120-P-2 complex with CD4



Virus was pre-incubated with increasing concentrations of P-2. These binary complexes were incubated with soluble CD4. The viral gp120 epitopes were captured with anti-gp120 monoclonal antibody coated wells as indicated on X-axis. The bound virus was lysed and p24 was estimated and plotted on Y-axis. Decreased p24 value in the experiment conducted with a particular antibody well indicates the potential binding region of peptide against that particular antibody since the peptide blocks the binding of antibody to the gp160 at that region.

Fig 5.6

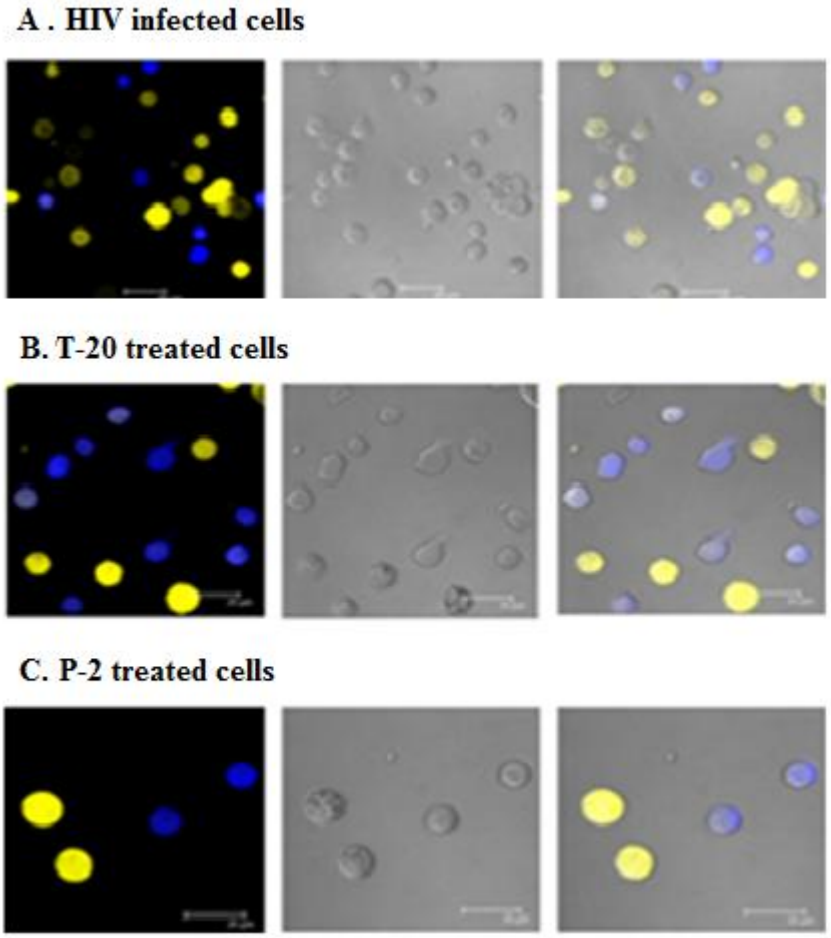
Interaction of virus surface gp120-CD4 complex with P-2



Virus was pre-incubated with soluble CD4. These binary complexes were incubated with increasing concentrations of P-2. The viral gp120 epitopes were captured with anti-gp120 monoclonal antibody coated wells as indicated on X-axis. The bound virus was lysed and p24 was estimated and plotted on Y-axis. Decreased p24 value in the experiment conducted with a particular antibody well indicates the potential binding region of peptide against that particular antibody since the peptide blocks the binding of antibody to the gp160 at that region.

Fig 5.7

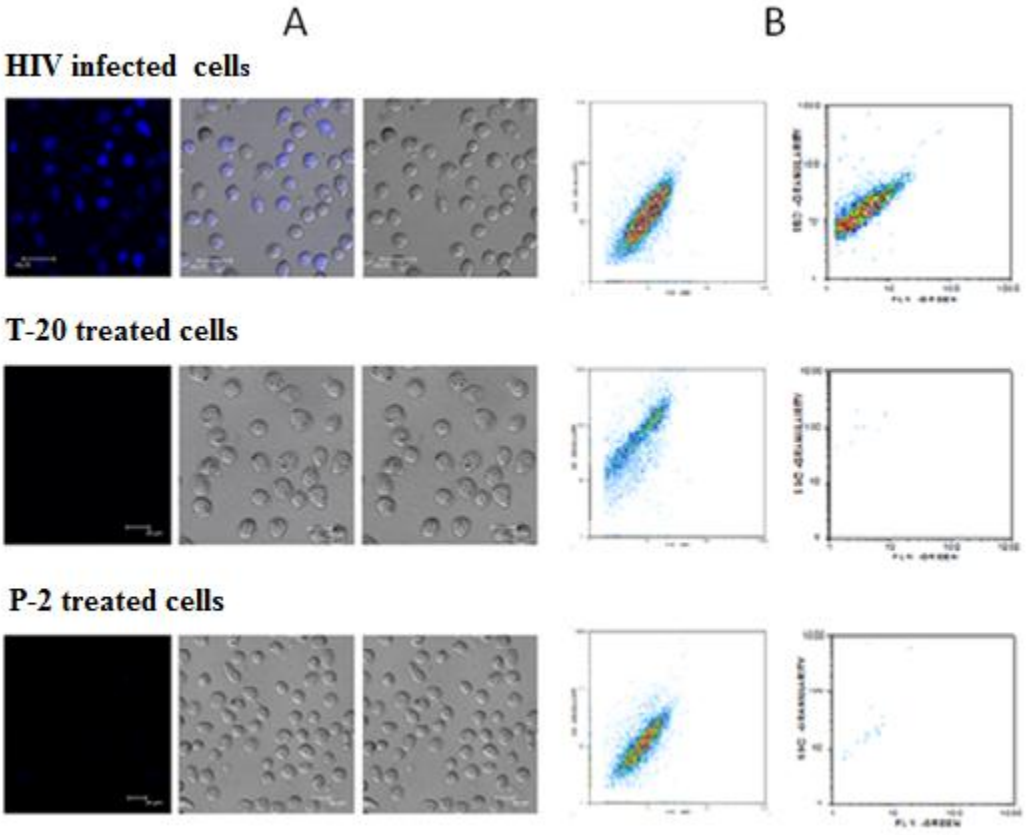
Inhibition of cell fusion by P-2



Dye transfer assay: Calcein Blue loaded Sup-T1 (Blue colour) and Calcein AM loaded HL2/3 (Yellow colour) cells were incubated in the presence of P-2. In the control assay (panel A) dye transfer took place between the cells whereas in the presence of T-20 (panel B) and P2 (panel C) there is significant reduction in the interaction of both cells which resulted in the inhibition of dye transfer.

Fig 5.8

Inhibition of viral entry by P2 in reporter cell line, TZM-BL



HIV-1 mediated β-gal expression in TZM-BL cells: TZM-BL cells were infected with HIV-1. HIV-1 entry allows tat mediated LTR based expression of β-gal and the fluorescent cells (due to azo dye) were counted by confocal microscopy and flow cytometry. Panel A indicates the confocal image of the TZM-BL cells which shows the expression of β- gal upon HIV-1 infection in control cells and the inhibition of expression by T-20(positive control) and P2. The Panel B shows the FACS analysis of the same assay to count the number of cells fluoresced.

Chapter 6

Design and Evaluation of peptides with enhanced activity

Introduction

Further molecular analysis of all the 4 peptides with gp120 revealed that all the major possible interactions lie in the first 15 residues. The docking was performed by taking the first 10-15 residues of all the 4 peptides with gp120. First peptide did not yield any significant interaction, while other 3 peptides P-2.1, P-3.1, P-4.1 showed 10, 7 and 1 interactions respectively. To experimentally confirm these interactions the above 3 peptides were synthesized commercially and analyzed for their anti-HIV activity and molecular action on gp120 mediated cell fusion .

Results

Molecular interactions of the peptides

Modeling was done for all the four initial peptides by talking the first fifteen residues where the maximum number of interactions was found. The first peptide which acquired a new structure could not bind to gp120. The second peptide increased the interactions to ten with gp120. And the third peptide has shown seven interactions with gp120. Fourth peptide was also omitted since it exhibited only one interaction with gp120. All the residues of the three peptides fall in the allowed regions of the Ramachandran's plot **(Fig 6.1, 6.2 and 6.3)**.

Analysis of cytotoxicity and anti-HIV activity of designed peptides

Cytotoxity of all the 3 peptides was analyzed in Sup-T1 cells at 50, 100 and 150 µg/ml concentrations by using MTT cell based proliferation assay. All the three peptides were considerably less toxic (<5%) **(Fig 6.4)**. Analysis of anti-HIV

activity of the peptides on HIV-1 93IN101 **(Fig 6.5)** and HIV-1 93RW024 **(Fig 6.7)** strains in Sup-T1 cells revealed that P2.1 and P3.1 could exhibit 80% inhibition whereas these peptides exhibited 60% activity on HIV-1 94 UG103**(Fig 6.8)** strain. P-4.1 was inactive.

Synergistic action of peptides

The synergistic action of both peptides P 2.1 and P3.1 in combination were tested for their HIV inhibition that resulted in 80% activity at 25ug concentration each (ie., total of 50ug) **(Fig 6.6)**. This confirmed that the peptides P2 and P3 have affinity to gp120 and anti-HIV active. Further analysis was taken up to analyze its molecular action.

Interaction of peptides with virus surface gp120.

The virus was captured using monoclonal antibodies spanning gp120 and gp41 regions in the presence of mixture of P-2.1 and P-3.1. The exposure of various epitopes of gp120 in the presence of peptides was monitored in terms of the amount of virus binding to corresponding antibody. The amount of bound virus was estimated by quantifying in terms of viral p24. The results of these experiments show that peptides binding to gp120 can block specifically at V3-F425 and 257DIV epitopic regions (**Fig 6.9**).

Action of P-2.1 and P-3.1 on Reporter based TZM-BL cells

Anti-HIV-1 activities of P-2.1 and P-3.1 were reconfirmed by reporter based assay. TZM-BL cells were challenged with HIV-1 in the presence and absence of

peptides. Expression of β-gal occured during HIV infection to the untreated TZM-BL cells and the reduction of X-gal took place, whereas the presence of T20, P-2.1 and P-3.1 inhibited the viral infection and the subsequent expression of β-gal (**Fig 6.10, Panel A**). The results of FACS analysis presented in **Fig 6.10 Panel B**, confirm that Peptides possess affinity to gp120 and prevented HIV-1 infection.

Discussion

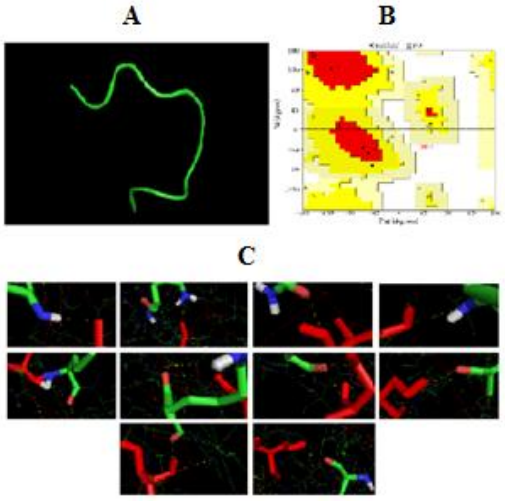
Modeling and docking of Epap-1 with trimeric gp120 showed the presence of specific interactions in stabilization of their binding. These molecular interactions with gp120 showed high affinity to V3 loop region. Along with this V3 loop there exists a potential interaction with other aminoacids in C2 (Glu268, Ile285 in P3.1), C3 (Asn340, Gln344, Ser347 in P3.1; Ser347, Ile339, Ile345, Ala346, Lys348. Arg350, Asn356 in P2.1) and C4 regions (Arg456 in P3.1). Indeed the insilico mutations of interacting aminoacids in P2.1 (Ser2, Ala5, Thr7, Ala8) and P3.1 (Val1, His14, Asp5, Cys6, Leu4, Ser10) abolishes the affinity of peptides to trimeric gp120. This suggests the significant role played by these amino acids in interacting with other residues spanning neighboring regions around V3 loop.

Even though the V3 region is immunodominant due to its extended nature and antibody accessibility (Huang et al., 2005) it is often masked by folding of the V2 loop along the V1/V2 stem (Wyatt et al., 1998). And the conformational shifts that occur in other variable regions make the binding of antibodies difficult in theses cryptic residues. On the other hand the recessed nature of other receptor binding sites contributes to their poor immunogenicity (Wyatt et al., 1998). But

the involvement of constant regions in virus neutralization need further attention as these regions may be unique and conserved among strains and thus may be involved in host recognition and pathogeneicity. One of the highlighting achievements in the present study is that the peptide not only binds to the V3 loop but also encompasses the adjacent constant regions. This is supported by the synergistic action of the two peptides. This kind of cooperative interdomain interactions offered by the peptides may provide some important clues in the development of efficient small molecule inhibitors.

Fig 6.1

Structure and Molecular interactions of P-2.1



Plot Statistics

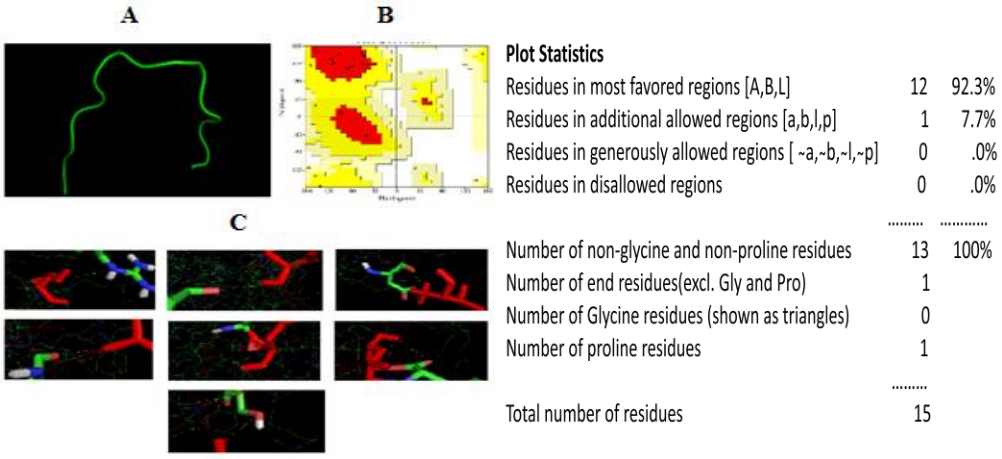
Residues in most favored regions [A,B,L]	5	62.5%
Residues in additional allowed regions [a,b,l,p]	2	25%
Residues in generously allowed regions [~a,~b,~l,~p]	0	.0%
Residues in disallowed regions	1	12.5%
Number of non-glycine and non-proline residues	8	100%
Number of end residues(excl. Gly and Pro)	2	
Number of Glycine residues (shown as triangles)	0	
Number of proline residues	0	
Total number of residues	10	

Panel A shows the structure of peptide, P-2.1 and the ramachandran plot of the same in panel B whereas the lower panel C indicates the number of interactions showed by peptide with gp120.Here peptide P-2.1 exhibited ten interactions with gp120 that were shown in the below table.

Sl. No.	Interacting Molecules	Bond Lengths in A
1	GLN352(NE2)-(O)ALA9	2.79
2	LYS348(O)-(O)THR7	3.24
3	ARG350(N)- (O)ALA8	3.14
4	ILE345(O)-(N)ALA8	2.50
5	ALA346(O)-(OG)SER2	3.32
6	SER347(N)-(O)ALA5	2.53
7	LYS357(O)-(O)ARG1	2.43
8	ASN356(OD1)-(N)THR 7	2.35
9	LYS357(O)-(OG1)THR 7	2.36
10	ILE339(N)-(OG1)THR 7	3.49

Fig 6.2

Structure and Molecular interactions of P-3.1

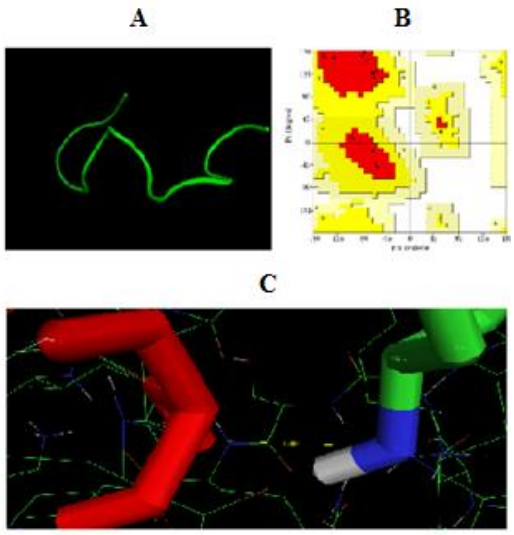


Panel A shows the structure of peptide, P-3.1 and the ramachandran plot of the same in panel B whereas the lower panel C indicates the number of interactions showed by peptide with gp120.Here peptide P-3.1 exhibited seven interactions with gp120 that were shown in the below table.

Interacting Residues of gp120 with epitope	Bond Distances in A
GLU268(O) – (N)VAL1	3.24
SER347(OG) – (O)LEU4	2.94
ASN340(O) – (N)ASP5	3.47
GLN344 (O) – (N)CYS6	3.21
PHE396(OXT) – (O)LEU7	3.37
ARG456(NE) – (OG)SER10	3.35
ILE285(O) – (O)HIS14	2.13

Fig 6.3

Structure and Molecular interactions of P-4.1



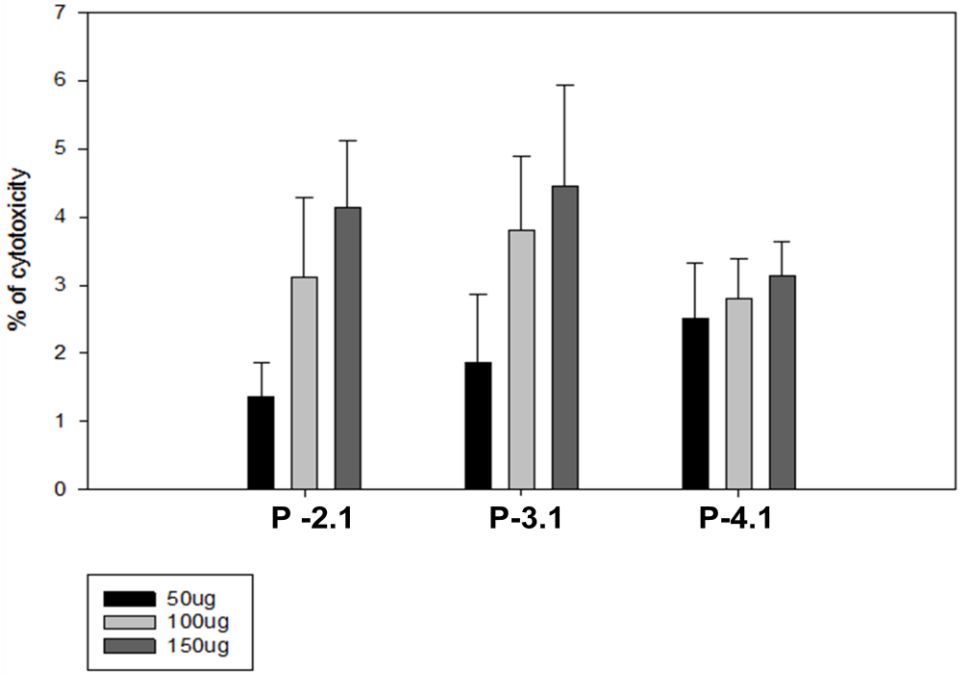
Plot Statistics

Residues in most favored regions [A,B,L]	12	92.3%
Residues in additional allowed regions [a,b,l,p]	1	7.7%
Residues in generously allowed regions [~a,~b,~l,~p]	0	.0%
Residues in disallowed regions	0	.0%
Number of non-glycine and non-proline residues	13	100%
Number of end residues(excl. Gly and Pro)	2	
Number of Glycine residues (shown as triangles)	0	
Number of proline residues	0	
Total number of residues	15	

Panel A shows the structure of peptide, P-4.1 and the ramachandran plot of the same in panel B whereas the lower panel C indicates the number of interactions showed by peptide with gp120. Peptide P-4.1 exhibited only one interaction at phe353 of gp120.

Fig 6.4

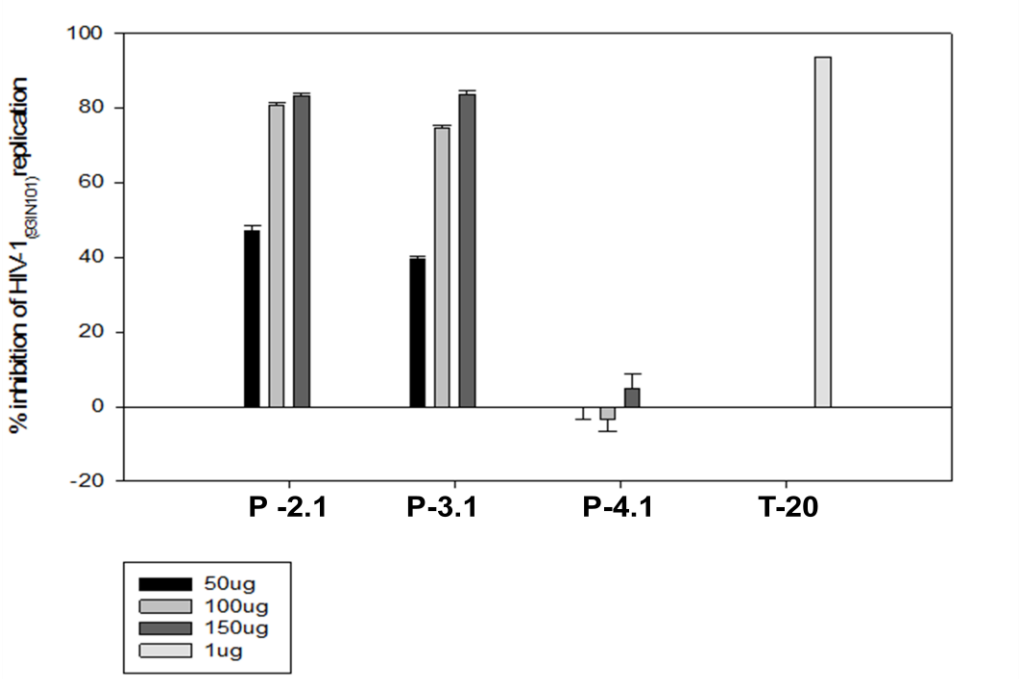
Cytotoxicity of peptides in Sup-T1 cells



Cytotoxicity of all the three peptides was tested using MTT cell proliferation assay as per mentioned protocol for 16 hours in four different cell lines. Absorbance was taken at 570 nm to quantitatively measure the purple color which indicates the percentage of live cells. All the three peptides were not cytotoxic and exhibited negligible cytotoxicity of 4% at 150µg concentration.

Fig 6.5

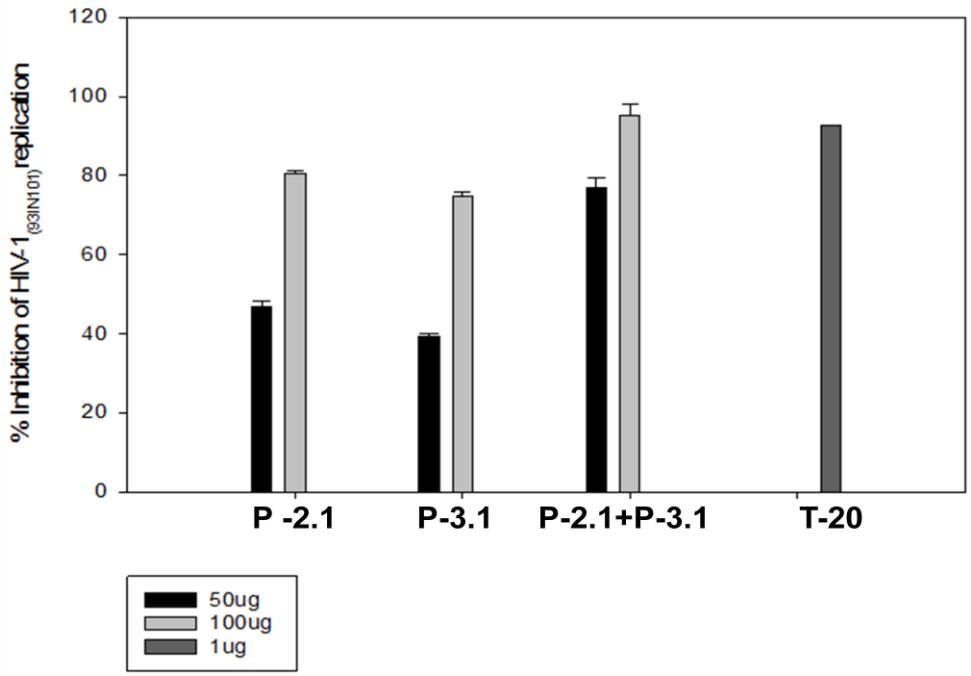
Anti-Viral assay of peptides in Sup-T1 cells



Sup T-1 cells were challenged with HIV-1 93IN101 in the presence and absence of Peptides for 2 hours. Cells were washed, re-cultured in fresh medium and amount of virus replicated at day 4 was estimated. Inhibition is calculated with reference to the control infection in the absence of virus, whose p24 was 8 ng/ml at day 4. Three concentrations 50, 100, 150 µg/ml of all three peptides were used. 1 µg/ml of T-20 was used as control. Out of 3 peptides P-2.1 and P-3.1 have shown 80% antiviral activities.

Fig 6.6

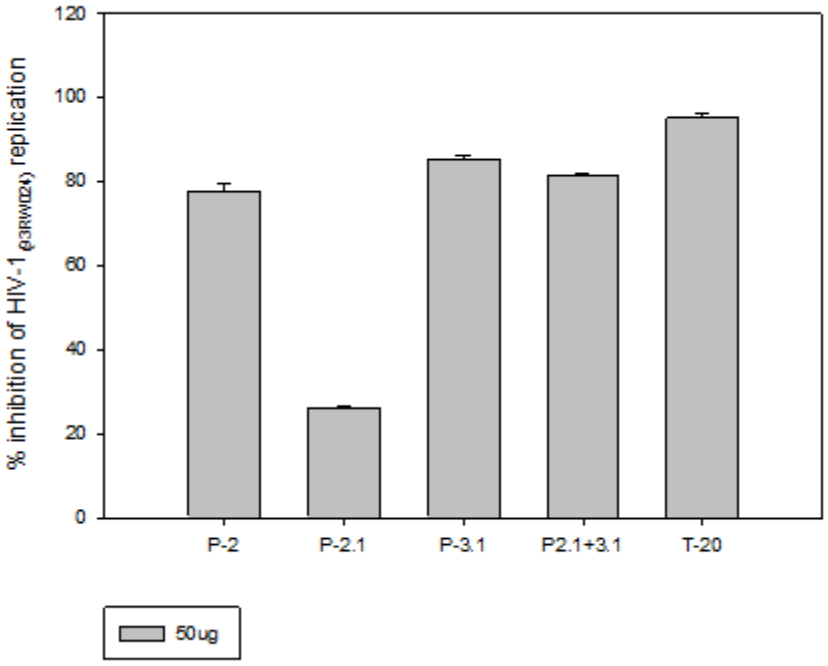
Synergistic antiviral action of peptides



Sup T-1 cells were challenged with HIV-1 93IN101 in the presence and absence of Peptides for 2 hours. Cells were washed, re-cultured in fresh medium and amount of virus replicated at day 4 was estimated. Inhibition is calculated with reference to the control infection in the absence of virus, whose p24 was 8 ng/ml at day 4. Two concentrations 50, 100µg/ml of the two peptides were used. 1 µg/ml of T-20 was used as control. To test the synergistic anti-viral action of peptides mixture of 25 µg/ml of each P-2.1 and P-3.1 were added . Here each peptide exhibited 40% activity at 50µg concentration individually whereas the mixture of both peptides of 50µg concentration exhibited 80% activity.

Fig 6.7

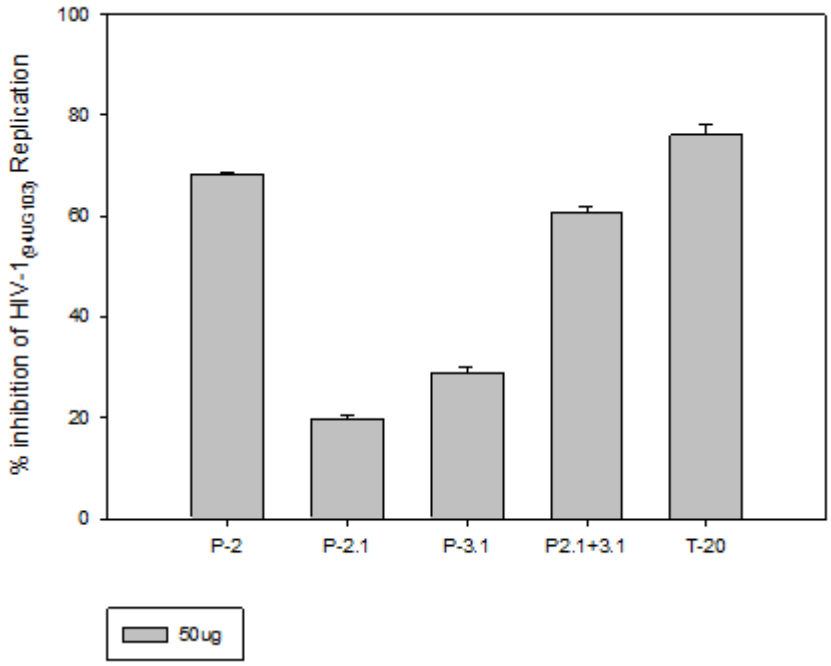
Anti-Viral assay of peptides on HIV-1 93RW024 strain



Sup T-1 cells were challenged with HIV-1 93RW024 in the presence and absence of Peptides for 2 hours. Cells were washed, re-cultured in fresh medium and amount of virus replicated at day 4 was estimated. Inhibition is calculated with reference to the control infection in the absence of virus, whose p24 was 8 ng/ml at day 4. All peptides were of 50 µg/ml concentration . 1 µg/ml of T-20 was used as control. Out of 3 peptides P-2 and P-3.1 have shown 80% antiviral activities along with the combination of P-2.1 and P-3.1.

Fig 6.8

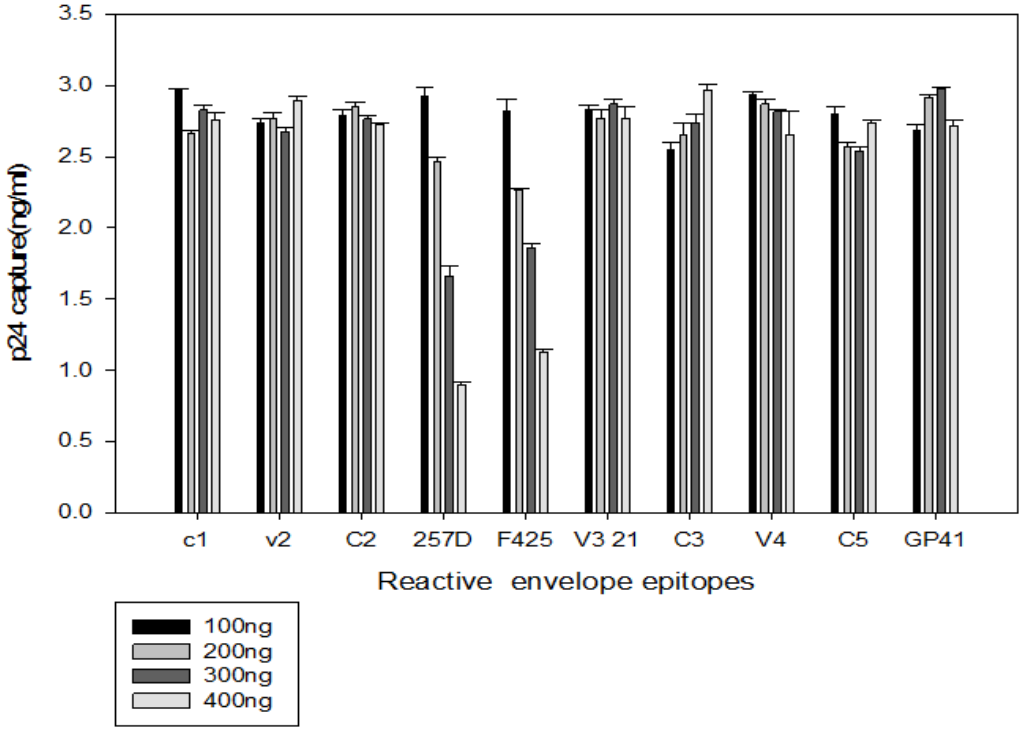
Anti-Viral assay of peptides on HIV-1 94UG103 strain



Sup T-1 cells were challenged with HIV-1 94UG103 in the presence and absence of peptides for 2 hours. Cells were washed, re-cultured in fresh medium and amount of virus replicated at day 4 was estimated. Inhibition is calculated with reference to the control infection in the absence of virus, whose p24 was 8 ng/ml at day 4. All peptides were of 50 µg/ml concentration. 1 µg/ml of T-20 was used as control. Out of 3 peptides P-2 and the mixture of P-2.1 and P-3.1 have shown 60% antiviral activities.

Fig 6.9

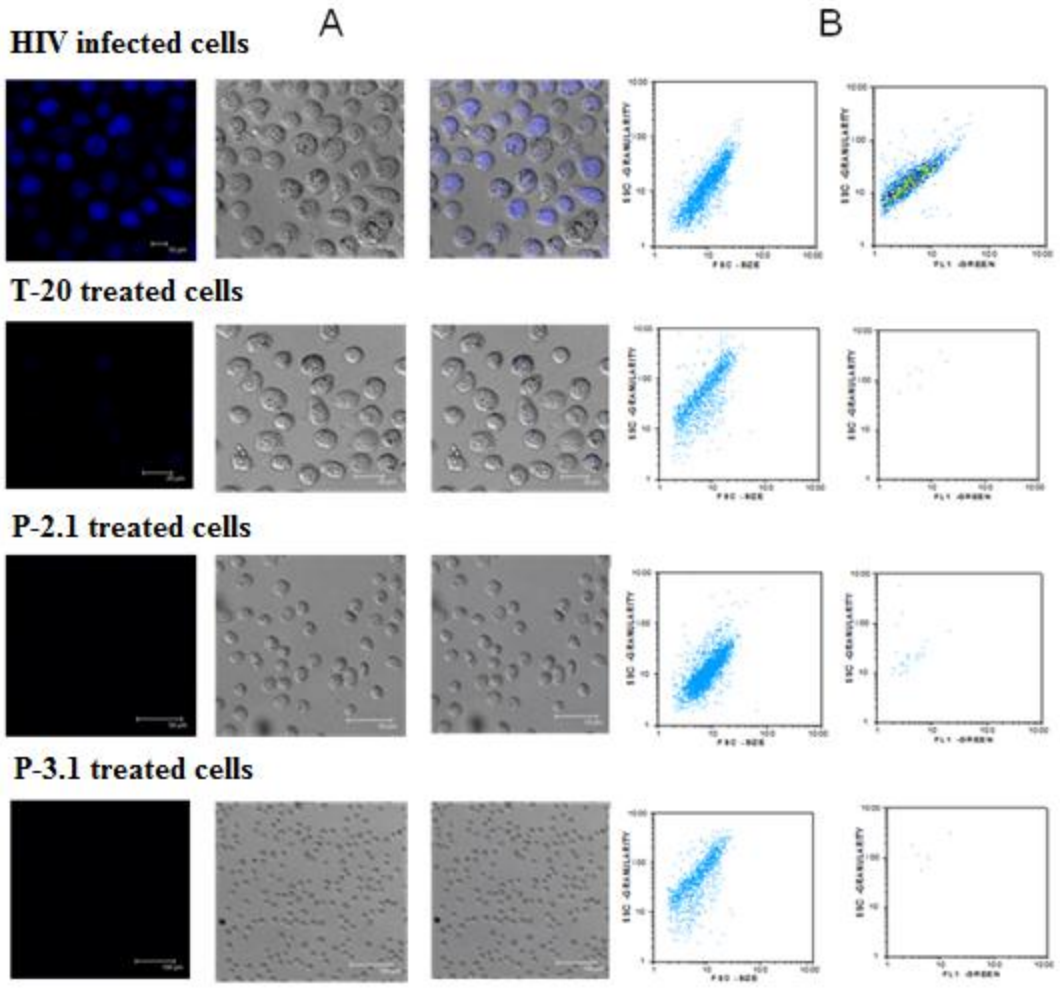
Interaction of peptides with virus surface gp120



Virus was incubated with increasing concentrations of P-2.1 and P-3.1 (in equal amounts). Virus was captured with gp120 monoclonal antibody coated wells as indicated on X-axis. The bound virus was lysed and p24 was estimated and plotted on Y-axis. Decrease in p24 value in the antibody well indicates the potential binding region of peptide against that particular antibody since the peptide blocks the binding of antibody to the gp160 at that region. Binding of the two peptides in combination to the same regions of gp120, 257D and F425 shows the synergistic effect of these peptides.

Fig 6.10

Inhibition of viral entry by peptides in reporter cell line, TZM-BL



HIV-1 mediated β-gal expression in TZM-BL cells: TZM-BL cells were infected with HIV-1. HIV-1 entry allows tat mediated LTR based expression of β-gal and the fluorescent cells (due to azo dye) were counted by confocal microscopy and flow cytometry. Panel A indicates the confocal image of the TZM-BL cells which shows the expression of β- gal upon HIV-1 infection in control cells and the inhibition of expression by T-20(control), P-2.1 and P-3.1 The Panel B shows the FACS analysis of the same assay to count the number of cells fluoresced.

Conclusions

- Tryptic digestion of both native and rec Epap-1 showed identical peptide pattern in MALDI analysis
- Trypsin Digest of rec Epap-1 showed significant anti-HIV-1 activity
- Trypsin Digest was separated by HPLC and the two Fractions could be isolated, in which Fraction-1 showed ~40% anti-HIV-1 activity, affinity to HL-2/3cells and inhibited the fusion reaction
- Epap-1 model was generated by threading technique using WURST server and docking was performed between Epap-1 and gp120 by HEX 5.6
- Out of four peptides obtained from docking studies, peptides 2 and 3 showed considerable interactions (8 and 7) with gp120
- All the 4 peptides showed binding to HL 2/3 cells and did not bind to Sup-T1 cells and showed considerably less cytotoxicity at 100µg concentration
- Peptide 2 and 3, which are having 8 and 7 interacting residues have shown anti-HIV activity, but peptide 2 (IC-50 - 59µg) possess higher activity compared to Peptide 3 in both Sup-T1 and PBMC's
- In studies conducted using soluble gp120 and virus, peptide 2 has shown a conserved interaction at V3 loop regions recognized by 257D and F425 antibodies.

- P-2 inhibited cell fusion mediated dye transfer between HL2/3 and Sup T1 cells suggesting that it blocks viral entry, which is further confirmed by its action on HIV infection mediated by Tat activated beta gal expression. These results together suggest that peptide 2 inhibits viral entry through interaction at V3 loop region

- Peptides (15mers) P-2.1 and P-3.1 have shown 10 and 7 interactions respectively with gp120 and were significantly anti-HIV active. They exhibited synergistic action when used in combination which is confirmed by anti-HIV assay and epitopic analysis using ELISA. They also inhibit the replication of sub type A viruses namely, HIV-1 93RW024 and HIV-1 94UG103 in Sup-T1 cells.

- Thus P- 2.1 and P-3.1 synergistically interacts with gp120 in V3 loop leading to the blockage of viral entry. The observation that P-2.1 and P-3.1 acting synergistically with 100 fold increase in activity suggests that binding sites of P-2.1 and P-3.1 in V3 region are distinctly different and cooperative. This suggests that the present study identified two new regions in the Epap-1 involved in the gp120 interaction, one is from Ile 339 to Asn 356 and the other is from Gln 268 to arg 456. Further these results indicate that Epap-1 interaction with gp120 is multidimensional in nature.

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