CELL-PENETRATING ANTIBODIES FOR TARGETING HIV-1 P24 CAPSID PROTEIN

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CELL-PENETRATING ANTIBODIES FOR TARGETING HIV-1 P24 CAPSID PROTEIN

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AIDS	Acquired immunodeficiency syndrome
Amp	Ampicillin
Antp	Antennapedia
APS	Ammonium persulfate
ATP	Adenosine triphosphate
α-HGA	α-hydroxy-glycineamide
BES	N, N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid
bp	Base pair
BSA	Bovine serum albumin
CA	Capsid molecule or *p*24
CAP-1	N-(3-chloro-4-methylphenyl)-N'-{2-[({5-[(dimethylamino)-methyl]-2-furyl}-methyl)-sulfanyl]ethyl}urea)
CAI	Capsid assembly inhibitor
CBD	Chitin-binding domain
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
CDRs	Complementarity-determining regions
CELISA	Cell-based enzyme-linked immunosorbent assay
CFU	Colony-forming unit
cm	Centimeter
CPP	Cell penetrating peptide
CV	Column volume
Da	Dalton
DC	Dendritic cells
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide

DNA	Deoxy ribonucleic acid
DSB	3-O-(3′,3′-Dimethylsuccinyl) betulinic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMEA	European Medicines Agency
Env	Envelope
EU	European Union
FBS	Fetal bovine serum
Fc	Constant fragment
FcR	Fc receptor
FDA	Food and Drug Administration
FPLC	Fast protein liquid chromatography
Gag	Group antigen
GAG	Glucoaminoglycans
G-NH_2	Glycineamide
gp	Glycoprotein
GPG-NH_2	Glycyl-prolyl-glycine-amide
HAART	Highly active antiretroviral therapy
HAMA	Human anti-mouse antibody
HEK293	Human embryonic kidney 293 cells
His	Histidine
HIV	Human immunodeficiency virus
hr	Hour
HRP	Horseradish peroxidase
HSPGs	Heparan sulfate proteoglycans
Ig	Immunoglobulin
IgG	Immunoglobulin gamma

IL-2	Interleukin-2
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropylthio-β-galactoside
kb	Kilo base
kDa	Kilo Dalton
KLH	Keyhole limpet hemocyanin
LB	Luria Bertani media
LTR	Long tandem repeats
MA	Matrix molecule or *p*17
MAb	Monoclonal antibody
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MβCD	Methyl-β-cyclodextrin
MDFF	Molecular dynamics flexible fitting
MBP	Maltose binding protein
MES	2-(*N*-morpholine)-ethanesulfonic acid
MHC	Major histocompatibility complex
mL	Mililiter
mm	Milimeter
mM	Milimolar
MTS	Membrane translocating sequence
MW	Molecular weight
NC	Nucleocapsid molecule or *p*7
Nc	Nitrocellulose
Nef	Negative factor
nm	Nanometer
OD	Optical density
ORF	Open reading frame

PCR	Polymerase chain reaction
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pg	Picogram
pH	Potential hydrogen
PHA	Phytohemagglutinin
PNA	Peptide nucleic acid
Pol	Polymerase
PTD	Protein transduction domain
RBCs	Red blood cells
RBS	Ribosome binding site
RE	Restriction enzymes
Rev	Regulator of expression of viral proteins
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase- polymerase chain reaction
scFv	Single chain variable fragments
sdAb	Single-domain antibody
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyarylamide gel electrophoresis
siRNA	Small interfering RNA
SP	Spacer peptide
TAE	Tris-Acetate-EDTA
Tat	Transactivator of transcription
TEMED	N,N,N\N'-tetramethylethylenediamine
TFA	Trifluoroacetic acid
US	United States

UV	Ultraviolet
Vif	Virus infectivity factor
Vpr	Viral protein r
Vpu	Viral protein u
WHO	World Health Organization
µm	Micrometer
µM	Micromolar
µL	Microliter
ºC	Degree Celsius

LIST OF PUBLICATIONS

Papers

Teow, S.Y. & Ali, S.A. A cell penetrating antibody targeting capsid protein (p24) inhibits the production of HIV-1 infectious particles. (Manuscript in preparation)

Teow, S.Y., Mualif, S.A., Omar, T.C., Wei, C.Y., Yusoff, N.M., & Ali, S.A. (2013). Production and purification of polymerization-competent HIV-1 capsid protein p24 (CA) in NiCo21(DE3) *Escherichia coli*. *BMC Biotechnol*. **13(107)**.

Conferences

Teow, S.Y., Yusoff, N.M., Tan, S.C., & Ali, S.A. Chemical engineering of cell-penetrating antibodies for potent intracellular targetings. UTAR National Postgraduate Fundamental and Applied Sciences Seminar (NPFASS), UTAR Kampar, Perak, 14-15th June, 2014.

Teow, S.Y., Yusoff, N.M., Tan, S.C., & Ali, S.A. Chemical engineering of cell-penetrating antibodies for targeting HIV-1 pathogenesis factor – p24 capsid protein. Monash Science Symposium, Monash University Malaysia, Bandar Sunway, Selangor, 18-19th June, 2014.

Mualif, S.A., **Teow, S.Y.**, Omar, T.C., Yusoff, N.M., & Ali, S.A. Construction of HIV-1 capsid protein p24 (CA) – specific single chain fv (scFv) phage display library: towards the development of novel therapeutic antibodies against HIV/AIDS. Monash Science Symposium, Monash University Malaysia, Bandar Sunway, Selangor, 18-19th June, 2014.

ANTIBODI PENEMBUS SEL UNTUK MENSASARKAN PROTEIN KAPSID P24 HIV-1

ABSTRAK

Protein kapsid *p24* memainkan peranan yang penting dalam peringkat awal dan akhir replikasi HIV. Justeru, kapsid HIV-1 dianggap sebagai sasaran penting dalam pembangunan ubat terapi baharu untuk merawat HIV/AIDS. Perencat peptida seperti *CAP-1*, *CAI* dan *NYAD-1* mampu menyekat fungsi kapsid HIV-1 dalam sel yang dijangkiti HIV. Walau bagaimanapun, sifat peptida yang kurang stabil dalam darah, menyebabkan ia cepat dilupuskan dari sistem malah kurang spesifik terhadap sasaran. Sebaliknya, antibodi monoklon (*MAb*) bersifat lebih stabil dan spesifik. Antibodi monoklon telahpun digunapakai sebagai modaliti terapi untuk merawat pelbagai penyakit termasuk kanser dan jangkitan virus. Namun, keberkesanan antibodi masih dipersoal kerana tidak dapat menembusi sel untuk menyasar sasaran dalam sel seperti kapsid HIV. Dengan ini, antibodi monoklon *anti-p24* telah dihasilkan secara kejuruteraan kimia dengan peptida penembus sel yang dapat mensasarkan sasaran *p24* dalam sel (*anti-p24-TransMAbs*). Dalam kajian ini, antibodi *anti-p24* telah dicantum dengan lapan jenis peptida penembus sel secara pengoksidaan *periodate*. Ujian *p24 ELISA* dan microskopi berpendafluor kemudiannya digunapakai untuk menilai tahap penembusan dalam sel. Antibodi *anti-p24* yang digabungkan dengan *MTS* mempunyai tahap penembusan sel yang paling berkesan. Penembusan sel ini adalah bergantung kepada kepekatan peptida dan hasil maksimum diperoleh selepas 24 jam rawatan. Rawatan setinggi 10µg/mL *anti-p24-TransMAbs* tidak menunjukkan sebarang kesan

toksik pada sel. Kajian mekanikal menunjukkan bahawa rawatan *anti-p24-TransMAbs* dalam monosit adalah bergantung kepada suhu and tenaga serta dikawal separa oleh endositosis *clathrin* dan *caveolae*. Sebaliknya, rawatan *anti-p24-TransMAbs* dalam limfosit T tidak bergantung kepada endositosis. Berdasarkan ujian sel *MAGI* untuk menyukat tahap jangkitan HIV-1, 10µg/mL *anti-p24-TransMAbs* berjaya mengurangkan tahap jangkitan HIV-1 sebanyak 66% dalam sel *Jurkat-T* dan 94% dalam sel *H9*, namun aktiviti lebih rendah dilihat dalam sel *THP-1* (38%) dan sel *U937* (27%). Tambahan pula, aktiviti virus dapat dikawal oleh antibodi ini dalam *PBMCs* (48%) dan sel *CD4*$^{+}$*-T* (60%). Sebagai kesimpulan, *anti-p24-TransMAbs* berpotensi sebagai terapi HIV/AIDS yang baharu. Strategi yang sama juga boleh digunapakai untuk menyasarkan protein HIV lain seperti *Nef*, *Vif* dan *Vpr*. Untuk meningkatkan aktiviti antibodi ini, kami memilih antibodi monoklon yang lebih reaktif dengan teknologi *phage-display*. Antibodi dalam format *scFv* ini sedang dihasilkan dan digabung dengan *MTS* secara molekular untuk menyasarkan kapsid dalam sel.

CELL-PENETRATING ANTIBODIES FOR TARGETING HIV-1 P24 CAPSID PROTEIN

ABSTRACT

The capsid protein plays seminal roles in both early and late stages of the HIV replication cycle. Hence, HIV-1 capsid protein is considered an important target for developing novel drugs to treat HIV/AIDS. Capsid molecule (CA)-targeting peptide inhibitors (CAP-1, CAI and NYAD-1) have been reported to disrupt the capsid protein functions in HIV-infected cells. However, the peptides are less stable in the blood, thus resulting in rapid clearance from the system and they can exhibit off-target activities. Monoclonal antibodies (MAbs) on the other hand, are stable and highly specific molecule, which have been successfully used as therapeutic modalities to treat various types of cancer and viral infections. Nonetheless, MAbs cannot penetrate into the cell and interact with intracellular target such as HIV-1 CA. To this end, anti-p24 MAbs were chemically engineered with cell-penetrating peptides (CPPs) to generate cell-penetrable (anti-p24-TransMAbs). In this study, anti-p24 MAbs were conjugated with 8 different CPPs through periodate oxidation. The cell penetration of anti-p24-TransMAbs was then examined by p24 ELISA and fluorescence microscopy. Anti-p24 MAbs conjugated with MTS (membrane-transduction sequence) resulted in most efficient penetration into the cells. The internalization of anti-p24-TransMAbs was concentration-dependent and maximum at 24 hours incubation. No toxicity was observed when the cells were treated with up to 10μg/mL anti-p24-TransMAbs. Mechanistic studies showed that the transportation of anti-p24-TransMAbs in promonocytic cell lines was

temperature- and energy-dependent, and partially mediated by clathrin and caveolae-dependent endocytic pathways. On the other hand, the transportation of anti-p24-TransMAbs in T-lymphocytic cell lines did not depend on endocytosis and resulted from CPP-mediated membrane perturbation. Using MAGI (multinuclear-activation galactosidase indicator) cell assay, the 10μg/mL anti-p24-TransMAbs successfully reduced the HIV-1 infectivity in Jurkat-T (66%) and H9 (94%) lymphocytic cell lines while lower inhibition was seen in infected THP-1 (38%) and U937 (27%) promonocytic cell lines. More promisingly, pronounced antiviral effects were seen in PHA-stimulated infected human PBMCs (48%), and infected primary human $CD4^{+}$-T-cells (60%). We conclude that anti-p24-TranMAbs targeting HIV-1 CA can be developed into a novel HIV/AIDS therapy. Similar strategy can also be potentially applied on targeting other intracellular HIV-1 protein such as Nef, Vif and Vpr. To further enhance the antiviral activity of antibody, we are currently selecting the monoclonal antibody against the capsid protein by phage-display technology. The antibody in scFv format are being generated and molecularly conjugated with MTS to target and block the intracellular CA.

1 INTRODUCTION

1.1. HIV/AIDS pathogenesis

1.1.1. Acquired Immunodeficiency Syndrome (AIDS)

AIDS is today's global life-threatening concern. Since its first identification in United States in 1981 (CDC Morbidity & Mortality Weekly Report, 1981), an increased number of infections and deaths attributed to AIDS have been reported. In 1983, Human Immunodeficiency Virus (HIV) was identified as the causative agent for AIDS (BarreÂ-Sinoussi *et al*., 1983). According to Joint United Nations Programme on HIV/AIDS (UNAIDS), approximately 35 million people worldwide were living with HIV/AIDS in 2013, and 19 million of them do not know that they have the virus. In 2013 alone, 1.5 million people have died of AIDS-related causes. Sub-Saharan Africa remains the region with highest number of reported cases in 2013 (24.7 million out of 35 million that accounts for 70.6% of global statistics) and more than 70% of all people of the country are living with HIV (UNAIDS Gap Report 2014). There are also a strikingly high number of AIDS-related cases in Malaysia. By the end of 2013, Malaysia had a cumulative number of 101,672 HIV-related cases, 20,235 reported AIDS and 16,340 AIDS-related deaths since the first occurrence in 1986 (Global AIDS Response Progress Report 2014).

In infected patients, the HIV presents either as free virus particles or lie within infected immune cells in biological fluids. HIV infects vital cells in the human immune system such as $CD4^+$-T-lymphocytes, monocytes/macrophages, and dendritic cells (DCs). Notably, a HIV-positive individual may not necessarily be AIDS-positive due to the clinical latency period that can last up to ten years or longer. During this period, the

virus wears down the immune system, rendering the body susceptible to ‘opportunistic infections’ and other medical complications (**Figure 1.1**). The latter usually happens when the total $CD4^+$-T-cells or "T-helper" cells, which are important component of the immune system, is less than 200 cells per cubic milliliter of blood (200 cells/mm^3). Healthy individuals usually have 500 - 1,500 cells/mm^3. The late stage of AIDS is often complicated by cancer and other opportunistic infections (**Figure 1.2**). These opportunistic infections are sufficient to cause death due to the non-functional immune system.

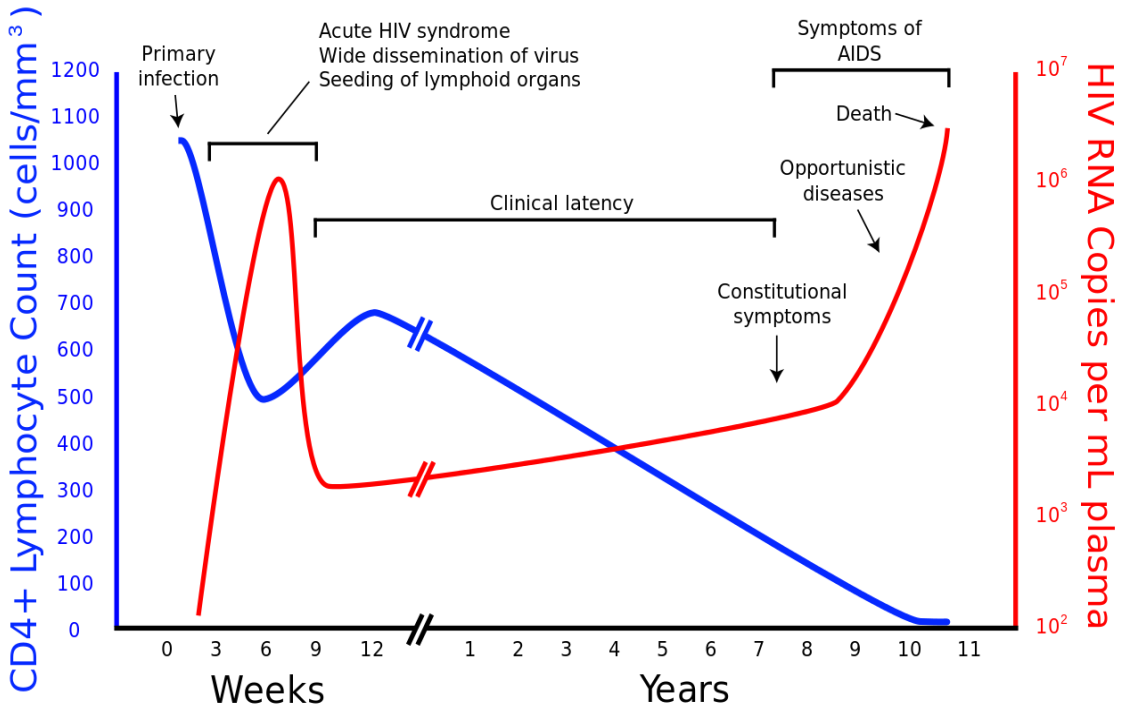

Figure 1.1. Levels of CD4$^+$-T-lymphocytes and HIV RNA copies throughout AIDS progression. The graph shows the general relationship of CD4$^+$-T-lymphocytes numbers (**blue line**) and viral load (**red line**) over the course of HIV infection. The CD4$^+$-T-lymphocytes number drops during the first 6-week of primary infection while the HIV replicates and spreads to multiple organs. The HIV number then significantly drops after the successful infection and gradually increases during the clinical latency while the CD4$^+$-T-lymphocytes drop every year. At the late stage of infection (CD4$^+$-T-lymphocytes number is less than 200 cells/mm^3), the immune system is compromised and attacked by multiple 'opportunistic' diseases. Due to the non-functional immune system, the HIV rapidly replicates to take over the body system.

Source: youngdayschool.edu.uy/webliceo/aids.html

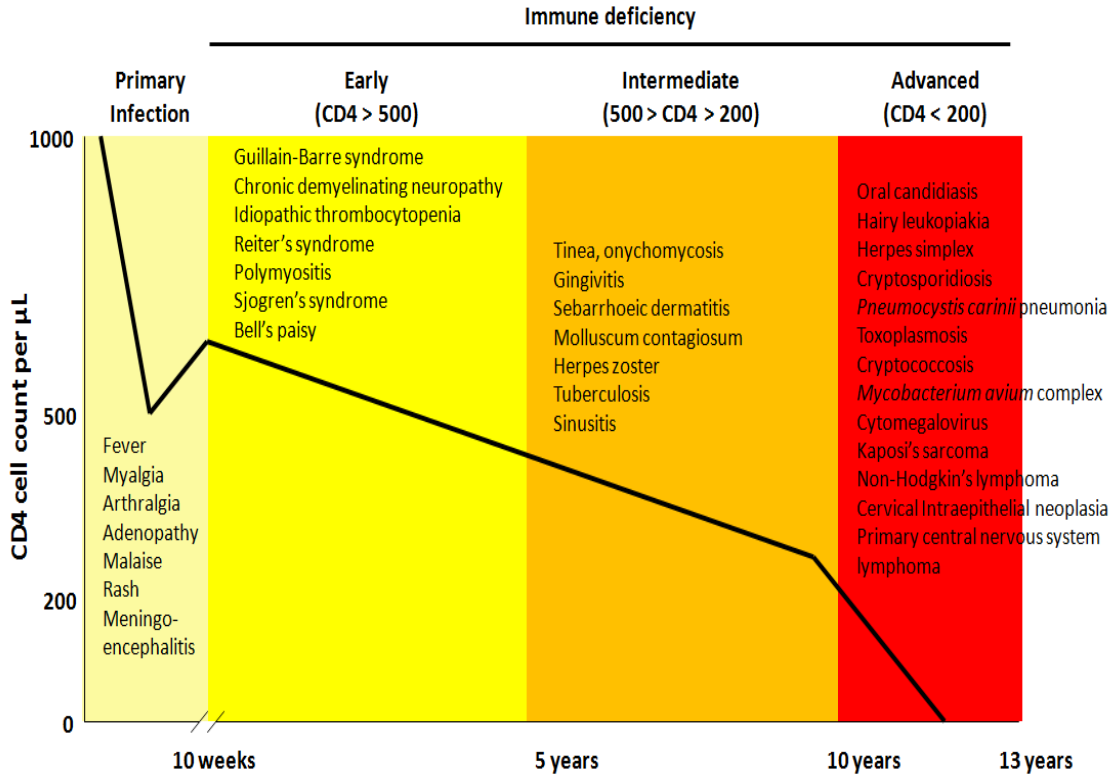

Figure 1.2. Occurrence of opportunistic infections and cancers over the course of HIV infection. During the primary infection, common symptoms of viral infections are seen such as fever and rashes. As the $CD4^{+}$-T-cell count drops due to the HIV infection that compromises the immune sytem, the host becomes infected by more deadly infections (tuberculosis, pneumonia, candidiasis, *etc.*) and cancer diseases (lymphoma, sarcoma, *etc.*).

Adapted and modified from southsudanmedicaljournal.com/archive/2009-08/untitled-resource.html

1.1.2. Human Immunodeficiency Virus (HIV)

HIV is an enveloped virus of 100-120nm containing two copies of approximately 9kb genomic RNA. HIV originates from *lentiviridae* subfamily of the *retroviridae* family, and there are two subtypes: HIV-1 and HIV-2. HIV-1 is more virulent and infective, thus contributing largely to the global infections whereas HIV-2 infection is only confined to West Africa (CDC Morbidity & Mortality Weekly Report, 2011). Upon viral entry into the target cells, the genomic RNA is reverse-transcribed into complimentary DNA (9.8kb) and gets integrated into the host genome. The virus then becomes latent and can reside in the host cells for a long period of time. HIV-1 is morphologically spherical and it encodes a total of three structural proteins, two envelope proteins, three enzymes, and six accessory proteins (Turner and Summers, 1999).

The viral genomic material is surrounded by an outer coat or viral envelope (**Figure 1.3**), composing of two layers of host cell-derived phospholipids during the budding of viral particles. The envelope also consists of cap-like protruded proteins made up of three molecules of knob-like glycoprotein 120 (*gp*120), and a stem consisting of three spike-like molecules known as glycoprotein 41 (*gp*41) that anchor the structure in the viral envelope. Both of these envelope-associated protein are cleaved by protease from glycoprotein 160 (*gp*160) coded by *env* gene (**Figure 1.4**). These protein structures play important roles in establishing the HIV infection during the attachment and entry steps (Clapham and McKnight, 2002).

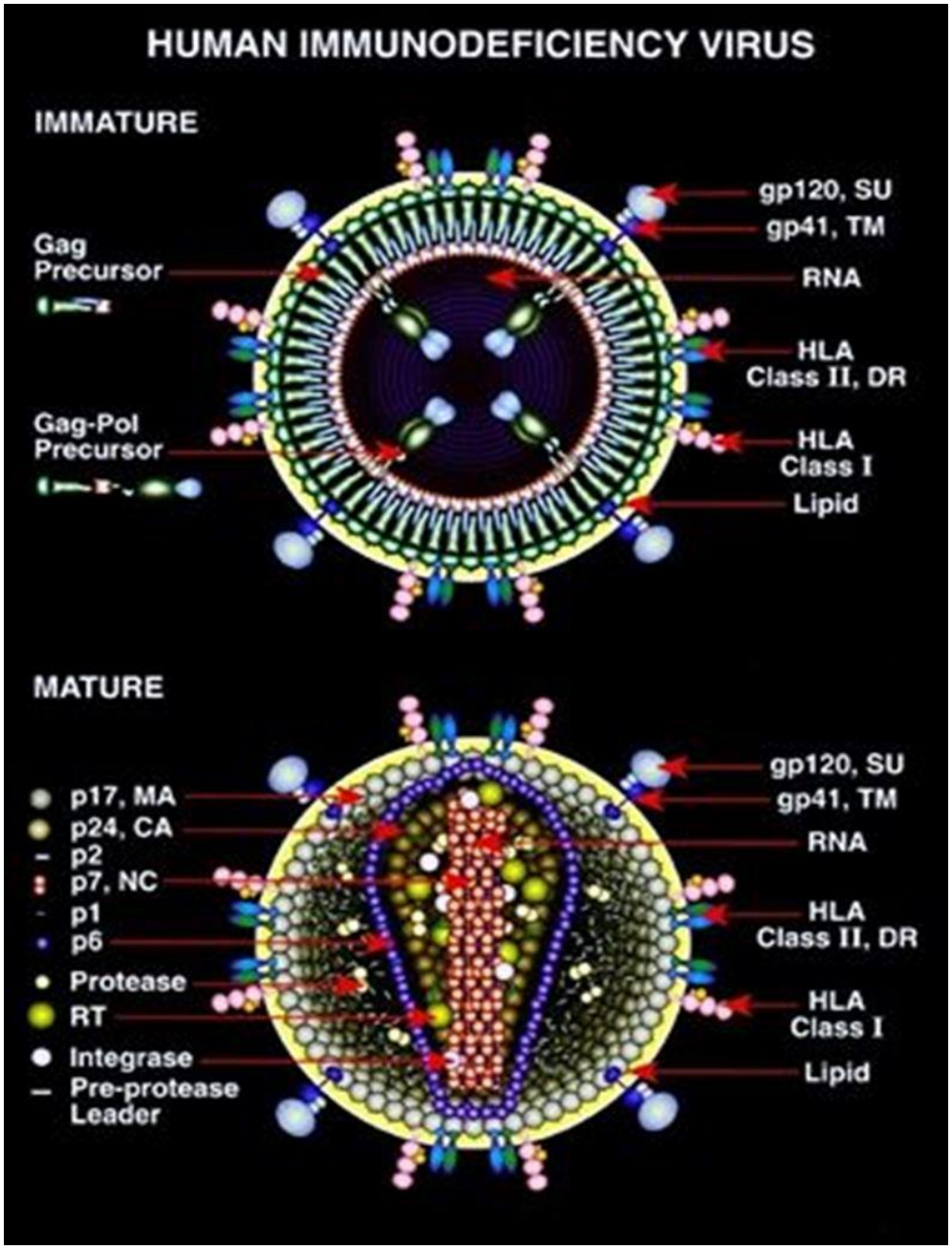

Figure 1.3. Structure of immature and mature forms of HIV-1. The mature HIV-1 contains a bullet-shaped core encapsulating RNA genome and viral enzymes whereas the immature HIV-1 has a 'hollow' centre due to the unprocessed *gag-pol* genes.

Source: mcdb-webarchive.mcdb.ucsb.edu/sears/ biochemistry/ presentations/f07-student-presentations/Justine-Swann/frames6.htm

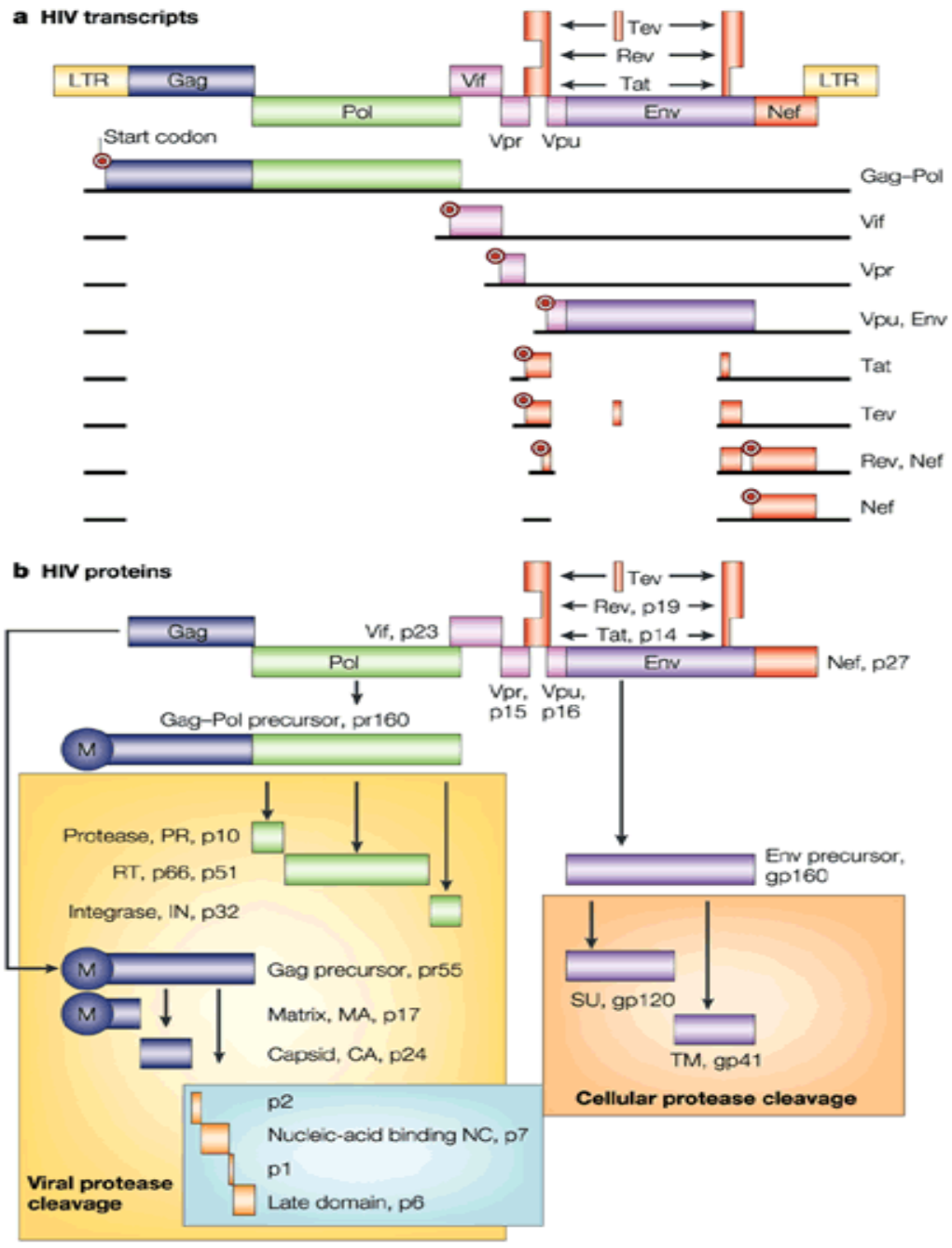

Figure 1.4. The HIV genome, transcripts, and proteins. (a) HIV transcripts consist of 10kb viral genome containing open reading frames for 16 proteins that are synthesized from at least 10 transcripts. **(b)** *gag*, *pol* and *env* precursors are processed by the viral proteases to form HIV proteins essential and accessory for AIDS progression.

Source: Peterlin and Trono, 2003.

Within the viral envelope, a layer of matrix protein composing of viral protein, MA or *p*17 helps to maintain the integrity of the virion particle. At the center of virus, there is a bullet-shaped core surrounded by a conical capsid made up of approximately 2,000 copies of the viral protein, CA or *p*24 (Luciw, 1996). The viral core contains two single strands of RNAs that encode the HIV-1 genome. These RNAs are tightly bound to the nucleocapsid proteins that are composed of NC or *p*7. The core also contains a few other viral enzymes such as reverse transcriptase, ribonuclease, proteases, and integrase that play important roles in the replication cycle (Freed, 2001). Three of the structural proteins (MA, CA and NC) are encoded by *gag* gene whereas *pol* gene encodes for the viral enzymes.

Other than *env*, *gag* and *pol* genes, which are indispensable for HIV-1 replication, the RNA genome also contains other regulatory genes such as *nef*, *tat*, *rev*, *vpu*, *vpr*, *vif*, and sometimes *tev* (fusion of *tat*, *env* and *rev*) (**Table 1.1**). Although they are not compulsorily required for replication, a majority of them have distinct functions that contribute to the enhanced viral infectivity and disease progression (Bour and Strebel, 2000; Steffens and Hope, 2001; Anderson and Hope, 2003; Seelamgari *et.al*., 2004). These regulatory genes can be separated into three categories – positive regulatory genes, negative regulatory genes and unspecified regulatory genes in which the exact functions are still largely unknown. The positive regulatory genes enhance and express regulatory proteins that promote formation of viral particles and viral infectivity while the negative regulatory genes control the level of viral infectivity (Epstein and Greene, 1991).

Table 1.1. Summary of functions of genes encoded in HIV-1 genome. *gag*, *env* and *pol* are precursors that produce essential proteins for HIV-1 replication. Other accessory genes indirectly regulate HIV-1 infectivity and AIDS progression.

Viral gene	Regulation	Activities towards HIV/AIDS pathogenesis
env	Structural	Encodes the outer protein coat *gp*160 which then processed by a protease into *gp*120 and *gp*41.
gag	Structural	Encodes internal proteins such as *p*17, *p*24, *p*7, and *p*9.
pol	Structural	Encodes the HIV core including *p*7, *p*9, RNA genome, reverse transcriptase, ribonuclease, integrase, and protease.
tat	Positive	A trans-activator of transcription which accelerates viral protein production of proviral genome several 1000-folds. Also up-regulates *rev*, *nef*, and itself.
nef	Positive	Affects cellular genes by down-modulating CD4/CD8 and MHC-I/II expression. Also negatively regulates *tat* and *rev*.
rev	Positive/ Negative	Increased level of *rev* enhances RNA expression and greater amount expression of Env proteins. Also down-regulates *tat* and itself and activates transport of viral mRNA from nucleus into cytoplasm.
vpr	Positive	A weak transcriptional activator that moderates stimulation of LTR, thus enhancing induction of HIV.
vpu	Negative	When *vpu* is defective, HIV replicates more quickly. Also suppresses CD4/Env interactions in the cell.
vif	Positive	Increases the infectivity of HIV and cell-to-cell (human-to-human) transmission. However, *vif*-deficient HIV still infects *in vitro*, but with lower kinetics.
tev	Positive/ Negative	A fusion protein resulting from a frameshift reading the mRNA--mixes up *tat*/ *env*/ *rev*--and apparently has full functional capacity of *tat* and *rev*.

MHC – major histocompatibility; LTR – long tandem repeat.

Adapted and modified from Haseltine, 1988; Epstein and Greene, 1991.

1.1.3. Disease control of AIDS

In the past ten years, various anti-HIV therapies targeting multiple components of the virus have been developed and some are currently under clinical trials (Maartens *et.al*., 2014). However, the ability of HIV to develop resistance towards currently available antiviral agents has rendered the drug development challenging. Some of antiviral drugs approved by the Food and Drug Administration (FDA) are summarized in **Table 1.2**. These therapeutic agents were designed based on the understanding of establishment of HIV-1 infection, which involves multiple steps in the replication cycle. The protein-protein interaction between the virus and the host cells are the keys to unlock new therapeutic avenues (Thompson *et.al*., 2012; Wilen *et.al*., 2012). The HIV-1 replication cycle and the targets for potential antiviral agents are represented in **Figure 1.5**.

Table 1.2. List of FDA-approved anti-HIV drugs. Different classes of anti-HIV drugs were designed to target multiple components of HIV in the replication cycle.

Drug Class	Generic Name	Brand Name	FDA Approval Date
Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)			
NNRTIs bind to and alter reverse transcriptase, an enzyme HIV needs to make copies of itself	Delavirdine (DLV)	Rescriptor	April 4, 1997
	Efavirenz (EFV)	Sustiva	Sep 17, 1998
	Etravirine (ETR)	Intelence	Jan 18, 2008
	Nevirapine (NVP)	Viramune	June 21, 1996
	Rilpivirine (RPV)	Edurant	May 20, 2011
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)			
NRTIs block reverse transcriptase, an enzyme HIV needs to make copies of itself	Abacavir (ABC)	Ziagen	Dec 17, 1998
	Didanosine (ddl)	Videx	Oct 9, 1991
		Videx EC	Oct 31, 2000
	Emtricitabine (FTC)	Emtriva	July 2, 2003
	Lamivudine (3TC)	Epivir	Nov 17, 1995
	Stavudine (d4T)	Zerit	June 24, 1994
	Tenofovir DF (TDF)	Viread	Oct 26, 2001
	Zidovudine (ZDV)	Retrovir	March 19, 1987
Protease Inhibitors (PIs)			
PIs block HIV protease, an enzyme HIV needs to make copies of itself	Atazanavir (ATV)	Reyataz	June 20, 2003
	Darunavir (DRV)	Prezista	June 23, 2006
	Fosamprenavir (FPV)	Lexiva	Oct 20, 2003
	Indinavir (IDV)	Crixivan	March 13, 1996
	Nelfinavir (NFV)	Viracept	March 14, 1997
	Ritonavir (RTV)	Norvir	March1, 1996
	Saquinavir (SQV)	Invirase	Dec 6, 1995
	Tipranavir (TPV)	Aptivus	June 20, 2005
Fusion Inhibitors			
Fusion inhibitors block HIV from entering CD4 cells of the immune system.	Enfuvirtide (T-20)	Fuzeon	March 13, 2003
CCR5 Antagonists			
CCR5 entry inhibitors block CCR5, a protein on the CD4 cells that HIV needs to enter the cells	Maraviroc (MVC)	Selzentry	Aug 6, 2007
Integrase Inhibitors			
Integrase inhibitors block HIV integrase, an enzyme HIV needs to make copies of itself	Raltegravir (RAL)	Isentress	Oct 12, 2007

Table 1.2. Continued.

Fixed-Dose Combination			
Fixed-dose combination tablets contain two or more anti-HIV medications from one or more drug classes.	Abacavir, Lamivudine	Epzicom	Aug 2, 2004
	Abacavir, Lamivudine, Zidovudine	Trizivir	Nov 14, 2000
	Efavirenz, Emtricitabine, Tenofovir DF	Atripla	July 12, 2006
	Elvitegravir, Cobicistat, Emtricitabine, Tenofovir DF	Stribild	Aug 27, 2012
	Emtricitabine, Rilpivirine, Tenofovir DF	Complera	Aug 10, 2011
	Emtricitabine, Tenofovir DF	Truveda	Aug 2, 2004
	Lamivudine, Zidovudine	Combivir	Sep 27, 1997
	Lopinavir, Ritonavir	Kaletra	Sep 15, 2000

HIV and its treatment – FDA-approved anti-HIV medications. U.S. Department of health and human services guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Reviewed on Aug 2012.

Adapted and modified from aidsinfo.nih.gov/guidelines.

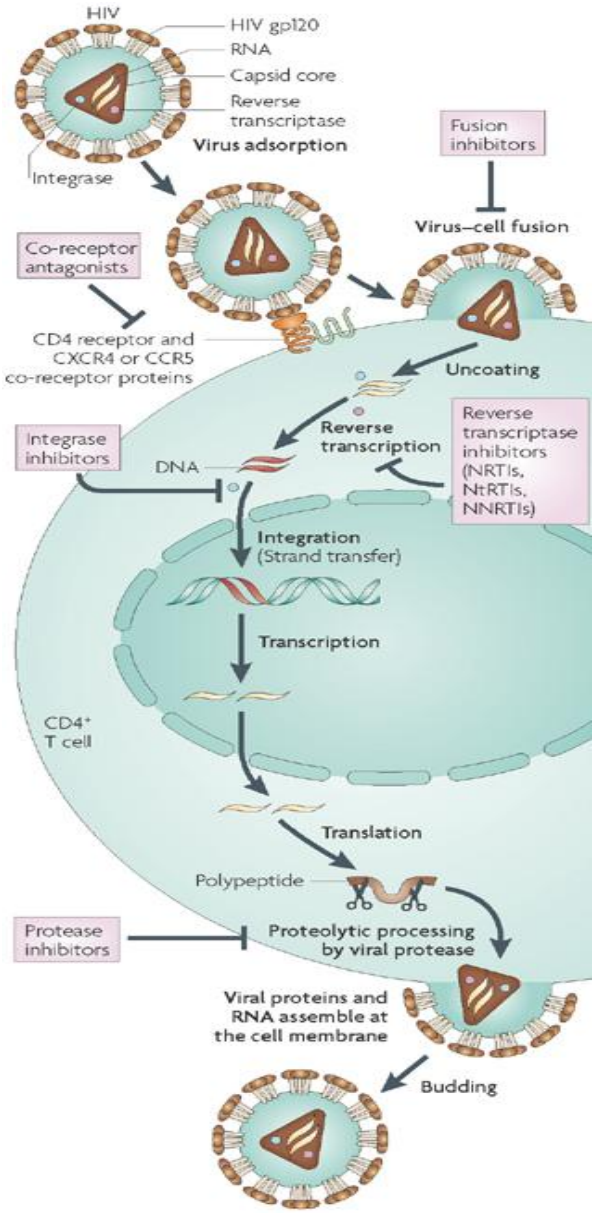

Figure 1.5. HIV-1 replication cycle and potential antiviral targets. A free virus interacts with a cell that contains essential receptors (CD4 and CXCR4 or CCR5) for establishment of infection. This interaction can be blocked by attachment and fusion inhibitors. Viral RNA is uncoated, reverse-transcribed, and integrated into host genome for viral protein production. Reverse transcription can be blocked by reverse transcriptase inhibitors whereas the DNA integration can be inhibited by integrase inhibitors. RNA and viral proteins assembles at the membrane for synthesis of a new viral particle. Upon the release and maturation of virus, protease acts on the Gag and Gag-Pol precursors to form an infectious particle. This step can be effectively blocked by protease inhibitors.

Adapted and modified from De Clercq, 2007.

Most anti-HIV therapies comprise single or dual agents, which target surface protein (*gp*41 and *gp*120) and viral enzymes (reverse transcriptase, integrase and protease) (Peters and Conway, 2011). The strikingly rapid resistance of HIV-1 remains a huge problem until highly active antiretroviral therapies (HAART) was introduced in 1995 (Hammer *et.al*., 1996, 1997). HAART consists of drug cocktails (combination of three to four different classes of drugs) that act on multiple targets of HIV-1 in order to interrupt the viral life cycle at different steps. Often, the drugs used in HAART are of reverse transcriptase inhibitors, protease inhibitors, and fusion inhibitors classes. This therapy has successfully controlled the infection by reducing total viral load and significantly delaying disease progression (Walensky *et.al*., 2006; Arts and Hazuda, 2012) while dramatically diminishing the mutation rate of HIV-1 strains (Piacenti, 2006). The clinical profile that exhibits increased survival rates after introduction of HARRT is depicted in **Figure 1.6**. However, the emergence of HAART-escaped HIV-1 mutant strains remains a major concern, and other relatively effective alternatives are still under progress such as vaccine development (Rerks-Ngarm *et.al*., 2009; Rolland *et.al*., 2012).

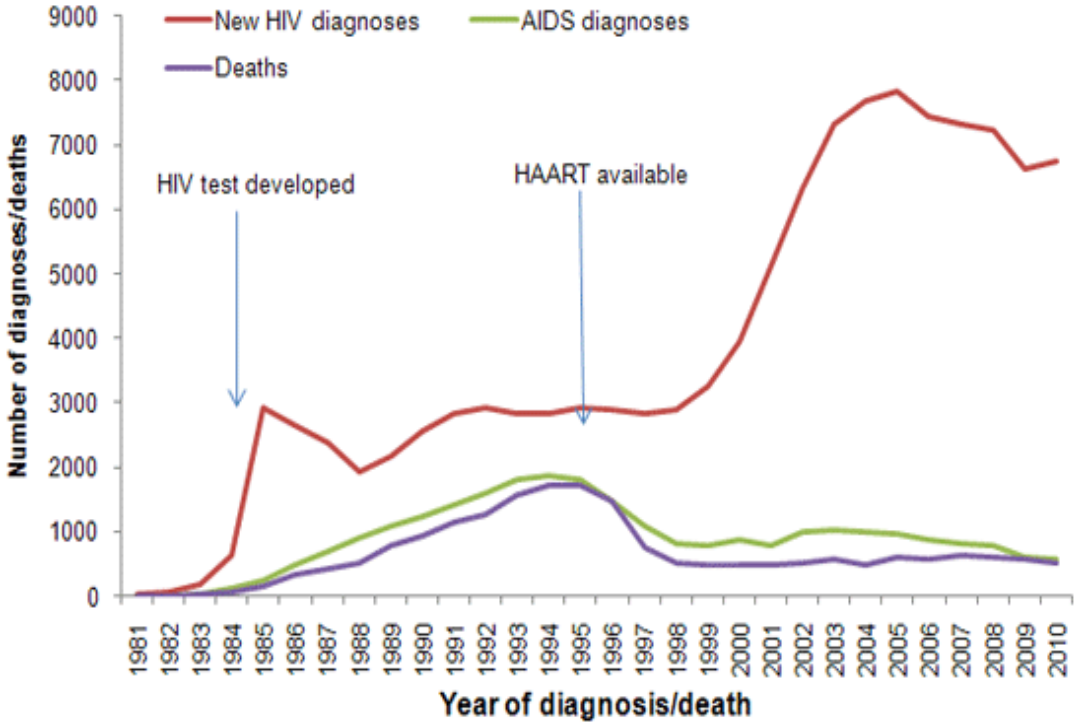

Figure 1.6. Increased survival rates of AIDS by HAART. The development of highly active antiretroviral therapy (HAART) has been the driving force behind a revolution in the treatment of HIV/AIDS. Since the introduction of HAART in 1996, the reported new AIDS cases and AIDS-related death cases were significantly reduced by at least 50%.

Source: hpa.org.uk/hpr/archives/2011/news2211.htm

1.1.4. Drawbacks of current therapies

Albeit the rate of disease progression being successfully suppressed by the HAART intervention (**Figure 1.6**), the drug resistance remains unresolved. Following a long exposure of drugs, the therapy will eventually become inefficient due to the acquired resistance of so-called 'escaped mutants'. The virus will strike back and progressively destroys the immune system. Furthermore, HAART may not be efficient to all AIDS patients due to poor compliance. The patients need to adhere strictly to a dosage schedule as HAART possesses a complicated regimen requiring patients to take a series of pills at multiple times of the day (Piacenti, 2006; Peters and Conway, 2011; Arts and Hazuda, 2012), which significantly affect their life qualities. Moreover, the cost of these multiple antiviral drugs is high (Schackman *et.al*., 2006).

HAART is also notorious of its short- and long-term side effects (**Table 1.3**). Patients under long-term treatment may also develop several clinical presentations such as lipodystrophy, hyperglycemia, diabetes, liver toxicity, and debilitating inflammatory syndrome (Jones and Núñez, 2012). These severe side effects may force the patients to skip the medication, rendering the therapy inefficient, and more crucially, promotes drug resistance. Due to these adverse effects, many cases have reported that the patients discontinued the HAART within the first year of medication (Robison *et.al.*, 2008; Cicconi *et.al.*, 2010; Woldemedhin and Wabe, 2012). With advents of these drawbacks and failure of vaccination, there is a pressing need to develop alternative therapies for controlling HIV infection.

Table 1.3. Side effects of HAART. Adverse effects of various drug types used in HAART.

Drug type	Adverse events
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	Nausea, diarrhea, headache, hypersensitivity syndrome, lactic acidosis, hepatic steatosis, pancreatitis, lipodystrophy, peripheral neuropathy, dyslipidemia, and renal insufficiency.
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	Nausea, diarrhea, headache, dizziness, Stevens-Johnson syndrome, toxic epidermal necrolysis, hyperlipidemia, hepatitis, and elevations in liver function tests.
Protease Inhibitors (PIs)	Nausea, diarrhea, headache, dyslipidemia, hyperglycemia, insulin resistance, hyperbilirubinemia, nephrolithiasis, lipodystrophy, and elevations in liver function tests.
Fusion Inhibitors	Injection site reactions, neutropenia, and possible increased frequency of pneumonia.
Chemokine Coreceptor Antagonists	Nausea, diarrhea, headache, hepatitis, joint or muscle pain, and elevations in liver function tests.
Integrase Inhibitors	Nausea, diarrhea, headache, muscle pain, and elevations in amylase and liver function tests.
Pharmacokinetic Enhancers	Nausea, diarrhea, headache, increased serum creatinine, and proteinuria

Adapted and modified from hivinsite.ucsf.edu/InSite?page=ar-05-01#S2X

1.2. Capsid protein in HIV-1 infectivity

1.2.1. Biological function of capsid protein

Assembly of viral particles and their maturation into infectious particles after viral budding are indispensable steps in HIV-1 replication cycle (**Figure 1.5**). Capsid molecule (CA) or *p*24 is known to play important roles in early (pre-integration) and late (post-integration) events of the replication cycle (Adamson and Freed, 2007; Briant *et.al*., 2011; Sundquist and Kräusslich, 2012). Upon cell entry, the capsid protein releases the RNAs and viral enzymes into the cell cytoplasm. Once the reverse-transcribed DNA is integrated into the host genome, viral proteins are produced and repackaged by the capsid protein for new infections.

CA is initially synthesized as a domain within a 55kDa Gag precursor polyprotein. Gag (*p*55) sometimes interacts with another large viral protein, Gag-Pol (160kDa) in the viral assembly. Approximately 2000-4000 copies of intact Gag polyprotein assemble at the cell membrane and bud to produce immature virus particles (Ganser-Pornillos *et.al*., 2008, 2012) (**Figure 1.7**). The intact Gag attaches to the inner cell membrane via the myristoylated N-terminus of matrix molecule (MA), one of the cleavage products of Gag. A proteolytic cleavage by viral protease then takes place and Gag is subsequently broken into CA, MA, nucleocapsid molecule (NC), and *p*6 molecules. Additionally, splicing of several spacer peptides (SP) that stabilizes CA hexamers occurs sequentially to complete the Gag proteolytic process. For instance, spacer peptide-1 (SP1) stabilizes CA and NC, whereas SP2 stabilizes NC and *p*6 (**Figure 1.7**). In Gag-Pol protein, the Pol domain is cleaved into viral enzymes such as reverse transcriptase, protease, and integrase proteins (Meng *et.al*., 2012).

CA develops into a cone-shaped capsid that encases the RNA genome and several viral enzymes, which then develops into mature infectious particles. Throughout the HIV-1 replication, capsid protein plays an important role in virus assembly and maturation. Without the proper assembly of capsid, mature virions cannot be formed hence affecting HIV-1 replication and infectivity. It has been reported that mutated CA in which the capsid stability was affected, significantly attenuated the HIV-1 replication (Neira, 2009; Blair *et.al*., 2010). In recent years, the essential role of capsid protein in HIV-1 replication cycle has drawn considerable attention of researchers to study its potential to be used as antiviral targets in HIV/AIDS therapy (Neira, 2009).

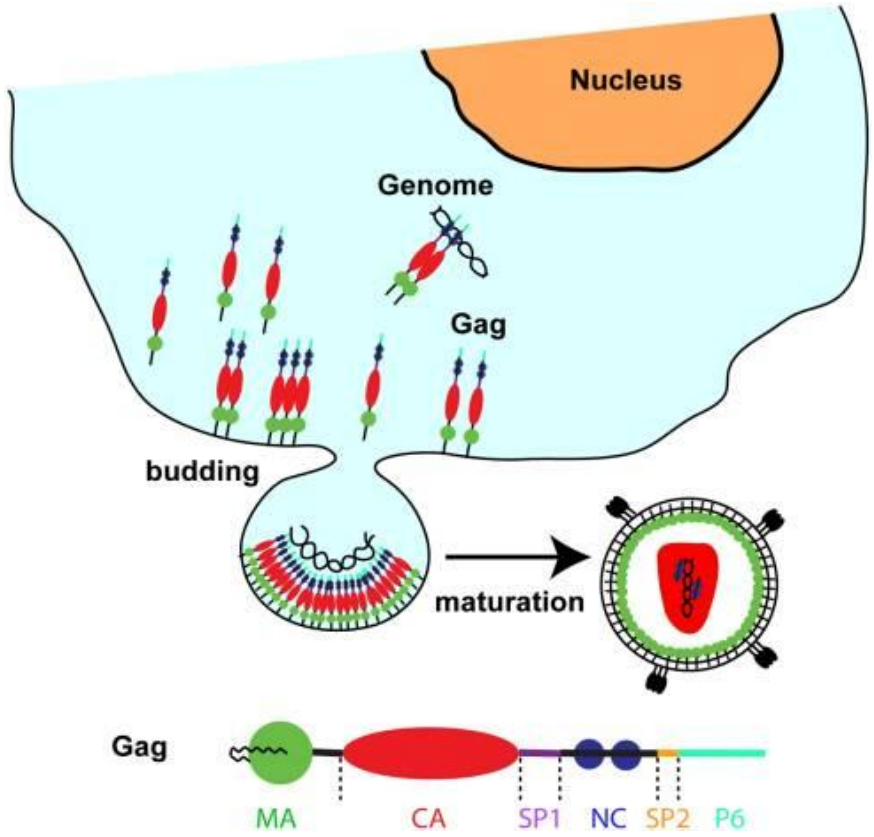

Figure 1.7. Assembly of Gag on cell membrane and its proteolytic cleavage. After protein synthesis, HIV-1 Gag assembles on the plasma membrane to form immature particles. The Gag precursors are cleaved by viral proteases into four structural proteins (MA – matrix molecule, CA – capsid molecule, NC – nucleocapsid molecule, and *p*6 - *p*6 molecule) upon maturation.

Source: Ghanam *et.al*., 2012.

1.2.2. Structure of capsid protein

In 2007, the three-dimensional structure of HIV-1 capsid was revealed by cryo-electron microscopy (cryoEM) (Ganser-Pornillos *et.al*., 2007), and the complete structure of capsid in both immature and mature forms were recently studied (Bharat *et.al*., 2012; Zhao *et.al*., 2013). CA forms a tube-like structure *in vitro* and assembles into spherical structure (**Figure 1.8**). This protein comprises two domains and has distinct roles in stabilizing the viral capsid architecture. They are N-terminal domain (NTD), residues 1-146 that forms hexamers and C-terminal domain (CTD), residues 147-231, which is dimeric in solution (Ganser-Pornillos *et.al*., 2007).

Each CA hexamer is composed of an inner ring of six NTDs and an outer ring of CTD (**Figure 1.9c**). Both of these independently folded domains are separated by a flexible linker but interacting closely with each other to form a compact structure (**Figure 1.9d**). Both domains predominantly compose of α-helical secondary structures. The arrowhead-like NTD contains seven α-helices (numbered 1–7) and an amino-terminal β-hairpin, while the globule-like CTD is made up of a single-turn 3_{10}-helix and four short α-helices (numbered 8–11) (**Figure 1.9d** and **1.9e**).

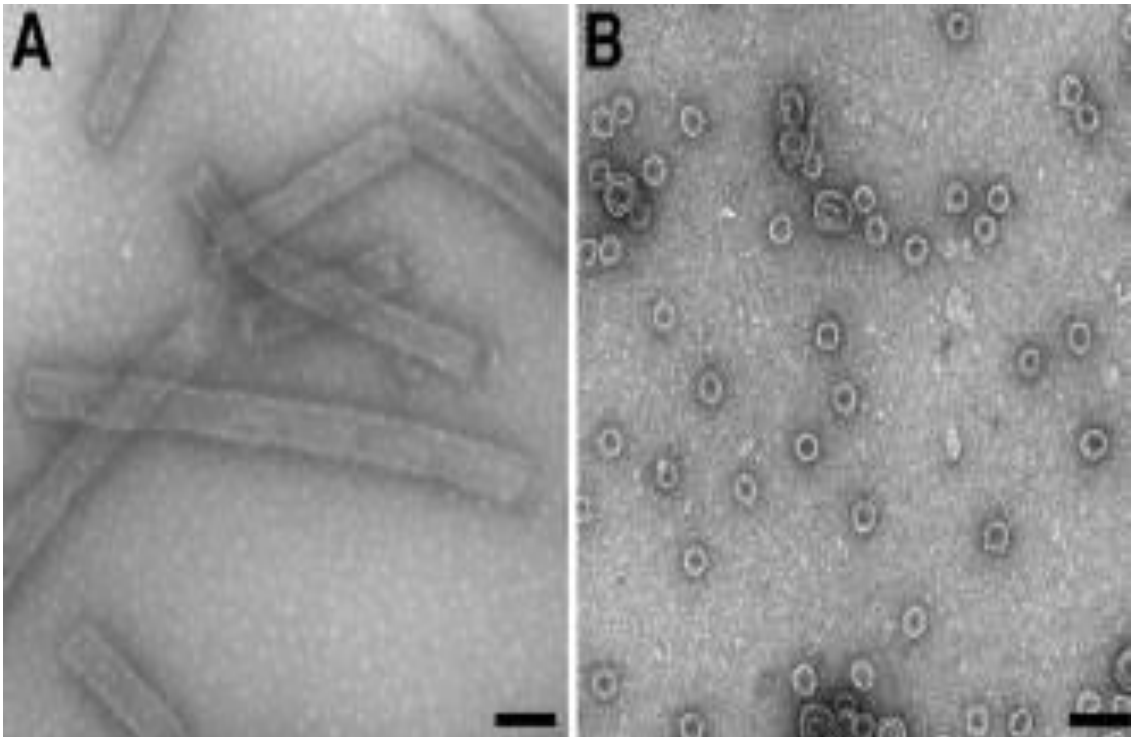

Figure 1.8. Characterization of HIV-1 CA hexametric lattice. Negative stain electron micrograph that shows **(A)** HIV-1 CA assembled into long and hollow tubes *in vitro* and **(B)** HIV-1 CA assembled into small spheres. The scale bar represents 100nm.

Adapted and modified from Ganser-Pornillos *et.al*., 2007.

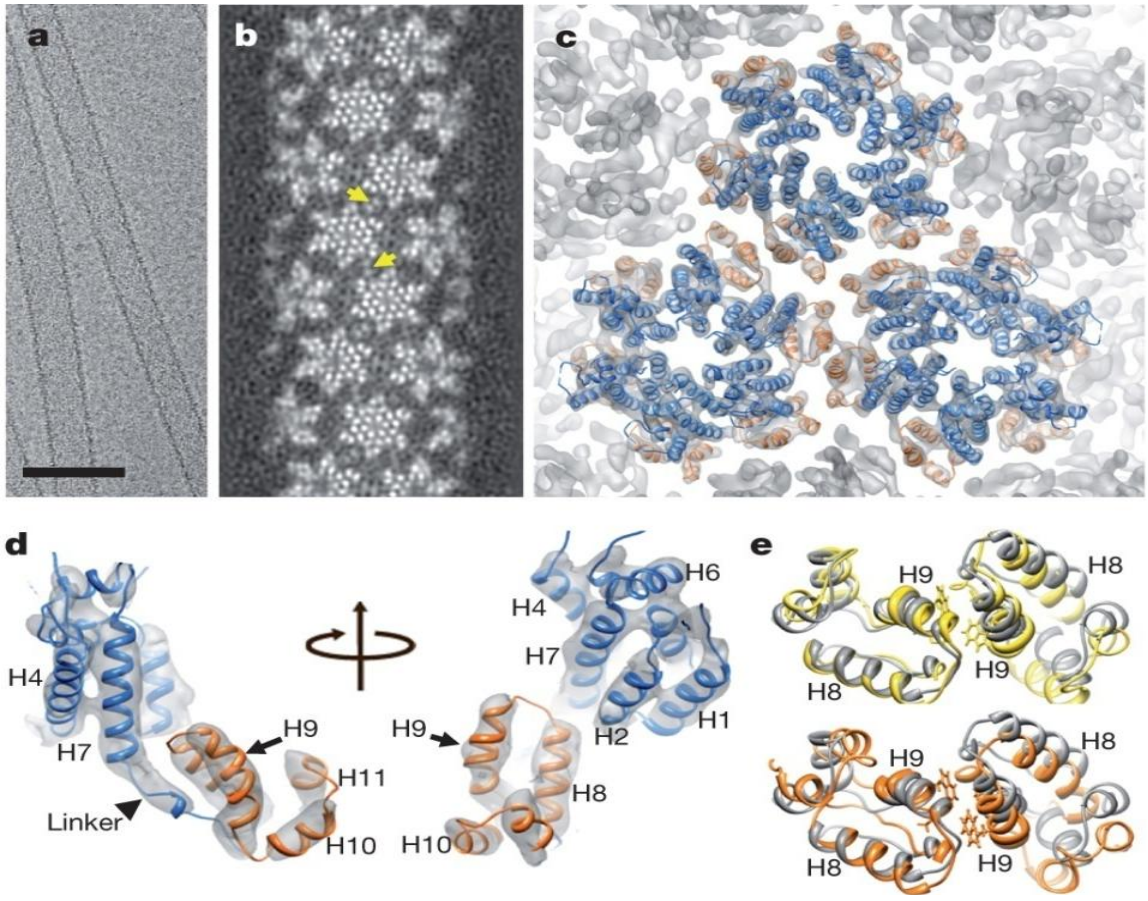

Figure 1.9. Cryo-EM reconstruction of HIV-1 CA tubular assembly. (a) A cryo-EM image of CA tubular assembly. Scale bar, 100nm. **(b)** Electron density map of the CA tube with helical symmetry, yellow arrows indicate pairs of helix H9, located between adjacent hexamers. **(c)** Molecular dynamics flexible fitting (MDFF) model of the HIV-1 capsid assembly, superimposed with the electron density map contoured at 4.0σ, three CA hexamers, with NTDs (**blue**) and CTDs (**orange**), are shown. **(d)** MDFF model of a CA monomer viewed from two angles. **(e)** Two CTD dimer structures along -1 (**orange**) and 11 (**yellow**) helical directions, superimposed onto the NMR solution dimer structure (**grey**, 2KOD).

Adapted and modified from Zhao *et.al*., 2013.

Structure of HIV-1 CA domains is highly associated with HIV-1 replication and infectivity. In the infectious virions, the mature CA assembles *in vitro* into three shapes: cylinders, cones, and spheres. This variation in shapes resulted from the distribution of 12 pentameric declinations within the hexameric lattice of CA. The 'cylindrical' capsids are obtained when tubular capsids are formed by 6 pentamers at each end of tube. An asymmetric distribution of the pentamers at the two ends results in 'conical' capsids while 'spherical' capsids arise from an even distribution of the pentamers throughout the hexameric lattice (Li *et al*., 2000; Ganser-Pornillos *et al*., 2004). It has been reported that the HIV-1 infectivity was reduced in the presence of virions with irregular core morphologies and heterogenous sizes of CA (Neira, 2009).

Furthermore, numerous mutagenesis studies have shown that amino acid substitutions in capsid protein lead to a reduced HIV-1 infectivity. It has been shown that mutation of arginine 18 to leucine at a site where helix-1 from the six NTDs are at their closest approach, altered the morphology of the *in vitro* assembled particles (Ganser-Pornillos *et al*., 2004). Alanine substitutions of tryptophan 184 and methionine 185 of helix-9 in the CTD diminish the dimerization *in vitro* and HIV-1 infectivity *in vivo* (Ganser-Pornillos *et al*., 2007). Furthermore, a single amino-acid deletion in HIV-1 CA CTD forms a 'domain-swapped' dimer in which its function in HIV-1 assembly and maturation was disrupted (Ivanov *et al*., 2007). These evidences suggest that the capsid protein is a highly viable target for anti-HIV therapy.