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The Effect of Alpha-HGA on HIV-1 Replication

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Cover picture: Electron micrograph of virions produced by pNL4-3 transfected cells. Picture was taken by Professor Stefan Höglund, Uppsala University.

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In The Name of God
The Most Gracious, The Most Merciful

To my family

ABSTRACT

Different classes of anti HIV-1 drugs are now available for treatment of HIV-1 infection. Although improved, these drugs show adverse effects and their long-term efficiency is severely hampered by the emergence of resistant viruses. Therefore, less toxic and more effective anti HIV-1 therapeutics agents are still needed. This thesis aimed to evaluate the anti HIV-1 activity of the small molecule, alpha-hydroxy glycineamide (αHGA), and to investigate its mode of action against HIV-1.

Here we showed that αHGA inhibits HIV-1 replication and that HIV-1 particles with aberrant core structures are formed in the presence of αHGA. We also showed that αHGA inhibits the replication of clinical HIV-1 isolates with acquired resistance to reverse transcriptase and protease inhibitors but has no effect on the replication of any of ten different RNA and DNA viruses. Alpha-HGA affected the ability of the HIV-1 capsid protein to assemble into tubular or core structures *in vitro* and *in vivo*.

In silica molecular modeling studies indicated a possible interaction of αHGA with the aspartate 51 (D51) of p24. This amino acid residue, located in the N-terminal domain of the capsid protein, has been shown to play a key role in virus assembly and maturation by forming a β-hairpin structure upon proteolytic cleavage of the Gag precursor protein. We introduced three different D51 substitution mutations into both prokaryotic and eukaryotic expression systems and studied their effects on *in vitro* CA assembly and virus infectivity. The results showed that substitutions of D51 with glutamate, glutamine, or asparagine, three amino acid residues that are structurally related to aspartate, could not rescue the structural integrity of the capsid nor viral infectivity. Surprisingly, in direct p24 binding studies αHGA was found to bind to the hinge region between the N- and C-terminal domains of the HIV-1 capsid protein and not to the p24 interaction surfaces. Importantly, αHGA only bound to dimerized p24 and not to monomeric protein. Binding to the flexible hinge region of p24 would indicate an allosteric effect of αHGA on the protein affecting its ability to assemble into capsids.

Since drug transport is an important aspect of drug function, we investigated the mechanism of [^{14}C]αHGA uptake by human T cell line. Uptake of [^{14}C]αHGA into H9 cells was time- and dose- dependent. The uptake properties showed low temperature dependency (Q_{10}<2) and the cellular uptake of [^{14}C] labeled αHGA was not inhibited by increasing concentrations of cold competitors. The metabolic inhibitors, NaN3 and NaF, had no effect on the cellular uptake of [^{14}C] labeled αHGA. Kinetic analysis of compound uptake, studies with metabolic inhibitors, saturation studies, and temperature coefficient value of αHGA uptake indicated that this compound enters H9 cells by a mechanism of passive diffusion.

Key words: *HIV-1, p24, capsid formation, alpha-HGA, antiretroviral, uptake.*

LIST OF PUBLICATIONS

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals:

I. Abdurahman S.*, **Youssefi M.***, Höglund S., Vahlne A.
Characterization of the invariable residue 51 mutations of human immunodeficiency virus type 1 capsid protein on in vitro CA assembly and infectivity. *Retrovirology*. 2007 Sep 28; 4:69.

II. Abdurahman S., Végvári A., **Youssefi M.**, Levi M., Höglund S., Andersson E., Horal P., Svennerholm B., Balzarini J., Vahlne A.
Activity of the small modified amino acid alpha-hydroxy glycineamide on in vitro and in vivo human immunodeficiency virus type 1 capsid assembly and infectivity. *Antimicrob Agents Chemother.* 2008 Oct; 52(10): 3737-44.

III. **Youssefi M.** and Vahlne A.
Cellular uptake of anti HIV-1 agent Alpha-Hydroxy Glycineamide (αHGA): Evidence for a mechanism of passive diffusion. *Manuscript*.

* These authors contributed equally to this work.

CONTENTS

LIST OF ABBREVIATIONS

aa	amino acid
AIDS	Acquired immunodeficiency syndrome
ART	Anti-retroviral therapy
αHGA	Alpha-hydroxy glycineamide
CA	Capsid protein
CRF	Circulating recombinant form
CTD	C-terminal domain
EC_{50}	Effective dose 50%
env	envelope gene
ER	Endoplasmic reticulum
FDA	Food and drug administration
gag	group associated gene
gp120	glycoprotein 120
GPG-NH_2	Glycine-prolyl-glycineamide
G-NH_2	Glycineamide
HAART	Highly active anti-retroviral therapy
HIV	Human immunodeficiency virus
kb	kilo base pair
LTR	Long terminal repeats
MA	Matrix protein
mAb	monoclonal antibody
nef	Negative regulatory factor gene
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NSI	Non-syncytium inducing
NTD	N-terminal domain
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PI	Protease inhibitor
PIC	Pre-integration complex
pol	polymerase gene
rev	regulator of virion proteins gene
RT	Reverse transcriptase
SI	Syncytium Inducing
SDS-PAGE	Sodium dodecyl sultafe-polyacrylamide gel electrophoresis
SP1, 2	Spacer peptide 1, 2
tat	trans activator of transcription gene
$TCID_{50}$	Tissue culture infectious dose 50%
TEM	Transmission electron microscopy
vif	virion infectivity factor
vpr	viral protein R
vpu	viral protein U
WB	Western blot
wt	wild type

1 INTRODUCTION

1.1 BACKGROUND

Human immunodeficiency virus type 1 (HIV-1) is the etiological pathogen causing the HIV/AIDS pandemic affecting millions of people around the world. Infection with HIV-1 initiates an intricate and fascinating series of host-virus interaction, the ultimate consequence of which is profound impairment of the host immune system. The knowledge about replication cycle of HIV-1 has resulted in the introduction of different classes of antiviral drugs. The advent of antiretroviral combination therapy (ART) has led to a dramatic reduction in morbidity and mortality due to HIV-1 infection. However, many patients have experienced failing therapy mainly due to the ability of the virus to become resistant to antiviral drugs. In addition to the antiviral resistance, drug toxicity and intolerance are significant problems. Therefore, there is still a need for new drugs with fewer side effects, less drug resistance and preferably with new antiviral mode of action. This thesis aims to describe a new potent anti HIV-1 agent, alpha-hydroxy glycineamide (αHGA).

1.2 HISTORY

In 1981, with the first reports of what came to be called AIDS, a new awareness of the threat of emerging infectious diseases arose [1-3]. The epidemiologic evidence suggested that a new infectious agent was responsible for cases of unusual opportunistic illness, indicative of severe immunosuppression. The pattern of AIDS case reports suggested that this disease agent was likely transmitted through sexual contact (homosexual and heterosexual), sharing of drug injecting syringe, contamination of the blood supply, and prenatally from mother to child. By 1984, a new retrovirus was isolated from AIDS patients and identified as causative agent [4-6]. Infection with HIV-1 results in a progressive loss of immune system function, ultimately leading to the opportunistic infections and malignancies of the acquired immunodeficiency syndrome (AIDS). The variability of disease expression depends on both host and viral factors. Important advances in treatment have significantly altered

the natural history of the HIV-1 infection, especially when combination antiretroviral therapy regimens are administered to the patients.

1.3 VIRION STRUCTURE

HIV-1 is a lentivirus, a member of the sub-family of retroviruses with complex regulation of gene expression and replication. HIV-1 is a ssRNA virus and the genomic RNA is present within viral particles in two copies [7]. The virions are initially assembled as immature particles containing unprocessed Gag and Gag-pol precursors of the proteins that eventually make up the mature virus. The immature virion morphology is spherical with a characteristic electron-lucent center. Upon maturation, the precursor proteins are processed and the structure and morphology of the virion changes dramatically. After processing of the Gag precursor, the capsid (CA) protein reassembles to form the electron dense conical core [8].

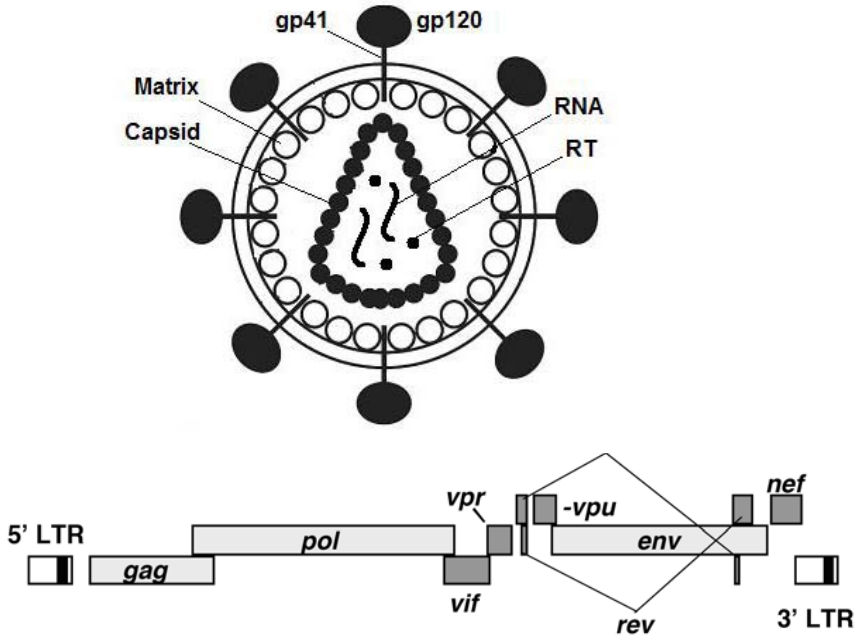

Figure 1. Schematic morphologic and genetic structure of HIV-1.

The virions exhibit a density of 1.16-1.18 g/ml in sucrose gradient. The virions are sensitive to heat, detergent, and formaldehyde. The stoichiometry of various gene

products in the virion are not very firmly established, but estimates suggest that about 4000-5000 Gag precursors are present per particle corresponding to approximately 4000-5000 molecules of Matrix protein, but the core of HIV-1 mature particle contains only 1000-1500 molecules of capsid protein, therefore not all the CA protein generated from Gag precursor becomes organized into the core [9, 10]. Levels of the pol proteins are typically less than the Gag proteins, estimated to be about 200 molecules per virion. The levels of the Env proteins are much lower, possibly as low as 72 trimers per virion [11]. The glycoprotein is composed of two subunits, a surface (gp120) and a transmembrane (gp41) subunit which are connected non-covalently [12, 13].

1.4 GENOMIC STRUCTURE

The genome of HIV-1 is about 9.2 kilo bases (kb) in size. The HIV genome has nine open reading frames (leading to nine primary translation products) but 15 proteins are made in all as a result of cleavage of three of the primary products.

The genome contains three common major genes: namely *gag*, *pol* and *env* which encode viral structural proteins or enzymes. The *gag* gene encodes the proteins: capsid (p24), matrix (p17), nucleocapsid (NC) and p6. The *env* gene encodes the envelope glycoprotein gp41 and gp120. The *pol* gene codes for viral enzymes: reverse transcriptase (RT), protease (PR) and integrase (IN).

To produce many proteins from a single primary transcript, the retroviruses use different strategies: generation and subsequent proteolytic processing of precursor polyproteins, ribosomal frameshifting, and alternative splicing of the primary transcript. For example, the *pol* gene is not expressed as a separate protein but it is expressed as a part of a larger Gag-Pol fusion protein. The *gag* and *pol* ORFs lie in different reading frames, and the formation of Gag-Pol polyprotein is mediated by a ribosomal frameshift mechanism [14]. Most of the time translation results in the formation of the Gag precursor; but approximately 5 to 10% of the time, as the translation approaches a specific homo polymeric site near the end of the *gag* ORF, the ribosome slips back one nucleotide (-1 frameshift) and proceeds onward in the new reading frame, the ribosome therefore passes through the stop codon out of the frame and so continues to synthesize protein using the codons of *pol* ORF [15, 16].

The expression of the various proteins as large precursors that are subsequently cleaved provides several advantages: it allows for many proteins to be made from one ORF, it ensures that the proteins are made at proper ratios, and it allows for many proteins to be targeted to the progeny virion during assembly as a single entity.
The *gag* gene and the *gag* and *pol* genes together are translated into large polyproteins which are then cleaved by a virus-encoded protease that is part of the Pol polyprotein.
Gag polyprotein is cleaved into proteins that are found in the mature virus: MA (matrix), CA (capsid), NC (nucleocapsid) and p6. Pol polyprotein is cleaved to: PR (protease), RT (reverse transcriptase), IN (integrase).
The *env* gene is translated to a polyprotein (gp160) which is then cleaved by a host cell protease, furin, that is found in the Golgi body. The gp160 is cleaved to surface (gp120) and transmembrane (gp41) subunits. The surface (SU) and transmembrane (TM) subunits remain attached to each other via non-covalent bonds.

Beside the three major genes *gag*, *pol*and *env* which are common in all retroviruses, the HIV-1 provirus contains six additional open reading frames which code for the two regulatory proteins (Tat and Rev) and the four accessory proteins (Vif, Vpr, Vpu and Nef). Three of these are incorporated into the virus (Vif, Vpr and Nef), while the others are not found in the mature virus. Tat increases the production of HIV-1 RNA synthesis and Rev controls the splicing pattern of HIV-1 RNA; the other proteins modulate interactions of the virus with its host and are needed for efficient virus replication and disease induction.

The nine genes are flanked by two repetitive regions called long terminal repeats (LTRs), which are important for both integration and transcription of the provirus. The 5′ LTR contains the site for initiation of viral transcription. Distinct functional parts within the LTR are known as U3, R, and U5. Cellular factors that modulate viral transcription interact primarily with sequences in U3. Some of these sequences connect the transcriptional activity of the provirus to the activation state of the host cell. For example, T-cell mitogens and cytokines increase nuclear factor-κB (NF-κB) levels in the nucleus that, in turn, act on sequences within the enhancer region of U3, thereby promoting the transcription from the LTR [17-20]. A *cis*-acting sequence designated the tat-response element (TAR), mediates the action of viral transactivator protein, Tat

[21-24]. The viral genome also contains the Rev-responding element (RRE) which binds to the Rev protein [25, 26]. The Rev protein stabilizes the viral RNA and facilitates the transport of unspliced and single spliced RNA species from nucleus to the cytoplasm [27]. Transcripts that encode Tat, Rev, and Nef do not contain the RRE and are multispliced species, while the other proteins are coded by unspliced and single spliced transcripts which contain the RRE (see below).

1.5 REPLICATION CYCLE

The infection is initiated by the binding of the virion gp120 to the surface receptor (CD4 molecule). This reseptor is expressed on T cells, macrophages, microglial cells, as well as, dendritic cells and Langerhans cells [28, 29]. In addition to CD4 as the primary receptor for HIV-1 the chemokine receptor molecules, CCR5 and CXCR4, have been identified as major co-receptors required for the post-binding step of membrane fusion and virus entry [30-33]. The interaction between gp120 and CD4 leads to a conformational change of gp120, leading to the exposure of gp120 binding site for the co-receptor molecules. The latter interaction in turn results in the exposure of the hydrophobic fusion domain of gp41 which leads to its insertion to the plasma membrane [34-37]. Because the external glycoprotein subunit is non-covalently linked to the transmembrane protein, it easily dislocates from the virion [38].

The reverse transcription of the viral RNA into a double-stranded DNA is defining hallmark of the retroviruses, and the step from which these viruses derive their names [39]. Reverse transcription normally begins soon after entry of virion core into the cytoplasm of the infected cell [40, 41]. The reaction takes place in a large complex, resembling the virion core, and containing Gag proteins including NC, as well as RT, IN, and the viral RNA [42]. The reverse transcriptase has three enzymatic activities: RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, and ribonuclease H (RNase H). The RNase H activity is responsible for the cleavage of the RNA strand in the RNA-DNA hybrid [43, 44].

The RT enzyme exhibits low fidelity, and the values of the error rate vary widely with the primer, template and type of the assay [45-47]. The mis-incorporation rate of RT under physiological conditions is about 10^{-4} errors per base incorporated. This rate

suggests that during replication there would be approximately one mutation per genome per reverse transcription cycle [48-52]. RT does not exhibit a 3' exonuclease proofreading activity, and misincorporated bases are not removed efficiently by RT as they are by cellular DNA polymerases [53, 54].

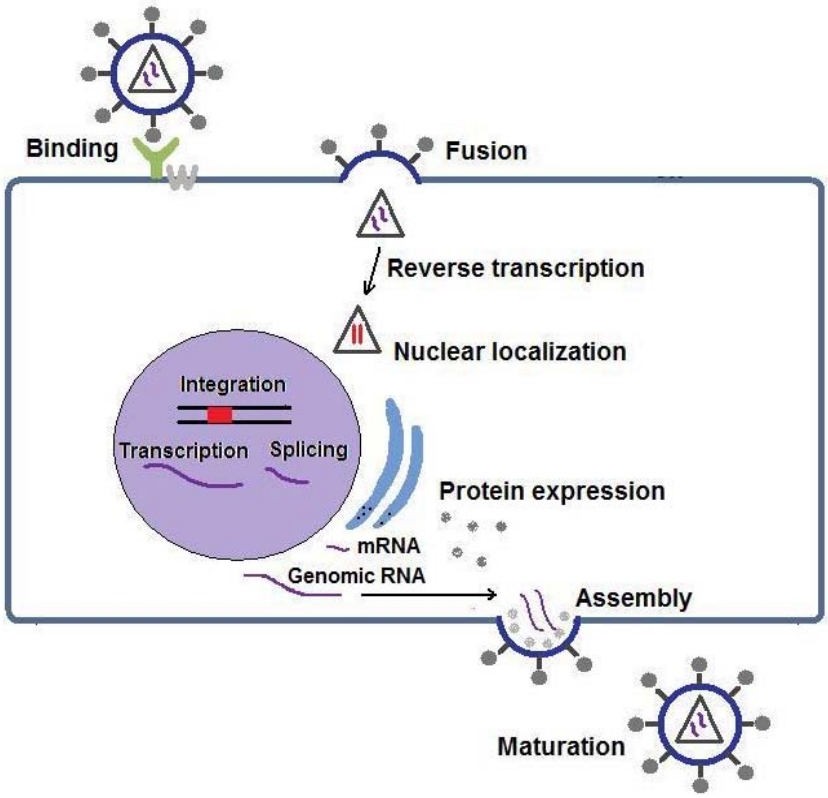

Figure 2. Schematic view of HIV-1 replication cycle.

The product of reverse transcription, the new double stranded linear DNA, remains associated with the nucleoprotein, and some other proteins: matrix, integrase, Vpr and RT; and forms the so called pre-integration complex (PIC) [55, 56]. The PIC of HIV-1 dose not contain detectable CA and uncoating of the viral core occurs at the nuclear pore upon RT completion [42, 57].

Once the viral DNA is actively transported to the nucleus of the infected cell [58-60], the linear retroviral DNA is integrated into the genome of host, preferably in actively transcribed genes, by the action of the viral integrase enzyme [61-64]. The viral DNA is permanently incorporated into the genome of the infected cell and is called provirus or proviral DNA at this stage. There is no mechanism by which provirus can be efficiently eliminated.

Synthesis of new viral RNA genomes and proteins is accomplished in a highly regulated manner utilizing host cell enzymes. Once integration has occurred, virus production depends on the presence of cellular and viral factors required for activation of viral promoters. For example, cellular activation, production of inflammatory cytokines, and cellular NF-κB and Sp1 enhance viral replication [65-70]. Using cellular enzyme RNA polymerase II, transcription of the provirus is initiated at the viral promoter, at the junction of the U3 and R regions in the LTR.

Transcription and translation of the provirus is highly regulated. Early after infection, activated cells produce 2-kb mRNAs coding for viral regulatory proteins and can be detected by Northern blot analysis or more sensitive RT-PCR technique [71, 72]. These messages represent the doubly spliced RNA for Tat, Rev, and Nef proteins. The Tat protein induces a markedly enhanced activity of the viral promoter, resulting in further increased RNA and protein production. With the accumulation of Rev protein, a switch to enhance unspliced and singly spliced mRNAs that code for the late viral proteins (Gag, Pol, Env, Vpu, Vpr, and Vif) is observed. The unspliced and singly spliced HIV-1 transcripts are exported from the nucleus to the cytoplasm by a transport mechanism mediated by Rev protein.

The unspliced species code for Gag and also Gag-Pol polyproteins; the Gag-Pol precursor is produced by ribosomal frameshifting from the same unspliced transcripts [15, 16]. The Env precursor protein is produced by the single spliced *vpu-env* transcripts [73]. The single spliced *vif* and *vpr* mRNA species are also produced by alternative splicing [74, 75]. The Gag and Gag-Pol polyproteins subsequently are cleaved by the viral protease to produce the mature viral structural proteins and enzymes. To do this, the Gag and Gag-Pol polyproteins are transported to the cell membrane where the activity of viral protease is initiated and virion assembly takes place [76-78].

The Env coding mRNA species contain a 5' leader sequences for ER targeting, the first translated amino acids contribute a hydrophobic signal peptide. Once this signal peptide is translated, the ribosome producing Env is targeted to ER, where production of precursor Env protein, gp160, takes place. The nascent Env protein is co-translationaly inserted into the ER. The protein undergoes glycosylation, folding and oligomerization

in ER [79-82] and then is exported to Golgi apparatus where it is cleaved to its functional components, gp120 and gp41, by a cellular protease [83-85]. The Env proteins are localized at the cellular membrane through the ER-Golgi-plasma membrane pathway.

Finally, as all viral components co-localize at the cell membrane, new virus particles are released. The progeny virus acquires its envelope from a portion of the plasma membrane enriched with viral glycoproteins as it buds. During the release or shortly after budding, the Gag and Gag-Pol precursors are cleaved by the viral protease, transforming the immature virus into a mature infectious virion with a characteristic conical core structure [77, 86].

1.6 VIRAL PHENOTYPES AND TROPISM

Apart from main receptor, CD4, HIV-1 requires a coreceptor, usually either CCR5 or CXCR4 to enter the target cell [87-89]. The virus strains that use either CCR5 or CXCR4 or both (called R5 or X4 or R5X4viruses, respectively), have been identified [90-92]. Viruses that use CCR5 are generally not syncytium inducing (NSI), and are sometimes referred to as monocyte/macrophage-tropic (M-tropic) viruses. Viruses that can use CXCR4 or both coreceptors, (X4 or R5X4 viruses) are generally syncytium inducing (SI), and can replicate and cause syncytia in MT2 cells [93-95]. This phenotypic distinction has biological importance: the *in vitro* SI capacity of HIV-1 isolates is associated with disease progression and means appearance of R5X4 or X4 viruses [96]. Early in the course of infection, the R5 viruses predominate, but eventually both R5 and X4 viruses can be found [97, 98]. The development of HIV-1 entry inhibitors that target CCR5 has heightened interest in coreceptor usage [99].

1.7 THE COURSE OF INFECTION

The infection initiates by sexual transmission, exposure to blood and mother to infant transmission. The natural course of HIV-1 infection has modified dramatically since the combination antiretroviral therapy has been introduced [100-103]. However, in populations without access to this therapeutic regimen, the course of infection has three distinct phases (Fig.3): primary (acute), asymptomatic (chronic) and the advanced [104, 105].

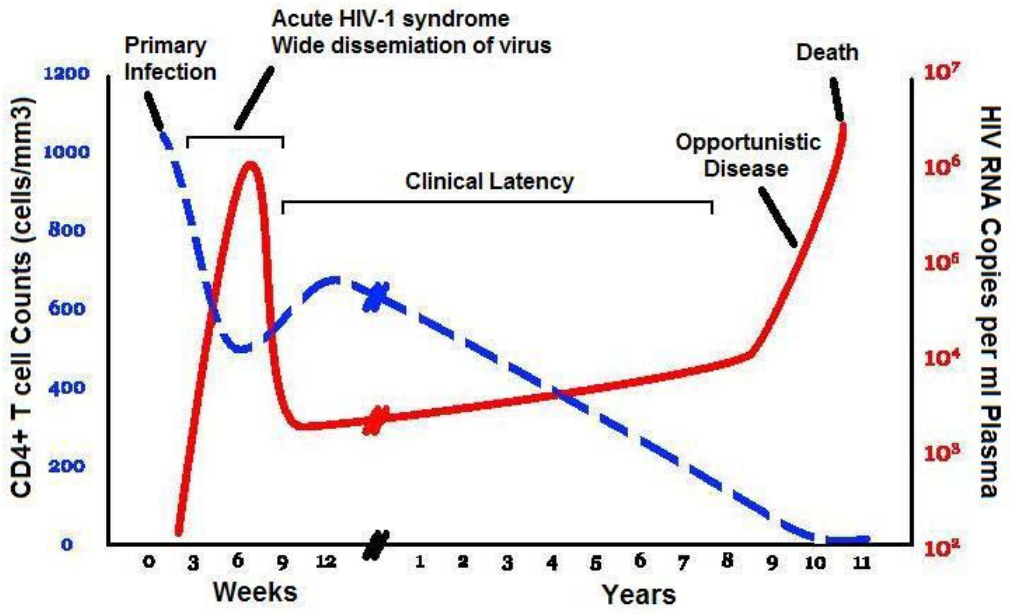

Figure 3. Graph showing the clinical course of HIV-1 infection. Dashed line indicates CD4+ T Cell counts and the solid line shows viral load.

The Acute phase: After infection, there is a rapid rise in plasma viremia within days [106-108], which is sometimes symptomatic. The self limiting symptomatic illness is characterized by maculopapular rash and flu like symptoms. This phase is fallowed by a remarkable reduction in virus load to steady state due to the vigorous host immune responses [109-112] .

The asymptomatic phase: By 3 to 6 months after infection, viral levels reach a steady state, sometimes called the viral set point. The degree of viremia at the viral set point is a direct predictor of how fast the disease will progress in a particular individual. That means the higher the viremia, the faster the progression. During this phase the number of CD4+ T-cells decrease at a steady rate. In the absence of therapy, the clinically latent phase may take 8-10 years in the majority of infected individuals [113, 114]. During this "chronic" phase, mild manifestations such as fatigue and lymphadenopathy can occur in some patients.

The symptomatic phase and AIDS: The "advanced" stage of the disease, when the infected person develops symptoms of AIDS, is characterized by a CD4+ T-cell count below 200 per µl and increased viral load. In this stage the opportunistic infections and malignancies due to HIV-1 infection are manifested [115].

1.8 CLASSIFICATION OF HIV-1 INFECTION

The CDC has developed a classification system for HIV-1 infection [116]. This classification system is based on three ranges of CD4+ T cell counts (≥500, 200-499, <200/μl) and three clinical categories (asymptomatic to sever AIDS indicator conditions) and therefore, the infection conditions is classified in nine categories, which represents the spectrum of HIV-1 infection. Using this system, any HIV-1 infected person with a CD4+ T cell count of <200/μl has AIDS by definition, regardless of the presence of symptoms or opportunistic disease. This classification system is used for reporting cases and scientific communications and categorization of the disease for epidemiologic purposes and public health strategies. It should be noted that this classification is not for management of infected individuals and the clinician should not focus on whether or not the patient fulfills the strict definition of AIDS, but should consider HIV infection as a spectrum ranging from primary infection to the advanced disease.

1.9 TREATMENT OF HIV-1 INFECTION

Combination antiretroviral therapy (ART) or highly active antiretroviral therapy (HAART) is the basis of management of patients with HIV-1 infection. Following the introduction of HAART, marked declines have been noted in the incidence of most AIDS-related conditions [117-119].

Although there are guidelines for treatment of HIV-1 infected patients [120, 121], the time for initiation of the therapy, the best initial regimen, and the time to change the regimen still differ between various countries. In general, the decision to start anti-retroviral therapy should be individualized, taking into account the relative risks and benefits to therapy. However, low CD4+ T cell counts, high viral RNA levels, a symptomatic disease, and late in pregnancy (for preventing transmission to child) are indications for initiation of therapy for the patient. Currently, most commonly used initial combination regimen consists of two nucleotide analogue RT inhibitors together with either a protease inhibitor or a non-nucleotide RT inhibitor.

The aim of therapy is to suppress the plasma HIV-1 RNA levels to undetectable limits. Changing therapy regimen should be considered if drug toxicity/intolerance occurs, if

the viral RNA levels monitoring shows poor virologic response to the therapy or a persistent decline in CD4+ T-cells is observed.

The phenotypic and genotypic resistance assays should be considered for patients who have experienced failure to therapy, for infected pregnant women with detectable viral RNA (to prevent mother to child transmission), and in the cases of recent acquisition of HIV-1 infection (due to the increased risk of transmission of drug-resistant HIV-1).

1.10 MECHANISMS OF RESISTANCE TO THERAPY

HIV-1 infection is a dynamic process, with high rates of replication [122-125] and a great potential for genetic variation mainly due to lack of proofreading activity of RT enzyme of the virus [126, 127].

The produced genetic variants populations of the virus are the main determinant for drug resistance. The genetic variants conferring resistance to a single antiretroviral agent may exist in the patient before any therapeutic regimen is initiated [128-130]. It has been estimated that 10^{10} virions of HIV are produced daily in an infected individual [124]. If each of these contains on average one mutation in a genome of 10^4 base pairs, then viruses with single drug-resistance mutations are likely to be generated daily. For example, a single nucleotide substitution that alters tyrosine to cysteine at amino acid residue 181 of the reverse transcriptase is enough to confer nevirapine resistance. This single mutation has been shown to exist in the quasispecies of untreated individuals [131]. When the selective pressure of the antiviral drug is applied in the infected individual, the preexisting minor viral species that are resistant to that drug become the predominant replicating species [132]. For some drugs like zidovudine and protease inhibitors, accumulation of three or more drug-resistance mutations in a single viral genome is needed to confer high level resistance [133, 134]. This means that resistant variants to these drugs emerge less quickly, and development of resistance to these drugs requires persistent viral replication in the presence of the selection pressure from drug treatment. If the replication is not slowed enough by the regimen, the mutations continue to accumulate [125, 135].

1.11 ANTI-RETROVIRAL DRUGS AND THEIR SIDE EFFECTS

The intense research and better understanding of HIV-1 replication has resulted in the introduction of different anti HIV-1 agents of which more than 20 are approved for use. These drugs can be classified into five main classes: the nucleoside/nucleotide analogue reverse transcriptase inhibitors (NRTIs), the non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), one integrase inhibitor and two entry/fusion inhibitors.

NRTIs: The NRTIs inhibit viral replication by acting as nucleoside analogues incorporated into the growing DNA chain, leading to premature chain termination, because they lack a 3'-hydroxyl group to form the binding with the incoming nucleotide [136]. Zidovudine which is an analogue to the nucleoside thymidine, is an example of NRTIs and was the first approved anti HIV-1 drug [137]. The major side effect of this drug is bone marrow suppression [138] and thus hematologic indices should be monitored during Zidovudine treatment. Less serious but more common side effects are headache, insomnia, fatigue, nausea, dyspepsia and myopathy/myalgia [139]. The other drugs of this class have been associated with pancreatitis, hepatotoxicity, peripheral neuropathy, diarrhea, abdominal discomfort, nausea and vomiting [140-143].

NNRTIs: This class includes drugs with different chemical structures that bind to the RT enzyme and inhibit its enzymatic activity [144-146]. These drugs have been associated with rash which may develop to Stevens Johnson syndrome, hepatotoxicity, and neurologic/psychiatric symptoms [147-149].

Protease inhibitors: These drugs inhibit the proteolytic cleavage of Gag and Gag-Pol polyproteins resulting in production of immature and defective viral particles [150]. Several metabolic side effects are associated with prolonged use of protease inhibitors. Most protease inhibitors induce hypertriglycemia, hypercholesterolemia, and hyperglycemia which in turn may be associated with an increase risk of myocardial infarction (MI) [151-156]. Abnormal fat deposits and lipoatrophy syndrome were first referred to protease inhibitors [157, 158], however, the body shape changes in HIV-1 infected patients seem to be a multi-factorial phenomenon [159]. Also osteopenia and osteonecrosis have been observed in patients treated with protease inhibitors [160].

Entry/fusion inhibitors: The fusion inhibitor, Enfuvirtide (T-20), is a 36-amino acid long peptide that is administered by subcutaneous injection. This peptide binds to gp41 and inhibits virus-cell membrane fusion. The most common adverse effects of Enfuvirtide have been local injection site reactions. Hypersensitivity reactions have also been reported but are rare. Also this drug may be associated with glomerulopathy and nephrotoxicity [161]. In 2007, FDA has approved the CCR5 antagonist, Maraviroc, for treatment of patients with CCR5-tropic HIV-1 (R5 viruses) [162].

Integrase inhibitor: Raltegravir was approved by FDA in 2007 as an integrase inhibitor. This drug is generally well-tolerated and the most common adverse experiences with this drug have been headache and dizziness [163, 164].

2 GAG PRECURSOR AND VIRUS ASSEMBLY

The expression of the Gag precursor is sufficient to mediate assembly of virus like particles [165]. Because of its central role in virus assembly, the Gag polyprotein has been subjected to intense analyses to define the domains required for various steps in the assembly process. Three domains of the precursor poly protein seem to be crucial: a membrane-binding (M) domain, an interaction (I) domain and a late assembly (L) domain [166].

The M domain is located at the amino terminus of Gag, in matrix. The Gag precursor is synthesized on cytosolic ribosomes [167] and becomes modified by covalently attachment of a myristic acid to its N-terminal Gly2 [168]. Myristic acid is a relatively rare 14-C fatty acid. The fatty acid is important for membrane localization and binding of the Gag precursor and increasing the hydrophobicity of the amino terminus of the protein [169, 170]. In addition, the N terminus of Gag contains residues which are crucial for Gag membrane targeting [171, 172].

The I domain is defined as a major region of Gag-interaction. This domain consists the one third of the C-terminal CA and the NC protein [173, 174].

The L domain lies in p6, at the C-terminus of Gag precursor and it contains the motif PTAP which is critical for virion release from infected cells [175, 176].

During and after release from the cell, the gag precursor is cleaved by the protease into a series of products present in the virion: the matrix protein (MA), the capsid protein (CA), the nucleocapsid (NC), p6, SP1 and SP2 [177, 178].

2.1 MATRIX PROTEIN (MA)

When the precursor Gag is myristylated at the amino terminus, the corresponding matrix protein retains the myristic acid and is attached to the membrane [179, 180]. In addition to myristilation, specific hydrophobic and basic residues in N-terminal of matrix are crucial for membrane binding [172, 181]. Single substitutions in the basic residues of MA causes virus assembly to be redirected to intracellular membrane, thus MA not only regulates membrane binding, but also determines the specificity of membrane targeting [182].

The matrix protein of HIV-1 has been shown to form trimers in crystallization studies [11] and can contribute to the ability of a larger Gag precursor to form trimers in solution [183]. In addition to regulation of membrane targeting and membrane binding, MA also contributes in incorporation of Env glycoproteins into the budding virions [184-186].

2.2 CAPSID PROTEIN (CA)

The capsid protein (CA) is a major component of the virion core [187]. This hydrophobic protein is relatively well conserved and contains a highly conserved motif among retroviruses, called the Major Homology Region (MHR). The function of this motif remains uncertain, although mutations in the region affect virion assembly [188]. CA is composed of two predominantly α-helical domains, the N-terminal (NTD) and C-terminal (CTD) domains, which are connected by a short flexible linker, the hinge region. The capsid protein has shown difficult to crystallize, and the structure of the complete protein is uncertain, but the structures of both NTD and CTD of HIV-1 CA have been determined [189, 190].

The NTD (residues 1 to 144) constitutes two third of the protein and contains a cyclophilin A binding site [191]. The CTD (residues 148 to 231) contains the MHR (aa 153 to 172). The structures of the NTD and CTD have been combined to predict the structure of intact capsid protein [192, 193].

Cyclophilin A is incorporated into HIV-1 virions through its association with HIV-1 CA [194, 195]. The interaction of cyclophilin A with CA was first identified using the Gal4 two-hybrid system [196].

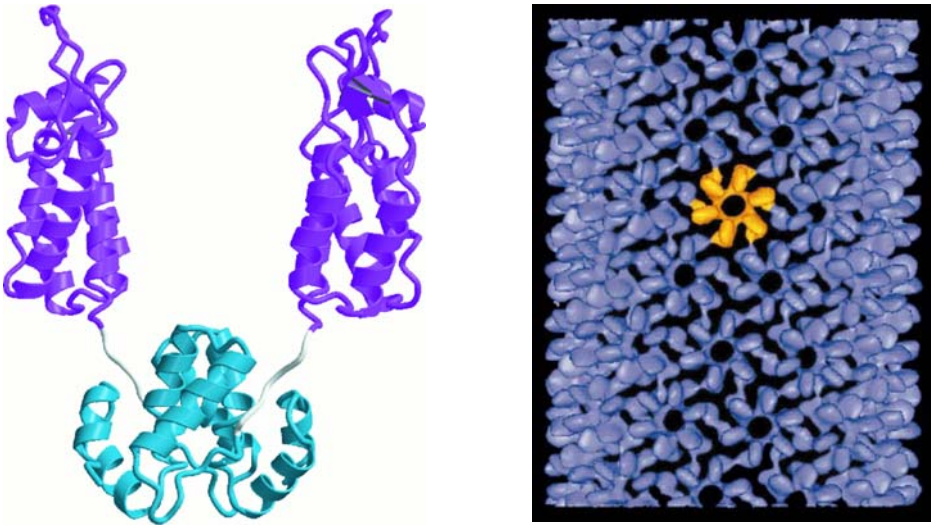

Figure 4. Left panel: The model of CA dimerization in which the C-terminal domains of the monomers interact *(adapted from Ref.190)*. Right panel: Hexameric model for CA assembly in which N-terminal domains forms the hexameric ring and C-terminal domains connect the hexameric ring with the adjacent rings *(adapted from Ref.199).*

CA can forms dimers in solution and is capable to assemble to form higher-order structures consisting of either tubes or cones [8]. Image reconstruction of electron micrographs, coupled to the sub-domain structures have led to suggesting models for the packing of capsid to form the large assemblies [197]. Different models for dimerization of HIV-1 capsid have been proposed [191, 192, 198]. However, the current general view is that the CA dimerization domain lies in C-terminus of the protein [190]. In the higher ordered assembled structures, CA is arranged in helical arrays in which the NTD forms hexameric rings that are linked into a continuous lattice by the C-terminal dimerization domain [199, 200]. This hexameric model for capsid assembly has been also shown in some other retroviruses [201-203].

CA assembly plays a critical role in HIV-1 replication and is considered as a promising target for the development of antiviral agents [204].

2.3 NUCLEOCAPSID PROTEIN (NC)

The Gag precursor binds to the viral genomic RNA and is responsible for its packaging into the budding virion particles. The NC domain is important for this function [205]. The NC domain also plays a critical role in Gag precursor multimerization [206, 207]. The NC which is located near the C-terminus of Gag precursor is obtained after the proteolytic cleavage of Gag precursor. Rich in basic residues (like Lys and Arg), the NC contains two copies of a zinc binding sequence, Cys-X2-Cys-X4-His-X4-Cys, referred to as retroviral Cys-His Zinc fingers [208, 209]. The basic residues confer a nucleic acid binding property [210] and the zinc fingers contribute to the specificity for the viral RNA [205]. During viral assembly the NC domain of the Gag precursor binds to the encapsidation site (psi site) near the 5′ end of the genomic RNA [211-213]. In addition NC exhibits a function known as "nucleic acid chaperon activity" which is critical for efficient reverse transcription process [214].

2.4 P6

The late domain, p6 which is at the C-terminus of Gag precursor is critical for the budding process [215]. This domain prompts the release of virions from the plasma membrane by interacting with host factors. It has been shown that p6 interacts with the cellular protein Tsg101 which is a component of the "endosomal sorting complexes required for transport" (ESCRT) machinery [216, 217]. The PTAP motif of p6 interacting with Tsg101is the dominant late domain function, however, a binding site for the cellular protein Alix which also interacts with the endosomal sorting machinery has been identified in p6 [218, 219]. In addition to its role in virion release, p6 is also important for incorporation of Vpr and Vpx into the virions [220].

3 AIMS OF THE STUDY

The main objective of this study was to investigate the role of αHGA on HIV-1 replication. The specific aims of the included studies were:

i) To investigate the antiviral activity of αHGA, its role on resistant variants, and the antiviral mechanism especially on CA assembly

ii) To investigate a possible interaction of αHGA with D51 of CA, and the role of this amino acid residue in HIV-1 CA assembly, both *in vitro* and *in vivo*

iii) To further determine the binding site of αHGA on CA using MALDI-MS

iv) To investigate the mechanism by which αHGA is taken up by cells

4 MATERIALS AND METHODS

4.1 PLASMID CONSTRUCTION

A DNA fragment consisting of 693 bp encoding amino acids 1 through 231 of p24 (CA) region of HIV-1 was generated from the proviral pNL4-3 clone [221] using PCR with 5′- ATG GAT CCA TAT GCC TAT AGT GCA GAA CCT CC-3′ and 5′-ATG GAT CCT ATC ACA AAA CTC TTG CTT TAT GGC C-3′ as forward and reverse primers, respectively. The primer sequences were flanked with a translational start codon in the 5′ primer (part of an introduced NdeI site) and two stop codons (TGA/TAG) in the 3′ primer of the coding sequences, and in addition two novel restriction sites in the 5′ primer (BamHI/NdeI) and one in the 3′ primer (BamHI).

The PCR product was subcloned into a TA cloning vector (Invitrogen, Groningen, NL) to generate the TA-CA plasmid. This vector was digested with NdeI and BamHI and the CA fragment was isolated and cloned into the pET11a vector (Novagen Inc., Madison, WI, USA), digested with the same restriction enzymes, resulting in the expression plasmid pET11a-CA. The pET11a-CA plasmid encodes recombinant CA protein with a predicted molecular mass of 25.6 kDa, containing an N-terminal Met in addition to the sequence of the protein. Protein sequence analysis showed that this N-terminal Met was removed in *E.coli*. The DNA sequence was verified by DNA sequence analysis.

4.2 SITE-DIRECTED MUTAGENESIS, SUBCLONING AND SEQUENCING

The high fidelity, PCR based QuickChange^{TM} Site-directed Mutagenesis Kit (Stratagene) was used to introduce the mutations into molecular clone pNL4-3 as well as protein expression vector pET11a-CA. The mutagenic primers were designed to alter D51 (aspartate) to N (asparagine), E (glutamate) and Q (glutamine) amino acids. The mutagenic oligonucleotides used were 5′-GCC ACC CCA CAA AAT TTA AAT ACC ATG-3′, 5′-GCC ACC CCA CAA GAG TTA AAT ACC ATG-3′ and 5′-GCC ACC CCA CAA CAA TTA AAT ACC ATG-3′ respectively. The mutations were verified by sequence determinations using an ABI 310 sequencer (Applied Biosystems) and the

BigDye cycle sequencing kit. The 1.3 kb *Apa* I to *Bss*H II fragment of each mutated pNL4-3 plasmid, containing the mutated p24, was then sub-cloned into the backbone of the wild type vector.

4.3 CELL CULTURE AND TRANSFECTIONS

HeLa-tat cells, 293T cells (a variant of Human Embryonic Kidney, HEK cells), COS 7 cells (a variant of kidney cells of African green monkey kidney) and reporter cell line, TZM-bl cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml Penicillin G and 100 µg/ml Streptomycin in a CO_2 incubator at 37°C in a humidified atmosphere. Transfection with wild type and mutated molecular clones were performed in 6-well culture plates (NUNC) using FuGENE 6 transfection reagent (Roche). The cells were lysed 48-72 h post transfection with RIPA buffer supplemented with protease inhibitor cocktail (Roche). The cell lysates were then subjected to western blot analysis.

4.4 VIRUS PURIFICATION

The virus containing supernatants from mutated and wild type pNL4-3 transfected cells were first clarified from cell debris by centrifugation at 1200 r.p.m for 10 min, filtered through 0.45 µm syringe filters and mixed (4:1 v/v) with Virafinity (CPG Inc). The mixture was centrifuged briefly and the viral pellets were washed in cold PBS. The viral pellets were then dissolved in 100 µl RIPA buffer and subjected to western blot analysis.

4.5 SDS-PAGE AND WESTERN BLOT ANALYSIS

SDS-PAGE was performed on transfected cells, as well as, purified virus preparations under reduced conditions. Electrophoretically separated proteins were transferred onto a nitrocellulose membrane using an electrotransfer system (Amersham Bioscience). The nitrocellulose membranes were blocked, and the immunoblot was performed using various primary antibodies or a pool of different HIV-1 positive sera. The bound antibodies were then detected with horseradish peroxidase (HRP)-conjugated

secondary antibodies. Finally, the appropriate chemiluminescent substrate (Pierce) was added to detect the bound HRP-labeled secondary antibodies.

4.6 IMMUNOFLUORESCENCE STAINING

HeLa cells were seeded on chambered slides (Nunc). The next day, cells were transfected with mutant and wild type pNL4-3. After 48 hrs, cells were washed and fixed in acetone/methanol (1:1). Immuno-staining was performed using a monoclonal anti-p24 antibody and a secondary FITC-conjugated rabbit anti-mouse IgG antibody (DAKO). To stain the nucleus, 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) was used. Finally, the slides were mounted and analysed under a Nikon Eclipse E600 fluorescent microscope.

4.7 VIRUS INFECTIVITY ASSAY

Wild type (wt) and mutated viruses were analysed for infectivity using two different assays. T cell lines H9 or MT4 cells were infected with wt virus or mutated viruses for 3 hrs. The cells were then pelleted and washed to remove unbound viruses. Thereafter, the cells were resuspended in fresh RPMI medium. The virus production was assayed by an in-house p24 ELISA on various days post-infection.

In another set of experiments, virus infectivity was assayed using the transgenic reporter cell line, TZM-bl cells. These cells are stably transfected with the reporter gene for luciferase that is under the control of HIV-LTR promoter [222]. After infection, HIV promoter is strongly transactivated by HIV-1 protein Tat and luciferase is produced which could serve as a marker of infection. TZM-bl cells were seeded the day before infection. The next day, the cells were treated with 20 μg/ml DEAE-dextran and infected for 2 hrs with wt and mutated viruses. Thereafter, unbound viruses were removed and the cells were cultured in fresh DMEM containing indinavir for 24 hrs. The viral infectivity was assayed by detection the luciferase activity in infected TZM-bl cells. The luciferase activity was quantified with a luciferase assay kit (Promega) and a microplate luminometer (Luminoskan Ascent, Thermo LabSystems).

4.8 IN-HOUSE P24 ELISA

Determination of HIV-1 p24 antigen was performed to evaluate the amount of HIV-1 virus in the culture supernatants. An in-house p24 capture ELISA was carried and p24 was detected by a double antibody sandwich system as described before [223]. The assay involved two different antibodies, one coated on the ELISA solid phase and the other conjugated with biotin. First, microwell plates (Nunc) were coated with anti-p24 antibody. The plates were then blocked with 3% BSA and serially diluted culture supernatant were added to the wells and incubated at 37°C for 1 hour. The plates were then washed and biotinylated anti-p24 was added. The plates were incubated for 1 hour followed by washing. Next, peroxidase-labeled streptavidin (Jackson ImmunoResearch Inc) was added to detect biotinylated antibodies. Finally the chromogenic substrate, 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) was added and the OD values were measured using a microplate photometer (Thermo Multiskan EX).

4.9 EXPRESSION AND PURIFICATION OF RECOMBINANT HIV-1 CA

Competent *E.coli* BL21 (DE3) cells were transformed with the pET11a-CA expression plasmid. Cells were grown in LB medium supplemented with 100 μg/ml ampicillin at 37 ºC to an A_{600} of 0.6. Subsequently, expression was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, followed by incubation at 37 ºC for an additional 4 h. Induced bacteria were collected by low-speed centrifugation and stored at –20 ºC. Recombinant CA protein was purified essentially as described before with a few modifications [224]. The CA protein, produced as inclusion bodies, was solubilized by treatment with 6 M Guanidine-HCl. After addition of distilled water to allow refolding the solution was dialysed over night with 50 mM Tris-HCl, pH 8 and centrifuged at 12,000×*g* for 10 min. The CA protein was precipitated by addition of ammonium sulphate to 30% saturation and collected by centrifugation for 10 min at 27,000×*g*. The precipitate was dissolved in 50 mM Tris-HCl buffer, pH 8, containing 30 mM NaCl and 1 mM EDTA. To remove nucleic acids a HiTrap DEAE column (Amersham Pharmacia Biotech) was run equilibrated with the same buffer using an ÄKTA FPLC chromatography system (Amersham Pharmacia Biotech). CA protein does not bind to the DEAE column under these conditions and was collected from the unbound material in the flowthrough fraction by addition of

ammonium sulphate to 50% saturation followed by centrifugation. The precipitate was redissolved in 50 mM Tris-HCl buffer, pH 8, containing 30 mM NaCl and 1 mM EDTA.

To purify the CA protein further gel filtration was performed on a HiLoad 16/60 Superdex 75 pg column (Amersham Pharmacia Biotech) equilibrated and eluted with 50 mM Tris-HCl buffer, pH 8, 30 mM NaCl and 1 mM EDTA at a flow rate of 1 ml/min. Fractions containing CA protein were pooled and the protein concentration was determined using a Bio-Rad *DC* Protein Assay Kit (Bio-Rad), aliquotated and stored at -85ºC until used.

4.10 ANTI-HIV ACTIVITY OF αHGA AGAINST CLINICAL HIV-1 ISOLATES

Stocks of clinical HIV-1 isolates were prepared by collecting the supernatants from infected cultures of PBMCs. The 50% tissue culture infective dose ($TCID_{50}$) was determined by infecting PBMCs with serial, 10-fold dilutions of the virus. PBMC (6×10^5 cells/ml) were infected with clinical isolates at 100 $TCID_{50}$ and cultured in medium containing various concentrations (1, 4, 16, 64, and 256 μM) of αHGA. Infected cultures without test compound served as controls, and each isolate was plated in duplicate. Medium was changed at days 5 to 7 post infection, and the supernatants were monitored for their p24 antigen contents until the termination of incubation at day 14.

The drug 50% effective concentrations (EC_{50}) were determined from graphs where p24 antigen production was plotted against drug concentrations used.

4.11 STUDY OF THE CA ASSEMBLY (*IN VITRO* AND *IN VIVO*)

Turbidity assay was used to study possible *in vitro* polymerization of p24. Polymerization of p24 was then followed spectrophotometrically, as the rate of p24 tube formation increases sample turbidity. The assay was performed at room temperature using a BioSpec-1601E spectrometer (Shimadzu) and the absorbance was set to 350 nm wavelength. Two-hundred μM of purified p24 was mixed with various concentrations of αHGA (5, 50, and 100 μM) and 50 mM Na-phosphate buffer (pH 8.0). Tubular p24 assembly was then induced by addition of 2.0 M NaCl solution, and

absorbance measurements were made every 10 s for up to 60 min. The assembly rate was then set by plotting the absorbance versus time.

Additionally, TEM analysis of *in vitro* assembled tubular structures was performed. The assembled p24 tubular structures were negatively stained with 2% ammonium molybdate at pH 8.0 and were analyzed by TEM. For *in vivo* (intracellular) p24 assembly, HeLa-tat cells (1×10^5) were seeded in single culture plates one day before and transfected with the CA expression plasmid, pC37M using FuGENE 6 transfection reagent (Roche). Forty-eight to seventy-two hours post-transfection, cells were fixed with freshly prepared 2.5% glutaraldehyde and subjected to TEM analysis.

4.12 TRANSMISSION ELECTRON MICROSCOPY (TEM)

Transfected HeLa-tat cells and virus infected H9 cells were fixed by freshly made 2.5% glutaraldehyde in phosphate buffer and postfixed in 1% OsO_4. The cells were embedded in Epon™ resin and poststained with 1% uranyl acetate. Epon sections were cut at approximately 60 nm thick to allow accommodation of the volume of the core structure parallel to the section plane. Duplicate sample preparations were performed, and minimal beam dose technique was employed throughout. Evaluation of morphology was carried out with series of electron micrographs to depict different categories of virus morphology.

Additionally, *in vitro* assembled CA proteins were negatively stained with 2% ammonium molybdate at pH 8.0 to study the CA tubular formation.

4.13 STUDY OF THE MECHANISM OF CELLULAR UPTAKE OF αHGA

Time course of cellular uptake of αHGA was investigated. H9 cells (1×10^6) were incubated in each 1.5 ml Eppendorf tube containing 400 µl RPMI 1640 medium. Transport of αHGA at 22°C was initiated by adding 25 µM [^{14}C]αHGA to the cells. After definite intervals, the uptake was terminated by centrifugation of the cells followed by rapid removal of the medium. The cell pellets were rapidly washed twice in cold phosphate buffer saline (PBS) supplemented with unlabeled αHGA to prevent compound efflux during the washing steps. The cell pellets were then re-suspended and solubilized in 70 µl of 0.5 *N* NaOH. Thereafter, pH was adjusted with 70 µl of 0.5 *N*

HCl and the cell homogenates were transferred into 6 ml scintillation tubes (NUNC) containing 3 ml scintillation cocktail (BECKMAN Coulter, USA). The cell associated radioactivity was quantified as counts-per-minute (cpm) in a liquid scintillation analyzer (1209 RackBeta Primo, Turku, Finland).

To reveal any possible transporter involved in the compound internalization, saturation studies were performed in two different ways. Cells were pre-incubated with 4 mM unlabeled αHGA for 30 min at 37°C. The cell incubations were then treated with increasing concentrations of [^{14}C]αHGA (25-100 μM). After one hour of incubation, the cells were harvested and the radioactivity was measured. In another set of experiments, the cells were incubated in the presence of 25 μM [^{14}C]αHGA together with various concentrations of unlabeled αHGA ranging from 0.25 to 2 mM corresponding to10-100 fold extra unlabeled compound at 37°C for one hour. The cells were then pelleted, washed and cell associated radioactivity was quantified.
Temperature coefficients (Q_{10}) are sometimes used in attempts to distinguish between simple and facilitated diffusion. The temperature dependence of αHGA uptake was investigated in incubations at different temperatures and a temperature coefficient (Q_{10}) for the compound uptake was determined. The effect of the metabolic inhibitors, sodium fluoride (NaF) and sodium azide (NaN_3), on uptake was also studied. Cells were treated with 1 mM NaF and/or 5 mM NaN_3 and the cellular [^{14}C]αHGA uptake was compared with the non-treated control.

In order to measure efflux of the compound, H9 cells were first loaded by exposure to 50 μM [^{14}C]αHGA at 37°C for one hour and subsequently pelleted and washed in PBS containing unlabeled compound. One sample served as the indicator of total intra cellular loaded radioactivity and the other samples were suspended in 1.2 ml fresh medium. Efflux of substance from the cells was determined from a comparison of the intracellular content of total initial radioactivity and after various time intervals.

5 RESULTS

Anti HIV-1 activity of αHGA (paper II)

The antiviral activity of αHGA was tested on 15 clinical HIV-1 isolates representing clades A, B, C, and CRF01-AE of both CCR5 and CXCR4 co-receptor usage. All isolates had acquired resistance against other antiretroviral drugs as determined by sequencing. The replication in PBMCs of all these clinical HIV-1 isolates was inhibited by αHGA. The 50% effective concentration (EC_{50}) of αHGA, ranged from 4.2 to 34 μM. From repeated experiments of the same isolate, the variation seen here between different isolates was most probably owing to test variations and not to actual differences in sensitivity to the drug. The EC_{50}-values for laboratory strains SF2, NL4-3 and HIV-1^{IIIB} tested in continuous cell lines were repeatedly between 1 and 8 μM.

Effect of αHGA on HIV-1 CA assembly both *in vitro* and *in vivo* (paper II)

To study possible effects of αHGA on *in vitro* p24 assembly, purified HIV-1 p24 was incubated with or without different concentrations of αHGA, after which 2.0 M NaCl was added to induce p24 tubular formation. Absence of αHGA resulted in an increased turbidity that was monitored spectrophotometrically at 350 nm. The p24 tubular formation decreased in an αHGA dose dependent manner. At 100 μM of αHGA, tube formation appeared to be completely inhibited. Glycine at 100 μM had no inhibitory effect on HIV-1 p24 assembly. In parallel, p24 assembled under similar conditions were analyzed with TEM. In the absence of αHGA, long cylinders of the p24 were observed. In contrast, no tubular structures could be detected when p24 was incubated in the presence of 100 μM αHGA. At 50 μM αHGA fewer and shorter tubular structures were observed.

The effect on intracellular p24 tubular formation was also investigated in HeLa-tat cells transfected with the p17-p24 expression plasmid and analyzed by electron microscopy. In the absence of αHGA, intracellular clusters of long tubular structures could be observed. Addition of 100 μM αHGA to the transfected cell cultures abolished the intracellular assembly of these tubular structures.

Effect of αHGA on virion morphology (paper II)

Untreated and αHGA treated H9 cells were infected with the HIV-1 SF-2 and progeny virus was analyzed by transmission electron microscopy (TEM). αHGA treatment at a concentration of 10 μM resulted in significant changes in virion morphology. Numerical analysis of 439 virus particles produced in untreated cells and 401 virus particles from αHGA treated cells were done focusing on the virus core morphology. Although a small percentage of viruses with aberrant core were present in the TEM images of untreated SF-2, approximately 62% of αHGA treated cultures showed virus with distorted cores.

Viral protein expression of HIV-1 capsid D51 mutations (paper I)

Three HIV-1 CA mutants affecting a possible binding site for αHGA, the D51N, D51E, and D51Q mutations, were tested for viral protein expression by initially transfecting HeLa-tat cells. The relative intracellular level of the Gag precursor with all mutants was comparable to that of the wild type, whilst the D51N and D51Q mutants displayed reduced levels of the CA protein. In western blot analysis of the precipitated viral particles, mature CA protein represented the major product; however, the level of this protein in both D51N and D51Q mutants was significantly reduced relative to the wild type and D51E mutant, correlating with the lower intracellular CA levels.

The viral protein expression profiles were further investigated by immunofluorescence staining using monoclonal antibody directed against CA protein. All mutants displayed a sharp specific signals concentrated near or at the plasma membrane. This feature was most pronounced in cells transfected with the three CA mutants and not with the wild type transfected cells. The staining pattern seen with the wild type control was mostly throughout the whole cytoplasm and the plasma membrane.

Effect of D51 mutations on virus infectivity (paper I)

The effect of the three CA mutations on virus infectivity was investigated with culture supernatants from transfected HeLa-tat III, 293T and COS7 cells. MT4 cells were infected with equal amount of cleared and filtered culture supernatants (normalized for p24 antigen) and assayed for p24 antigen contents with p24-ELISA three days post-

infection. While none of the three mutant viruses were able to replicate, as expected, the wild type virus replicated in this cell line. Similar results were also seen when the infectivity of mutant viruses was tested in H9 cells.

We also analyzed the infectivity of the wild type and mutant viruses in a single replication cycle infectivity assay using the TZM-bl reporter cell lines. While the Tat-induced luciferase activity could not be detected in cells infected with mutant D51N and D51Q virions, only a subtle amount of luciferase activity was observed repeatedly in cells infected with the D51E virions. On the other hand, the level of Tat-induced luciferase activity was significantly higher in cells infected with the wild type virus.

Effect of D51 mutation on virus release & morphology of virions (paper I)

The effects of CA mutations on Gag assembly and virus particle release were also analyzed by measuring the p24 antigen contents released into the culture medium of transfected HeLa-tat III, 293T and COS7 cells. The virion release of D51N and D51Q was dramatically reduced in all three cell lines. However, the reduction in virus production and release of the D51E mutant particles was less pronounced and was only reduced by 2- to 6-fold as compared to the wild type.

Morphogenesis of all mutant viruses and the wild type control were analyzed by transmission electron microscopy. The D51N and D51Q mutant virions showed mostly particles devoid of the typical HIV-1 core structure. Instead, heterogeneous virus populations with aberrant core structures were observed. Additionally, the D51N virions showed a large pool of intra-vesicular viruses that were deficient of the electron dense core structure. A limited number of immature-like viruses and occasionally mature-like viruses but with aberrant core were observed with the D51E mutant. Only the wild type control produced viruses with typical immature- and mature-like virions.

Effects of D51 mutations on purified CA assembly (paper I)

Turbidity assay was used to study a salt-induced self-assembly process of CA. In this assay, polymerization of CA is monitored spectrophotometrically, as the rate of CA tube formation increases sample turbidity. An increase in the rate of sample turbidity was observed for both D51N and D51E mutant CA proteins, however, the kinetics of CA assembly which is reflected by the increase in sample turbidity was lower than the

wild type control. In marked contrast, the rate of increase in sample turbidity for the D51Q mutant CA protein was higher than the wild type control, proceeding immediately upon addition of the salt as indicated by an increase in the optical density (OD) measurement.

The effect of CA mutations on *in vitro* CA assembly was further investigated by transmission electron microscopy. Thin-sections of the polymerized material used in turbidity assay was prepared and analyzed by TEM. Long tubular structures were observed in both D51N and D51E mutant CA proteins. Additionally, the morphology of the tubes formed by these two was comparable to the structures formed by wild type CA. In contrast, no structures that resembles CA tubular formation was observed with the D51Q mutant CA protein under same conditions.

Interaction of αHGA with CA (paper II)

A series of experiments were designed to investigate whether αHGA can interact with HIV-1 p24. Recombinant p24 proteins were incubated with and without αHGA and then enzymatically cleaved with trypsin and subjected to MALDI-MS analysis. If the interactions occurred are strong, a mass shift of one or more of the generated peptide fragments obtained after incubation with αHGA compared to the untreated control could reveal the positions where αHGA interacts with the protein.

With the wild type p24, five characteristic long peptide fragments were readily detected in all MALDI-MS experiments. A new signal appeared in the vicinity of the fragment MYSPTSILDIR, with mass value increased 72 Da in all samples upon incubation of p24 with αHGA as compared to non-treated p24. Similar results were also observed with three p24 mutants, D51N, D51E, and D51Q. The MYSPTSILDIR peptide corresponds to the 10 amino acids residues (143 to 153) in the hinge region connecting the NTD and the CTD of p24.

This interaction, however, was not detected when similar experiments were performed with the C-terminal domain (amino acids 151-231) or p24 containing mutations in the C-terminal dimer interface (W184A+M185A).

Cellular toxicity and mitogenecity of αHGA (paper II)

At concentrations up to 1,000 μM αHGA had no effect on cell viability on PBMC or any of the cell lines tested. αHGA had no mitogenic activity against human PBMCs at concentrations of up to 2,000 μM. In contrast, 2 μg/ml PHA markedly stimulated dThd incorporation into PBMC DNA. No effect on PHA-induced stimulation of DNA synthesis (cell proliferation) was observed when αHGA was added to PBMCs at concentrations up to 400 μM. However, at 2,000 μM, the PHA-induced stimulation was markedly inhibited.

Cellular uptake properties and kinetics for αHGA (paper III)

The time course of [^{14}C]αHGA uptake was monitored after incubation of cells with the labeled compound for different time points. αHGA was taken up by the cells in a time- and dose- dependent manner. The uptake exhibited an initial rapid phase which was followed by a gradual approach to the steady state. During the course of experiments, a negative association between compound dose and equilibration time was observed.

Mechanism by which αHGA is taken up by cells (paper III)

To establish whether uptake of αHGA is a saturable process, inhibition studies were carried out. Cells were incubated with [^{14}C]αHGA either alone or together with unlabeled compound in 10-100 fold excess over tracer. Accumulation of radio-labeled αHGA in the cells was not affected even by as high concentration as 2 mM of cold competitor. The dose-uptake pattern of [^{14}C]αHGA remained linear after pre-incubation with a high concentration (4 mM) of unlabeled compound.

Experiments were also carried out to determine if metabolic inhibitors such as sodium fluoride (NaF) and sodium azide (NaN_3) would appreciably affect the uptake of αHGA. Treatment of the cells with high concentrations of such energy inhibitors did not affect cellular internalization of the compound.

In order to reveal a possible carrier-mediated mechanism for αHGA transport, parallel experiments were performed at 4, 22 and 37°C. Temperature dependence of uptake was analyzed and a temperature coefficient (Q_{10}) for αHGA uptake was calculated. Uptake of αHGA exhibited low temperature dependency with a Q_{10} value of < 2.

Efflux of αHGA from the cells (paper III)

The efflux of [^{14}C]αHGA from the cells was also tested by loading the cells with the radioactive compound and subsequently re-suspending the cell pellets in a large volume of drug free medium and was shown by the loss of cell associated radioactivity over time at 22°C. Rapid efflux of [^{14}C]αHGA from H9 cells was observed. Within 10 min, 82% of radioactive compound was released from the cells to the surrounding media. The remaining 18% radioactivity remained associated with cells even over longer incubations.

6 DISCUSSION

Alpha-hydroxy glycineamide (αHGA), a small uncharged molecule (MW 90), is the active derivative of pro-drug tripeptide GPG-NH_2 (Abdurahman S., *et al.* Retrovirology *In press*). Treatment of infected cells by GPG-NH_2 , inhibits HIV-1 in the late phase of its replication cycle [225, 226]. Electron microscopy studies have indicated a possible interaction of GPG-NH_2 derivatives with viral capsid assembly [227]. It has been demonstrated that GPG-NH_2 is metabolized in two steps into the active antiviral compound. First GPG-NH_2 is metabolized to glycine-amide (G-NH_2) through the specific action of CD26/dipeptidyl-peptidase IV [228-230]. G-NH_2 in turn is converted into αHGA, the actual antiviral compound through enzymatic oxidation of its α-carbon (Abdurahman S., *et al.* Retrovirology *In press*).

Here, the anti HIV-1 activity of αHGA and its effect on HIV-1 CA assembly was investigated. The EC_{50} values for αHGA on the laboratory strain HIV-1 SF-2 was approximately 5 μM and ranged between 4 and 34 μM for clinical HIV-1 isolates resistant to other antiretroviral drugs, indicating that the compound had no cross-resistance to the anti HIV-1 drugs.

Consistent with previous results [227, 228] obtained with GPG-NH_2 and G-NH_2 ,we found that virus particles generated in the presence of αHGA exhibit aberrant HIV-1 core structures with varying morphology indicating a possible interaction with HIV-1 CA assembly. Alpha-HGA had no antiviral activity against a variety of DNA and RNA viruses with different size, genome and morphology, emphasizing the specificity in the αHGA on HIV-1 replication. Interfering with CA assembly of HIV-1 is an interesting anti HIV-1 target. Some other compounds that inhibit or interfere with the HIV-1 p24 maturation or assembly have recently been reported. PA-45, is a compound that binds to the proteolytic cleavage site of the p24 precursor (p25/CA-SP1) and thereby affects its maturation to p24 [231]. However, αHGA did not affect p25 to p24 processing. It has been also reported that compound CAP-1 binds to the N-terminal domain of p24 [232]. CAP-1, which has a molecular mass of approximately four times that of αHGA, has an EC_{50} value for HIV-1 replication of approximately 75 μM [232]. A 12-mer long peptide has been also shown to interfere with recombinant p24 dimerization, but not with HIV-1 replication in cell culture [233, 234].

αHGA could inhibit both *in vitro* and intracellular p24 tubular assembly correlating well with aberrant core structures seen in TEM studies. The interaction of αHGA with p24 assembly suggested that this molecule could bind to HIV-1 p24 and thereby interfere with its assembly. In attempts to determine the binding site in p24 for αHGA, molecular modeling studies were carried out, and suggested D51 of p24 as a possible binding site.

The CA protein reassembles following Gag cleavage. This structural rearrangement of CA is a highly conserved phenomenon in most retroviruses [235]. In HIV-1, it has been shown that a β-hairpin structure is formed by a salt-bridge between Pro1 and Asp51 (D51) of CA, which is important for conformational stability of the N-terminal CA structure [236] which is involved in hexamerization of p24 during capsid assembly.

Initially, a study was designed to evaluate the possibility of interaction of αHGA with Pro1/Asp51 ionic bond formation. To do this, mutants where D51 was replaced with more or less conservative substitutions, were tested for capsid formation, infectivity and binding of p24. The study demonstrated that substitution of D51 with glutamate (D51E), asparagine (D51N), and glutamine (D51Q) (three amino acids which have similar properties as aspartate; Glu > Asn > Gln) could partly restore *in vitro* CA assembly but not the infectivity of the virus particles.

The intracellular levels of CA protein in different cell lines transfected with D51N and D51Q were generally reduced. This could not be explained by the lack of recognition by the antibody used for immunoblotting, since similar results were obtained with mouse anti-p24, rabbit anti-p24 or a pool of sera from HIV-infected patients. The reduced levels of CA with D51N and D51Q mutations might be a consequence of protein instability. We also investigated the intracellular CA distribution with indirect immunofluorescent staining using mouse anti-p24 antibody. This analysis revealed a strong staining pattern near or at the plasma membrane (PM) of cells transfected with all mutants, indicating that there was no defect in intracellular transport of the Gag precursor to its steady-state destination [78] where activation of the viral protease takes place [77]. It seems that the three D51 mutations did not affect the intracellular level of the Gag-precursor because the stabilizing β-hairpin structure in the N-terminal domain of CA is only formed at maturation and was also shown to be absent or disordered in an immature-like virus [237].

TEM analysis revealed that all mutants were assembly competent but produced virus particles with aberrant core morphology. The infectivity of the virus particles was severely reduced or absent. Self-associative properties of HIV-1 CA protein have been previously shown [224]. However, depending on the protein concentration, salt, and the buffering pH, the morphology of the assembled structures or the rate of assembly may be variable [238, 239]. The effects of D51 mutations on *in vitro* CA assembly was monitored spectrophotometrically, and as expected, the assembly rate of both D51N and D51E mutants were substantially reduced relative to the wild type protein. However, the ability of these mutants to form tubular structures was confirmed by thin-section transmission electron microscopy (TEM). Thus, it seems likely that the D51N and D51E mutations impose less structural changes than the D51A mutation described before [236]. Although no tubular structure was observed with the D51Q mutant by TEM analysis, an increased optical density measurement that reflects the assembly kinetics was repeatedly observed. One could speculate that the increased OD may result as a consequence of amorphous aggregates that are resistant for stable higher-order CA tube formation.

The data provided in this study and the previous observations [236, 240] suggest that the invariable D51 residue of HIV-1 CA is crucial for formation of the β-hairpin structure in matured protein and even substitution of D51 with such a similar residue as with glutamate could not restore the integrity of this structure. Although our data demonstrated that D51N and D51E substitutions could restore the *in vitro* tubular formation; all mutations were non-infectious, indicating that D51 is an indispensable residue.

To define the αHGA binding site on p24, we investigated the interaction of αHGA with p24 capsid protein, both with NMR-titration and MALDI-MS analysis. NMR-titrations experiment of αHGA with N-terminal domain of p24 and full length p24, having mutations at the p24 dimer interfaces (p24W184A+M185A), failed to show any interactions between αHGA and p24. The two mutated residues in the p24 used in these experiments were introduced to inhibit interactions necessary for p24 C-terminal dimer formation. In MALDI-MS, however, αHGA repeatedly was shown to interact with the wild type p24 and three other p24 mutants (D51N, D51E, and D51Q) having

intact p24 dimer interfaces. Analysis with MALDI-MS also indicated that αHGA bound to the hinge region of the p24 molecule. Based upon the increased mass of the additional observed signal in mass spectra following incubation of the proteins with αHGA (Mw 90), a condensation reaction may be assumed due to the nucleophilic amino group on αHGA. Although, the chemical character of αHGA may allow such reactions it is unclear on which residue it takes place. Interestingly, p24 having mutations at the p24 dimer interfaces was not found to bind αHGA in the MALDI-MS experiments, corroborating the NMR results, indicating that binding of αHGA can only bind to p24 when as a dimer. Binding of αHGA to the flexible hinge region and not to the p24-p24 interaction surfaces also indicate that the consequence of αHGA binding to p24 may be an allosteric effect on the protein, hindering its proper conformation for capsid assembly.

To affect capsid assembly, αHGA must be taken up by the HIV-1 infected cell. To better understand the dynamics of αHGA on the HIV-1 replication cycle, the cellular pharmacokinetics of αHGA was investigated using [^{14}C] labeled compound. The cell incubations with radio-labeled αHGA showed a rapid uptake. With 25 μM radioactive substance, the uptake established a steady state after 20 min of incubation. The number of molecules crossing the membrane per unit area per unit time is dependent on the concentration difference across the membrane and on physicochemical factors of the permeant. During the course of experiments, a negative association between the compound concentration and steady state time was observed. This kinetics is consistent with a passive diffusion phenomenon.

Temperature coefficients (Q_{10}) are frequently used in attempts to distinguish between a passive or facilitated transport. Uptake of αHGA exhibited low temperature dependency with a Q_{10} value of less than two, hence a finding characteristic for a mechanism of passive diffusion for αHGA transport.

Further studies were carried out in the presence of known energy inhibitors. Such treatment resulted in no significant decrease in the ability of the cells to take up αHGA. In addition, no effect on uptake was observed when cells were pretreated with both of the inhibitors, NaF and NaN_3, simultaneously. These results tend to rule out an energy requirement for αHGA accumulation in the cells.

Competition studies were also performed to reveal possible facilitation of uptake by the transporters. Uptake of labeled αHGA, however, was not competed by excess unlabeled material, indicating that it is unlikely that specific cell surface binding sites were involved in the uptake process. Moreover, dose-uptake pattern of [^{14}C]αHGA remained linear after pre-incubation with a high concentration of unlabeled competitor. This observation also corroborates the other findings, suggesting a passive mechanism for αHGA transport.

The efflux experiments showed a fast efflux of >80% internalized compound. A portion of the remaining radioactivity might have been owing to a new steady state with the non-radioactive surrounding medium and another portion might have been a result of the non-specific binding of the radio-labeled substance to the cells or radioactivity trapped in the cell membrane or other cellular compartments. The data provide evidence for a passive diffusion uptake mechanism for αHGA.

In conclusion, this study demonstrated that substitution of one invariable residue of CA protein of HIV-1 to a similar amino acid is sufficient to disrupt core assembly, viral replication and infectivity. Therefore interfering with the HIV-1 capsid formation, which is a result of multiple semi-stable protein interactions, is a promising target for antiviral therapy. Alpha-HGA, an easily adsorbed compound could serve as a lead compound for such interventions.

7 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In summary, the work presented in this thesis has demonstrated that:

1) The small molecule, αHGA, inhibits the replication of HIV-1 including different strains and clinical isolates with or without resistant to RT inhibitors and protease inhibitors through interfering with viral capsid assembly.

2) The substitution of invariable D51 residue of HIV capsid to three structurally related amino acids impairs the viral replication.

3) αHGA binds to the flexible hinge region between the N-terminal and C-terminal domains of the p24 capsid protein.

4) αHGA only binds to p24 after its dimerization.

5) αHGA probably has an allosteric effect on p24 dimer, hindering its right conformation for capsid assembly.

6) The mechanism by which αHGA enters the target cells is passive diffusion.

The findings that αHGA has anti HIV-1 activity *in vitro* against a wide range of HIV-1 strains including the drug resistant variants and that its antiviral effect is specific for HIV-1 makes it an interesting molecule for further investigations. The CA protein could serve as a promising target for HIV-1 therapy and therefore new attempts for crystallization of the protein to provide more structural information would be of interest. The effect of substances with chemical similarity to αHGA on HIV-1 replication can also be investigated. A complete and detailed "pharmacological profile" for the compound should be prepared. Any attempt to introduce a new anti HIV-1 is appreciated and αHGA might prove to be a lead compound for a new class of anti HIV-1 agents.

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APPENDIX (ARTICLES)