UNIVERSITA' DEGLI STUDI DI TORINO

Ph.D IN COMPLEXITY IN POST-GENOMIC BIOLOGY

XXI CYCLE

DOCTORAL DISSERTATION

Development of HIV-1 infectivity peptide inhibitor: A possible role of Tat in the virus entry process

Candidate: Raffaella Bagnod

Faculty Supervisor:

Prof. Federico Bussolino

External Supervisor:

Dr. Silvio Traversa

Academic Years 2005/2006-2006/2007-2007/2008

Scientific Field: BIO/11

I would like to express my gratitude to all those who gave me the possibility to complete this manuscript.

First of all I wish to express my gratitude to my supervisor, Prof. Federico Bussolino of the Institute of Cancer Research and Treatment of University of Turin, for his the cooperation throughout this work.
I am deeply grateful to my supervisor, Dr. Silvio Traversa for his sage advice and constructive comments.
My thanks are also due to Dr. Fumero and CREABILIS *therapeutics* that gave me the opportunity to follow the PhD course.
I warmly thank all CREABILIS *therapeutics* team. In particular, I am extremely really grateful to Luisa, Giulia, Valentina, Luca and Elena for their continuous supported.
Lastly, and most importantly, I wish to thank my parents and Andrea. They encouraged me, support me, believed in me and loved me.
To them I would dedicate my thesis.

This research was partially supported by a grant from ASP (Associazione per lo Sviluppo Scientifico e Tecnologico del Piemonte).

TABLE OF CONTENTS

1. Introduction to HIV and AIDS

On June 5, 1981, Morbidity and Mortality Weekly Report (MMWR) published a report of *Pneumocystis carinii* pneumonia in five previously healthy young men in Los Angeles, California [Gottlieb MS, 1983; Kenton KA, 2006]. More complete evaluation of the patients showed that they had in common a marked deficiency in cellular immune responses and a significant decrease in the subpopulation of T cells that carry the CD4 marker (T helper cells) [Kubly, Immunology]. These cases were later recognized as the first reported cases of Acquired Immunodeficiency Syndrome (AIDS) [Kenton KA, 2006].

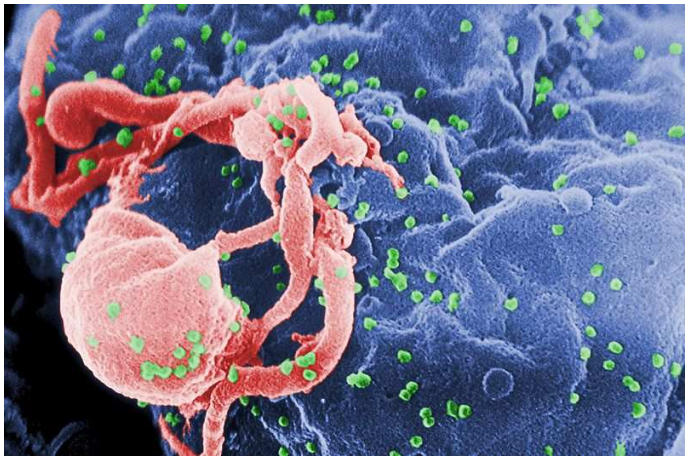


Fig. **1** - HIV Scanning electron micrograph of HIV-1 budding from cultured lymphocyte. This image has been colored to highlight important features. Multiple round bumps on cell surface represent sites of assembly and budding of virions. CDC/ C. Goldsmith, P. Feor

In 1983 the electron microscopist of the Pasteur Institute in Paris, Charles Dauguet, demonstrated a new virus in a sample from an AIDS patient. The French group reported the isolation of a T-lymphotrophic retrovirus, but as they wrote "the role of this virus in the etiology of AIDS remains to be determined" [Kallings LO, 2008; Barre-Sinoussi F, 1983]. In 1984, the French group and researchers at the US National Institutes of Health, led by Robert C. Gallo, published several papers that established, with

virological and epidemiological evidence, that the virus know as HIV was the causative agent of AIDS [Fauci AS, 2003; Montagnier L, 2002] Fig.1. It was eventually agreed that the French were the first to report the discovery of the virus, and Americans the first to convincingly show that it caused AIDS [Fauci AS, 2003].

1.1. The AIDS pandemic

Since its discovery, AIDS has increased to epidemic proportions throughout the world [Kubly, Immunology] . The Joint United Nations Programme on HIV/AIDS (UNAIDS) and World Health Organization (WHO) estimates that about 33.2 million people worldwide are living with HIV. In 2007 alone, there were about 2.5 million new HIV infections and 2.1 million AIDS deaths [2007 AIDS Epidemic Update; Cohen M. S., 2008].

1.2. Clinical course of infection

HIV can infect exposed individuals through mucosal surfaces or due to direct inoculation into the bloodstream.

1.2.1. Primary HIV infection

The first stage of infection, the primary or acute infection, is a period of rapid viral replication that immediately follows the individual's exposure to HIV leading to an abundance of virus in the peripheral blood with levels of HIV commonly approaching several million viruses per mL. This response is accompanied by a marked drop in the numbers of circulating CD4+ T cells Fig.2. This acute viremia is associated in almost all patients with the activation of CD8+ T cells, which kill HIV-infected cells, and subsequently with antibody production, or seroconversion. The CD8+ T cell response is thought to be important in controlling virus levels, which peak and then decline, as the CD4+ T cell counts rebound to around 800 cells per mL

(the normal blood value is 1200 cells per mL). A good CD8+ T cell response has been linked to slower disease progression and a better prognosis, though it does not eliminate the virus. During this period (usually 2-4 weeks post-exposure) most individuals (80 to 90%) develop an influenza or mononucleosis-like illness called acute HIV infection, the most common symptoms of which may include fever, lymphadenopathy, pharyngitis, rash, myalgia, malaise, mouth and esophagal sores, and may also involve, but less commonly, headache, nausea and vomiting, enlarged liver/spleen, weight loss, candidiasis and neurological symptoms. Infected individuals may experience all, some, or none of these symptoms. The duration of symptoms varies, averaging 28 days and usually lasting at least a week. Because of the nonspecific nature of these primary symptoms, they are often not recognized as signs of HIV infection. Even if patients go to their doctors or a hospital, they will often be misdiagnosed as having one of the more common infectious diseases with the same symptoms. However, recognizing the syndrome can be important because the patient is much more infectious during this period.

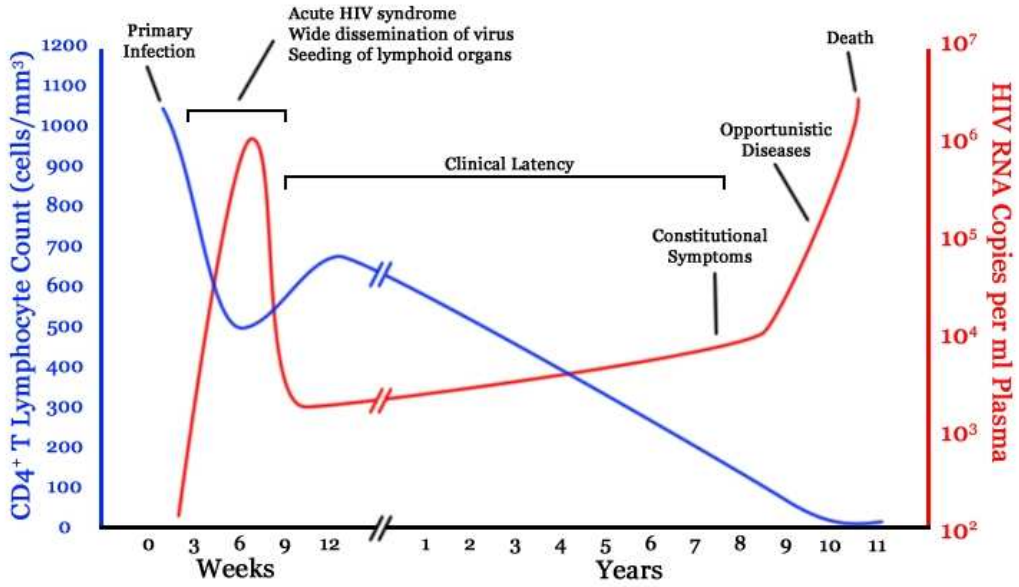


Fig. 2 - Graph showing HIV copies and CD4 counts in a human over the course of a treatment-naive HIV infection (Source: Wikipedia)

1.2.2. Latency stage

A strong immune defense reduces the number of viral particles in the blood stream, marking the start of the infection's clinical latency stage. Clinical latency can vary between two weeks and 20 years. During this early phase of infection, HIV is active within lymphoid organs, where large amounts of virus become trapped in the follicular dendritic cells (FDC) network. The surrounding tissues that are rich in CD4+ T cells may also become infected, and viral particles accumulate both in infected cells and as free virus. Individuals who are in this phase are still infectious. During this time, CD4+ CD45RO+ T cells carry most of the proviral load.

1.2.3. AIDS

When $CD4^+$ T cell numbers decline below a critical level, cell-mediated immunity is lost, and infections with a variety of opportunistic infections appear. The first symptoms often include moderate and unexplained weight loss, recurring respiratory tract infections (such as sinusitis, bronchitis, otitis media, pharyngitis), prostatitis, skin rashes, and oral ulcerations. Common opportunistic infections and tumors, most of which are normally controlled by robust CD4+ T cell-mediated immunity then start to affect the patient. Typically, resistance is lost early on to oral Candida species and to *Mycobacterium tuberculosis*, which leads to an increased susceptibility to oral candidiasis (thrush) and tuberculosis. Later, reactivation of latent herpes viruses may cause worsening recurrences of herpes simplex eruptions, shingles, Epstein-Barr virus-induced B-cell lymphomas, or Kaposi's sarcoma, a tumor of endothelial cells that occurs when HIV proteins such as Tat interact with Human Herpesvirus-8. Pneumonia caused by the fungus *Pneumocystis jirovecii* is common and often fatal. In the final stages of AIDS, infection with cytomegalovirus (another herpes virus) or *Mycobacterium avium complex* is more prevalent. Not all patients with AIDS get all these infections or tumors,

and there are other tumors and infections that are less prevalent but still significant.

1.3. The Human Immunodeficiency Virus – 1 (HIV-1)

HIV-1 is a lentivirus belonging to the retrovirus family [Nielsen, 2005; Klimas,2008; Chiu, 1985]. Two genetically distinct viral types of HIV have been identified [Klimas,2008; Butler, 2007]. HIV-1 variants are classified into three major groups: group M (main), group O (outlier), and group N (non M/non O). Group M, which is responsible for the majority of infections in the worldwide HIV-1 epidemic, can be further subdivided into 10 subtypes, or clades (A to K) [Klimas,2008; Simon, 2006; Buonaguro, 2007].

1.3.1. The viral structure

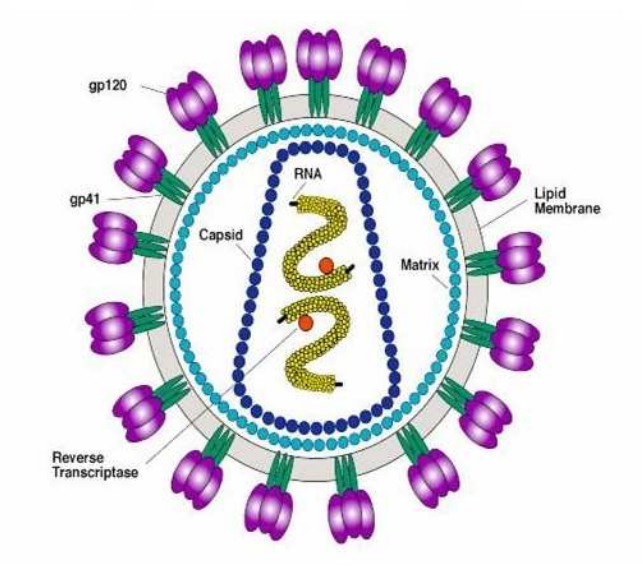


Fig. 3 - Graphical representation of the HIV-1 virus (Source NIAID)

Mature HIV virions have an overall spherical shape of about 110nm diameter Fig.3. Each virion expresses 72 glycoprotein projections

composed of gp120 and gp41. The gp41 molecule is a transmembrane molecule that crosses the host-derived lipid bilayer of the viral envelope [Lythgo, 2004]. gp120 is associated with gp41 and serves as the viral receptor for CD4 on host cells.

The envelope surrounds a dense truncated generally cone-shaped nucleocapsid (core) which includes a layer of the protein MA (matrix protein or p17) and an inner layer of the protein CA (capsid protein or p24). Within the capsid of the viral particle are two identical 9.2kb single-stranded positive-sense RNA molecules, viral protease (PR or p10), reverse transcriptase (RT or p64), integrase (IN or p32), Vpu, Vif, Vpr and Nef, and some cellular factors [Briggs, 2006; Sierra, 2005; Lippincott, 1996].

1.3.2. The replication cycle

1.3.2.1. HIV-1 entry

The first step in the life cycle of HIV, attachment to the surface of the target cells, has been attributed to a variety of cell-surface molecules, including heparin sulfate proteoglycan, LFA-1 and nucleolin [Nisole, 2004]. In addition, dendritic cell-specific DC-SIGN is also thought to mediate transient adhesion and transfer of HIV-1 virions from dendritic to T cells thereby augmenting HIV-1 propagations in virus-naïve cells [Markovic, 2006]. As the affinity of HIV envelope glycoproteins for CD4 is relatively low, especially in the case of primary virus isolates, the existence of other attachment factors may serve to concentrate the virus on the target cell surface prior to specific receptor engagement.

Following successful initial attachment, gp120 bind to CD4 receptor on the surface of lymphocytes expressing CD4. This causes unmasking of a

second gp120-binding site for target chemokine co-receptors CXCR4 and CCR5 and binding of gp120 to either or both co-receptors. Although CXCR4 and CCR5 are the major co-receptors, other receptors can interact with gp120 in some settings. The completion of co-receptor binding leads to the fusion-active conformation of the viral transmembrane fusion protein gp41. The ectodomain of gp41 contains two heptad repeat regions: HR1 (proximal to the N terminus) and HR2 (proximal to the C terminus). The hydrophobic fusion peptide region enters into the host cell membrane, whereas the HR1 regions of gp41 form a trimeric coiled coil structure. HR2 regions then fold back within the hydrophobic grooves of the HR1 coiled coil, forming a hairpin structure containing a thermodynamically stable six-helix bundle that draws the viral and cellular membranes together for fusion. The exposure of gp41 and conformational changes leading to fusion are not instantaneous processes. A transient pre-hairpin intermediate stage can last for several minutes, causing exposure of the N terminus of gp41 [Matthews, 2004].

Recent in vitro data implicated cell-surface associated oxido-reductase (i.e., protein disulfide isomerase, PDI) in HIV-1 entry, which acts post CD4 binding but prior to, or concomitant with, coreceptor engagement. Inhibitors of this enzyme prevent HIV-1 fusion and infection without impairing normal intracellular functions. These findings are supportive of the viral entry mechanism where Env conformational restructuring and viral-cell fusion depend on receptor binding, PDI redox action and coreceptor binding, operating sequentially to provide the activation energy needed for fusion proteins to transition from a metastable to a stable, low energy state [Markovic, 2006]. Below is an outline of the model of the HIV-1 entry process as suggested by Ryser and Flückiger in a review published by Drug Discovery Today in 2005.

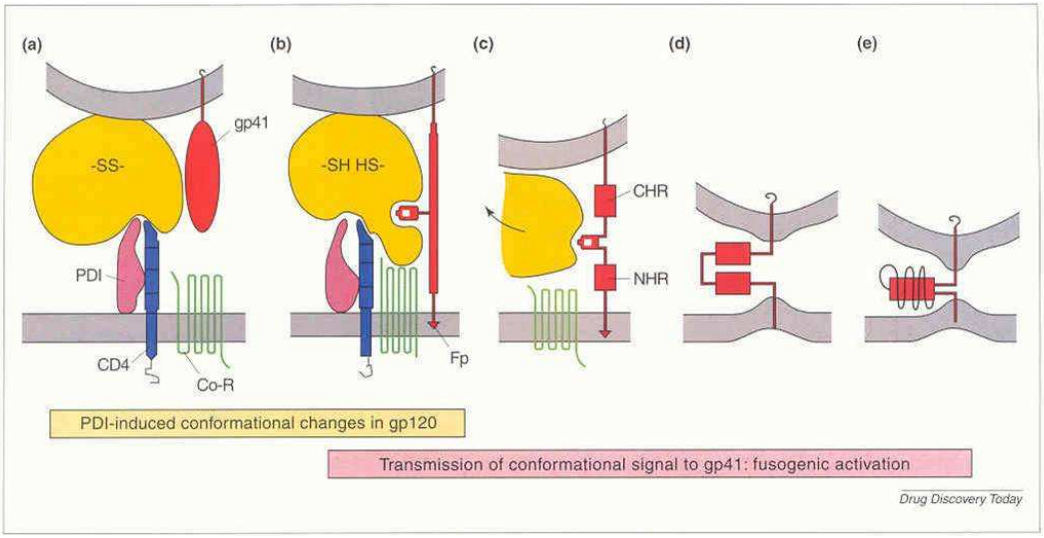


Fig. 4 - Model of PDI-induced conformational changes leading to activation of gp41. (a) PDI present at the surface of target cells binds to D3 domain of CD4, while CD4 (D1) binds to a conserved pocket of gp120. PDI makes contact with gp120 in the region of the CD4-gp120 binding site. The disulfide bonds of gp120 are intact (-SS-) and both envelope glycoproteins are in their native conformation. (b) Structure-stabilizing disulfide bonds have been reduced (-SH + SH-) leading to conformational changes that increase the gp120 interaction with co-receptors and with a small conserved loop of gp41. That interaction generates major conformational changes in gp41 that elongate the molecule on both sides of the small loop and form the N-terminal fusion peptide (Fp) that inserts into the cell membrane (c,d) Activation of gp41 includes the formation of helices in the heptade repeat section of NHR and CHR that bend to assume an antiparallel position. The distance between virus and cell is decreased and gp41 becomes the only link between them. (e) The CHR helix winds itself around the NHR helix. In the trimeric states of gp41 the three CHR helices wind themselves around three NHR helices that form a central coiled-coil, giving rise to a six-helix-boundle. (Source: Drug Discovery Today)

1.3.2.2. Replication, integration and transcription

Following viral and cellular membrane fusion, the nucleocapsid released into the cytoplasm undergoes initial uncoating and the viral RNA genome is retrotranscribed into a full-length cDNA by the viral RT [Warrilow D, ; Nisole S,2004; Isolated HIV-1 core is active for reverse transcription, 2007]. Reverse transcription yields the pre-integration complex (PIC), composed of double-stranded cDNA, integrase IN, matrix protein MA, viral protein Vpr, reverse transcriptase and host proteins. The PIC moves toward the nuclear membrane using the microtubule network and enters

the nucleoplasm through the nuclear pore [hivinsite; Bukrinsky, 2004]. Viral integrase proteins (IN) insert the linear double-stranded viral genome into the host chromosome, where the integrated provirus may remain latent until some regulatory signal starts the expression process. Once transcription factors stimulate transcription of proviral DNA by cellular RNA polymerase II, multiply spliced followed by unspliced and singly spliced viral transcripts are transported to the cytoplasm and translated into various viral proteins by host-cell ribosomes [Sierra, 2005; Lythgo, 2004; Lippincott, 1996].

1.3.2.3. Assembly and Release

In the final steps of the viral cycle, three viral structural protein precursors – group-specific-antigen protein (Gag), Gag-polymerase (Gag-Pol) and the envelope protein (Env) – are translated into the cytoplasm, and transported to the plasma membrane by vescicular, cytoskeletal or other routes. Nascent virions are assembled adding to these proteins full-size genomic RNA, cellular tRNAlys3 primer and other cellular compounds. Later on, this complex buds through the plasma membrane producing an immature virion. Finally, maturation of the virions, which is triggered by the viral protease, results in a drastic reorganization of the core and the acquisition of virus infectivity [Naghavi, 2007; Sierra, 2005]. After maturation the virus is ready for another round of infection [Briggs, 2006; Nielsen,2005].

2. Anti-retroviral Therapy

2.1. Overview of drugs currently approved by FDA

Twenty years after its discovery, HIV-1 remains a threat to public health and a challenge for drug development.

Current therapeutic intervention in HIV infection relies upon 32 different drugs. Despite the impressive efficacy shown by these drugs, we are confronted with an unexpected frequency of adverse events, such as mitochondrial toxicity and lipodistrophy, and resistance, not only to individual drugs but to entire drug classes [FDA, 2008] Tab.1.

DRUG CLASS	BRAND NAME
Multi-class Combination Products	*Atripla*
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	*Combivir, Emtriva, Epivir, Epzicom, Hivid, Retrovir, Trizivir, Truvada, Videx EC, Videx, Viread, Zerit, Ziagen*
Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs)	*Intelence, Rescriptor, Sustiva, Viramune*
Protease Inhibitors (PIs)	*Agenerase, Aptivus, Crixivan, Fortovase, Invirase, Kaletra, Lexiva, Norvir, Prezista, Reyataz, Viracept*
Entry Inhibitors (EIs)	*Selzentry,Fuzeon*
HIV integrase strand transfer inhibitors	*Isentress*

Tab. 1 - AIDS treatments approved by FDA, 2008

Thus , there is now a great need for new antiretroviral drugs with reduced toxicity, increased activity against drug-resistant viruses and a greater capacity to reach tissue sanctuaries of the virus. Two different molecules have long been selected as a targets of drug inhibition: reverse transcriptase and protease. Targeting the interactions between the HIV envelope and the cellular receptor complex represents a novel and more recent approach against HIV and has raised great interest and hope

because of the potential activity against multi-drug-resistant viruses [Castagna, 2005].

2.1.1. Nucleoside analogue reverse transcriptase (NRTIs)

The first HIV drugs to reach the market were nucleoside reverse transcriptase inhibitors (NRTIs). NRTIs are structurally similar to the building blocks of nucleic acids (RNA, DNA) but differ from their natural analogues by the replacement of the hydroxyl group in the 3' position by another group that is unable to form the 5' to 3' phosphodiester linkage that is essential for DNA elongation. NRTIs block reverse transcriptase activity by competing with the natural substrates and incorporating into viral DNA and they act as chain terminators in the synthesis of proviral DNA. To exert their activity, NRTIs must first be intracellularly phosphorylated to their active 5' triphosphate forms by cellular kinases. Tenofovir is the only NRTI that does not require phosphorylation, since it already contains a phosphate molecule in its structure [Temesgen Z, 2006; Decision Research Report, 2001]. A major problem with NRTI monotherapy is that HIV can mutate, resulting in drug-resistant strains of the virus. However, HIV that is resistant to one NRTI (such as zidovidine) may respond to certain other NRTIs. Researchers have found that combination therapy with two or three NRTIs is preferable to monotherapy in the treatment of HIV-infected individuals.

2.1.2. Non-Nucleoside analogue reverse transcriptase NNRTIs

NNRTIs bind directly and non-competitively to the enzyme reverse transcriptase. Although the drugs differ chemically from each other, they all bind to the same site: a site that is distinct from the substrate binding

site. They block DNA polymerase activity by causing a conformational change and disrupting the catalytic site of the enzyme. Unlike nucleoside analogues, NNRTIs do not require phosphorylation to become active and are not incorporated into viral DNA [Temesgen Z, 2006]. In general, NNRTIs have better toxixologixal profiles than do NRTIs. Hower, a major problem with NNRTIs has been the ability of HIV to rapidly develop resistance to these drugs. For example, HIV resistance to nevirapine can develop within a month when it is used as monotherapy. Interest in NNRTIs waned for a period of time because of the viral resistance problem. However, as NRTIs and protease inhibitor (PI) combination therapies have increasingly failed, interest in NNRTIs has been renewed, particularly when used in combination with NRTIs. Initial use of combined NRTI and NNRTI drugs allows physicians to delay the use of the more potent HIV PIs, which we discuss next [Decision Resources, 2001].

2.1.3. Protease Inhibitors

Protease Inhibitors (PIs) exert their antiviral effect by inhibiting HIV-1 protease. HIV-1 protease is a complex enzyme that is composed of two identical halves (i.e., a symmetrical dimer) with an active site that is located at the base of the cleft. It is responsible for the cleavage of the large viral precursor polypeptide chains into smaller, functional proteins, thus allowing maturation of the HIV virion. This process takes place in the final stages of the life cycle. Inhibition of the protease enzyme results in the release of structurally disorganized and non-infectious viral particles. PIs are metabolized by the CYP system and are themselves, to varying degrees, inhibitors of this system [Temesgen Z, 2006]. The PIs available today are potent antiviral agents. However, these PIs are not used as monotherapies because they can trigger the development of resistant strains of HIV and thus lose effectiveness. PIs are used in combination with NRTIs, but even when used in combination therapy, they may

eventually fail as the virus develops resistance. Adverse effects associated with PIs include gastrointestinal problems such as nausea, vomiting and diarrhea. Adverse effects that may be associated with the class of PIs include elevated triglyceride (and sometimes elevated cholesterol) levels, development of hyperglycemia and insulin resistance (and occasionally diabetes), and lipodystrophy (a redistribution of fatty tissue from subcutaneous location and an accumulation of visceral fat) [Decision Resources, 2001].

2.1.4. Entry Inhibitor

At the moment there are only two inhibitors of HIV entry commercially available worldwide: FUZEON® and SELZENTRYTM.

FUZEON® (enfuvirtide) is the first, and thus far the only, fusion inhibitor to be approved by the FDA (2003). Enfuvirtide is a linear 36-amino acid synthetic peptide that mimics the HR2 domain of gp41. It binds to the HR1 region of gp41 and blocks the formation of the six-helix bundle structure, which is critical for the fusion process. The clinical efficacy and safety of enfuvirtide was demonstrated in the TORO1 and TORO2 clinical trials, in which the virological and immunological benefits of adding enfuvirtide along with the optimized antiretroviral regimen in multidrug-experienced patients was demonstrated. Injection site reactions are the most common adverse events that were reported. Their manifestation includes erythema, induration, ecchymosis, nodules or cysts, and may present symptoms of pruritus, pain or discomfort. A needle-free drug delivery system that may decrease the impact of injection site reactions is under investigation [Temesgen Z, 2006; FUZEON (Roche)].

In August 2007, the FDA granted approval to the new HIV-1 entry inhibitor Pfizer's Selzentry (Maraviroc). Maraviroc is a CCR5 antagonist for the treatment of HIV infection in combination with other antiretroviral drugs [Hyland, 2008]. On the basis of its mechanism of action it is

expected that this drug will be effective only in a subpopulation of HIV-1 infected people, namely those harboring only the R5 virus. It is potent, orally available and has been shown to be effective and well tolerated in pre-clinical and clinical studies. The wide use of this drug is currently hampered by lack of readily available R5 virus only determination tests (tropism test) and by insufficient scientific insight into the dynamics of R5 and X4 viruses during infection [Hyland, 2008; Vandekerckhove, 2008]. Along with its desired effects, maraviroc may cause some unwanted effects. The most common side effects seen in studies so far include cough, fever, dizziness, headache, lowered blood pressure, nausea, and bladder irritation. Two Phase III studies reported possible liver problems and cardiac events, an increased risk for some infections, and a slight increase in cholesterol levels [AIDSinfo, 2008; Pfizer report].

2.1.5. HIV integrase Inhibitor

Raltegravir, the first integrase inhibitor, acts specifically to inhibit integration of reverse transcribed HIV DNA into the genome of host cells. The advantage of this drug target is that integrase is an essential and highly conserved enzyme. However, one disadvantage is that moderate-level to high-level resistance to this and other integrase inhibitors can follow after only one or two amino-acid mutations [Flexner, 2007]. In clinical trials, raltegravir has been shown to be a potent drug with a good pharmacokinetic and side-effect profile, both in treatment-naive and -experienced patients, and has achieved high rates of virological suppression even in those with limited treatment options. Raltegravir was approved by the US FDA in October 2007 and by the European Commission in December 2007 to be used in combination therapy in previously treated HIV-1-infected individuals [Cahn, 2007].

2.1.6. Multi-class Combination Products

ATRIPLA® is the first multi-class antiretroviral drug available in the United States and represents the first collaboration between two U.S. pharmaceutical companies to combine their patented anti-HIV drugs into one product. This product contains a fixed dose combination of two NRTIs (emtricitabine and tenofovir disoproxil fumarate) and one NNRTI (efavirenz). Combining the three drugs into a single, once-daily pill reduces pill burden and simplifies dosing schedules, and therefore has the potential to increase adherence to antiretroviral therapy [ATRIPLA website; HIV]. The most common side effects of ATRIPLA are the same as with the drugs it contains. They include headache, diarrhea, nausea, vomiting, vivid dreams, anxiety, rash, dizziness, insomnia, and loss of appetite [aidsinfonet].

2.2. Overview of HIV entry inhibitors under development

Currently, FUZEON® (Enfuvirtide, Roche Pharmaceutical) and SELZENTRY™ (Maraviroc, Pfizer) are the only two HIV entry inhibitors to reach the market but several other compounds are at different development stages. (Table: List of entry inhibitors) [Esté, 2007] Tab.2.

	Status (last update)*	Target	Developer	ClinicalTrials.gov Identifier†
Enfuvirtide (Fuzeon, T20)	Approved	gp41	Trimeris/Roche	..
Maraviroc	Phase III (2007) expanded access	CCR5	Pfizer	NCT00426660
Vicriviroc	Phase III (2007)	CCR5	Schering-Plough	NCT00243230
Aplaviroc	Phase II (2006) discontinued	CCR5	GlaxoSmithKline	NCT00321438
INCB9471‡	Phase II (2007)	CCR5	Incyte Corp	NCT00393120
PRO 542	Phase II (2006) (not under active development)	CD4	Progenics	NCT00055185
SP01A	Phase II (2006)	Cholesterol binding	Samaritan Pharma	NCT00299897
BMS-488043	Phase II (n/a)	gp120	Bristol Myers Squibb	n/a
TNX-355	Phase II (2006)	CD4	Tanox/Biogen	NCT00089700
AMD11070 (AMD070)	Phase II (2007) halted	CXCR4	Genzyme	NCT00089466
PRO 140	Phase I (2005)	CCR5	Progenics	NCT00110591
Sifurvitide	Phase I (n/a)	gp41	Fusogen	n/a

Tab. 2 - HIV entry inhibitors (Source: Estè 2007)

n/a=not available.

*Last update shown at the US NIH Clinical Trials information service.

† ClinicalTrials.gov Identifier=US NIH Clinical Trials information service.

‡ Additional information can be found at http://www.incyte.com/drugs_product_pipeline.html.

3. Viral and Host molecules involved in HIV-1 entry

3.1. 2.1. Viral molecules

3.1.1. Envelope Glycoproteins (gp120 and gp41)

The HIV-1 envelope glycoproteins, gp120 and gp41, of HIV-1 mediate viral entry and are the primary targets of neutralizing antibodies [Center, 2000]. On the cell and virion surface, the glycoproteins are organized in trimers via non-covalent gp120-gp41 interactions [Sanders,2000]. The surface envelope glycoprotein gp120 is a heavily glycosylated protein with carbohydrates accounting for about 40% to 50% of the molecular weight. It is composed of five constant regions (C1-C5) interspersed with five variable regions (V1-V5). gp120 structure has been deduced from functional analysis of variant viruses, topographical mapping based on monoclonal Ab binding analysis, crystallographic and Nuclear Magnetic Resonance (NMR) studies of small portions of gp120, and molecular modelling with reference to homologous viral proteins of known structure. These studies suggested that the conserved regions of gp120 form a central core composed of 25 β-strands, 5 α-helices, and a loop segment and it is folded into a heart-shaped globular structure. The core is formed of an inner domain and an outer domain that are linked by a four-stranded sheet termed the bridging sheet Fig 5. Among different clades of HIV-1, the inner domain is more conserved than the outer domain. The variable regions, with the exception of V5, are bracketed with cysteine disulfide bonds and form four loops that emanate from the surface of the protein [Poignard, 2001]. The inner domain is believed to interact with gp41 envelope glycoprotein, while the outer domain, which is quite variable and heavily glycosylated, is believed to be exposed on the

assembled envelope glycoprotein trimer. The "proximal" side of the gp120 core, which includes the N- and C-termini, is thought to reside near the viral membrane after CD4 binding occurs [Wyatt, 2002]. On gp120, the CD4 binding site is located in a depression formed at the interface of the outer and inner domains with the bridging sheet. The conformational changes initiated by gp120 binding to sCD4 do not appear to be sufficient to activate gp41. The primary receptor CD4 that binds the virus to the cell has separate and adjacent binding sites for gp120 and PDI. Several of the nine conserved disulfide bonds of gp120 are vulnerable to reduction by CD4-bound PDI as they are situated in the immediate vicinity of the gp120-CD4 binding site [Ryser, 2005].

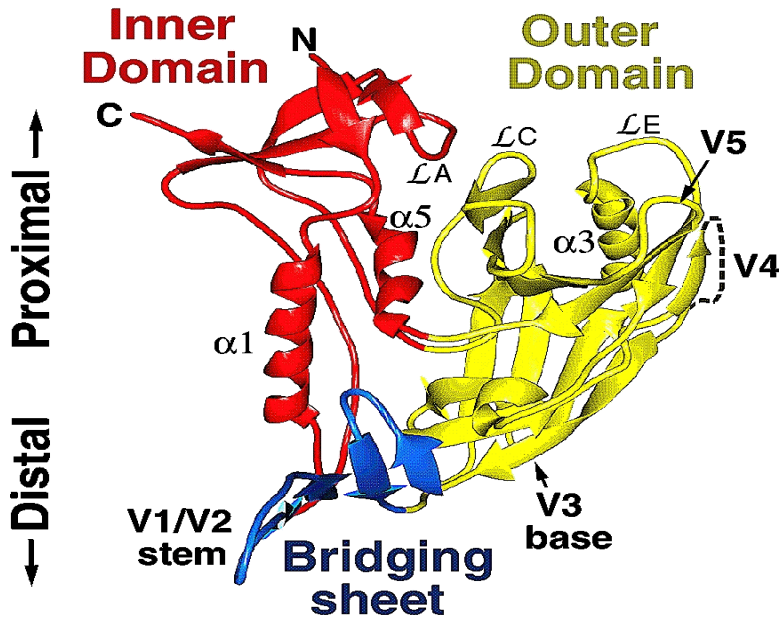


Fig. 5 – Core gp120 showing the inner domain, outer domain and bridging sheet

Subsequent to the gp120-CD4 interaction, extensive conformational changes ensue in the gp120 that involve exposure of V3 and relocation of V1/V2 hypervariable loops of gp120, as well as formation of a highly conserved region comprised of residues adjacent to and within the bridging sheet. While the V3 loop is important for the specificity of

coreceptor utilization, conservation of the bridging sheet-region seems to also contribute to gp120 interacting either with CCR5 or CXCR4 [Markovic, 2006].

gp41 molecule consists of extracellular, transmembrane, and cytoplasmic domains. Its extracellular domain (ectodomain) contains four major functional regions: a hydrophobic, glycine-rich fusion peptide (FP), an N-terminal heptad repeat (NHR) (or HR1), a C-terminal heptad repeat (CHR) (or HR2), and a tryptophan-rich region. The fusion-active gp41 core structure is a stable six-helix bundle (6-HB) folded by its trimeric NHR and CHR. Peptides derived from the CHR region of HIV-1 gp41 are potent fusion inhibitors that block viral and cellular membrane fusion by targeting the NHR region [He, 2008].

3.1.2. Tat

The Transactivator of transcription (Tat) is a small protein of 86 to 101 amino acids (depending on viral strain) produced by HIV-infected cells and conserved in the genomes of all primate lentiviruses [Poster].

Tat is synthesized from mRNA joined by two coding exons. The first exon encodes amino acids 1-72 and the second exon encodes amino acids 73-101 [*Jeang*]. The combined results from many laboratories have permitted an arbitrary demarcation of "domains" in Tat Fig. 6. Tat protein can be subdivided into 5 functional domains. The N-terminus, called Acid N-terminal Domain (Met1 to Ala21), contains 13 amino acids with amphipatic characteristics organized in α-helix. Proline residues are responsible for the bent of this structural domain. The N-terminus domain together with the following two domains, generates the transactivating domain of Tat. The second domain (Cys22 to Cys37), called Cysteine Rich Domain, contains seven Cysteines highly conserved between different isolates of HIV-1s. Individual mutation in six of the seven cysteines abolishes Tat function.

Although originally proposed as a metal-chelating dimerization domain, this region was recently shown to be used for intra-molecular disulfide bond formation.

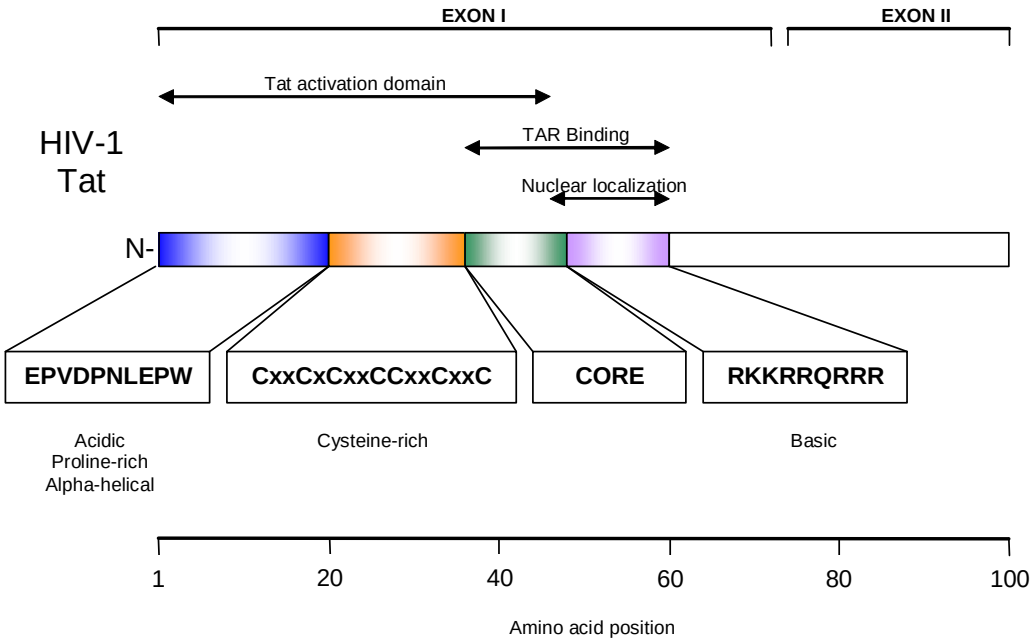


Fig. 6 – Tat functional domains

The third domain (Phe38 to Tyr48), also called Core Domain, contains a RKGLGI motif that is conserved between HIV-1, HIV-2, SIV and also EIAV. The fourth domain, also called Basic Domain (Gly48 to Arg57), contains the nuclear targeting signal Gl48-Arg49-Lys50-Lys51-Arg52, which functions as an NLS (Nuclear Localizator Signal) placed on a heterologous protein. The basic domain is involved in RNA binding. The core domain either also binds directly to TAR or influences the structure of the basic region of Tat and thereby affects TAR binding. The C-terminal domain of Tat (Ala58 to Gly72) appears to contribute to both nuclear localization and RNA binding activity of this viral transactivator [Lippincott; Neuveut, 1996]. The second coding exon of Tat is transduced in only one domain called Cell Adhesion C-Terminal Region (from 73 to 101 residues), encompassing RGD sequence that is used as a cell adhesion signal for binding to cellular integrins and an ESKKKVE motif which is conserved in

most HIV-1 Tat proteins and is partially preserved in HIV-2 and SIV Tats. Recently Prof. Bussolino and co-workers have proven that the second exon (particularly from Gln73 to Glu86) is involved in the binding to gp120 envelope protein of HIV-1.

Although the crystal structure of HIV-1 Tat is still unavailable, three-dimensional nuclear magnetic resonance (NMR) data have revealed that this protein is largely unfolded. The absence of a defined secondary structure probably plays a key role in the various biological properties that characterize this protein.

Tat's main function is to enhance the transcription of viral RNAs, allowing the production of new viral particles and the consequent spreading of the infection. Due to its peculiar amino acid sequence and according to the principle of viral economy, Tat has been demonstrated to modulate several other processes. As a nuclear factor, Tat can activate the transcription of host genes such as the cellular receptors and co-receptors for HIV-1 itself, CD4 and the chemokine receptors CCR5 and CXCR4. Tat can also be released by infected cells both *in vitro* and *in vivo*, and can enter surrounding cells, interfering with their gene expression. Finally, when present in the extra-cellular compartment, Tat acts as a growth factor, inducing various cellular pathways through binding to trans-membrane receptors. For example, Tat stimulates angiogenesis by binding to VEGF Receptors-2, Flk-1.

All the listed biochemical effects have been related to clinical outcomes: a large amount of data suggest that Tat could contribute to the onset of disorder associated with HIV-1 infection [Poster].

3.2. Host molecules

3.2.1. Cluster of Differentiation 4

Cluster of Differentiation 4 (CD4) is a 55 kDa transmembrane protein that belongs to the immunoglobulin (Ig)-like superfamily [Markovic, 2006].

CD4 consists of an extracellular portion (residues 1-371), a transmembrane segment (372-395) and a cytoplasmatic tail (396-433). The analysis of amino acidic sequence of CD4 showed that the extracellular region consists of four Ig-like domains, D1 to D4 [Matthias, 2002; Barclay, 1993].This Ig-like protein is expressed on the surface of some T cell subsets such as helper and pro-inflammatory T cells, as well as on monocytes and macrophages, where it provides help for antibodies and enhances T cell responses by binding class II major histocompatibility complex (MHC). In addition, CD4 serves as a primary receptor for HIV-1, HIV-2 and Simian Immunodeficiency Virus (SIV) attachment.
D1, the outermost domain of CD4 (in particular the amino acids Phe 43 and Arg 59), has been demonstrated to be involved in the binding to gp120 and a recent molecular docking study indicates that the D3 domain binds to PDI [Ryser, 2005; Borkow,2005].

3.2.2. Chemokine Receptors (CCR5 and CXCR4)

Several types of chemokine receptors support HIV infection including CCR2, CCR3, CCR8, CX3CR1, GPR1, GPR15, STRL33, CHemR23 and APJ but do so with poor efficiency and for a relatively small number of viral strains [Kazmierski, 2006]. The chemokine receptors CXCR4 and CCR5, members of the G protein-coupled receptor superfamily, have been identified as main coreceptors for HIV-1 [Berger, 1999]. Primary clinical infections are caused by HIV-1 strains that utilize CCR5 (R5 tropism) but, during the course of infection, the virus can adapt to bind also to CXCR4 (R5X4 dual tropism) or to CXCR4 only (X4 tropism) [Ryser, 2005].
These receptors normally guide hematopoietic cells to distinct sites via specific chemokine gradients. CCR5 is the specific receptor for the β-chemokines RANTES, MIP-1α and MIP-1β, while CXCR4 is the specific

receptor for only one chemokine, the stromal-cell derived factor alpha (SDF-1α).

The site for gp120 binding on CCR5 involves the amino terminus and three extracellular loops of the receptor, while on CXCR4 the binding site is located on the extracellular loops of CCR5 and CXCR4 are also involved in gp120 binding, and their interaction with gp120 might vary between different HIV-1 subtypes.

The importance of CCR5 has been demonstrated by the discovery that a homozygous 32bp deletion in the CCR5 gene confers resistance to HIV infection. Indeed R5 viruses are prevalent during the early infection. Furthermore, heterozygosity for the CCR5Δ32 deletion, delays progression to disease, probably because of a decrease in coreceptor expression [Poignard, 2001].

3.2.3. Protein Disulfide Isomerase

Protein Disulfide Isomerase (PDI; EC 5.3.4.1) is an essential 57 kDa eukaryotic enzyme. This member of the thioredoxin superfamily catalyzes both oxidation and reduction of disulfide bond as well as disulfide isomerazation.

PDI has four distinct structural domains (a, b, b', a) and a C-terminal region (c) as deduced from its primary and tertiary structure. The a-type domains usually contain two cysteines in a CXXC active-site motif with an intervening Gly-His sequence being the most common in the PDIs. The b-type domains do not have cysteines in the active site and are therefore not redox active. In addition to these domains, PDI has a cationic C-terminal region where the endoplasmic retrieval sequence –KDEL, necessary for retaining the enzyme in the lumen of the ER, resides [Kersteen, 2003; Appenzeller-Herzog, 2008].

PDI has an important role in the folding of secreted proteins in the biosynthetic pathway forming disulfide bonding. On the surface of the cell,

it has been shown to cause structural modifications of proteins attached to the cell acting as a reductase that cleaves disulfide bonds Fig.7. Independently of its catalytic role, PDI exhibits chaperone activity by inhibiting the aggregation of unfolded proteins and is a member of at least two mammalian multimeric enzyme complexes: prolyl 4-hydroxylase (P4H) and a microsomial triglyceride transfer protein (MTP) [Kersteen, 2003; Ferrari, 1999].

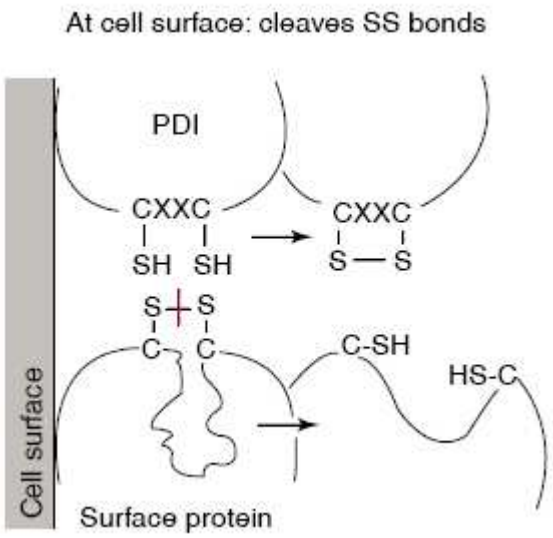


Fig.7 – Model showing the effect of PDI at the cell surface, where it acts as a reductase cleaving disulfide bonds of proteins attached to the cell membrane (Source: Ryser, 2005)

Recently it has been demonstrated that PDI strongly binds to CD4 as shown by various co-precipitation experiments. This view is confirmed by molecular docking studies indicating that PDI interacts strongly with the D3 domain of CD4 and less strongly with D2. Moreover PDI and CD4 have been immuno-detected in the same surface areas of target cells, although evidence of their immuno-co-localization was sparse in the absence of gp120, suggesting that gp120 might enhance PDI-CD4 interaction and the formation of a PDI-CD4-gp120 complex [Ryser, 2005]. Clustered on the lymphocyte surface in the vicinity of CD4-enriched regions, PDI may influence the conformational modifications that occur during the interaction of HIV-1 gp120-gp41 complex with the target cell surface

receptors through a partial reorganization of the network of the disulphide bonds of the viral protein [Barbouche, 2003].

Inhibition of the activity of PDI at the cell surface of target cells prevents the activation of gp41, the entry of HIV-1 strains into target cells and envelope-mediated cell-cell fusion, confirming the critical role of PDI in HIV-1 entry [Ryser, 2005].

4. A new mechanism of HIV-1 entry into target cell and peptide inhibitors

Up to now only one single mechanism of viral entry (the so-called fusion mechanism) has been universally accepted but over the last decade new hypotheses have put forward.

Intensive studies on a potential (never previously investigated) direct implication of the Tat protein in HIV entry and spreading of infection, have recently led Prof. Bussolino and co-workers to the postulation of a totally novel mechanism of viral entry into host cells featuring the Tat protein as the main player. Data related to this new model of viral entry were published in "Blood" in April 2005.

4.1. A novel mechanism of HIV-1 entry

Bussolino's experiments proved that HIV-1 infected cells release Tat into the micro-enviroment and that Tat is then sequestered by heparan sulphate proteoglycans on the surface of surrounding cells (infected and not) both *in vitro* and *in vivo*. In particular, the presence of soluble and surface bound Tat protein was observed in the medium of human PBMCs (Peripheral Blood Mononuclear Cells) of healthy donors infected with two different laboratory-adapted strains (IIIB and Ba-L) and in PBMCs from HIV-1-positive patients naïve for antiretroviral therapy (ARV). Moreover, the group led by Prof. Bussolino set up a co-cultured system in which U937/Tat expressing cells (U937 cell line transduced with vector carrying Tat_{86} gene) were seeded in the lower compartment of a cell culture trans-well, and C8166 T-lymphocyte cells were seeded in the upper compartment. The two compartments were divided by a membrane that allows the exchange of secreted proteins. In this system they used immunostaining to show the presence of surface-bound Tat on Tat-

producing and nearby untrasduced cells. The Tat-region involved in surface binding was identified to be the basic domain.

Based on above data, they hypothesized that the cell surface could function as a reservoir for Tat, which, in that context, could enhance virus entry.

This hypothesis was explored using lentiviral vectors (LVs), which are modifications of the wild type HIV-1 virus. Interestingly, recombinant Tat was proved to enhance HIV-1 env LVs entry into C8166 cells, as well as the entry of the both HIV-1 CXCR4 dependent strain IIIB and CCR5-dependent HIV-1 strain Ba-L in PBMCs cells from healthy donors. This effect was completely annulled if the cells were treated with the enzyme Heparanase III, confirming the involvement of membrane-bound Tat in this process. Moreover, the entry into permissive cells of lentivirus coated by different envelopes, for example VSV (Vescicular Stomatitis Virus) env, were uninfluenced by the presence of Tat. These data demonstrate that the increase in cell entry is strictly dependent not only on surface-bound Tat but also on the presence of the HIV-1 envelope protein.

In accordance with the results reported above, it was shown that Tat binds to the HIV-1 gp120 envelope protein with a Kd = 8.1nM ± 0.3. Evaluation of Tat/gp120 interaction was performed by BIAcore technology using recombinant proteins.

Fig. 8 – Amino acidic sequence of Tat expressed by HIV-1 $_{HXB2}$ laboratory adapted strain

In order to identify the region(s) of Tat required for gp120 binding, different Tat variants were produced: full-length Tat (Tat_{86}), Tat mutants lacking the C-terminal (Tat_{72}), Tat mutants containing mutated basic

domains (Tat_{BasMut}) or cysteine-rich domains (Tat_{CysMut}). All variants were able to bind gp120 with the exception of Tat_{72}, showing that the interaction between Tat and gp120 is mediated by the C-terminal portion of Tat (aa 73-86).

These results led Bussolino's group to suggest a new model of pathogenesis in which HIV-1 infected cells release Tat into their environment; then Tat binds the surface of surrounding, still uninfected cells, thus rendering those cells more permissive to HIV-1 infection. This amplification possibly causes an acceleration in the spreading of the virus.

Until these studies were performed, not enough was known about Tat functions to confirm its direct involvement in HIV-1 infection. Bussolino's group demonstrated for the first time that these events depend on enhanced Tat-driven virus entry into the cells, due to a specific interaction between gp120 and Tat at the cell surface.

4.2. Peptides inhibitors of HIV-1 entry and spreading

Once the mechanism of Tat-mediated increase in infection had been demonstrated, Bussolino and co-workers started searching for inhibitors, by screening phage display libraries on U937/Tat-expressing cells. Two different libraries were used, $CX_{10}C$ and $CX_3CX_3CX_3C$, where C is Cys and X is any amino acid. Interestingly, by searching for similarities in the BLAST-NCBI database, they found out that some of the peptides selected by phage display technology showed high similarity with different regions of the HIV-1 gp120env protein. In particular, three out of 13 peptides selected from the $CX_3CX_3CX_3C$ library and two out of 5 from the $CX_{10}C$ library showed almost complete homology with regions of gp120env. They called these peptides A, B, D (from $CX_3CX_3CX_3C$ library), C and E (from $CX_{10}C$ library). Since some of the peptides map in regions not completely conserved among different viral strains, a consensus sequence of NL4-3 (a

laboratory adapted HIV-1 strain) was selected for comparison. As shown in Fig. 9 peptides A and E identify a region at the beginning of loop V2, C maps immediately after loop V3 and D is localized in the fusion peptide of gp41env. Further studies revealed that B, D and C peptides were not specific for Tat-binding or HIV-1 inhibition, thus it was decided to focalize attention on A and E peptides.

They then synthesized cognate soluble peptides (CT303 = CSFNIT; CT304 = RDKVKK; CT319 = CSFNITTEIRDKVKK) and tested their ability to compete for gp120 binding to Tat.

Fig. 9 – gp120 amino acidic sequence of HIV-1 NL4-3 strain. (Source: Marchiò, 2005)

To do this, they evaluated Tat86 binding to rgp120-coated microwells in the presence of increasing concentrations. All the peptides inhibited Tat-gp120 binding, with CT319 showing the highest efficiency. In these experiments, CT304 showed a complete dose-response effect, while the displacement was not increased by adding concentrations higher than 10nM and 1mM for CT319 and CT303, respectively. Fig. 10

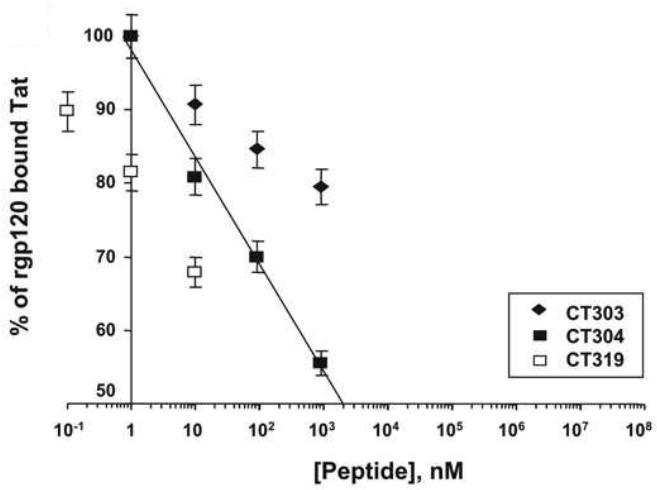


Fig. 10 – Binding of GST-fused Tat_{86} on gp120-coated microwells in the presence of the gp120 like synthetic peptides.

Finally, they demonstrated that synthetic gp120-mimic peptides could block not only the Tat-driven HIV-1 entry but also spreading. Among the selected soluble peptides, our candidate lead, CT319 showed the best results, even if PBMCs infected with HIV-1 IIIB and treated with a single administration of CT319 (1nM) gave different effects on the spreading of the infection depending on the administration day. Administration on day 7 led to a delayed infection, administration on day 10 inhibited the infection, while administration on day 14 completely blocked it. Fig. 11

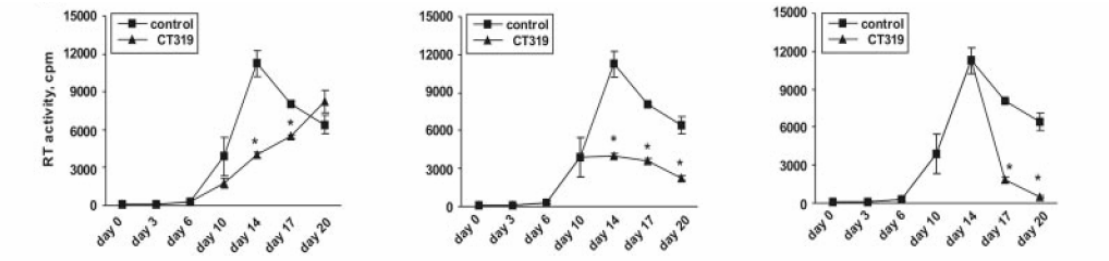


Fig. 11 – Single peptide administrations have different outcomes on the spreading of the infection. PBMCs were infected with HIV-1 III B strain, and 1nM CT319 was administered at the indicated time points. Similar results were obtained for Ba-L infection. Each value indicates mean ± SD of 3 experiments in triplicate.

5. Results and Discussion

5.1. Project rationale

Development of this innovative strategy against AIDS starts from the studies carried out by Prof. Bussolino and his co-workers. They suggested and proved experimentally a totally novel and never previously described, direct, non transcriptional function of Tat as mediator of HIV-1 entry.

As described in detail in Section 4, they started from the observation that Tat localized at the surface of both Tat-producing and neighboring cells, in an experiment where Tat-producing cells were co-cultured with non-producing cells. Moreover, they demonstrated that membrane-bound Tat specifically enhances the entry of lentiviruses presenting HIV-1 gp120 on their envelope. In fact, there was a dose-dependent increase in gp120-LV entry into C8166 cells incubated with rTat.

Based on the fact that extracellular Tat is partially sequestered by heparan sulphate proteoglycans, thus remaining concentrated at the cell surface in its active form, well protected from proteolytic degradation, Bussolino and co-workers postulated that Tat concentrated at the cell surface could bind specifically to HIV-1 gp120 envelope protein and that this interaction could enhance virus entry into the host cells. Consequently, Tat-gp120 interaction was analyzed by Surface Plasmon Resonance (BIAcore). The measured dissociation constant (Kd= 8.55 nM) demonstrated high specific binding.

Therefore, a pool of peptides binding specifically to Tat was generated by the phage display technique, with the intention of interfering with Tat/gp120 interaction. This screening of Tat-binding peptides led to the design of our candidate lead, CT319. This is a 15 L-amino acid peptide (Sequence: CSFNITTEIRDKVKK; MW: 1782.1 Da) mimicking the gp120 V1/V2 loop.

Intriguingly, CT319 was able to specifically and selectively inhibit the entry of the virus *ex vivo*, reducing global infectivity. In fact, a single

administration at different time points of 1nM CT319 to PBMCs previously infected with HIV-1 IIIB strains gave the following results:

- Addition on day 6: spreading of infection delayed
- Addition on day 10: spreading of infection partially inhibited
- Addition on day 14: spreading of infection completely blocked

Having selected CT319 as our hit, we reasoned on the fact that peptides containing one or more free cysteine residues are unstable. They can easily dimerize or polymerize. Therefore, in order to avoid peptide dimerization and the possible loss of activity, new cognate peptides were synthesized where Cys was replaced with methyl cysteine (CT321), with Serine (CT324), with Threonine (CT325), and with Methionine (CT323), while in CT326 a Cys residue was added to the C-terminal, thus triggering cyclization by disulfide bond formation, whereas in CT322 the cysteine was deleted Tab. 3.

Creabilis Code	Generic Code	Sequence	Molecular Weight
CT319		NH2-CSFNITTEIRDKVKK-COOH	1782,1 Da
CT321	C(Me)15K	NH2-C(Me)SFNITTEIRDKVKK-COOH	1795 Da
CT324	S15K	NH2-SSFNITTEIRDKVKK-COOH	1765 Da
CT325	T15K	NH2-TSFNITTEIRDKVKK-COOH	1779 Da
CT326	C16C	NH2-CSFNITTEIRDKVKKC-COOH	1882 Da
CT322	S14K	NH2-SFNITTEIRDKVKK-COOH	1678 Da
CT323	M15K	NH2-MSFNITTEIRDKVKK-COOH	1809 Da

Tab. 3 – List of synthesized peptides

All the mentioned peptides were tested for inhibition activity in the same ex vivo model previously used for CT319. The comparison of the data obtained revealed that CT319 is the most active peptide, and consequently we chose to continue the development of this molecule as our candidate lead.

In an *in vitro* stability study performed in mouse blood, CT319 showed a half-life of about 1 hour. Finally, a preliminary pharmacokinetics (PK) study in the mouse demonstrated that 15 minutes after intraperitoneal administration of 10mg/kg of CT319, the peptide disappeared from blood flow.

This project has the purpose of developing CT319 (or any derivative/analogue with improved PK characteristics) as an efficient and totally innovative inhibitor of HIV-1 entry to be used in the treatment of AIDS and in particular at the stage of massive spreading of the infection.

5.2. Aim of the experimental activities

CT319 is a 15 amino acid peptide selected as a candidate lead for further development and optimization.

This peptide is able to specifically and selectively decrease the entry of the virus *ex vivo*, reducing global infectivity.

As expected, previous stability and preliminary PK data showed that the gp120-peptidomimetic is characterized by short half life and poor PK profile. Based on these results, a CT319 derivative or analogue with improved PK performance is needed.

With the aim of confirming the *ex vivo* proof of concept and of developing a CT319 (derivative or analogue) as an efficient treatment of AIDS, the following activities were performed:

- Tat-CT319 bond characterization by MALDI-TOF
- Tat-CT332 and Tat-CT333 binding study by MALDI-TOF
- Evaluation of HIV-1 spreading inhibition by peptides

5.3. Tat-CT319 bond characterization by MALDI-TOF

Previous *ex vivo* studies investigating CT319 cognate peptides ability to inhibit HIV-1 spreading in PBMCs revealed that the Cys-bearing peptide CT319 had the highest activity. This led us to hypothesize that the Cys at the N-terminal of CT319 could form a disulfide bond with one of the 7 Cys that compose Tat Cys-domain.

To get an insight into the nature of Tat/CT319 binding (covalent or not) we planned a series of MALDI-TOF analyses in denaturing and reducing conditions. Thus, rTat/CT319 equimolar solutions in 20nM ammonium bicarbonate buffer (pH 7.4) were prepared. Incubation at 37°C in ammonium bicarbonate buffer allows the analysis to be performed in conditions (particularly temperature and pH) mimicking physiological ones. In addition, the aqueous ammonium bicarbonate solvent is well tolerated by MALDI-TOF mass spectrometry up to a 20nM concentration.

5.3.1. Bond characterization in absence of denaturing or reducing conditions

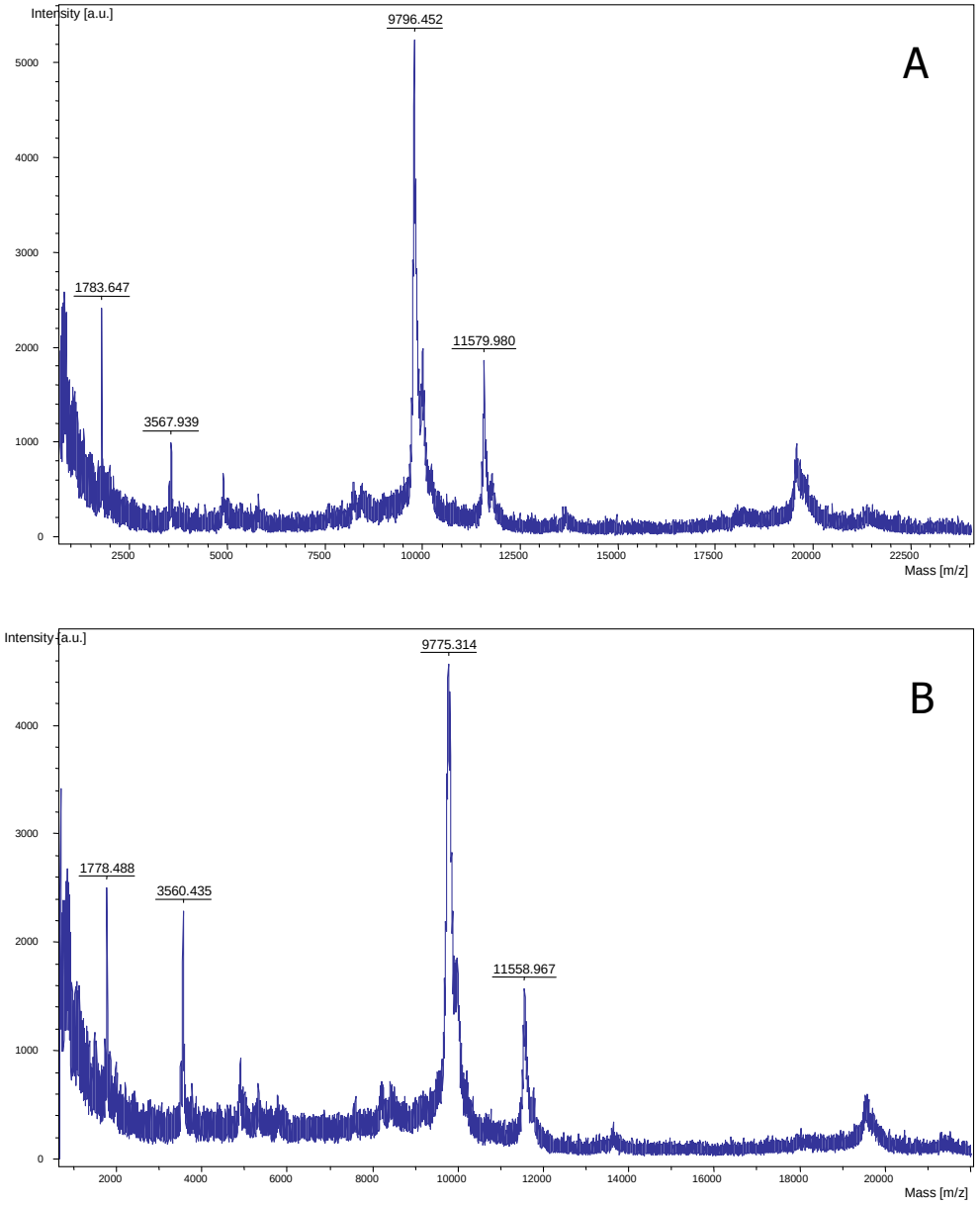
MALDI-TOF mass spectrometry is a fast method for the analysis of proteins but is rarely used for the direct analysis of protein-protein interactions. The principal reason is that the normal sample preparation conditions and the laser desorption process itself would disrupt the non-covalent interactions of protein complexes.

Consequently, mass spectrometry analysis performed in the standard conditions could highlight the presence of a covalent bond in a protein complex disrupting all the weak interactions.

Therefore, to exploit the presence of a disulfide bond between rTat and CT319 a equimolar solution of the two molecules was prepared as

described in Chapter 8. The solution prepared was kept in a thermostated water bath set at 37°C.

Since data related to the binding kinetics of the two molecules was not available, we collected samples at the following different time points: 1 hour, 2 hours, 3.5 hours, 26 hours Fig. 12.



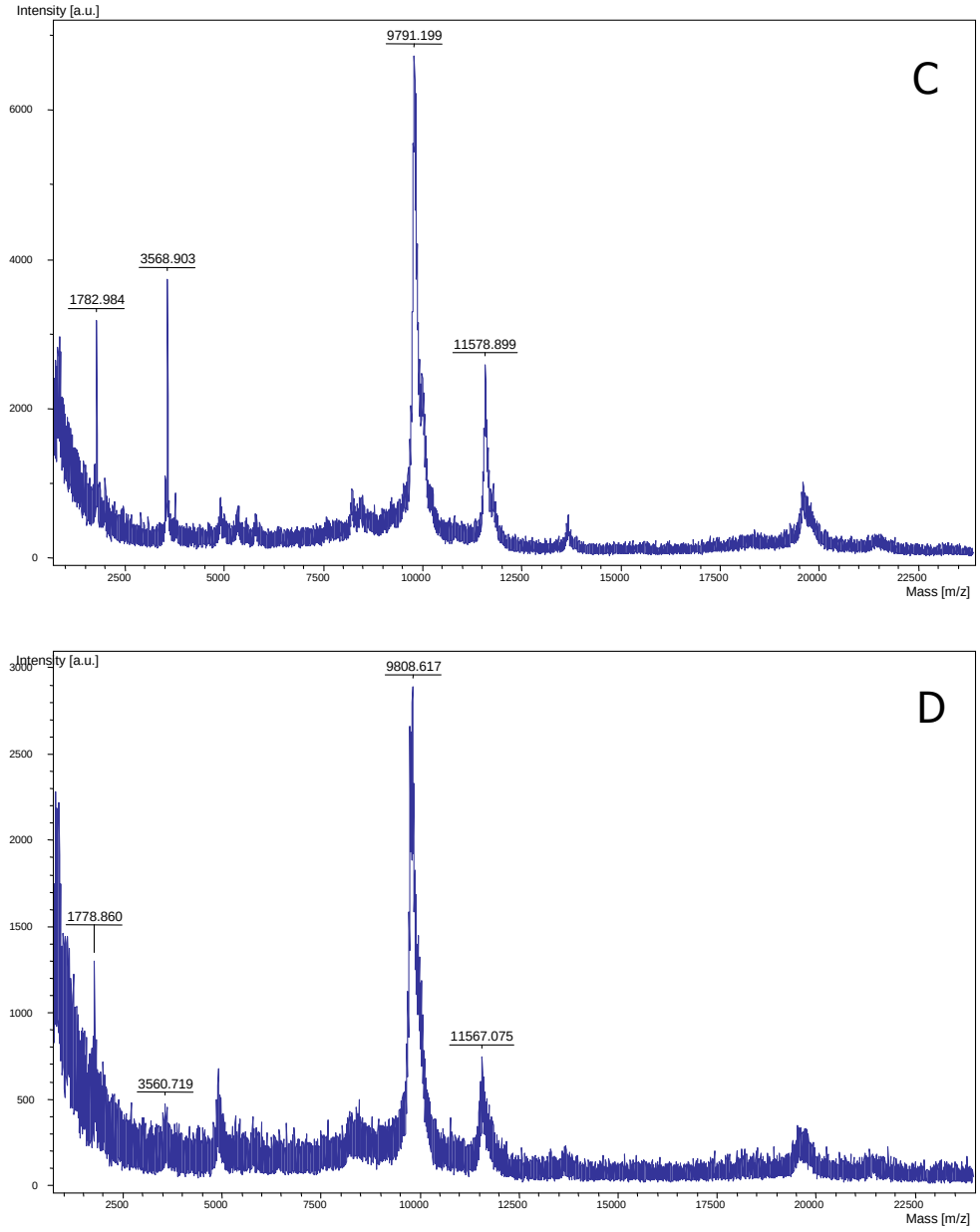


Fig. 12 - MALDI Spectra of 50µM CT319/rTat samples collected at the following incubation times: A) 1 hour; B) 2 hours; C) 3 hours; D) 26 hours

All the collected samples presented the same peaks in their spectra. In particular, the spectra displayed the presence of five main peaks corresponding to CT319 peptide (1780,994 m/z), CT319 dimer (3564,499 m/z), rTat (9792,895 m/z), rTat-CT319 adduct (11571,230 m/z) and Tat dimer (19624,855).

The presence of the peak at 11558,726m/z corresponding to Tat-peptide adduct molecular weight supports our hypothesis of the presence of a disulfide bond linking the two molecules.

5.3.2. Bond characterization in presence of a denaturing agent

Aiming to support the hypothesis of the adduct presence in a strong denaturing condition we tried to perform a preliminary test to evaluate the maximum SDS (Sodium Dodecyl Sulfate) percentage tolerated by MALDI-TOF.

Since SDS is an anionic surfactant that is able to disrupt non-covalent bonds in and between proteins, MALDI analysis of sample in its presence guarantees a complete disruption of all weak interactions. Unfortunately, samples for MALDI analysis should be as pure as possible (no detergent and phosphate), and have very low concentrations of salts, glycerol, urea, guanidine, tris etc. The presence of these substances could lead to the detection of a signal with a intensity so high that it suppresses out the sample spectrum.

As a consequence, 66,6μM CT319 samples were prepared in SDS-ammonium bicarbonate buffer in order to have an SDS percentage (w/v) equal to: 0, 0.2, 1 and 2. Subsequently, the four solutions were analyzed with MALDI-TOF.

The analysis of the sample spectra acquired display that exclusively in the CT319 sample lacking SDS a peak at 3571,540 m/z (corresponding to CT319 dimer mass) appears. Unfortunately, in the resulting spectra of the other samples containing SDS the signal-to-noise ratio was too low, likely due to SDS interference with the energy transfer from matrix to peptide. (Data not shown)

These data led us to conclude that it is not possible to study rTat/CT319 adduct formation with MALDI-TOF mass spectrometry in presence of a denaturing agent such as SDS.

5.3.3. Bond characterization in presence of a reducing agent

To finally confirm that a disulfide bond is involved in rTat-CT319 binding, we repeated the experiment described in 5.3.1 section adding the reducing agent DTT (Dithiothreitol) to the sample. To do this, we prepared 30µL of a fresh equimolar rTat-CT319 solution as previously described. After 1 hour's incubation at 37°C in a thermostated bath we collected a 5µL sample to perform mass spectrometry analysis Fig. 13. 10µL of DTT concentrated 1mg/kg was added to the remaining 25µL of mixture which was kept for another hour at 50°C.

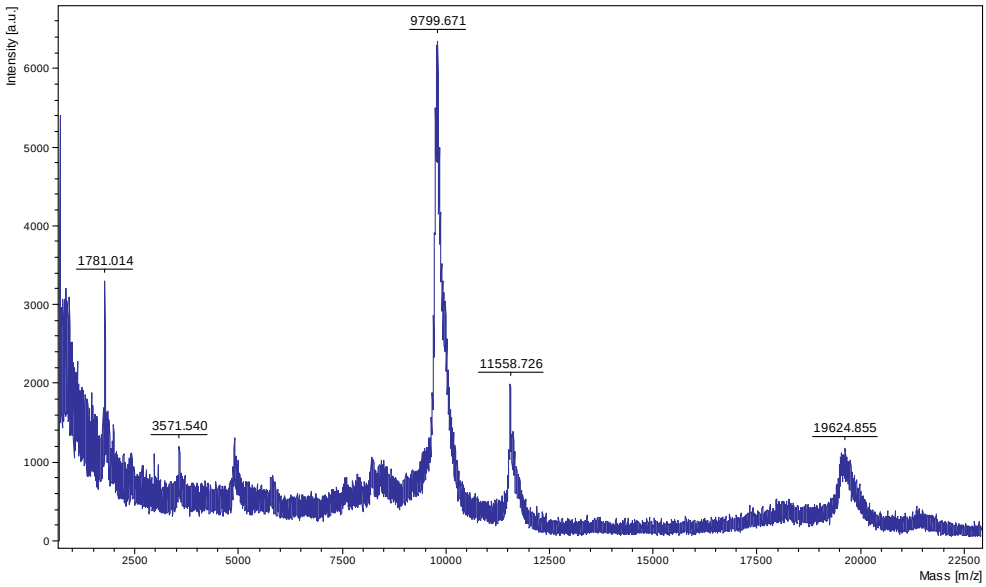


Fig. 13 - MALDI Spectra of 50µM CT319/rTat solution in absence of reducing agent

Following collection, the samples were analyzed by MALDI-TOF mass spectrometry. The spectrum of rTat-CT319 sample recorded before DTT addiction displayed had exactly the same profile as in the previous experiment. As can be seen from Fig. 13 five main peaks appeared in the

spectrum at 1781.014, 3571.540, 9799.671, 11558.726 and 19624.855 m/z corresponding respectively to CT319 peptide, CT319 dimer, rTat, adduct rTat-CT319, rTat dimer mass. Conversely, the analysis of the same sample added with the reducing agent showed dimers (CT319-CT319 and rTat-rTat) and adduct (rTat-CT319) peaks had completely disappeared Fig. 14. The only remaining peaks emerging from the background noise were the following: 1788.698 and 9793.557 m/z, respectively corresponding to CT319 and rTat molecular weights.

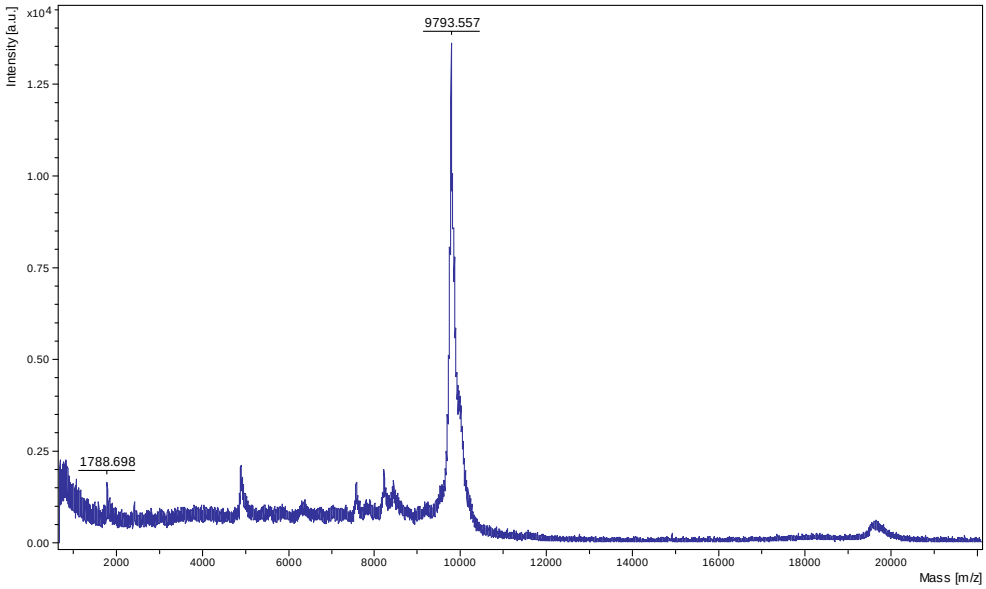


Fig. 14 - MALDI Spectra of 50µM CT319/rTat solution in presence of the reducing agent DTT

In conclusion, the disappearance of the adduct peak in reducing conditions confirms our starting hypothesis that CT319 and rTat are held together by a disulfide bond.

5.4. CT319 sequence specificity study

With the aim to evaluate CT319 sequence specificity, two derivatives were synthesized and assessed by mass spectrometry for the ability to form the adduct in presence of rTat.

5.4.1. Tat-CT332 binding study

In order to understand the role of cysteine position in CT319, a new derivative peptide named CT332 was produced. This is a 15-L-aminoacid analogue of CT319 with the same sequence but with the cysteine moved to the C-terminus (NH2-SFNITTEIRDKVKKC-COOH).

After sample preparation as described in Section 8.4.1, the mixture containing rTat and peptide was incubated at 37°C.

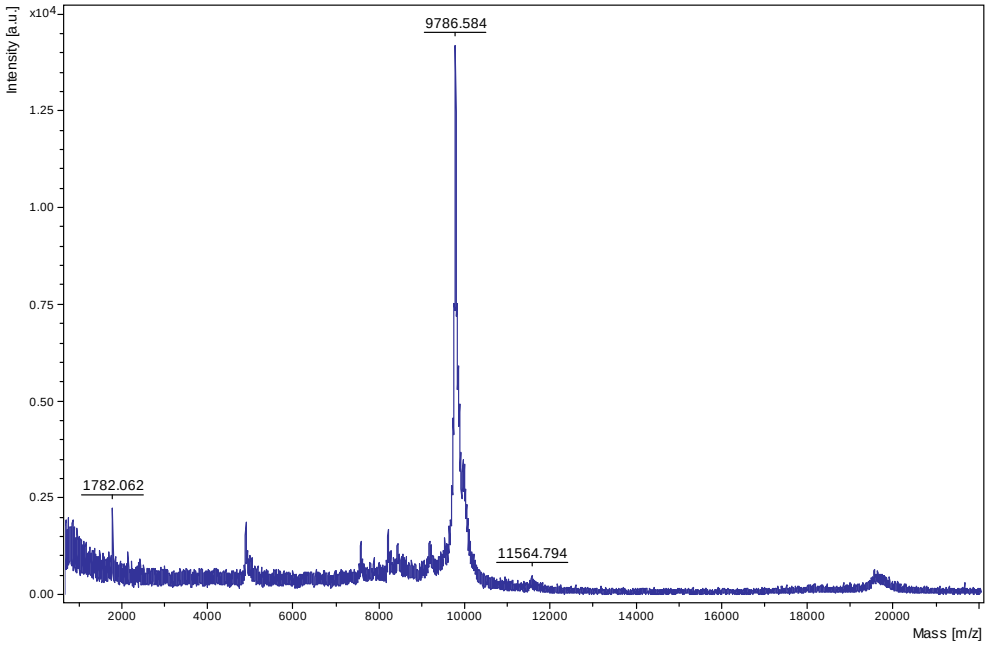


Fig. 15 - Tat-CT332 binding study. MALDI spectra of 50µM rTat/CT332 solution after 1 hour incubation.

The sample spectrum acquired by MALDI-TOF mass spectrometry after 1 hour incubation showed the main peak at 9786.58 m/z corresponding to rTat mass Fig. 15. The absence of the peak corresponding to the adduct led us to conclude that the positioning of the cysteine at the N-terminal plays a crucial role in the binding formation.

5.4.2. Tat-CT333 binding study

Based on the fact that scrambled peptides can assess the influence of residue sequence and on the observation that cysteine residue at the N-terminus plays a pivotal role, a new CT319 peptide analogue named CT333 was synthesized. CT333 is a 15-L-aminoacid with a Cys at the N-terminus and all the other amino acids of CT319 in a randomly scrambled position.

After sample preparation as described in Section 8.4.1, the mixture containing rTat and peptide was incubated at 37°C.

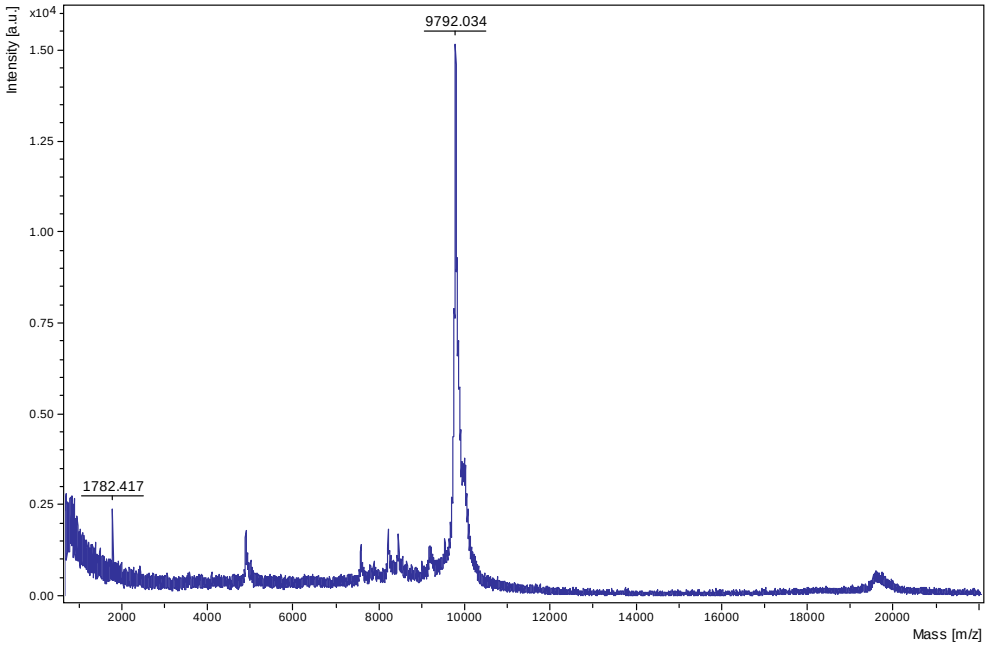


Fig. 16 - Tat-CT332 binding study. Spectra of 50µM rTat/CT332 solution after 1 hour incubation**.**

The spectrum of rTat-CT333 equimolar solution recorded after 1 hour incubation showed the main peak at 9792.034 m/z corresponding to rTat ion and the absence of any rTat-CT333 adduct Fig. 16. These results strongly support the involvement of all CT319 sequence in rTat recognition.

5.5. Evaluation of HIV-1 spreading inhibition by peptides

The purpose of this present study was to confirm the activity of our hit compound CT319 and compare it with CT332 in an ex vivo infectivity assay.

Peripheral Blood Mononuclear Cells (PBMCs) from two healthy donors (Donors A and B) separated on a Ficoll-Hystopaque gradient, were cultured in a RPMI-1640 supplemented medium.

After phytoemagglutinin-stimulation, the freshly isolated PBMCs were exposed to HIV-1 R5 (HIV-1_{BaL}) and HIV-1 X4 (HIV-1_{IIIB}) strains at 0.1 molteplicity of infection (moi). Infected leucocytes were plated in 96 microwells and monitored for 24 days after infection.

The reconstituted test compounds (CT319 and CT332) were added at 0.1, 1 and 10nM concentrations as a single or repeated additions to parallel cultures of virus-exposed cells at different time points, following the schedule below:

A. During primary infection and every 72 hour (sempre)

B. During primary infection (giorno 0)

C. On day 3 post-infection (giorno3)

D. On day 6 post-infection (giorno 6)

E. On day 9 post-infection (giorno 9)

F. On day 12 post-infection (giorno 12)

For the evaluation of virus propagation, HIV-1 Retrotranscriptase (RT) activity was quantified in the cell supernatants collected and kept at -20°C with a standard single assay as described. This is based on the production of radiolabeled enzyme product that can be detected and quantified by β-counter analysis.

The experiment was performed in quadruple.

5.5.1. Evaluation of HIV-1 spreading inhibition by CT319 peptide

The results showed a CT319 inhibitory activity on both PBMCs donors infected with R5 strain after the following peptide treatment schedule :

During primary infection and every 72 hours

During primary infection

On day 3 post-infection

On day 6 post-infection.

The viral replication inhibition reached 60-80% values. Treatment on days 9 and 12 post-infection did not show any activity of the compound. Moreover, we did not observe any dose-dependency in the 1 and 0,1 nM concentrations range, while the highest concentration tested (10 nM) showed a dual phase effect on viral replication (increase and then inhibition) in "sempre" conditions in A donor culture Fig.17.

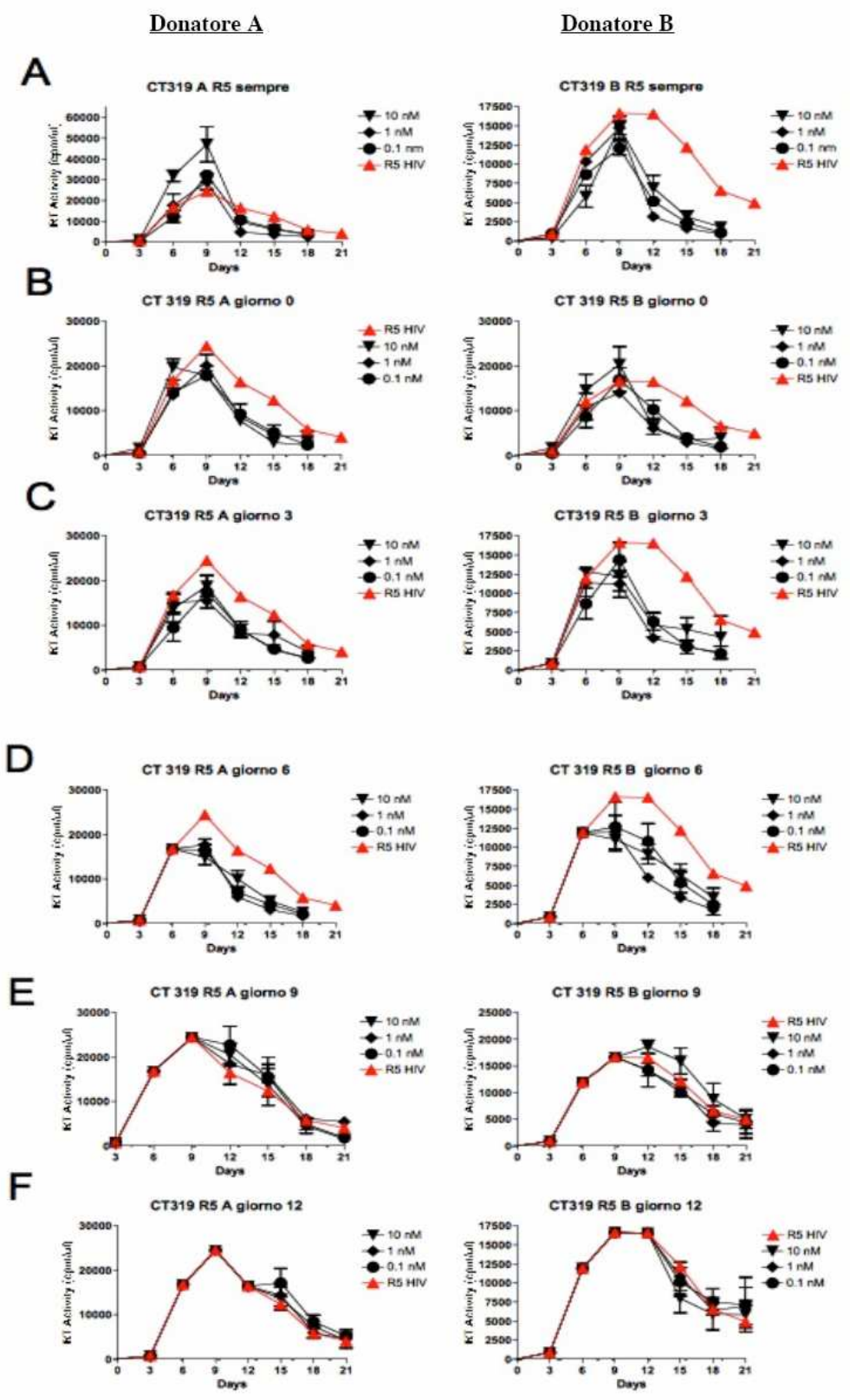


Fig. 17 - HIV-1_{BaL} (R5) viral spreading in presence/absence of CT319 peptide at 0.1,1 or 10nM concentrations

The evaluation of CT319 peptide activity on HIV-1 X4 spreading inhibition showed a weak viral replication inhibition (<50% with respect to the control) on both A and B donors cultures Fig.18. A slight increase in viral replication was observed (<50% compared to the control) in some points of the kinetics of infection in the cell culture of both donors:

Donor A cell culture showed an increase in the viral replication on day 6 post-infection in the "giorno 0" condition

Donor B cell culture showed an increase in the viral replication on days 3 and 6 in the "giorno 0" condition

The addition of CT319 peptide to both cell donor cell on day 3 did not modulate the viral spreading in the "giorno 3" condition.

It was not possible to evaluate effects of following experimental conditions since X4 viral replication returned to basal levels, likely due to target cell depletion.

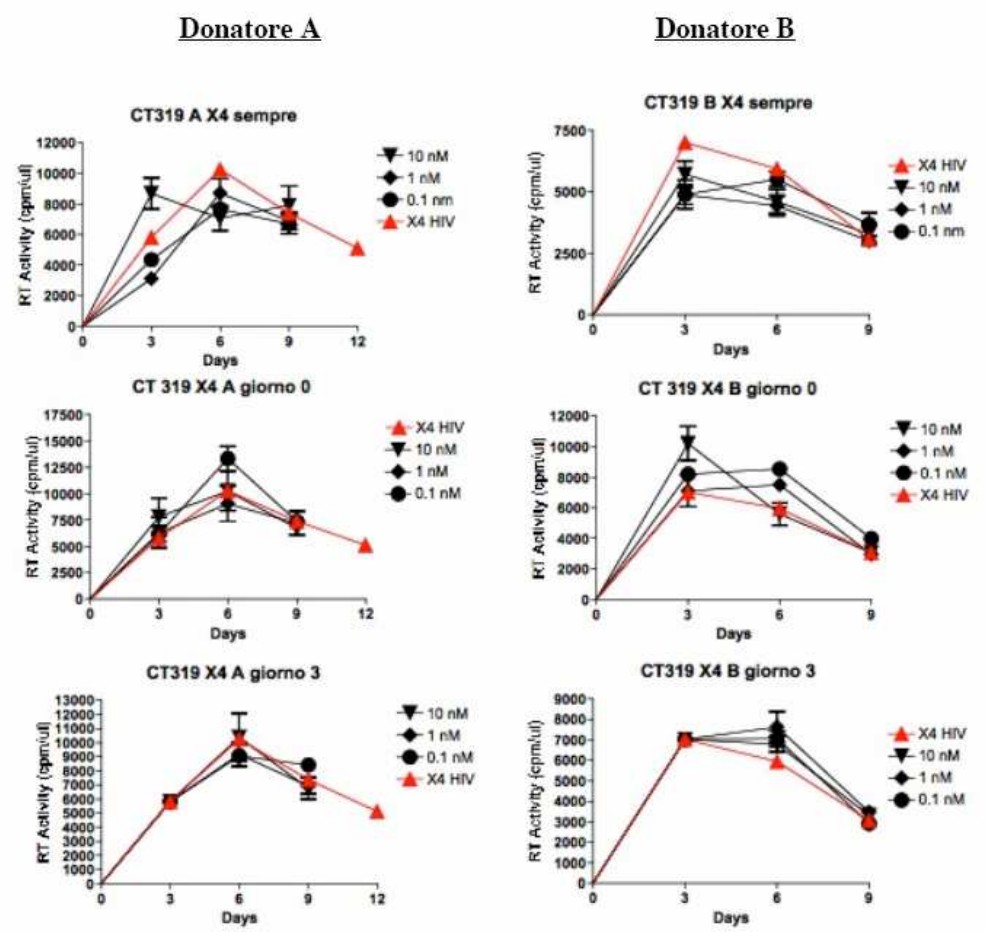


Fig. 18 - HIV-1_{IIIB} (X4) viral spreading in presence/absence of CT319 peptide at 0.1,1 or 10nM concentrations

5.5.2. Evaluation of HIV-1 spreading inhibition by CT332 peptide

CT332 is a 15-aminoacid long peptide of the same CT319 sequence but except the cysteine residue that the C-teminus instead of being at the N-terminus.

The addition of the test compound to both PBMCs donors cell cultures exposed to HIV-1 displayed a dual phase viral replication in "sempre" and "giorno 0" conditions. Indeed, an increase was observed followed by inhibition of the viral spreading Fig.19.

The addition of CT332 on days 3 and 6 post-infection showed, followed a CT319-like effect on the progression of HIV-1 replication.

No effect was observed after addition of the peptide on 9 and 12 days post-infection.

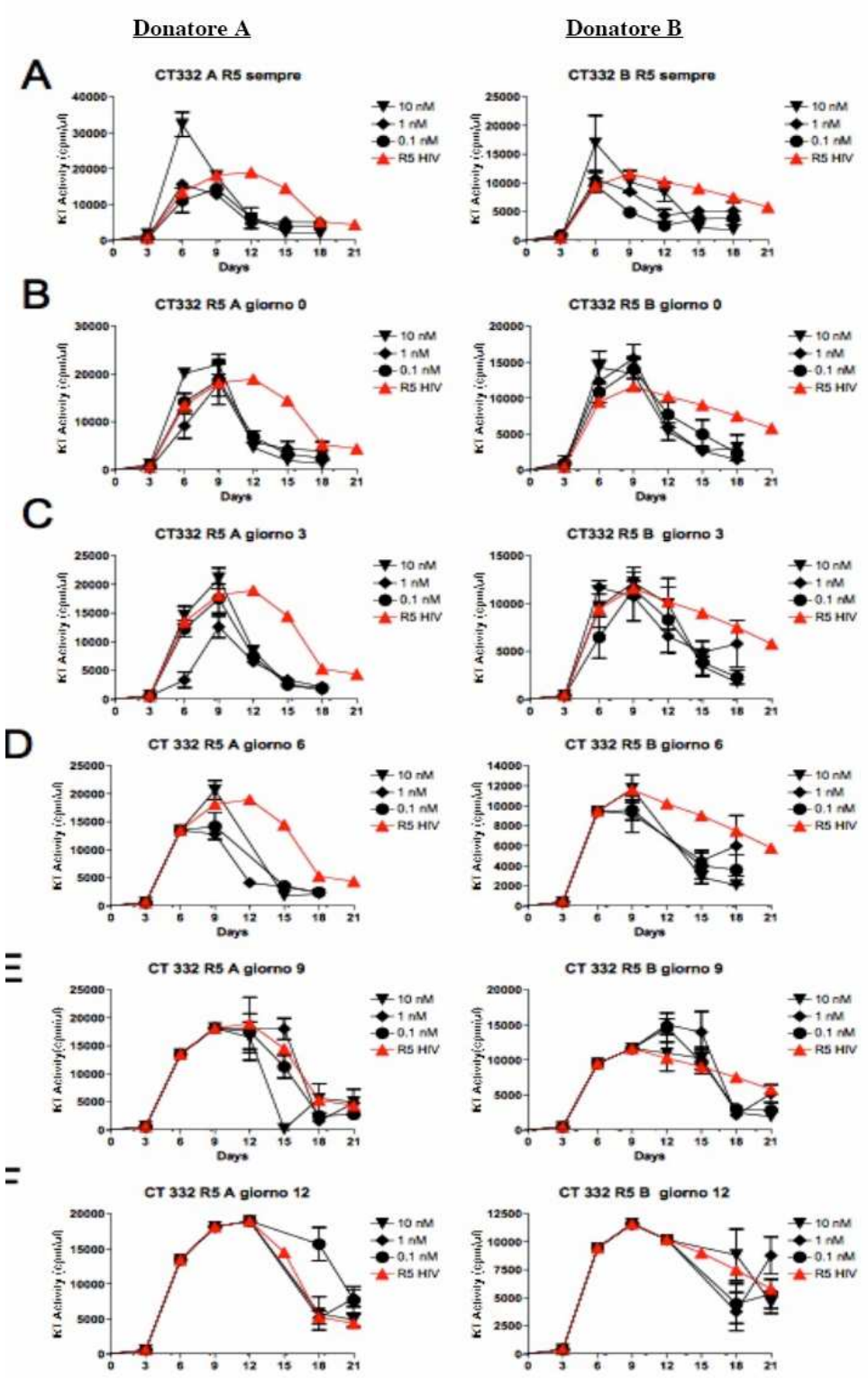


Fig. 19 - HIV-1_{BaL} (R5) viral spreading in presence/absence of CT332 peptide at 0.1,1 or 10nM concentrations

The evaluation of CT332 activity on HIV-1 X4 spreading inhibition did not show significant activity in any of conditions tested. The only exception could be an increase in viral replication in "sempre" conditions on Donor A cell cultures Fig.20.

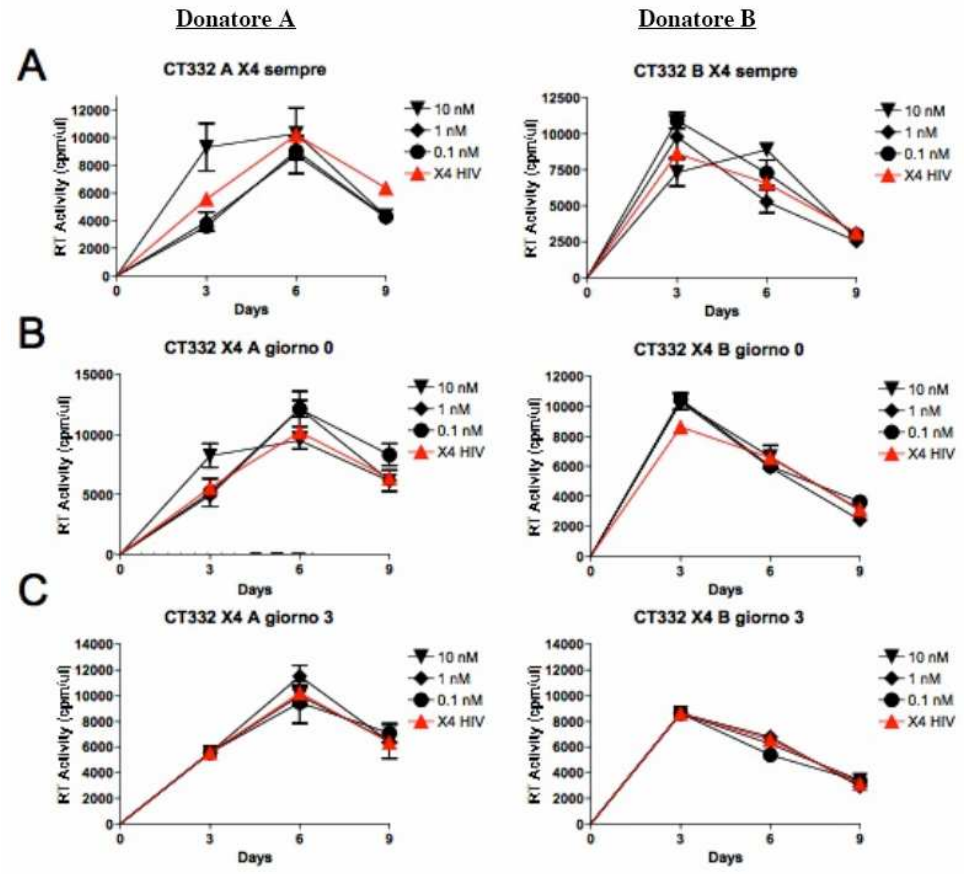


Fig. 20 - HIV-1_{IIIB} (X4) viral spreading in presence/absence of CT332 peptide at 0.1, 1 or 10nM concentrations

It was not possible to evaluate effects of following experimental conditions since X4 viral replication returned to basal levels, likely due to target cell depletion.

6. Discussion

HIV-1 infection is characterized by early viremia followed by a long period of clinical latency. After years, when a clinically apparent disease develops, this steady state is unbalanced toward an exponential increase in viral burden.

The endogenous HIV-1 transactivator of transcription Tat is released by infected cells both *in vitro* and *in vivo* and seems to play several functions in the extracellular microenviroment. Interestingly, a direct non-trascriptional functions of Tat in the setting of a spreading of viral infection has also been suggested. Indeed, circulating anti-Tat antibodies correlate with low or undetectable viral load in HIV-1 seropositive patients. Despite the large body of scientific literature regarding the role of extracellular Tat, an insight into the molecular mechanism responsible for Tat-driven spreading of infection has so far remained elusive.

The starting point in the development of this innovative strategy against AIDS is represented by a recent finding published in Blood where the authors showed that HIV-1 Tat is sequestered by glycosaminoglycan (GAG) at the surface of surrounding cells (either HIV-1 infected or uninfected) through its basic domain. Membrane-bound Tat acts as a novel, specific receptor for gp120 envelope protein. The specific interaction between the C-terminal portion of Tat (aa72-86) and gp120 V1/V2 loop (K_d = 8nM), never described before, enhances virus entry into permissive cells. This mechanism is independent of the viral strain, having a similar outcomes on infection by R5-tropic (Ba-L) and X4-tropic (III B) viruses. These data suggests that membrane-associated Tat is a novel modulator of virus entry and that the Tat/gp120 interaction is a critical step in HIV-1 spreading during the course of infection.

Based on these findings, the group led by Prof. Bussolino suggested a simple model of pathogenesis: 1) HIV-1 infected cells release Tat into

their enviroment; 2) Tat, concentrated on cell surface proteoglycans and protected from proteolytic degradation, remains in a biologically active form; 3) membrane-bound Tat binds gp120 viral envelope protein; 4) this interaction enhances virus attachment and entry into cells. In addition to the well known fusion mechanism, this newly discovered mechanism could be utilized by the virus in the aggressive spreading phase of the infection.

Consequently, aiming at the development of an innovative and very specific anti-AIDS drug, the group led by Prof. Bussolino screened several peptides for their ability to bind Tat and inhibit HIV-1 spreading. This screening led to the identification of a series of inhibitor peptides that shared homology with a portion of the gp120 V1/V2 loop, among which our candidate molecule, CT319, was selected for further development. CT319 is a 15 L-amino acid synthetic peptide (MW:1782.1 Da) able to specifically and selectively inhibit the entry of the virus reducing the global infectivity in a PBMC *ex vivo* model. An international patent application related to this new mechanism of virus entry and its inhibitors has been published [Patent no. PCT/EPO3/10162].

In a preliminary pharmacokinetic study, CT319 showed a poor profile after single intraperitoneal administration at a dose of 10mg/kg. Since the stability study in mouse blood revealed a half life of about 1h, the fast disappearance of the peptide from blood flow seems to be due not only to preteolytic degradation but also to fast renal clearance. It is of note that CT319 molecular weight (MW:1782.1 Da) is far below the renal glomerular cut-off (MW:60000 Da). As a consequence, rapid excretion of the peptide was expected.

In addition to information from the previous study, this work provides an insight into the nature of Tat/CT319 binding. The MALDI-TOF mass spectrometric analysis of an equimolar solution of Tat and CT319 reveals the presence of a peak corresponding to the sum of Tat and CT319 mass (11570 m/z). Based on this result and on the fact that the MALDI-TOF

approach led to disruption of weak interactions we could assume that the two moieties of the Tat/CT319 adduct are linked by a covalent bond.
Furthermore, the addition to the same solution of the reducing agent dithiothreitol caused the disappearance of the peak corresponding to the adduct. This evidence further supports our starting hypothesis of the presence of a covalent bond between CT319 cysteine and a Tat cysteine.
We also investigated CT319 sequence specificity for target molecular recognition. For this reason, we synthetized and tested two CT319 derivative peptides for their ability to form an adduct with the same approach and in the conditions as of the previous experiments. Interestingly, both CT332 (15-L-amino acid peptide with the N-terminal cysteine moved to the C-terminal) and CT333 (15-L-amino acid peptide with the cysteine at the N-terminal and all the remaining amino acids randomly scrambled in position) did not form the adduct. Taken together these data suggest that the molecular recognition of Tat by CT319 gp120-like region drives the formation of a covalent bond between the free N-terminal cysteine of the peptide and one of the 7 free cysteines of Tat Cystein Rich Domain.
Until now CT319 has been tested on 6 PBMCs donors infected with R5 and 7 donors infected with X4 at the following concentrations: 0.1, 1, and 10 nM. Here we present data related to the evaluation of HIV-1 spreading inhibition on 2 other donors. These preliminary results show a weak inhibitory activity of the peptide on R5 and X4 HIV-1 strains. In particular, the spreading inhibition by CT319 seems to be dependent on the time of treatment. According to literature data [Anzinger, 2008], in our results it is also possible to observe an high variability on HIV-1 spreading in different PBMCs donors. As a consequence, we are planning to enlarge the donors sample size with the aim to obtain a statistically significant results of CT319 activity.
While our work was proceeding, an interesting review by Ryser and Flückiger was published in Drug Discovery Today. It was widely known

that the interaction of gp120 with CD4 was crucial for viral infection but was not sufficient to allow the gp120 conformational changes leading to binding with the co-receptor and subsequent viral entry into target cells [Ryser, 2005; Dianzani U, 1995]. In this paper the authors describe experimental evidence that demonstrate the involvement of a new player in the HIV-1 entry process. Protein Disulfide Isomerase is a 57 kDa enzyme able to catalyze both oxidation and reduction of a disulfide bond as well as disulfide isomerization. On the cell surface it has been shown to act as a reductase that cleaves disulfide bonds through its catalytic domains containing the CXXC motif (where C stands for cysteine and X for any amino acid). Ryser and Flückiger's group proved that PDI gives the opportunity to the PDI catalytic domain to reduce 1 of the 9 disulfide bonds of gp120 and consequently starts the cascade of events leading to the fusion of the 2 membranes.

Based on these recent findings and taking into account previous studies on Tat viral protein we noted some parallelisms. Both CD4 and Tat can bind gp120 with similar affinity (K_d in the nM range). Both CD4 and glycosaminoglycan-associated Tat can anchor HIV-1 in the proximity of the cell surface. Both PDI and Tat bear CXXC motifs in their primary sequence. Taken together these data led us to formulate a new hypothesis of Tat's role in the HIV-1 entry process. In particular, the Tat/glycosaminoglycan complex (binding, anchoring and reducing gp120 disulfide bond) could be a functional substitute for the CD4/PDI complex in the exponential phase of infection leading to a CD4-independent entry mechanism. However we cannot exclude that Tat alone acts as a PDI substitute.

In conclusion, we suggest that the formation of a covalent bond between the CT319 N-terminal cysteine and one of the cysteines of the Tat CXXC motif causes the loss of Tat capability to mimic PDI. This hypothesis fits in well with the "plateau" behaviour of dose/effect CT319 curves evidenciated in the previous study performed by the group led by Prof.

Bussolino. In fact, once all Tat molecules are inactivated by covalent bond there is no use in increasing the peptide concentration. Moreover, since CT319 activity is due to its peculiar capability to form a covalent bond with Tat, it is possible that the inhibition of infection is not only due to competition with gp120 binding, but mainly to inactivation of Tat redox function.

7. Current and Future Development

Preliminary PK data showed that CT319 is characterized by a poor pharmacokinetic profile mainly due to glomerular filtration other than proteolytic degradation. To improve the pharmacokinetic performance of this totally innovative inhibitor of HIV-1 entry different approaches are currently under evaluation: retroinverso peptides, peptidomimetics, drug delivery systems, dendrimers and pegylation.

In retroinverso peptides the residues are aligned in the reverse order and D-amino acids replace L-amino acids. These peptides assume a 3D conformation very close to their corresponding L-forms but proteases become unable to recognize cleavage sites resulting in an increased *in vitro* and *in vivo* half-life. Unfortunately, this strategy does not solve the kidney clearance problem as well as there remaining a possible immunogenicity risk [Pescarolo MP, 2000; Lynch MP, 2005; Fromme B, 2003].

Another strategy to overcome the limitations of developing a peptide as a therapeutic agent could be the generation of peptidomimetics. Peptidomimetics are protein-like chains designed to mimic the conformational profile of a peptide. They may arise from modification of the peptide backbone or incorporation of non-natural amino acids keeping structural and functional properties of their native parental molecules. These organic molecules are featured by a stiffer structure than the parent peptides and reproduce peptidic configuration that exerts the best binding with the targets. This strategy does not solve the clearance problem but is likely to improve the pharmacokinetic profile. Interestingly, peptidomimetics show enhanced resistance to enzymatic degradation and lack of immunogenicity.

Among the several potential strategies for an improving of the pharmacokinetic parameters we have to consider the use of controlled release drug delivery systems, such as:

Local delivery

Micro/Nano particles of polymeric or lipidic nature. Thanks to the high volume of these particles they are retained by glomerular kidney filtration. Moreover reduced degradation keeps the drug concentration constant in the blood flow

Liposomic formulation

Dendrimers are hyperbranched macromolecules having a tree-like structure, consisting of a core molecule and alternating layers of monomers. They can be synthesized by divergent and convergent growth methods. During synthesis, properties such as dendrimer size, molecular mass and surface group can be controlled and configured to the desired need. The ability of dendrimers to encapsulate and bind the guest molecule can be used for solubility and bioavailability enhancement, sustained release and drug delivery applications. For pharmacological use they have to be highly purified, non immunogenic, biocompatible, biodegradable and selected for size. We have to remember that internalization mechanism does not work for particles up to 100nm size since it is unlikely that particles up to 50nm leave the blood flow. Biocompatibility depends on the charge and the external conformation. Biocompatibility is higher for molecules with round surfaces. The possibility of accurately pre-determining and controlling properties such as dimension are essential in design molecule, in order to find the best size to overcome the kidney clearance problem.

[Singh I, 2008;Palmiotto G, 2005]

One of the most interesting strategies that is being evaluated is the PEGylation approach. PEGylation is the process of covalent attachment of poly(ethylene glycol) PEG chains to another molecule. The conjugation of small proteins and peptides with PEG has become an increasingly common

method of improving the half-life of biological products, mainly through reducing urinary excretion of the molecule, but also by reducing enzymatic degradation due to the increased steric bulkiness. Moreover, the addition of the PEG moiety can have beneficial effects on the immunological profile of a molecule by reducing the ability of the compound to raise antibodies in humans. Unfortunately, it can influence the binding affinity of the therapeutic moiety to the cell receptors and can alter the absorption and distribution patterns. To minimize this effect it is possible to optimize the conjugate structure using appropriately selected: linear PEG, branched PEG or linkers conveniently chosen [Veronese, 2005;Webster, 2007; Roberts, 2002; Molineux, 2003].

Moreover, a deeper understanding of the complex biology of HIV-1 entry and CT319 mechanism of action could support the development of the best new product candidate.

As a consequence we are planning to investigate wether the viral protein Tat exerts a reductase activity like PDI. For this purpose we have contacted Prof. Emmanuel Fenouillet of the CNRS in Marseille, one of the leading experts working on the model of PDI-induced conformational changes triggering gp41 activation, to seek collaboration to demonstrate our hypothesis [Barbouche, 2005; Barbouche, 2003; Barbouche, 2002; Fenouillet, 2001]

We are evaluating also the possibility of mapping the Tat cysteine responsible for bond formation with CT319 applying a simple methodology for differentiating between cysteine (sulphydryl) and cystine (two disulfide bond-linked cysteines). This approach employs a specific reaction between free sulphydryls and 2-nitro-5-thiocyanobenzoic acid (NTCB) to selectively cyanylate cysteine thiols. The N-terminal peptide bond of the modified cysteinyl residue can then be cleaved under alkaline conditions to form an amino-terminal peptide and a series of 2-imothiazolidine-4-carboxyl peptides which can be mapped to the sequence by MALDI-TOF mass spectrometry. The use of the NTCB reagent is unique in that it specifically

targets the sites being analyzed. Other advantages include fast analysis, easy operation and high sensitivity [Wu, 1996; Daniel, 1997].

Once determined the cysteine residue that can bind CT319 we could confirm the crucial role of this amino acid expressing the Tat mutant (CysXX→SerXX) and testing it in comparison with wild type Tat for the ability to enhance HIV-1 entry.

Finally, in order to further investigate CT319 *in vitro* inhibitory activity we are planning to repeat the infectivity assay on an enlarged sample of about 25 different human PBMCs donors.

8. Materials and Methods

8.1. Materials

Recombinant Tat (HIV-1); diatheva; Product Number REP0002b; Lyophilized form; Purity >50%; Lot 005;

CT319 (Sequence: Cys- Ser-Phe-ASN-Ile-Thr-Thr-Glu-Ile-Arg-Asp-Lys-Val-Lys-Lys; Lot number: T84-1//165-085; Grade: 95%; Molecular weight: 1782.1; Multiple Peptide System);

CT332 (Sequence: Ser-Phe-ASN-Ile-Thr-Thr-Glu-Ile-Arg-Asp-Lys-Val-Lys-Lys-Cys; Part number: SP060028; Lot number: CF05246; Grade: 95%; Molecular weight: 1781.9; NeoMPS);

CT333 (Sequence: Cys-Arg-Ile-Thr-Ser-Asn-Val-Lys-Lys-Lys-Phe-Asp-Ile-Glu-Thr; Part number: SP060029; Lot number: AW07419; Grade: 95%; Molecular weight: 1781.7; NeoMPS);

SDS, Sodium Dodecyl Sulphate, C12H25NaO4S, SIGMA.

DTT ((DL)-dithiothreitol FW 154.25 SIGMA Lot D9779-5G//024K0606)

8.2. Isolation and culture of human PBMCs

Peripheral Blood Mononuclear Cells (PBMCs) from HIV-seronegative healthy donors separated on a Ficoll-Hystopaque gradient, were washed and cultured in RPMI-1640 supplemented with 15% FCS and 200 U/ml Interleukin-2 (as described in the paper Marchiò, 2005). Patient samples were obtained following approval by the institutional review boards of San Raffaele Scientific Institute in Milan; and written informed consent was obtained from all patients and healthy donors in accordance with the Declaration of Helsinki.

8.3. Infectivity assay

After 3 days of phytoemagglutinin-stimulation, the freshly isolated PBMCs ($1x10^6$ cells/mL) were exposed to 0.1 moi (infectious unit per cell) of HIV-1 R5 (III B) or HIV-1 X4 (Ba-L) strains for 2 hours at 37°C. Following the incubation period, the cells were washed to remove residual free virus.
The reconstituted test compounds (CT319 and CT332) were added at 0.1, 1 and 10nM as a single or repeated addition to parallel cultures of virus-exposed cells at different time points after primary infection (0, 3, 6, 9, 12 days post-infection). For evaluation of virus propagation, HIV-1 retrotranscriptase (RT) activity in the cell supernatants was quantified with a standard assay as described. [Schmidtmayerova H, 1998]

8.4. Mass spectrometry

8.4.1. Sample preparation

rTat solution

100µM rTat solution was prepared dissolving the 50µg of lyophilized protein in 36µL of 20mM ammonium bicarbonate (NH4HCO3) solution, previously prepared and adjusted in order to reach the physiological pH (pH 7.4).

Peptide solutions

1mg of CT319 or CT332 or CT333 powder was reconstituted in about 5.6mL of ammonium bicarbonate (NH4HCO3) in order to prepare a 100µM solution. 20mM ammonium bicarbonate (NH4HCO3) solution has been previously prepared and adjusted to reach the physiological pH (pH 7.4).

rTat-peptide mixture

A 1:1 rTat-peptide mixture was prepared mixing an equal volume (15µL) of the two 100µM solution previously prepared (rTat solution and peptide

solution). The final concentration of the 30µL equimolar mixture was 50µM.

CT319-SDS mixture

CT319 66,6 µM solutions was prepared mixing CT319 100 µM solution with ammonium bicarbonate supplemented with different volume of 6% SDS solution in ammonium bicarbonate to reach the following final SDS percentages: 0%, 0,2%, 1%, 2%.

CT319-DTT solution

Prepared 30µL of a rTat-CT319 equimolar solution as described above. After 1 hour incubation at 37°C in a thermostated bath, 5µL of sample was collected for mass spectrometry analysis. The remaining 25µL of mixture were added with 10µL of 1mg/mL of DTT. The mixture was kept for another hour at 50°C. Following the sample were analysed by MALDI-TOF mass spectrometry.

All the solutions used were freshly prepared for the analysis.

8.4.2. MALDI TOF Analysis

Positive-ion spectra were recorded in linear mode with time of flight delayed extraction using a MALDI-TOF Reflex III (Bruker Daltonics, Bremen, Germany) instrument, equipped with a pulsed-N2 operating at 337nm. A satured solution of α-cyano-4-hydroxycinnamic acid in 70% acetonitrile (ACN) and 0.2% trifluoroacetic acid (TFA) was prepared to dilute the sample being analyzed. 0.5 µL of this solution were spotted onto MALDI plate. After the sample spot dried completely, MALDI plate was inserted into the instrument. The detection was conducted in reflector mode. To enhance the reproducibility, 200 laser shots from the laser were averaged for each mass spectrum. The instrument calibration was

performed in 1045 Da – 3346 Da molecular range with an external peptide mixture.

9. References

AIDSinfo 2008: www.aidsinfo.nih.gov

AIDSinfonet: www.aidsinfonet.org

Anzinger JJ, Olinger GG, Spear GT Donor variability in HIV binding to peripheral blood mononuclear variability Virology Journal 2008;5:95

Appenzeller-Herzog C, Ellgaard L. In vivo reduction-oxidation state of protein disulfide isomerase: the two active sites independently occur in the reduced and oxidized forms Antioxid Redox Signal. 2008;10(1):55-64.

Atripla website: www.atripla.com

Barbouche R, Lortat-Jacob H, Jones IM, Fenouillet E. Glycosaminoglycans and protein disulfide isomerase-mediated reduction of HIV Env. Molecular Pharmacology 2005; 67(4):1111-8

Barbouche R, Miquelis R, Jones IM, Fenouillet E. Protein-disulfide Isomerase-mediated reduction of two disulfide bonds of HIV envelope glycoprotein 120 occurs post-CXCR4 binding and is required for fusion 2003; 278(5):3131-3136

Barbouche R, Miquelis R, Jones IM, Fenouillet E. Protein-disulfide isomerase-mediated reduction of two disulfide bonds of HIV envelope glycoprotein 120 occurs post-CXCR4 binding and is required for fusion. Journal of Biological Chemistry 2003; 278(5):3131-6.

Barclay AN, Brady RL, Davis SJ, Lange G. CD4 and the immunoglobulin superfamily Philosophical transactions of the royal society biological sciences 1993; 342(1299):7-12 (Abstract)

Barré-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vézinet-Brun F, Rouzioux C, Rozenbaum W, Montagnier

L. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS) Science: 868-71.

Berger EA, Murphy PM, Farber JM Chemikine receptors as HIV-1 coreceptors: roles in viral entry, tropism and disease. Annu Rev Immunol 1999; 17:657-700

Borkow G, Lapidot A Multi-targeting the entrance door to block HIV-1 current Drug Targets-Infectious disorders 2005; 5:3-15

Briggs JAG, Grünewald K, Glass B, Föster F, Kräusslich HG, Fuller S. The mechanism of HIV-1 core assembly insight from three-dimensional reconstructions of authentic virions Structure 2006; 14:15-20

Bukrinski M, A hard way to the nucleus Molecular medicine 2004; 10:1-6

Buonaguro L, Tornesello ML, Buonaguro FM. Human immunodeficiency virus type 1 subtype distribution in the worldwide epidemic: pathogenetic and therapeutic implications. J Virol. 2007;81(19):10209-19.

Butler IF, Pandrea I, Marx PA, Apetrei C. HIV genetic diversity: biological and public health consequences Curr HIV Res 2007;5(1):23-45.

Cahn P, Sued O. Raltegravir: a new antiretroviral class for salvage therapy. Lancet. 2007;369(9569):1235-6.

Castagna A, Biswas P, Beretta A, Lazzarin A. The appealing story of HIV entry inhibitors Drugs 2005; 65(7):879-904

Center for Disease and Control Prevention (CDC) Pneumocystis Pneumonia --- Los Angeles. Morbidity and Mortality Weekly Report (MMWR) 1981; 30:250—2.

Center for Disease and Control Prevention (CDC) Twenty-Five years of HIV-AIDS --- United States, 1981--2006. Morbidity and Mortality Weekly Report (MMWR) 2006; 55(21):585—589.

Center RJ, Earl PL, Lebowitz J, Schuck P, Moss B The Human Immunodeficiency Virus Type 1 gp120 V2 Domain Mediates gp41-Independent Intersubunit Contacts Journal of Virology 2000; 74(10):4448-4455

Chiu IM, Yaniv A, Dahlberg JE, Gazit A, Skuntz SF, Tronick SR, Aaronson SA. Nucleotide sequence evidence for relationship of AIDS retrovirus to lentiviruses. Nature. 1985;317(6035):366-8

Cohen MS, Hellmann N, Levy JA, DeCock K, Lange J The spread, treatment, and prevention of HIV-1: evolution of a global pandemic The journal of clinical investigation2008; 118(4):1244-1254

Daniel R, Caminade E, Martel A, Le Goffic F, Canosa D, Carrascal M, Abian J. Mass spectrometric determination of the cleavage sites in Escherichia coli dihydroorotase induced by cisteine-specific reagent The journal of biological chemistrym 1997; 272(43):26934-26939

Decision Research Report 2001

Dianzani U, Bragardo M, Buonfiglio D, Redoglia V, Funaro A, Portoles P, Rojo J, Malavasi F, Pileri A. Modulation of CD4 lateral interaction with lymphocyte surface molecules induced by HIV-1 gp120 European Journal of Immunology 1995; 25(5):1306-11

Estè J, Telenti A HIV entry inhibitor Lancet 2007; 370:81-88

Fauci A HIV and AIDS: 20 years of science Nature Medicine 2003; 9(7):839-843

FDA 2008: www.fda.gov/oashi/aids/virals.html

Fenouillet E, Barbouche R, Courageot J, Miquelis R. The catalytic activity of protein disulfide isomerase is involved in human immunodeficiency virus envelope-mediated membrane fusion after CD4 cell binding. Journal of Infection Disease 2001; 183(5):744-52

Ferrari DM, Söling HD. The protein disulphide-isomerase family:unravelling a string of folds Biochemical Journal 1999; 399:1-10

Flexner C. HIV drug development: the next 25 years Nature reviews Drug discovery 2007; 6:959-966

Fromme B, Eftekhari P, Van Regenmortel M, Hoebeke J, Katz A, Millar R. A novel retro-inverso gonadotropin-releasing hormone (GnRH) immunogen elicits antibodies that neutralize the activity of native GnRH Endocrinology 2003; 144(7):3262-3269

Fuzeon Roche: www.fuzeon.com

He Y, Liu S, Li J, Lu H, Qi Z, Liu Z, Debnath AK, Jiang S. Conserved salt bridge between the N- and C-terminal heptad repeat regions of the human immunodeficiency virus type 1 gp41 core structure is critical for virus entry and inhibition. J Virol. 2008;82(22):11129-39.

HIV-insite: www.hivinsite.org

Hyland R, Dickins M, Collins C, Jones H, Jones B. Maraviroc: in vitro assessment of drug-drug interaction potential. Br J Clin Pharmacol. 2008;66(4):498-507.

Isolated HIV-1 is active for Reverse Transcription 2007

Kallings LO The first postmodern pandemic: 25 years of HIV/AIDS Journal of internal medicine 2008; 263: 218-243

Kazmierski WM, Kenanin TP, Gudmundsson KS. Peptide, peptidomimetici and small-molecule drug discovery targeting HIV-1 host-cell attachment and entry through gp120, gp41, CCR5 and CXCR4 Chemical Biology and Drug Design 2006;67:13-26

Kersteen EA, Raines R Catalysis of protein folding by protein disulfide isomerase and small-molecule mimics Antioxidants & redox signaling 2003; 5(4):413-424

Kindt, Goldsby, Osborne. Immunology Sixth Edition 2007

Klimas N, O'Brien Koneru A, Fletcher MA Overview of HIV Psycosomatic Medicine 2008 70:523-530

Luciw PA, Human Immunodeficiency Viruses and their replication Fields Virology Lippincott-Raven Publishers 1996

Lynch MP, Rawale SV, Behery AA,Hudleson WP, Takenouchi N, Yao K, Jacobson S and Kaumaya PTP Novel Retro-inverso Envelope Peptide Mimetic Fusion Inhibitors as a Potential Therapy for HTLV-1 Infected Individuals Understanding Biology Using Peptides 2005; 9:527-528

Lythgo PA Molecular Virology of HIV-1 and Current Antiviral Strategies Molecular Virology of HIV and Current Antiviral Strategies 2004; 2:81-87

Marchiò S, Alfano M, Primo L, Gramaglia D, Butini L, Gennero L, Devivo E, Arap W, Giacca M, Pasqualini R, Bussolino F. Cell surface-associated Tat HIV-1 modulates HIV-1 infection and spreading through a specific interaction with gp120 viral envelope protein Blood 2005; 105(7):2802-2811

Markovic I, Stantchev TS, Fields KH, Tiffany LJ, Tomiç M, Weiss CD, Broder CC, Strebel K, and Clouse KA Thiol/disulfide exchange is a prerequisite for CXCR4-tropic HIV-1 envelope-mediated T-cell fusion during viral entry Blood 2004; 103(5): 1586-1594.

Matthews T, Salgo M, Greenberg M, Chung J, DeMasi R, Bolognesi D. Enfuvirtide: The first to inhibit the entry of HIV-1 into host CD4 lymphocytes Neture reviews 2004; 3: 215-225

Matthias LJ, Yam PTW, Jiang XM, Vandergraaff N, Li P, Poumbourios P, Donoghue N, Hogg PJ. Disulfide exchange in domain 2 of CD4 is required for entry of HIV-1 Nature Immunology 2002; 3(8):727-732

Molineux G PEGylation: Engineering improved biopharmaceuticals for oncology Supplement to pharmacotherapy 2003; 23(8):3S8S

Montagnier L A History of HIV Discovery Science 2002; 298:1727-1728

Naghavi MH, Goff SP. Retroviral proteins that interact with the host cell cytoskeleton.Curr Opin Immunol. 2007;19(4):402-7.

Neuveut C, Jeang KT Recombinant human immunodeficiency Virus type 1 genomes with tat uncostrined by overlapping reading frames reveal residues in Tat important for replication in tissue culture Journal of Virology 1996; 70(8):5572-5581

Nielsen MH, Pedersen FS, Kjems J. Molecular strategies to inhibit HIV-1 replication Retrovirology. 2005; 16;2:10.

Nisole S and Saïb A. Early steps of retrovirus replicative cycle Retrovirology 2004; 1:1-20

Palmiotto G. I dendrimeri tra preente e futuro NCF 2005; 108-113

Patent No. PCT/EP03/10162 A novel mechanism for HIV-1 entry into host cells and peptides

Pescarolo MP, Bagnasco L, Malacarne D, Melchiori A, Valente P, Millo E, Bruno S, Basso S, Parodi S. A retro-inverso peptide homologous to helix 1 of c-Myc is a potent and specific inhibitor of proliferation in different cellular systems The FASEB Journal 2000

Pfizer report: www.pfizer.com

Poignard P, Ollmann Saphire E, Parren PWHI, Burton DR. gp120: Biologic aspects of structural features Annual Reviews of Immunology 2001; 19:253-274

Poster Cmommunication at Pep Talk (Jan 11th - 13th 2006, Sna Diego, CA) "A new peptide inhibitor of HIV-1 entry" Authors: Marchiò S, Bagnod R, Traversa S, Fumero S, Bussolino F

Roberts MJ, Bentley MD, Harris JM Chemistry for peptide and protein PEGylation Adbvances Drug Delivery Reviews 2002; 54:459-476

Ryser HJ, Flückiger R. Progress in targeting HIV-1 entry. Drug Discov Today. 2005 10(16):1085-94

Sanders RW, Schiffner L, Master A, Kajumo F, Guo Y, Dragic T, Moore JP, Binley JM. Variable-loop-deleted variants of the human immunodeficiency virus type 1 envelope glycoprotein can be stabilized by an intermolecular disulfide bond between the gp120 and gp41 subunits. J Virol. 2000;74(11):5091-100.

Sierra S, Kupfer B, Kaiser R. Basics of the virology of HIV-1 and its replication Journal of Clinical Virology 2005; 34:233-244

Simon V, Ho DD, Abdool Karim Q. HIV/AIDS epidemiology, pathogenesis, prevention an treatment Lancet 2006; 368:489-504

Singh I, Rehni AK, Kalra R, Joshi G, Kumar M. Dendrimers and their pharmaceutical applications--a review. Pharmazie. 2008;63(7):491-6

Shmidtmayerova H, Alfano M, Nuovo G, Burkrisky M. Human Immunodeficiency Virus Type 1 T-lymphotropic strains enter macrophages via a CD4 and CXCR4 mediated pathway: replication is restricted at a postentry level. J Virol 1998; 72(6):4633-4642

Temesgen Z, Warnke D, Kasten MJ. Current Status of antiretroviral therapy Expert Opinion 2006; 7(12):1541-1554

UNAIDS ADIS epidemic update 2007

Vandekerckhove L, Verhofstede C, Vogelaers D. Maraviroc:integration of a new antiretroviral drug class into clinical practice Journal of antimicrobial chemotherapy 2008; 1-4

Veronese FM, Pasut G PEGylation, succesful approach to drug delivery DDT 2005; 10(21):1451-1458

Warrilow D, Harrich D. HIV-1 replication from after cell entry to the nuclear periphery. Curr HIV Res. 2007;5(3):293-9.

Webster R, Didier E, Harris P, Siegel N, Standler J, Tilbury L, Smith D PEGylated proteins: Evaluation of their safety in the absence of definitive metabolism studies Drug Metabolism and disposition 2007; 35(1):9-16

Wu J, Gage DA, Watson T A strategy to locate cysteine residues in proteins by specific chemical cleavage followed by matrix-assisted laser desorption/ionization Time-of-flight mass spectrometry Analytical biochemistry 1996; 235:161-174

Wyatt R, Kwong PD, Hendrickson WA, Sodrosky JG Structure of the core of the HIV-1 gp120 exterior glycoprotein 2002