Resistance to HIV entry inhibitors: signature mutations as tool guide for the identification of new antiviral agents

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Resistance to HIV entry inhibitors: signature mutations as tool guide for the identification of new antiviral agents.

Memòria presentada per Emmanuel González Ortega per optar al títol de doctor per la Univeristat de Barcelona

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"Somos lo que hacemos,
sobre todo lo que hacemos
para cambiar lo que somos."
E. Galeano

Resumen.

Existen varias razones para celebrar los avances recientes en el tratamiento de la infección por VIH. De acuerdo al Programa Conjunto de las Naciones Unidas sobre el VIH/SIDA, el número de nuevas infecciones bajó un 15%; también hubo una disminución del 22% en el número de muertes asociadas al VIH/SIDA. Sin embargo, existen retos emergentes, tales como la transmisión de cepas de VIH-1 resistentes a algunos fármacos; por lo que hay una exigencia a la investigación continua de agentes antiretrovirales nuevos y más potentes. La entrada del VIH en las células implica una serie de pasos complejos y bien orquestados que involucra moléculas tanto virales como celulares y que finaliza con la producción de nuevas partículas virales. La glicoproteína gp120 del VIH se une al receptor celular CD4 y a un receptor de quimiocinas, induciendo rearreglos estructurales que prosiguen con la fusión de las membranas celular y viral mediada por la glicoproteína del VIH gp41. De tal manera, la entrada del VIH es un paso esencial en la replicación viral que ofrece una vía para el diseño de nuevos compuestos antivirales y que pueden ser incluidos en el repertorio de fármacos utilizados en el tratamiento de la infección por VIH. En coincidencia con la información reciente y relevante sobre el mecanismo de fusión que ocurre durante la entrada viral, el diseño de nuevos inhibidores de la fusión se ha convertido en una de las áreas más prometedoras y debatidas en el estudio de inhibidores de entrada. ADS-J1 fue seleccionado para unirse a gp41 e inhibir la fusión de las membranas. Por medio de varios ensayos, incluida la generación de cepas resistentes a ADS-J1, nuestro laboratorio demostró que ADS-J1 interactúa con gp120 y no con gp41. Una publicación posterior sugirió que ADS-J1 se une a la 'pocket-region' de gp41, previniendo la infección por el virus. En el presente trabajo, nosotros confirmamos que ADS-J1 interactúa con gp120 y no con gp41. La recombinación de gp120 en un VIH silvestre restituyó el fenotipo resistente. Ensayos de tiempo de adición demostraron claramente que ADS-J1 no interactúa con gp41.

VIRIP fue identificado como un péptido natural presente en el hemofiltrado humano capaz de inhibir la fusión de membranas mediada por gp41 del VIH. Se sugirió que VIRIP interactúa con el péptido de fusión en gp41, bloqueando la fusión de las membranas. Con el objetivo de determinar el modo de acción de VIRIP, generamos un virus resistente a VIR-353, un análogo de VIRIP. Adicionalmente, determinamos la combinación de mutaciones que generan el fenotipo resistente. Estudios recientes mostraron la efectividad de VIR-576, un péptido con alta similitud a VIRIP y VIR-353 en un ensayo clínico fase I/II. La resistencia a VIRIP/VIR-353 requirió un periodo de tiempo largo para emerger, lo cual sugiere una elevada barrera genética a la resistencia. Las mutaciones responsables del fenotipo resistente afectaron en gran escala la capacidad replicativa del virus, sin embargo, varias mutaciones compensatorias restauraron la capacidad replicativa, manteniendo inalterada la resistencia a VIR-353. La actividad antiviral de T20 no parece estar afectada por VIR-353, la combinación de los dos inhibidores de fusión mostraron un efecto aditivo en la inhibición de la replicación. Desafortunadamente, no pudimos determinar de manera precisa el sitio en el que VIRIP/VIR-353 actúa en gp41, sin embargo nuestros resultados sugieren que debe ser diferente al sitio de interacción que originalmente se había postulado: el péptido de fusión en gp41. En general, nuestros resultados evidencian la plasticidad de las glicoproteínas de la envuelta del VIH. Esta plasticidad es realzada cuando el virus replica bajo la presión selectiva impuesta por fármacos que inhiben la replicación viral, lo cual añade una barrera genética adicional a ser superada por el virus.

Resum.

Existeixen diverses raons per celebrar els avanços recents en el tractament de la infecció per VIH. D'acord al Programa Conjunt de les Nacions Unides sobre el VIH/SIDA, les noves infeccions van disminuir un 15% i també va haver-hi una reducció del 22% en el nombre de morts associades al VIH/SIDA. No obstant, existeixen reptes emergents, com la transmissió de soques de VIH-1 resistents a certs fàrmacs; que exigeix una cerca de nous agents antiretrovirals més potents. L'entrada del VIH en les cèl·lules implica una sèrie de passos complexos i ben orquestrats que impliquen molècules tant virals com a cel·lulars i que finalitzen amb la producció de noves partícules virals. La glicoproteïna de l'envolta gp120 del VIH s'uneix al receptor cel·lular CD4 i a un receptor de quimiocines, induint reordenaments estructurals que condueixen a la fusió de les membranes cel·lular i viral operada per la glicoproteïna gp41 del VIH. D' aquesta manera, l'entrada del VIH és un pas essencial en la replicació viral que ofereix una via pel disseny de nous compostos antivirals que poden ser inclosos al repertori de fàrmacs utilitzats en el tractament de l'infecció pel VIH. En coincidència amb la información recent i rellevant sobre el mecanisme de fusió que té lloc durant l'entrada viral, el disseny de nous inhibidors de la fusió s'ha convertit en una de les àrees més esperançadores i debatudes en l'estudi d'inhibidors d' entrada. ADS-J1 ha estat seleccionat per la seva capaçitat d'unir-se a gp41 i inhibir la fusió de les membranes. A través de diversos assajos, incloent la generació de soques resistents a ADS-J1, el nostre laboratori va demostrar que ADS-J1 interactua amb gp120 i no amb gp41. Una publicació posterior va suggerir que ADS-J1 s'uneix a la 'pocket-region' de gp41, prevenint l'infecció pel virus. En el present treball, nosaltres confirmem que ADS-J1 interactua amb gp120 i no amb gp41 i que la recombinació de gp120 en un VIH silvestre restitueix el fenotip resistent. Assajos de temps de addició van demostrar clarament que ADS-J1 no interactua amb gp41.

VIRIP va ser identificat com un pèptid natural present en el hemofiltrat humà capaç d'inhibir la fusió de membranes operada per gp41 del VIH. Es va suggerir que VIRIP interactua amb el pèptid de fusió de gp41, bloquejant la fusió de les membranes. Amb el objectiu de determinar el mode d'acció de VIRIP, nosaltres hem generat un virus resistent a VIR-353, un anàleg de VIRIP. Addicionalment, hem determinat la combinació de mutacions que generen el fenotip resistent. Estudis recents van mostrar l'efectivitat de VIR-576, un pèptid amb alta similitud a VIRIP i VIR-353 en un assaig clínic fase I/II. La resistència a VIRIP/VIR-353 va requerir un període de temps llarg per emergir, la qual cosa suggereix una elevada barrera genètica a la resistència. Les mutacions responsables del fenotip resistent van afectar en greument la capacitat replicativa del virus, no obstant això, diverses mutacions compensatòries van restaurar-ne la capacitat replicativa, mantenint intacta la resistència a VIR-353. L'activitat antiviral de T20 no sembla afectada per VIR-353, la combinació dels dos inhibidors de fusió van mostrar un efecte additiu en la inhibició de la replicación. Malgrat que no vam poder determinar de manera precisa el lloc en el qual VIRIP/VIR-353 actua a gp41, els nostres resultats suggereixen que ha de ser diferent al lloc d'interacció que originalment s'havia postulat: el pèptid de fusió a gp41. En general, els nostres resultats evidencien la plasticitat de les glicoproteïnes de l'embolcall del VIH. Aquesta plasticitat es realça quan el virus replica sota la pressió selectiva imposada per fàrmacs que inhibeixen la replicació viral, la qual cosa afegeix una barrera genètica addicional a ser superada pel virus.

Summary.

There are several reasons to celebrate the latest advances in the treatment of the infection with HIV. According to the Joint United Nations Programme on HIV/AIDS, the number of new infections dropped by 15%; there is also a decrease by 22% in the number of deaths related to HIV/AIDS. Nevertheless, there are new emerging challenges, i.e. the transmission of drug-resistant HIV-1 strains. Therefore, there is a demand for the continued research for new and more potent antiretroviral agents. The entry of HIV into the cell implies a complex and well-orchestrated series of steps in which both viral and cellular molecules are implied, ending with the production of new viral particles. The HIV gp120 glycoprotein binds to the cellular CD4 receptor and to a chemokine receptor, inducing structural rearrangements that continue with the cellular and viral membrane fusion mediated by the HIV glycoprotein gp41. Hence, the entry of HIV is an essential step of the viral replication that offers an open path for the design of new antiviral compounds that could be added to the repertory of drugs used in the treatment of HIV infection. In coincidence with the recent and highly relevant information of the fusion mechanism occurring during the viral entry, the design of new fusion inhibitors has become one of the most promising and debated areas in the study of entry inhibitors. ADS-J1 was originally selected to bind to gp41 and to inhibit the fusion of membranes. In several assays, including the generation of HIV strains resistant to ADS-J1, our laboratory has proved that ADS-J1 interact with gp120 instead of gp41. A more recent publication suggested that ADS-J1 binds to the pocket region of gp41 preventing the infection by the virus. Here, we confirmed that ADS-J1 interacts with gp120 instead of gp41. Recombination of gp120 into a wild type HIV-1 backbone restored the resistant phenotype. Moreover, time of addition assays clearly demonstrated that ADS-J1 does not interact with gp41.

VIRIP was identified as a natural peptide present in human hemofiltrate that inhibits the HIV gp41-mediated membrane fusion. It was suggested that VIRIP interact with the fusion peptide in gp41, therefore blocking the fusion of membranes. With the objective to determine the precise mode of action of VIRIP, we generated a HIV-1 virus resistant to VIR-353, an analogue of VIRIP. Additionally, we determined the most relevant combination of mutations for the resistant phenotype. Recent studies have shown the effectivity of VIR-576, a peptide closely related to VIRIP and VIR-353 in a clinical trial phase I/II. The resistance to VIRIP/VIR-353 took a long time to emerge, suggesting a high genetic barrier to resistance. The mutations responsible for the resistant phenotype affected in large scale the replicative capacity of the virus, nevertheless, several compensatory mutations restored the viral fitness, while the resistance to VIR-353 was unaltered. The antiviral combination of VIR-353 and T20 showed an additive effect in inhibiting viral replication, indicating that VIR-353 appeared no to affect the binding of T20 to gp41 in its antiviral activity, the combination of the two fusion inhibitors showed an additive effect in inhibiting viral replication. Unfortunately, we could not precisely determine the site in which VIRIP/VIR-353 exerts its activity in gp41, nevertheless, our results suggest that it may be different than the one originally postulated: the fusion peptide of gp41. In general, our results evidence the plasticity of the HIV envelope glycoproteins. This plasticity is highly remarked when the virus replicates under drug selective pressure, which imposes an additional genetic barrier for the virus to overcome.

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1. General introduction.

1.1 The human immunodeficiency virus.

The human immunodeficiency virus (HIV) is the etiologic agent of the acquired immune deficiency syndrome (AIDS) (19, 98, 156). AIDS was identified in 1983 and is characterized by a drastic immunodeficiency, marked by a quantitative loss of CD4+ T cells and cells of the monocyte/macrophage lineage (54, 73).

HIV has been classified into the Lentivirus genus of the Retrovirus family. Retrovirus are so called because their RNA genome is transcribed into DNA inside the host cell using the viral enzyme reverse transcriptase (RT) and later, mediated by the viral enzyme integrase (IN), the retro transcribed DNA is integrated into the genomic DNA of the host cell as a provirus (47). The Lentivirus genus includes virus infecting primates, felids and a variety of wild and domestic mammals. In non-human primates these infections appear to be clinically silent (161).

1.2 HIV pandemic.

In humans, AIDS is caused by two lentiviruses: HIV-1 and HIV-2. While HIV-2 has been restricted to West Africa, HIV-1 has spread around the world. (200). Based on phylogenetic analyses primarily of the envelope genes of different isolates, HIV-1 has been classified in three groups: M, N and O. The group M (for Major) includes the majority of HIV-1 strains, and has been associated with the pandemic. Groups O (for outlier) and N (for non-M, non-O) are restricted to West Africa (48, 53).

UNAIDS reported that during 2009 the estimated number of persons living with HIV worldwide was 33.3 million (31.4 - 35.3 million), the number of new infections was of 2.6 million, and the number of deaths due to AIDS was 1.8 million. It is also estimated that during 2009 there were over 7000 new HIV infections each day, most of them occurred in the low and middle-income countries. Sub-Saharian Africa is the region of the world most densely affected by the pandemic, accounting for 67% of all the people living with HIV and more than 70% of the deaths derived from AIDS (http://www.unaids.org) (77). (Figure 1)

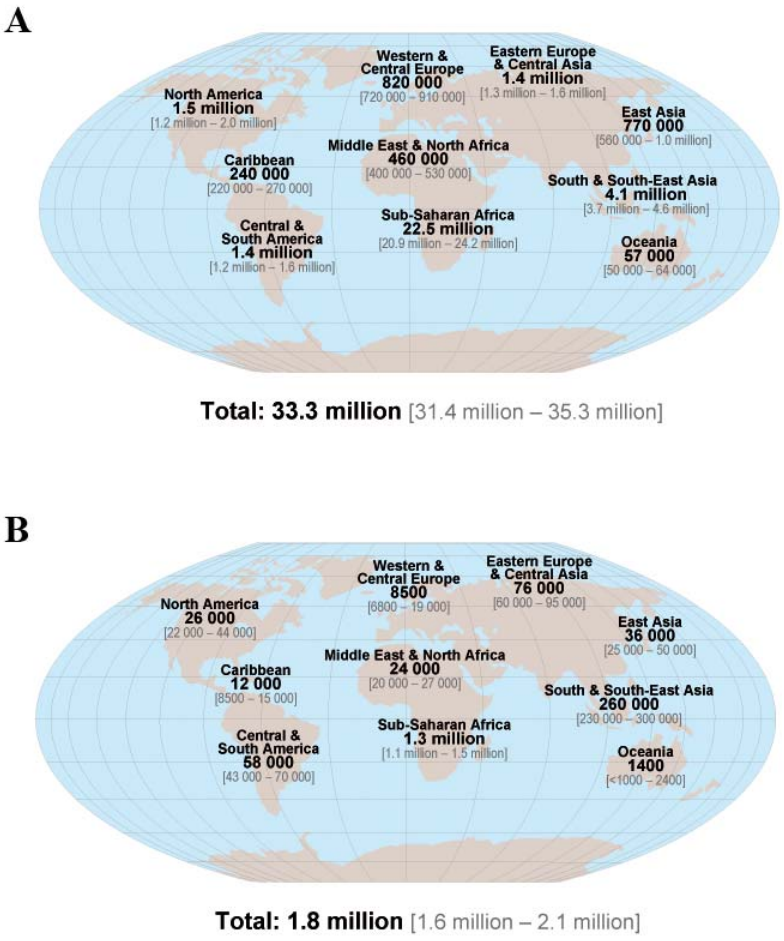


Figure 1. Summary of the AIDS pandemic in 2010. A) Estimated worldwide population who lived with AIDS. B) Estimated deaths from AIDS. Taken from UNAIDS (1)

1.3 Organization of the HIV genome and structure of the virion.

The HIV genome is composed of a RNA molecule of approximately 9.8 kilobases (kb) which comprises nine different open reading frames; three genes: *gag*, *pol* and *env* encode for the structural proteins Gag, Pol and Env, which are subsequently proteolyzed into individual proteins and are essential for the correct formation of the virion.

The *gag* gene encodes for four different proteins: MA (matrix or P17), CA (capsid or P24), NC (nucleocapsid or P7) and p6, these proteins are the structural components of the viral capsid that form the core of the virion (54). The enzymatic functions of the HIV are performed by the proteins codified by the *pol* gene: PR (protease or P11), RT (reverse transcriptase or P66/51) and IN (integrase or P32). Two glycoproteins: SU (surface or gp120) and TM (transmembrane or gp41) are the products of the *env* gene.

There are also the so called accessory genes *vif, vpr, nef, vpu* which codify for the proteins Vif, Vpr, Nef, Vpu, respectively and they are not essential for the replication of the viral particle (Figure 2).

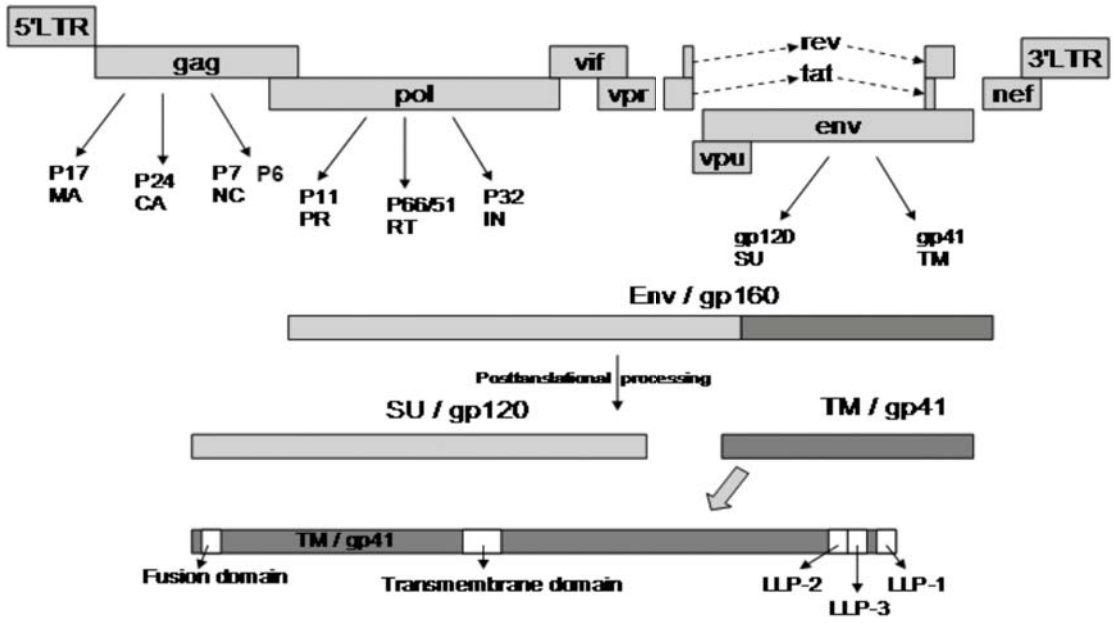


Figure 2 Schematic representation of the organization of the HIV-1 genome and the transcriptional splicing. Adapted from (50).

The HIV-1 particle has an icosaedral morphology (179) with an average size of approximately 145 nm (34). The envelope is formed by a lipidic bilayer, acquired from the host

cell, in which approximately 10 trimeric envelope glycoproteins (gp120 and gp41) are inserted (54). Inside the lipidic bylayer, there are the Gag proteins: MA, which is involved in the plasma membrane targeting of the Gag proteins and in the assembly of the virion. The CA protein is involved in the assembly of the virion. In the core formed by the CA protein are included the genome molecules and the viral proteins: protease, reverse transcriptase, integrase, as well as the viral accessory proteins. The NC protein is involved in the packaging of the viral RNA (33, 91, 99).

The budding and maturation processes of the virion initiates with the activity of the viral protease (PR) on the Gag polyprotein to produce the MA, CA, NC, p6, and the proteolytic activity over the Gag polyprotein generates the PR, RT and IN proteins (33, 90, 91, 99).

The Reverse Transcriptase (RT) catalyzes the synthesis of double-stranded DNA from the single-stranded viral RNA molecule (48). The ribonuclease H (RNase H) activity is also catalyzed by RT, the RNA from the RNA/DNA replication intermediate is degraded to make nascent strand DNA available as template for the synthesis of a new strand of DNA synthesis (103, 171).

Integrase (IN) catalyzes the integration of the reverse transcribed DNA of the virus into the DNA of the cell host (126). The integration process is essential for the replication of HIV for two important reasons. First, the DNA molecules form the virus are not able to replicate autonomously as episomes and the integration of the viral genome into the host cell chromosome, avoids its degradation. Second, the integration is important for an efficient transcription of viral DNA into new copies of the viral genome and mRNA that encode viral proteins (22, 48, 91, 126).

The Vif protein is required for productive *in vivo* HIV infection and for infection of primary CD4+ T cells, monocytes, and macrophages *ex vivo* (4). Vif acts by inhibiting the activity of APOBEC3G, a cellular cytidine deaminases protein with antiviral activity. APOBEC3G deaminates the cytidines of the DNA helix synthesized by the viral protein reverse transcriptase (4).

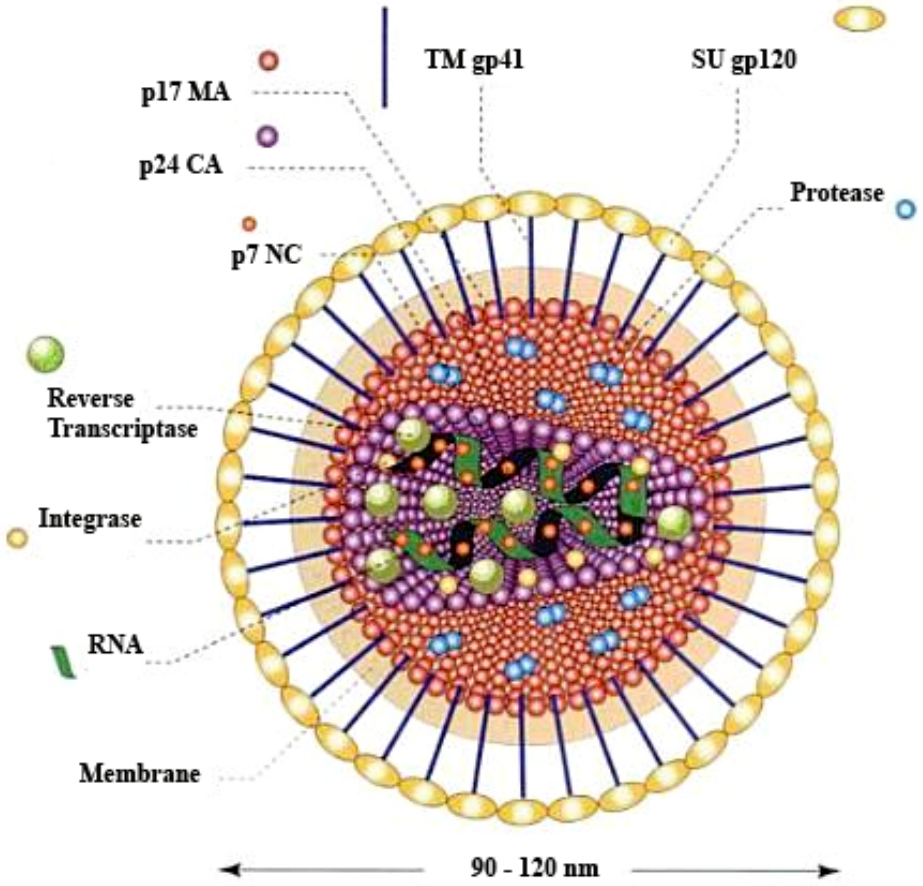
Vpr mediates many processes that aid HIV-1 infection, evasion of the immune system, and the persistence in the host, contributing to the morbidity and mortality of AIDS. The main function of Vpr is the import of the viral pre integration complex to the nucleus of the host cell, but it is also involved in the induction of the G2 cell cycle arrest and the transcriptional co activation of viral and host genes (204).

Vpu promote the degradation of CD4 which is in complex with Env glycoprotein (gp160), it is synthesized previously in the endoplasmic reticulum (ER), thus allowing Env transport to the cell surface (204). The CD4 receptor is rapidly degraded by the proteasome (221). Vpu also regulates the detachment of virions from the cell surface by down regulation of BST-2, also referred as tetherin, an interferon-inducible protein which restrict the release of virions (175).

Nef is a multifunctional accessory protein, necessary for the enhancement of the HIV-1 infectivity (90), this viral protein drastically reduces the level of CD4 on the cell surface following degradation via the endosomal/lysosomal under a clathrin-dependent pathway (204). Nef is also able to down-modulate the MHC class I molecules (198). This down regulation enables the infected cell to evade the destruction by the immune system during active viral replication (90).

The HIV-1 regulatory proteins Tat and Rev are RNA binding proteins (210). Tat is a transcriptional activator implicated in the activation of cellular genes such as TNF-β as well as in the inhibition of genes implicated in the apoptosis pathway (189, 210). The Rev protein is required for the replication of the viral proteins Gag, Pol and Env from the integrated proviral DNA and its transport from the nucleus to the cytoplasm (Figure 3) (34, 105).

A



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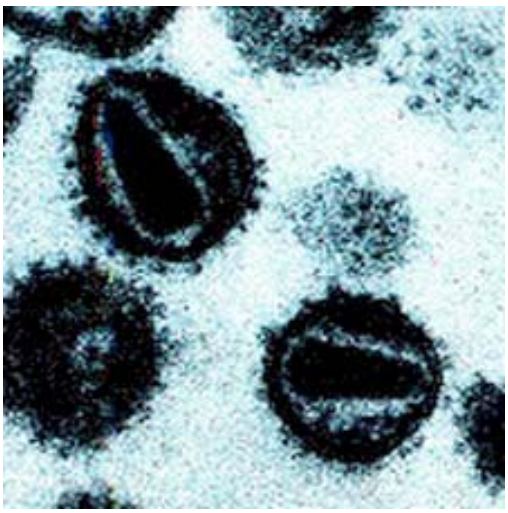

Figure 3 Representation of the structure of the HIV-1 virion. (A) Modified from (119) and (B) taken from www.virology.net.

1.4 Structure of the Env glycoproteins

The Env glycoprotein is composed by two non-covalently associated subunits: SU gp120 and TM gp41 (52), which are produced after the gp160 polyprotein is processed by the proteolysis by cellular furin or furin-like proteases in the Golgi apparatus (Figure 4) (54, 92).

After this cleavage, the interactions between gp120 and gp41 are maintained by non-covalent interactions and form heterotrimeric spikes on the viral surface (70, 141, 168). The study of the diversity of the *env* gene among HIV-1 isolates has uncovered a high variability in discontinuous segments within the gp120 glycoprotein which may arose through recombination and point mutations, as well as by insertions and deletions of nucleotides (51, 54, 56, 145). Env glycoproteins can exhibit up to 35% of amino acid diversity between subtypes and 20% within a subtype, being gp120 the most variable region (145). The Env glycoproteins play an essential role in the initial stages of the HIV-1 infection, mediating the attachment of the virion to the cells, the fusion of the viral and cellular membranes (52).

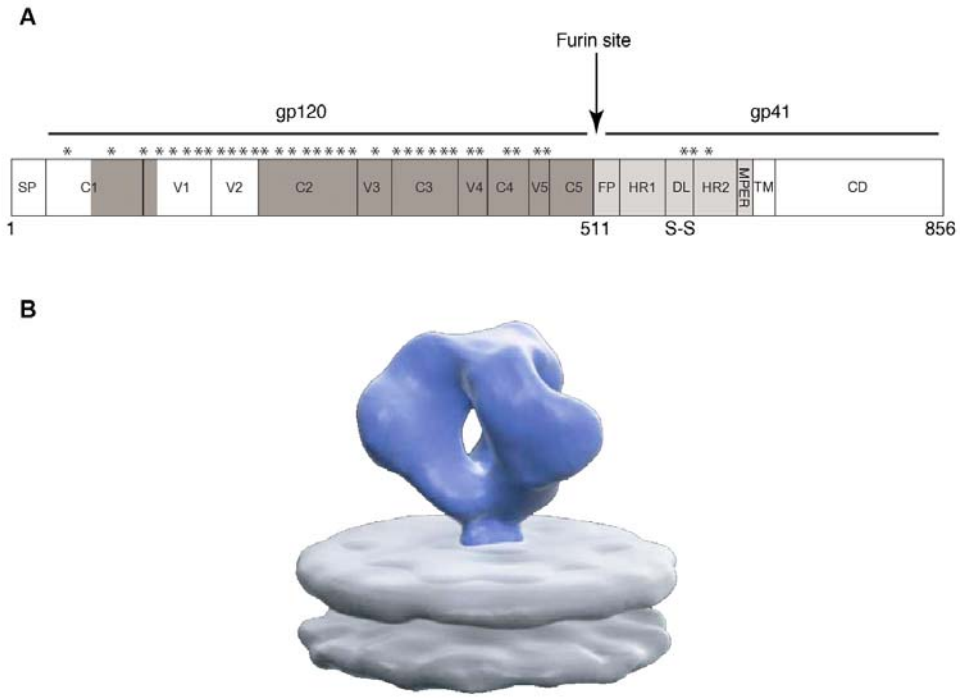

Figure 4. A) **Organization of HIV-1 envelope proteins**. Numbering corresponds to HIV HxB2 strain. Putative glycosylation sites are denoted by asterisks. Domain abbreviations SP, signal peptide; C1-C5, conserved domains 1 to 5; V1-V5, variable domains 1 to 5; FP, fusion peptide; HR1 and HR2, heptad repeat 1 and 2; DL, disulfide loop; MPER, membrane proximal external region; TM, transmembrane domain; CD, cytoplasmic domain. High resolution structural information available for gp120 (darker gray) and for gp41 (lighter gray) B) Representation of the 3D structure of the trimeric glycoprotein spike on native HIV-1. Adapted from (38) and (141).

1.4.1 gp120

The gp120 glycoprotein has five variable domains (V1-V5) interspersed with five relatively conserved domains (C1-C5) (38, 188, 222). The gp120 and gp41 proteins are highly glycosilated and alterations on the N-linked glycosilation patterns in the Env proteins alter the infectivity and cytopathogenicity of the virus (86, 139, 167, 212). It has been also demonstrated the essential role of glycosilation in HIV-1 survival and in immune evasion, by altering the sensitivity to neutralizing antibodies (195, 223).

The V1/V2 domain in gp120 has been shown to be determinant in the progression to AIDS. Chohan *et al* determined that some signatures in the V1/V2 loop of gp120 glycoprotein, maybe be relevant markers for the replication levels, the pathogenicity and the progression to AIDS. These hallmarks included an enlargement of the length of the V1/V2 domain and an increase in the number of predicted N-linked glycosilation sites that may promote the escape from the host immune response (51, 54, 56).

The V3 domain in gp120 is the major neutralizing domain of HIV-1. V3 is a loop-form region, composed of 35-40 amino acids, and has a high degree of sequence variability (145). V3 defines in great manner the specificity of the coreceptor used by the virion (CCR5 or CXCR4) to attach to the host cell (40, 121, 177). Freed *et al* showed that V3 is also involved in the fusion of viral and cellular membranes (94, 180).

Several crystallographic and mutagenic studies have evidenced gp120 as one of the glycopeptide structures with more plasticity in nature. Concerning to immune evation, the diversity of potential conformations of gp120, i.e. allowing the hiding of the CD4 binding site, which is target of neutralizing antibodies. Additionally, the flexibility of the spikes is related to the emergence of resistance to neutralization (183).

1.4.2 gp41

Fusion of the viral lipid envelope and the host cell membrane is mediated by the ~340 amino acid transmembrane (TM) glycoprotein. gp41 has a molecular mass of 41 kDa and is more conserved than gp120. The glycoprotein has three major functional domains: the extracellular domain (aa 512 to 683; numbering is based on HIV-1 HXB2), a transmembrane domain (aa 684 to 705), and a cytoplasmic tail (aa 705 to 856) (Figure 5) (168).

The extracellular domain (ectodomain) contains the regions that are directly involved in the membrane fusion process: a fusion peptide (FP), which is a hydrophobic region located at the N- terminal region of gp41 (aa 512 to 527); the heptad regions 1 (aa 546 to 581) and 2 (aa 628 to 661) (HR1 and HR2); a disulfide hydrophobic loop; a tryptophan rich domain which is known as membrane-proximal external region (MPER) (aa 660 to 683), and a transmembrane domain (TM) at the C-terminus of the protein. The intracellular part of gp41, called cytoplasmic tail is also involved in the fusion of the viral and cellular membranes (54, 96).

The 28 amino acid length hydrophobic region of gp41, where the fusion peptide (FP) is located, is relevant for the formation of syncitia, the transport and secretion of the envelope glycoprotein (93). The FP is transiently exposed while the interactions between gp120 and CD4 are taking place. Additionally, It has been proposed that the FP induces the destabilization of the cellular membrane which results in the membrane fusion (168).

The heptad repeat regions HR1 and HR2, also named N-helix and C-helix, or N-HR or C-HR respectively, are two leucine zipper-like 4-3 repeat regions forming the characteristic six-helix structures. The leucine zipper structure was originally identified as DNA-binding proteins.

In this type of proteins, a leucine is repeated every seven residues. In the heptad repeat regions, the amino acids have been labelled as *a, b, c, d, e, f,* and *g*. At position *a* in the HRs, there is a hydrophobic amino acid, including a valine, isoleucine, in addition to leucine (97, 191). The hydrophobic interactions between the hydrophobic amino acids in HR1 and HR2 give stability to a dimer of the helix structure. Nevertheless, some hydrophilic amino acids also conform the six-helix bundle (52, 217). This region forms a helical trimer of antiparallel dimers (144). The six-helix bundle conformed by the heptad repeats is the core of gp41 (Figure 5B) (217).

Three N peptides are arranged in helices of the trimeric-coiled coil. The C peptides form outer helices that are packed into the highly conserved hydrophobic grooves of the coiled coil, in an anti parallel manner (52). The arrangement of the six-helix bundle provides the energy necessary to set closeness of the membranes and to drive the fusion of the viral and host cell membranes (Figure 5B) (154, 217).

The membrane-proximal external region (MPER) is highly conserved among HIV-1 strains and is characteristically enriched with hydrophobic residues, mainly tryptophan (Trp). It has been observed that mutation of these Trp reduced the amount of gp41 incorporated to virions (217). MPER has been shown to be essential for the fusion process. It was also demonstrated that the MPER domain interacts with the lipids of the membranes (i.e. cholesterol) by acting as signal for targeting lipid domains and promoting the fusion of the membranes (213).

The MPER has received major attention since the isolation of the three broadly neutralizing antibodies (bNtAb) 2F5, 4E10, and Z13. These neutralizing antibodies recognize epitopes in the membrane proximal external region, blocking the infection of a wide variety of HIV-1 clades, most probably by interfering one of the determinant steps in the entry of the virus to the cell. It was also demonstrated that a domain of the MPER interacts with the lipids of the membranes by acting as signal for targeting lipid domains and promoting the fusion of the membranes (194, 213).

The transmembrane region (TM) anchors the Env glycoprotein into the lipid membrane. Currently it is not completely understood if the TM has a direct role in membrane

fusion (168). Due to the hydrophobic nature of this region, it has not been elucidated due to problems with the expression and purification of this kind of proteins.

The gp41 cytoplasmic tail (CT) is approximately 150 aa in length and is involved in the conformation of gp120 and the gp41 ectodomain (72, 203) nevertheless, the biological functions of this domain of gp41 are diverse and the complete understanding of the different roles *in vivo* such as cell to cell transfer, host interacting proteins remains to be addressed (168).

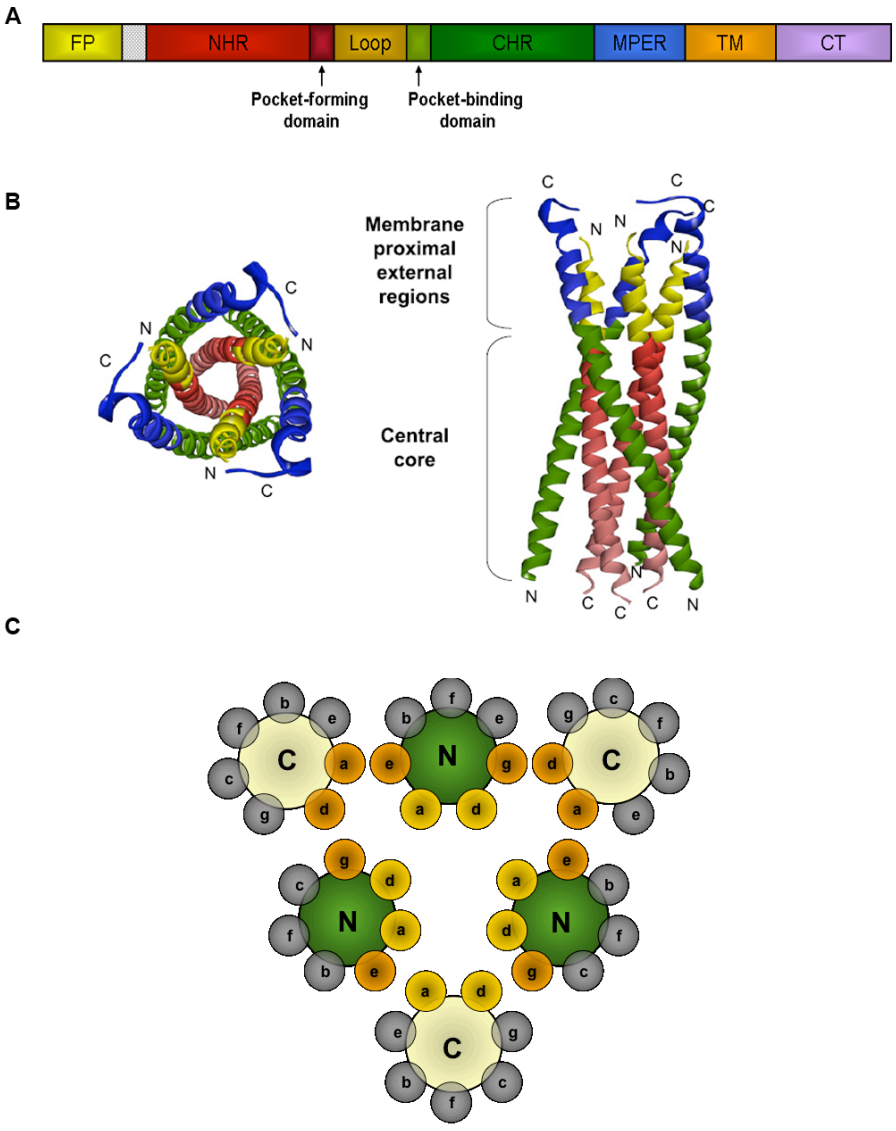

Figure 5. A) **Organization of the gp41 subunit in the HIV-1 Env gene**. Fusion peptide (FP), N-terminal Heptad Repeat (NHR), C-terminal Heptad Repeat, membrane proximal external region (MPER), transmembrane domain (TM), cytoplasmic tail (CT) B). Top and lateral views of the hairpin structure of gp41. C) Cross-sectional view of the HIV gp41 six-helix bundle. Heptad-repeat residues are shown to indicate positions in the heptad that contribute to six-helix bundle stability. '*a*' and '*d*' positions in N-heptad are especially important for stabilizing trimeric, coiled-coil core, while residues in the '*e*' and '*g*' positions in N-heptad frequently interact with residues in the '*a*' and '*d*' positions in the C-heptad. Modified from (16) and (216)

1.5 Cellular factors involved in the attachment of the virus to the host cell.

1.5.1 CD4

HIV-1 is able to actively infect different cell types: CD4+ T lymphocytes, dendritic cells and macrophages (54). These cell types express the CD4 surface receptor, a transmembrane glycoprotein that is a member of the immunoglobulin super family. CD4 binds to histocompatibility class II acting as a co-receptor for the T-cell antigen receptor (TCR) (class II MHC) (31, 188). HIV-1 utilizes the CD4 receptor to anchor and enter the host cell. The presence of CD4 in the cell surface is necessary but not sufficient for the virus to enter the cell (60, 211).

1.5.2 Chemokine receptors

Although CD4 is the primary receptor for the attachment of HIV-1 to the host cell, the virus also utilizes the chemokine receptors CCR5 or CXCR4 to bind to cells (Figure 6) (7, 20, 85). The chemokine receptors CCR5 and CXCR4 have received this classification based on the molecule that are sensing. The nomenclature of the chemokines was assigned according to the number and orientation of N-terminal cysteine motifs. C Chemokines have a single cysteine residue, C-C chemokines, C-X-C chemokines and C-X3-C chemokines have 0, 1, or three cysteine residues, respectively. The C-C and C-X-C are the only chemokines related with HIV-1 infection (173).

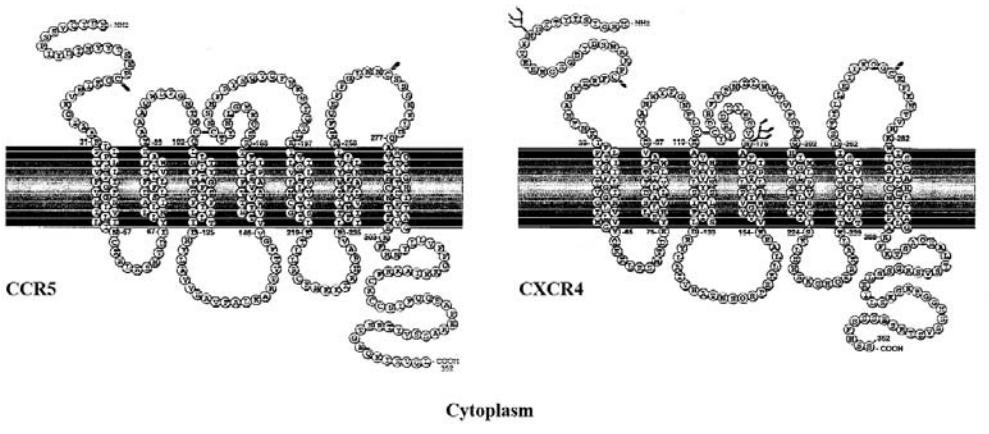

Figure 6. Representation of the predicted membrane topology of the HIV-1 coreceptors CCR5 and CXCR4. Adapated from (66)

Viruses that use CCR5 coreceptor to enter the cell are named R5-viruses, while the virus that use CXCR4 to attach to the cell are named X4 viruses (102). These receptors belong to the family of seven-transmembrane G-protein coupled receptors (GPCRs). The GPCRs induces signal cascades when small and frequently specific ligands are bound to these receptors (32). The ligands of CCR5 are the chemokines CCL3, CCL4 and CCL5. The natural ligand of CXCR4 is SDF-1/CXCL12 (6, 178). The ligands of CCR5 and CXCR4 are suppressors of the HIV-1 infection.

1.6 HIV-1 coreceptors and viral tropism.

The tropism of a HIV-1 strain refers to the ability of the virus to establish infection in different CD4+ cell types and is determined by the cell coreceptor usage (102). In the early and chronic stages of infection, the CCR5-using viruses are preferentially transmitted. In the late phases of infection, and in approximately 40% to 50% of infected individuals, a switch in the coreceptor usage precedes the progression to AIDS, from R5-viruses to virus using CXCR4 or both CCR5 and CXCR4 viruses (duo-tropic virus) (170) although many patients progress to late stages of infection harbouring exclusively R5 viruses (Figure 7) (85).

Although the complete molecular mechanisms involved in coreceptor switching are not totally understood, it is known that amino acid mutations and deletions in the V3 and V1/V2 loops in gp120 of HIV-1 subtype B are required for coreceptor switching (46, 85, 176). Additionally, the loss of a N-linked glycosilation site in V2 reduced the ability to infect by late stages CCR5-using viral isolates (28) and an increase in the positive charge in V1/V2, V4/V5 resulted in the reduced sensitivity to entry inhibitors (176, 186).

The finding that subjects that are homozygous for the CCR5Δ32 allele are protected from HIV-1 infection (117, 196) prompted the development of small molecule inhibitors of CCR5, ending with the licensing the clinical use of Maraviroc in 2007 (220).

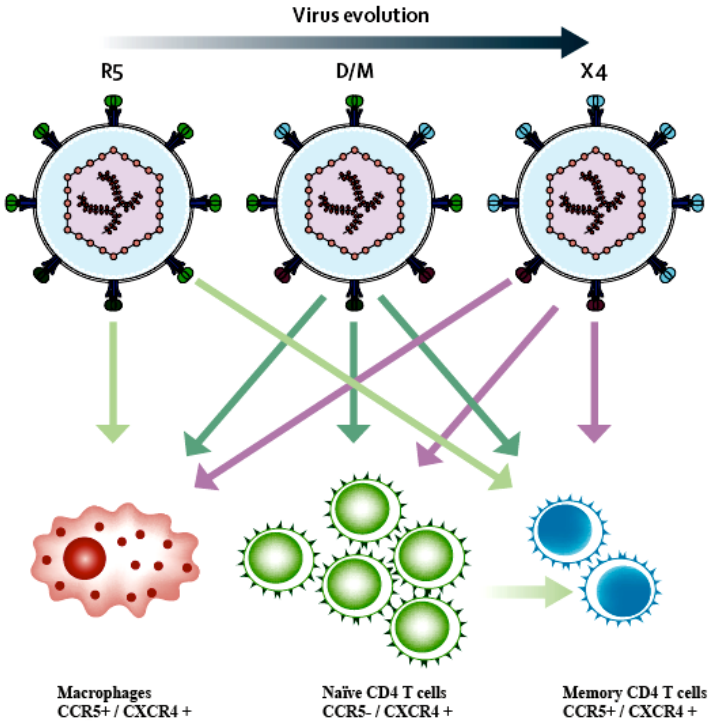

Figure 7. **Schematic representation of the evolution of the coreceptor switching**. CCR5- tropic strains can infect macrophages and memory CD4+ T cells. Duo-tropic strains can utilize both CCR5 and CXCR4 coreceptors. X4-tropic strains can infect macrophages and CD4+ T cells. From (85).

1.7 The HIV-1 entry and membrane fusion processes.

The current model for the entry of HIV-1 into the host cells involves multiple steps involving both cellular and viral factors. Unspecific interactions between positively charged domains of gp120 and negatively charged proteoglycans in the cell membrane may be the first events in the entry process (206). As CD4 is the primary receptor for HIV, the HIV gp120 glycoprotein interacts with a conserved region in the N-terminus of CD4 (226), this interaction triggers the reconfiguration of the gp120 glycoprotein, in which the V1,V2 and V3 loops are exposed and the binding of gp120 to the N-terminus of the coreceptor -CCR5 or CXCR4- takes place (132).

Binding of gp120 to the coreceptor produces rearrangements in the envelope glycoprotein such as the exposure of the fusion peptide, which insert and destabilize the cell plasma membrane. After the insertion of the FP, several energetically-favorable rearrangements produce the formation of hairpins by the heptad repeat domains 1 and 2 (HR1 and HR2), which are arranged into a coiled-coil and set the transmembrane protein close to the fusion protein which ultimately bring in close proximity the cellular and viral membranes, the formation of the pore occurs and the viral capsid is able to enter the cell (Figure 8) (52, 154, 217).

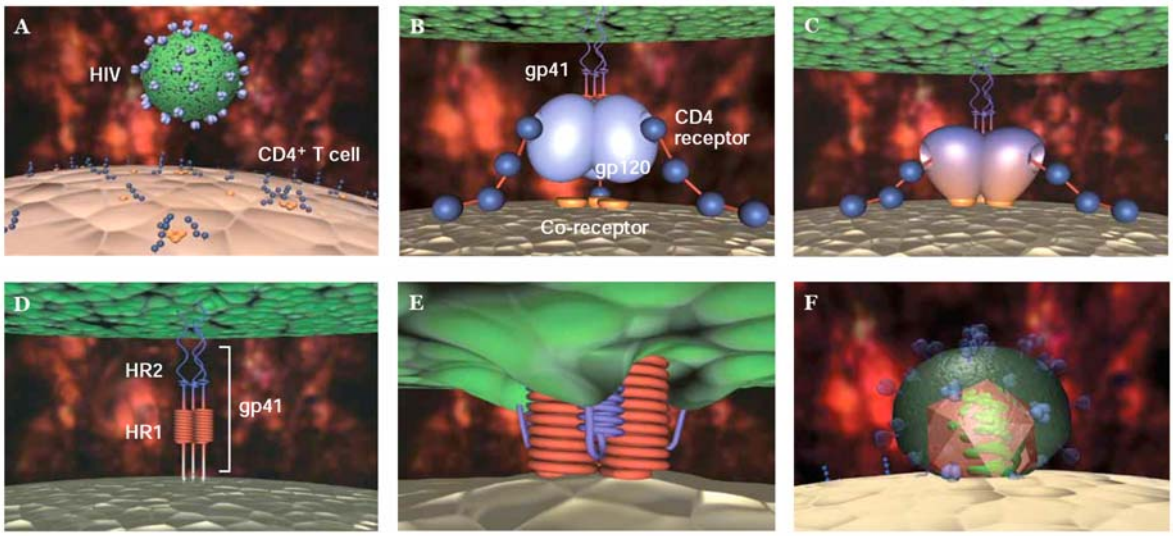

Figure 8. **Schematic representation of the entry of HIV-1 into the host cell**. A-B) HIV gp120 interacts with the CD4 receptor. C) The interaction of gp120 with the coreceptor triggers major conformational changes of the Env glycoprotein allowing the insertion of the fusion peptide. D-F) Arrangements of the HR1 and HR2 bring together the viral and cellular membranes and ultimately the membrane pore is formed. Modified from (150).

1.8 Overview of the replication cycle of HIV-1.

The HIV-1 replication cycle is a multi-step process that depends on both viral and host cell factors (3). It starts with the binding of gp120 to CD4, this process trigger a conformational change in gp120 in which the affinity for the coreceptor CCR5 or CXCR4 is enhanced. Further conformational changes in both gp120 and gp41 provoke the fusion of the viral and cellular membranes which ultimately takes to the uncoating of the virion and its delivery into the cytoplasm (153). The viral core that is composed by the capsid protein (CA) that encapsidate the dimeric, single-stranded viral RNA which is in complex with the nucleocapsid (NC) and the viral enzymes reverse transcriptase and integrase (RT and IN respectively) (3).

HIV-1 enters host cells by two different mechanisms: by direct attachment and fusion with the viral membrane or by endocytosis mediated by clathrin, followed by fusion with the membrane in the endosome (162).

Following the entry into the cytosol, the viral RNA is reverse transcribed into double-stranded DNA by the RT. The viral DNA is translocated across the nuclear pore and once in the nucleus, the IN catalyses the integration of the DNA into the host cell chromosomal DNA.

The integrated DNA is transcribed into viral RNAs, which are transported to the cytoplasm for being translated. A new viral particle is assembled with the newly synthesized viral proteins and two molecules of unspliced viral RNA. The PR cleaves the Gag and GagPol polyprotein precursors as the viral particle is released from the infected cell, which turns the immature particle into a mature virion, which can initiate a new cycle of infection (48) (Figure 9).

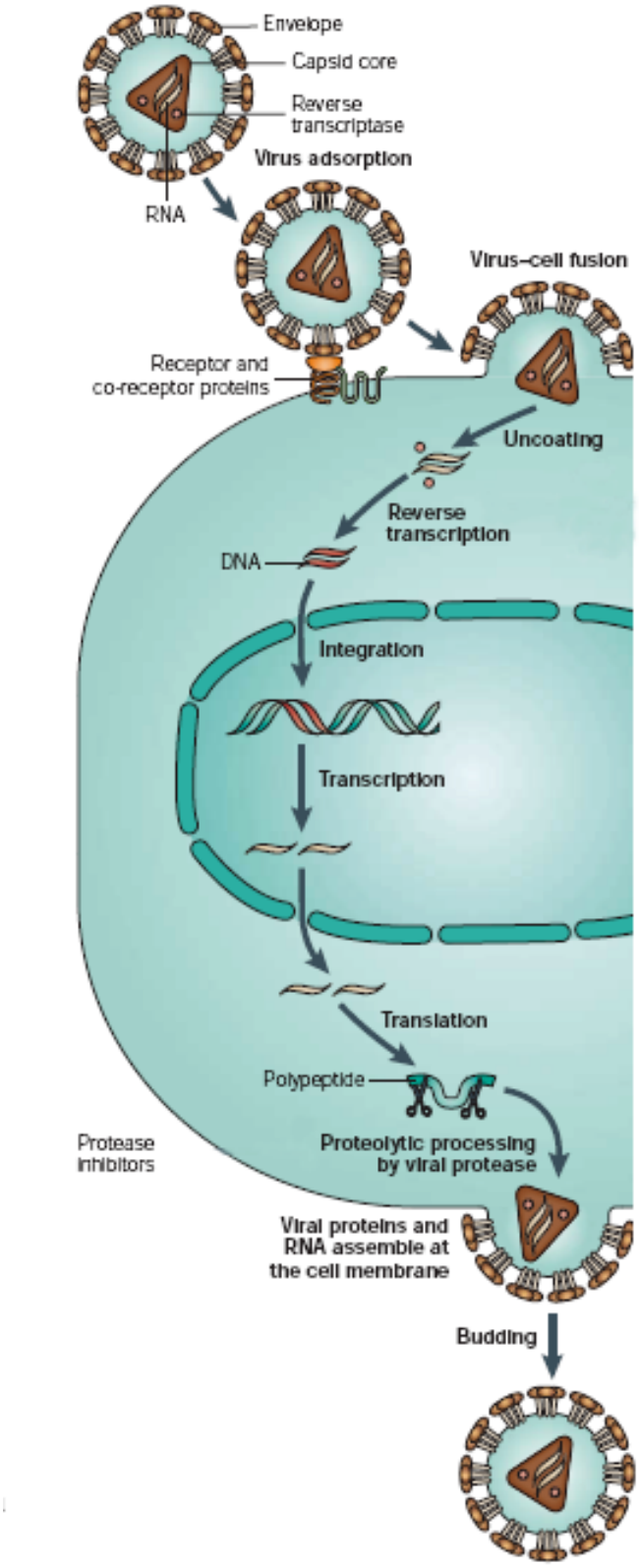

Figure 9. Schematic representation of the replication cycle of HIV-1. Modified from (150)

1.9 Time-course of the infection with HIV-1.

The infection with HIV-1 is characterized by three clinical stages: acute viremia, a latency phase, and the AIDS phase (50, 69).

The first phase is characterized by a high viral load, a sharp drop in peripheral blood CD4+ T cell count (49, 197), the development of HIV-1 specific immune response and the establishment of a reservoir of latently infected CD4+ T cells (57).

The chronic infection phase is characterized for being generally an asymptomatic condition, which can last in average ten years. A drop in the viral load is observed, and a slight recovery of the peripheral blood CD4+ T cell count which then begin to fall slowly again. This stage of infection is also marked by a high level of activation of CD4+ and CD8+ T cells (138), the viremia count go up slightly and the depletion of T cells is subtle but continued.

As peripheral blood CD4+ T cells count decline to less than 200 cells/μl, the AIDS stage of the infection is present: the immune system is collapsed and the so called opportunistic infections and tumours are evident (Figure 10).

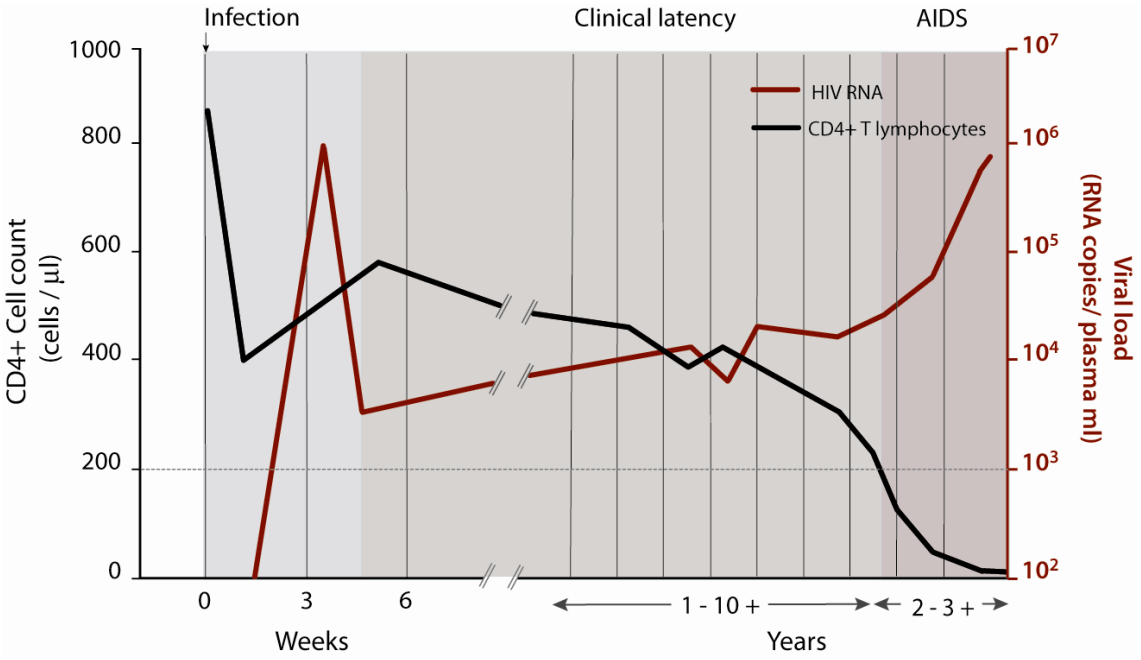

Figure 10 Diagram representing the time course of the infection by HIV-1. Correlates the evolution of the peripheral blood CD4+ T cell count and the viral load. Adapted from (50).

1.10 Brief overview of the current status of the antiretroviral drugs and HIV-1 treatment.

The implementation of highly active antiretroviral therapy (HAART) in high-income countries, which started in 1996 (43), has yielded an increase in the life expectancy among HIV-infected patients (2). Nevertheless, these benefits have not reached all the infected people around the world; especially those living in low and middle-income countries where the number of new HIV infections is over 5000 each day. In addition, recent economic crises menace to cut down the funds for prevention programmes, patient care treatments and research on HIV/AIDS.

Over the years, HAART has been gradually evolved from drug regimens with more than 20 pills daily in 1996, to three pills daily in 2003, to two pills daily in 2004, and finally to one pill daily in 2006 (43).

The objective of the antiretroviral therapy is to achieve a sustained suppression of the viral replication: below the level of detection of the most sensitive diagnostic tests currently approved (less than 50 viral RNA copies/ml) and in this manner to allow the reconstitution of the immune system by the increase of CD4+ cells and reduce the emergence of HIV-1 resistance (77).

Nowadays there are more than 30 antiretroviral drugs, single agents and multiple FDCs (fixed dose combinations) approved for clinical use by the United States of American FDA (Food and Drug Administration) (174). Based in the knowledge of the molecular mechanisms involved in each step of the replication cycle of the virus the antiviral drugs are classified as follows: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), CCR5 coreceptor inhibitors (CIs), fusion inhibitors (FI), integrase inhibitors (INI). Most of the licensed drugs are RT or protease inhibitors (43, 156) (Table 1 and Annex V).

Table 1. Anti HIV-1 drugs approved by the USA FDA. Adapted from Naeger, DeClerq and Paredes

Brand name®	Generic name	Mechanism of action	USA FDA approval year
	Nucleoside reverse transcriptase inhibitor (NRTI)		
Retrovir®	Zidovudine (AZT)	NRTIs are chain terminators. They are incorporated into the nascent chain of viral DNA. Because they lack a 3 hydroxyl group, no additional nucleotides can be appended.	1987
Videx®	Didanosine (ddI)		1991
Hivid®	Zalcitabine (ddC)		1992
Zerit®	Stavudine (d4T)		1994
Epivir®, Zeffix®	Lamivudine (3TC)		1995
Combivir®	Lamivudine + Zidovudine		1997
Ziagen®	Abacavir (ABC)		1998
Trizivir®	Abacavir + Lamivudine + Zidovudine		2000
Emtriva®	Emtricitabine ((-)FTC)		2003
Epzicom®	Abacavir + Lamivudine		2004
	Nucleotide reverse transcriptase inhibitor (NtRTI)		
Viread®	Tenofovir disoproxil	NtRTIs are chain terminators. They are incorporated into the nascent chain of viral DNA. Because they lack a 3 hydroxyl group, no additional nucleotides can be appended.	2001
Truvada®	Tenofovir disoproxil fumarate + Emtricitabine		2004
Atripla®	Tenofovir disoproxil fumarate + Emtricitabine + Efavirenz		2006
	Protease inhibitor (PI)		
Norvir®	Ritonavir	PIs mimic the structure of the natural viral substrates of the HIV PR, competing with them for binding to active site of the enzyme.	1996
Crixivan®	Indinavir		1996
Viracept®	Nelfinavir		1997
Fortovase®	Saquinavir Soft Gel Cap		1997
Agenerase®, Prozei®	Amprenavir		1999
Kaletra®	Lopinavir + Ritonavir		2000
Reyataz®	Atazanavir		2003
Lexiva®	Fosamprenavir		2003
Aptivus®	Tipranavir		2005
Prezista®	Darunavir		2006
	Non-nucleoside reverse transcriptase inhibitor (NNRTI)		
Viramune®	Nevirapine	Small molecules with high affinity for a hydrophobic pocket near the catalytic domain of HIV RT. Binding of the inhibitor alters the flexibility of the enzyme, impeding the synthesis of DNA.	1996
Rescriptor®	Delavirdine		1997
Sustiva®, Stocrin®	Efavirenz		1998
Intelence®	Etravirine		2008
	Fusion inhibitor (FI)		
Fuzeon®	Enfuvirtide (T20)	T20 is a 36-mer synthetic oligopeptide. Binds to the trimeric HR-1 complex, preventing the association of HR-1 with HR-2 and inhibiting fusion.	2003
	Co-receptor inhibitor (CRI)		
Selzentry®	Maraviroc	The conformational state of the CCR5 receptor is altered by the antagonist, inhibiting the binding of gp120 to CCR5 by an allosteric mechanism.	2007
	Integrase inhibitor (INI)		
Isentress®	Raltegravir	DNA strand-transfer inhibitors block the joining of the processed viral DNA ends into the host chromosome.	2007

The guidelines for the treatment of HIV-1 infected adults recommend to start the anti HIV-1 treatment with two NRTIs and a NNRTI or a PI (9, 156), this strategy is assumed after considering several factors as: transmitted resistance, pill burden, tolerability profile and long-term toxicity, among others (9).

The latest updates of the guidelines tend to recommend an earlier start of the antiretroviral treatment after weighing the potential risks compared to the benefits of that decision, to be taken when the CD4 counts drop below 350 cells/μl. Some of the benefits considered for advancing the start of the antiviral therapy are not only the reduction of the AIDS-related symptoms but also the reduction of cardiac, hepatic and renal diseases (9, 77).

Unfortunately, adjustments in the treatment regimens are the common rule in the treatment of HIV-1, and these are due to several reasons being the emergence of resistant viruses one of the major factors contributing to therapy failure. HIV has a high rate of mutagenesis (~10^{-4} to 10^{-5} mutations per nucleotide and cycle of replication) and approximately 10^{9} viral particles are produced every day (156). HIV-1 resistance is a fundamental strategy of the virus for its survival (185) and is caused by the emergence of mutations in its genome due to the selective pressure imposed by a drug.

Fortunately, physicians from the middle and high-income countries have a wide range of options of anti-HIV drugs for designing new combination therapies that can effectively suppress viral replication in the context of the emergence of resistant strains.

The guidelines for the treatment of HIV-1 infection have been evolving during the last years according to the experience gained from the clinical trials and cohorts (9). Nevertheless, the implementation of new drugs in the treatment of patients opens new windows of opportunity for new treatment regimens and for reaching major success of HAART but simultaneously, new challenges are imposed such as the drug-drug interactions, the improvement of the adherence to treatment via the simplification of the regimens, the reduction of the side effects of the new drugs, and to guarantee the access to HAART in the resource-limited countries.

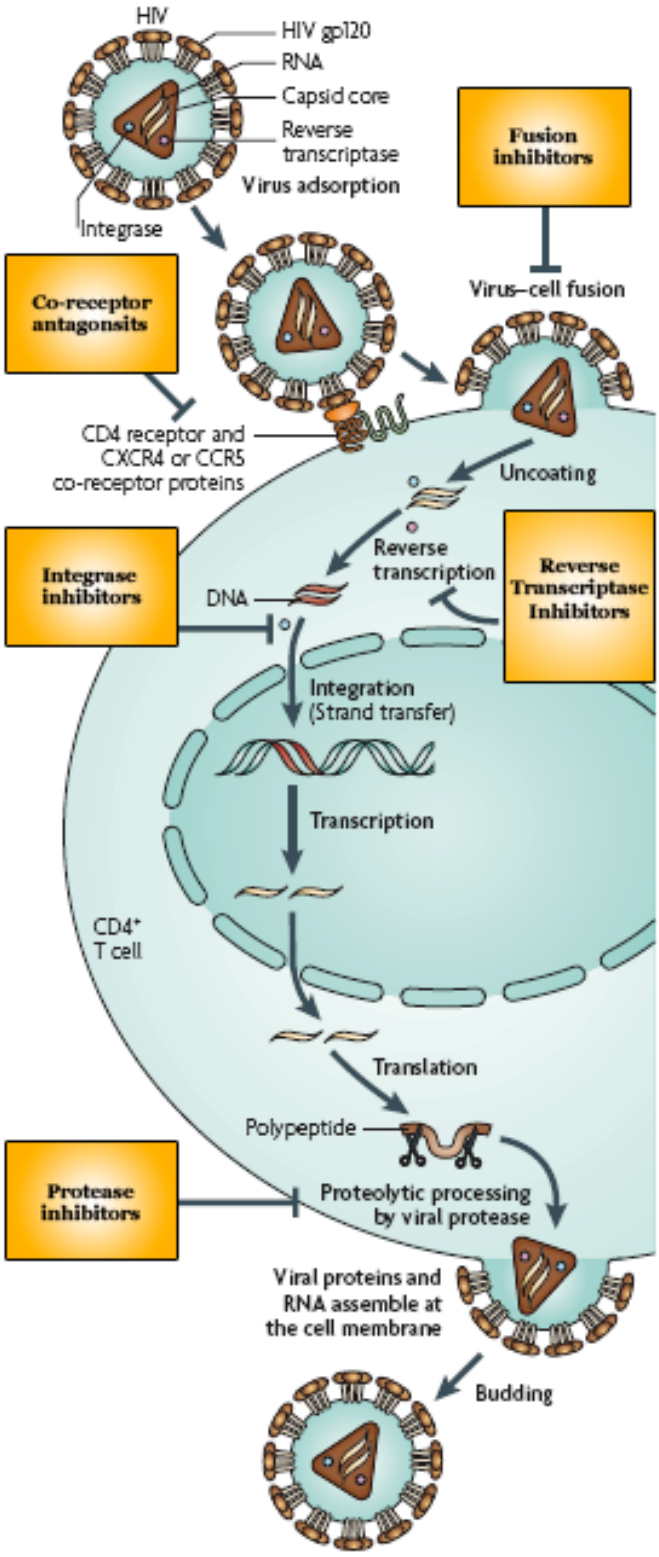

Figure 11. Diagram representing the life cycle of HIV-1 with the targets for therapeutic intervention highlighted. Modified from (43).

1.11 HIV-1 entry inhibitors.

The treatment of HIV with HAART has been successfully in many patients but due to several disadvantages in the treatment regimes such as the long-term toxicity, the emergence of drug resistant strains, and the drug-drug interactions; the need to identify new drugs that effectively inhibit the viral replication is mandatory (85).

Entry of HIV into the cells is a multi-stage and complex process which can be intervened by pharmacological compounds in order to inhibit the replication of the virus since the very beginning of the cycle, abrogating the necessity for the drugs to cross the cellular membrane (130). Recent advances in the understanding of the viral entry process via structural biology studies, mutagenic analyses and computational design, are opening new targets for therapeutic intervention (38).

The HIV entry inhibitors discovered to date are divided in three classes: drugs targeting the interaction between gp120 and CD4, drugs blocking gp120-coreceptor interaction, and drugs that block the membrane fusion mediated by gp41.

1.11.1 Blocking the gp120-CD4 interaction.

The evidence that relatively high concentrations of soluble CD4 (sCD4) can inhibit the entry of laboratory-adapted HIV-1 strains led to the design of several molecules that mimic the inhibition process. The first generation of sCD4 failed to inhibit the viral entry *in vivo*, and this was due to the inability to neutralize primary isolates of HIV, while in the other hand, the capacity to enhance viral replication at low concentrations. The concentration of sCD4 required to neutralize primary isolates *in vitro* was up to 1000-fold higher than laboratory-adapted strains (15, 16, 58, 114). A second generation of sCD4 derivatives effectively inhibits HIV-1 replication by changing the conformation of the gp120 glycoprotein to a non-functional molecule (15, 123, 149, 206).

BMS-378806 (referred as BMS-806) is part of a small-molecule family including BMS-488043 and VIL/JC095 (BMS-155), and was designed to block viral entry (140, 206). Although the mechanism of action of these compounds remains imprecisely defined, some studies suggested that BMS-806 target the binding site to CD4 in the gp120 glycoprotein. The BMS compounds inhibit X4 and R5 viruses, and Lin *et al* suggested that BMS-488043 inhibit gp120-CD4 interaction by changes in the conformation in the CD4 and CCR5 binding regions (114).

1.11.2 Blocking the gp120-coreceptor interaction.

The human chemokine receptors CCR5 and CXCR4 are attractive targets for the design of small-molecules that inhibit HIV-1 infection. The observation in a group of patients heterozygous for CCR5: Δ32-ccr5, which implied high-level resistance to HIV-1 infection, led to major studies to find molecules capable to block of HIV-1 entry by CCR5 coreceptors (143, 196).

The naturally occurring chemokines CCL3 (MIP-1), CCL4 (MIP-1) and CCL5 (regulated on activation, normal T-cell expressed and secreted, RANTES) have antiviral activity by binding to CCR5, inducing its internalization from the cell surface and therefore, inhibiting the infection by CCR5-using HIV-1 strains (45). Some N-terminal modified analogues of RANTES such as PSC-RANTES and others are now being developed as microbicides (42).

The natural ligand of CXCR4 is the chemokine stromal cell-derived factor 1 (SDF-1), which inhibit the infection by HIV-1 CXCR4 tropic viruses (26, 178). Small-molecule CXCR4 antagonist were developed, being AMD-3100 the most representative (74). AMD-3100 is a bicyclam analogue that showed high *in vitro* activity against X4-using strains (61, 78). The development of this compound and other derivatives as an antiviral agents was halted due to a lack of significant viral load reduction and cardiac tissue alterations (112).

ADSJ-1 is a non-peptidic compound selected by computational docking supposedly as a potential inhibitor of the membrane fusion mediated by gp41 (64); instead, we have found that ADSJ-1 inhibited the replication of HIV-1 at a time/site similar to agents blocking gp120. Molecular modelling suggested the gp120 V3 loop as the preferential binding site for ADSJ-1 (148). Moreover, strains resistant to ADSJ-1 showed mutations in gp120 coding sequence, mainly in the V3 loop confirming ADSJ-1 as a gp120-blocking agent (12, 38, 100).

1.11.3 Blocking the gp41-mediated membrane fusion.

During the course of natural infection by HIV-1, a large amount of antibodies (Abs) are generated. Due to the exposure on the viral surface, the Env glycoproteins gp120 and gp41 are the principal targets of these antibodies, and it has been suggested that the selective pressure exerted by theses antibodies account for the extensive variability of *env* in comparison to other HIV genes (192). However, since HIV-1 accounts with many strategies to escape to the susceptibility of neutralization by antibodies, i.e. high levels of viral replication and mutation

rates, the shedding of Env glycoproteins, decoy forms of HIV Env; the appearance of escape mutants is frequently observed (166). Then, the immune system produces new Abs to counteract viral escape mechanisms.

A very few amount of the antibodies produced by the immune system have the ability to inactive the virus in a highly specific manner and are called broadly neutralizing monoclonal antibodies (bNtMAbs). Eventually, a vaccine with Env as target could potentially block the infection with HIV-1 by eliciting bNtMAbs (131). So far, few bNtMAbs have been discovered for gp120, while only three for gp41 (2F5, Z13 and 4E10) (152, 202, 208, 209).

Most of the gp41 structure is occluded by gp120 in the native viral spikes. Nevertheless, gp41 may be briefly exposed during different stages of the fusion process. This is reflected by the strong reactivity of the Ab against gp41 (115).

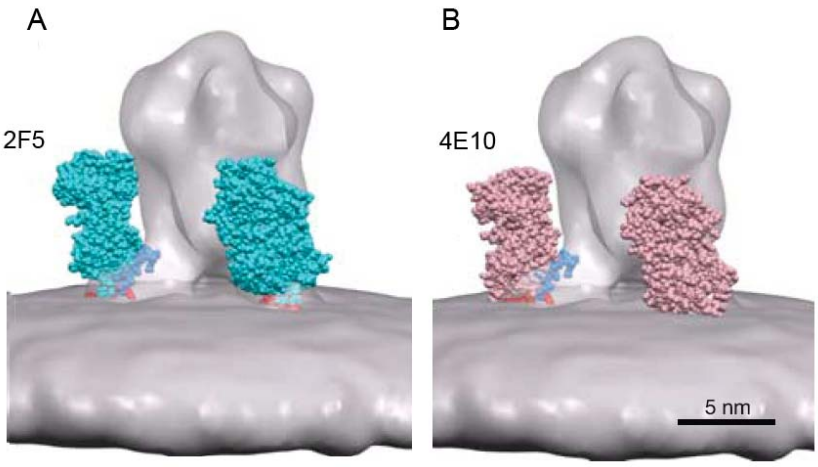

Figure 12. Model of the Envelope spike showing the proposed placement of the broadly bNtAbs 2F5 and 4E10 over MPER of HIV-1 gp41. Modified from (227).

Enfuvirtide (also named ENF, T20, Fuzeon) is the first anti-HIV drug targeting the fusion of membranes mediated by gp41, approved by the FDA and recommended for treatment-experienced patients, but because of the lack of bioavailability, it has to be administered via subcutaneous injection. T20 is a 36 amino acid, linear synthetic peptide, discovered during research aimed to vaccine development (150). The T20 sequence is identical to part of the CHR region of gp41, overlapping both the CHR and MPER.

T20 binds to the gp41 coiled-coil in NHR and form a peptide/protein hybrid structure, blocking the six-helix bundle formation and preventing the progression from a pre-hairpin to a hairpin conformation finally resulting in inhibiting the virus-cell membrane fusion (Figure 13) (127).

T20 imposes a low genetic barrier to the emergence of HIV-1 resistant strains, after few weeks under treatment with T20, mutations were introduced in the highly conserved amino acid motif of the NHR domain at positions 36-41 (GIVQQQNNLL) in gp41, being the most common sites of mutations the position 36 (G36E, G36D, G36S) and 38 (V38A, V38G, V38M) of gp41 (43, 193).

The research for second and third generation of fusion inhibitors with improved efficacy and pharmacokinetics has been ongoing, considering as a major objective the activity of these new inhibitors against T20-resistant strains (122).

T-1249 is a second-generation peptidic fusion inhibitor engineered to give more stability to the interactions between the heptad repeats HRs. The structure of T-1249 includes a lipid-binding domain in its sequence, which is also present in T20. Additionally T-1249 includes a pocket-binding domain, also present in C34, another fusion inhibitor peptide with a sequence similar to the CHR (11, 52, 190). T-1249 has shown greater efficacy and longer half-life than T20. Additionally, T-1249 showed antiviral activity against T20-resistant strains (75, 133). Nevertheless, the development of T-1249 was halted by the pharmaceuticals, arguing challenges with the formulation of the antiviral.

Sifuvirtide is another fusion inhibitor, which showed a different mechanism of action from that of T20, this was confirmed by its activity against T20 resistant strains. In clinical studies, Sifuvirtide showed well tolerability and a longer half-life than T20. Combinatorial assays between T20 and Sifuvirtide have shown a synergistic activity in the inhibition of the infection by HIV-1, suggesting that the combination regimes of fusion inhibitors could improve the resistance profile and reduce the dosage (109, 182).

Although peptides with sequence similar to the CHR are potent inhibitors of infection, its fully application in the clinics is constrained due to proteolytic degradation. Alternative approaches for the design of new fusion inhibitors are under study. The constant generation of new data about the structure and fusion process mediated by gp41 opens previously unexplored possibilities for the design of new inhibitors.

The highly conserved deep hydrophobic pocket formed by the N-trimer of hairpins of gp41 has been pointed as an attractive target for the design of new fusion inhibitors.

Using a mirror-image phage display, a first generation series of D-peptides were discovered and synthesized. The called D-peptide pocket-specific inhibitors of entry (PIEs) target the hydrophobic pocket formed by the N-trimer region of gp41 and modestly inhibited the HIV-1 infection (71).

In the mirror phage display technique, a phage library is used against a mirror-image version of the target protein, which is synthesized using D-amino acids. By symmetry, mirror images (D-peptides) of the discovered sequences will bind to the natural L-peptide target (71).

D-peptides present several advantages that make them attractive to be used as fusion inhibitors: they are not substrate for cellular proteases, the half-life could be larger than that of the L-peptides and the D-peptides can be absorbed when taken orally (71, 219).

A second generation of 8-mer PIE D-peptides was generated. These D-peptides showed an increased activity comparing with the first generation of PIE peptides, being the trimer version of PIE7 the most active peptide against laboratory-adapted and primary HIV-1 strains (219). A third generation of D-peptide named PIE-12 trimer, which was engineered to improve by 100000-fold the binding to its target, compared to the previous PIE7 (225).

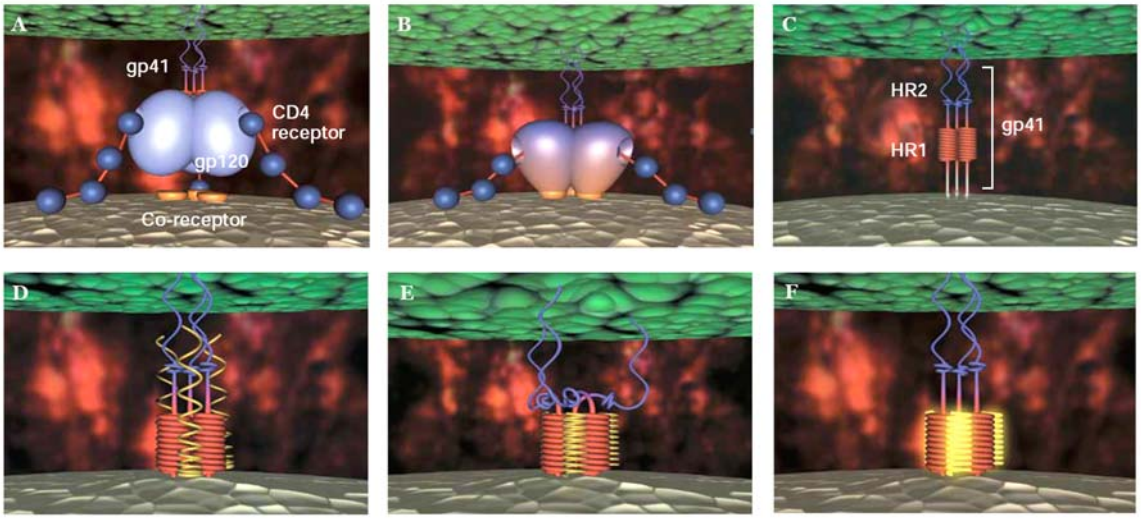

Figure 13 Blockade of HIV-1 entry by a fusion inhibitor (i.e. T20). T20 interacts with the NHR, preventing the formation of the 6-helix bundle (6HB) and thereby, impeding the infection by HIV. Modified from (150).

Chapter 2. Scope of this Thesis.

Scope of this Thesis.

In order to overcome the pressure imposed by the microenvironment, the HIV-1 envelope glycoprotein exhibits a high degree of plasticity. This characteristic, which was acquired through an evolutive process, is highly remarked when the virus replicates under drug selective pressure. This Thesis discusses the results obtained in three different studies aimed to explain the resistant profile generated by the two entry inhibitors of the human immunodeficiency virus (HIV).

The principal results presented here are:

We confirmed that the small polyanionic compound ADS-J1 does not interact with gp41 but instead appears to block a previous step of the viral entry, probably driven by the HIV gp120 glycoprotein alone.

The development of a HIV strain resistant to the peptidic fusion inhibitor VIRIP/VIR-353 and the acquisition of relevant evidence that indicate that this peptide may not interact with the fusion peptide of HIV gp41, as originally suggested.

We studied the effect of the mutations of the VIR-353-resistant virus over its replicative capacity. Additionally, we evaluated the combined activity of VIR-353 and T20 in inhibiting the replication of HIV.

Globally, our results provide interesting information of the mechanisms used by HIV-1 to overcome the selective pressure imposed by drugs that target a not yet completely understood step of the viral replication but continue to be a promising therapeutic target: the entry of the HIV-1 into the cells.

Chapter 3. ADS-J1 inhibits HIV-1 entry by interacting with gp120 and does not block the fusion active gp41 core formation.

3.1 Objectives of this Study.

It was published by Wang and collaborators that ADSJ-1, a polyanionic compound, could be used as a template compound for the design of new non-peptidic HIV-1 fusion inhibitors. In that study, it was shown that ADSJ-1 could bind to a synthetic peptide mimic of the pocket region of gp41, inhibiting the infection by HIV-1 (214).

Previously, our group demonstrated in several assays, that ADS-J1 inhibited HIV-1 replication at a different step than the gp41-mediated membrane fusion (12).

We decided to further study the inhibiting activity of the polyanionic compound ADS-J1.

Especifically:

A. To evaluate the activity of ADS-J1 against HIV strains resistant to entry inhibitors that target either gp120 or gp41.
B. To determine the target gene responsible for the resistance to ADS-J1 by molecular strategies.
C. To accurately determine the time/site of antiviral activity of ADS-J1, by comparing its inhibiting activity with that of other anti-HIV-1 compounds.

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3.2 Resumen.

Previamente, nuestro grupo había demostrado que la resistencia a ADS-J1 está asociada a cambios de aminoácidos en la glicoproteína de la envuelta, dichos cambios están localizados mayoritariamente en la región codificante para gp120. Ensayos de Tiempo de Adición y de transferencia endocítica de virus, claramente demostraron que ADS-J1 se comporta como un inhibidor de gp120. El virus resistente a ADS-J1 presenta resistencia cruzada al polianión dextran sulfato. Adicionalmente, el fenotipo de resistencia a ADS-J1 fue recuperdado únicamente mediante la recombinación de gp120. En resumen, ADS-J1 bloquea un paso inicial de la entrada del virus que parece estar conducida únicamente por gp120.

3.2 Abstract.

We had shown that virus resistance to ADS-J1 was associated to amino acid changes in the envelope glycoprotein, mostly located in the gp120-coding region. Time of addition and endocytic virus transfer assays clearly demonstrated that ADS-J1 behaved as a gp120 inhibitor. ADS-J1-resistant virus was cross-resistant to the polyanion dextran sulfate and recombination of gp120 only recovered the ADS-J1-resistant phenotype. In summary, ADS-J1 blocks an early step of virus entry that appears to be driven by gp120 alone.

The essential steps of HIV-1 entry in the host cell offer several potential targets for the development of novel antiviral agents (64, 84, 128, 169). Agents that disrupt gp41-mediated membrane fusion, collectively called fusion inhibitors, were the first entry inhibitors to be approved for the treatment of HIV infection.

Enfuvirtide (T20, Fuzeon™) is a 36 amino acid synthetic peptide with a sequence identical to a part of the C-terminal heptad repeat 2 (HR2) region of gp41 that binds to the N-terminal heptad repeat 1 (HR1) in an antiparallel manner, forming a coiled-coil structure during the prefusion step. Mutations in the highly conserved amino acid motif 36-45 in the HR1 domain confer resistance to T20 (146), providing strong evidence that HR1 is the site of interaction of T20. However, mutations in other regions of HIV-1 envelope (Env) have been also associated to T20 resistance (88).

Several small molecular weight (SMW) compounds have been identified as blockers of the initial steps of virus entry, including CCR5 coreceptor (128, 169). However, the identification of SMW compounds targeting gp41 has been elusive. A polyanionic compound, ADS-J1, was previously identified *in silico* as a potential candidate and shown to bind to gp41 peptides and interfere with the formation of the gp41 coiled-coil domain in an in vitro ELISA model of HR1/HR2 interaction (63, 120, 124).

Conversely, we had shown that ADS-J1 blocked the binding of HIV particles to lymphoid MT-4 cells and inhibited HIV replication at a time/site of interaction similar to the polyanion dextran sulfate (DS), a well described, non-specific inhibitor of virus entry (12). Moreover, at least 4 HIV-1 strains resistant to ADS-J1 were generated. The resistance to ADS-J1 was associated to gp120 based on the fact that the majority of the mutations were located in the gp120 coding sequence, mainly in the V3 loop region. Although, three of the resistant strains contained mutations in gp41, one of them, HIV-1 ARA45C, did not (12). In addition, molecular modelling suggested that the gp120 V3 loop was the preferential binding site for ADS-J1 onto HIV-1, and mutations induced by the inhibitor significantly changed the stereoelectronic properties of the gp120 surface, justifying a marked drop in the affinity of ADS-J1 towards an ADS-J1-resistant HIV-1 strain (147). At that time, we considered as conclusive the evidence of the mode of action of ADS-J1.

Later, Wang et al, suggested that ADS-J1 could bind directly to a trimeric peptide containing the gp41 pocket region (IQN17) in a surface plasmon resonance (SPR) assay, and indicated that ADS-J1 can be used as a lead compound for the design of novel HIV-1 fusion

inhibitors (214). Therefore, we thought relevant to provide further evidence of the mode of action of ADS-J1.

3.3 ADS-J1 -resistant HIV is cross-resistant to agents targeting gp120.

We evaluated the activity of ADS-J1 against a panel of HIV strains resistant to entry inhibitors, targeting either gp120 or gp41 (Table 1) that have been described elsewhere (12, 13, 30, 37, 80, 83). Anti-HIV activity and cytotoxicity measurements in MT-4 cells were done as described elsewhere (18, 159, 164)**.** The AR177-resistant strain (80) was selected to generate ADS-J1 resistance in order to bypass the activity of ADS-J1 as a gp120-CD4 interaction inhibitor and to evaluate its properties as a gp41 fusion inhibitor(12). The AR177-resistant virus is hypersensitive to T20 (Table 1 and 2) due to a change of an aspartic acid to glycine at gp41 position 34 that occurs after virus culture in the absence of selective pressure.

ADS-J1 blocked the replication of viruses resistant to the gp41 fusion inhibitors C34, T20 and sifuvirtide (SFV) at concentrations similar to that of the wild type NL4-3 strain. Although ADS-J1 was similarly active against a BMS-155-resistant virus, it was 17-fold less potent when tested against the AMD3100-resistant strain. AMD3100-resistant HIV has been shown to be cross-resistant to ADS-J1 and to DS (81, 83), suggesting that mutations that confer resistance to AMD3100 affect the sensitivity to other agents targeting gp120. The ADS-J1 resistant strains Ara49 and Ara45C were clearly cross-resistant to the gp120 blocking agent DS (130) (200 and >300 fold change to the wild type NL4-3 virus), while T20 and C34 resistant virus remained sensitive to ADS-J1. This result suggests that ADS-J1 targets gp120 instead of gp41.

Table 1. Anti-HIV activity of selected compounds against virus strains made resistant to entry inhibitors

Compound	EC_{50}[a] (μM)		EC_{50}[a] (μM) [Fold-resistance][b]					
	HIV-1 NL43	AR177-res	ADS-J1-Res Ara49	ADS-J1-Res Ara45C	BMS-res	AMD3100-res	T20/C34-res	SFV-Res
ADS-J1	0.1± 0.04	2.04 ±0.24	14.6 ± 2.4 **[146]**	12.3 ± 1.4 **[123]**	0.2 ± 0.002 **[2]**	1.7 ± 0.44 **[17]**	0.4 ± 0.16 **[4]**	0.08 ± 0.01 **[1]**
BMS-155	0.02 ± 0.004	0.005 ± 0.004	0.1 ± 0.04 **[5]**	0.05 ± 0.01 **[3]**	>2.7 ± na **[>350]**	0.03 ± 0.008 **[2]**	0.031 ± 0.004 **[2]**	0.007 ± 0.0007 **[0]**
AMD3100	0.002 ± 0.002	0.01 ± 0.006	0.003 ± 0.0006 **[2]**	0.006 ± 0.0005 **[3]**	0.004 ± 0.002 **[2]**	0.7 ± n.a. **[350]**	0.003 ± 0.002 **[2]**	0.002 ± 0.0008 **[1]**
Dextran Sulfate	0.05 ± 0.02	0.37 ± 0.26	10.07 ± 7.12 **[200]**	17.2 ± 1.74 **[344]**	0.005 ± 0.003 **[0]**	>25 ± n.a. **[>500]**	0.05 ± 0.03 **[1]**	0.005 ± 0.003 **[0]**
C34	0.0002 ± 0.0003	0.0005 ± 0.0003	0.0007 ± 0.0006 **[2]**	0.001 ± 0.001 **[5]**	0.001 ± 0.0003 **[3]**	0.005 ± 0.0008 **[25]**	0.01 ± 0.003 **[60]**	0.15 ± 0.03 **[750]**
T20	0.09 ± 0.04	0.004 ± 0.004	0.5 ± 0.06 **[5]**	0.5 ± 0.49 **[5]**	0.2 ± 0.10 **[2]**	0.06 ± 0.01 **[1]**	>2.5 ± n.a. **[>20]**	0.9 ± 0.04 **[10]**
Sifuvirtide	0.001 ± 0.00005	0.0004 ± 0.0005	0.002 ±0.003 **[2]**	0.002 ±0.002 **[2]**	0.002 ± 0.001 **[5]**	0.008 ± 0.001 **[4]**	0.01 ± 0.01 **[10]**	1.03 ± 0.04 **[>1000]**
AZT	0.007 ± 0.004	0.007 ± 0.008	0.015 ±0.01 **[2]**	0.012 ± 0.007 **[1]**	0.001 ± 0.006 **[1]**	0.004 ± 0.005 **[1]**	0.007 ± 0.001 **[1]**	0.003 ± 0.0008 **[0]**

[a] EC_{50}: 50% effective concentration, or the concentration needed to block replication of the wild-type NL4-3 HIV-1 in MT-4 cells.

[b] Fold change in EC_{50} compared to that of the wild-type HIV-1 NL4-3 strain. Data represent the means and standard deviations of results of at least two independent evaluations done in triplicate. n.a., not available.

[c] res, HIV-1 strain resistant to the corresponding drug.

3.4 Recombination of ADS-J1 resistant gp120 into wild type HxB2 confers resistance to ADS-J1.

To identify the gene responsible for the resistance to ADS-J1, gp120 and/or gp41 from ADS-J1 resistant virus and the virus passaged in parallel but without any selective pressure (Arwt45B2 virus), were recombined into the pJJ5-Δenv HXB2 backbone (55) as described before (14) (Figure 1). HXB2-env clones were transfected into MT-4/CCR5+ cells with the Amaxa Nucleofector system (Lonza, Madrid, Spain). Viral stocks were generated and proviral DNA was extracted to confirm chimeric env sequences. As shown in Table 2, the recombination of the full envelope containing the gp120 and gp41 sequence of the ADS-J1-resistant strains conferred resistance to ADS-J1 (109-fold resistant compared to wild type NL4-3, similar to that shown in Table 1).

Recombination of gp120 from the ADS-J1-resistant strains and wild type gp41 was sufficient to confer resistance to ADS-J1. Recombination of the gp41 coding sequence of the ADS-J1 virus alone did not induce any change or modified the sensitivity to the fusion inhibitors tested (T20 and C34), confirming that resistance depends on the gp120 coding sequence and did not affect gp41.

3.5 ADS-J1 interferes in gp120 but not gp41 function in a time of drug addition assay.

The time/site of drug addition (TOA) experiments allow the determination of the last step blocked by an anti-HIV drug (12, 41). The time delay before the addition of a drug is an estimate of its mode of action. Compounds with dual mechanism (e.g. inhibition of entry and reverse transcription, RT) would be interpreted as inhibitors of the last step. To identify the time/site of interaction of ADS-J1, drugs acting at different steps of virus entry, as well as combinations of these compounds with BMS-155 or ADS-J1 were added at various times post-infection. BMS-155 lost its activity if added later than 7 minutes post infection. The addition of the CXCR4 antagonist AMD3100 could be delayed up to 14 minutes, whilst addition of T20 could be delayed up to 35 minutes. AZT remained completely active when added up to 2 hours post infection (Figure. 2A). When ADS-J1 was tested alone, its activity was lost at minute 14 post infection (Figure. 2A). To demonstrate that the TOA assay shows the effect on the latest step inhibited, combinations of anti-HIV agents were tested. Addition of BMS-105 155 (Figure 2B) or ADS-J1 (Figure 2C) with AMD3100, T20 or AZT recapitulate the activity of AMD3100, T20 or AZT alone indicating that ADS-J1 did not share a mode of action with AMD3100, T20 or AZT.

Table 2. Recombination of gp120 from ADS-J1 resistant virus restores ADS-J1 resistance

Compound	EC_{50}[a] (μM)		EC_{50}[a] (μM) [Fold-resistance][b]						
	Wt NL4-3	AR177-res [c]	gp 120 Wt and gp41 Wt	gp120 ARA49 and gp41ARA49	gp120 ARA45C and gp41 ARA45C	gp120 ARA49 and gp41 Wt	gp120 ARA45C and gp41 Wt	gp120 Wt and gp41 ARA49	gp120 Wt and gp41 ARA45C
ADS-J1	0.1 ± 0.04	2.04 ± 0.24	2.35 ± 0.2 **[1]**	10.88 ± 0.26 **[6]**	11.1 ± 0.26 **[6]**	10.73 ± 0.3 **[6]**	12.23 ± 0.11 **[6]**	1.9 ± 0.1 **[1]**	2.01 ± 0.04 **[1]**
BMS-155	0.007 ± 0.004	0.005 ± 0.004	0.02 ± 0.001 **[4]**	0.01 ± 0.003 **[2]**	0.008 ± 0.001 **[2]**	0.008 ± 0.003 **[2]**	0.02 ± 0.01 **[4]**	0.008 ± 0.003 **[2]**	0.02 ± 0.007 **[4]**
AMD3100	0.002 ± 0.002	0.01 ± 0.006	0.02 ± 0.004 **[2]**	0.01 ± 0.001 **[0]**	0.003 ± 0.0005 **[0]**	0.004 ± 6e-4 **[0]**	0.003 ± 0.001 **[0]**	0.017 ± 0.004 **[2]**	0.02 ± 0.003 **[2]**
Dextran Sulfate	0.05 ± 0.01	0.3 ± 0.26	0.08 ± 0.005 **[0]**	2.2 ± 0.6 **[7]**	1.66 ± 0.18 **[6]**	1.93 ± 0.86 **[6]**	2.5 ± **[8]**	0.08 ± 0.001 **[0]**	0.077 ± 0.03 **[0]**
C34	0.0003 ± 0.0003	0.0003 ± 0.0003	0.0007 ± 0.0003 **[2]**	0.0004 ± 0.0005 **[1]**	0.0001 ± 0.0004 **[0]**	0.0003 ± 0.0004 **[1]**	0.0004 ± 0.0002 **[0]**	0.0008 ± 0.0002 **[3]**	0.001 ± 0.0004**[4]**
T20	0.09 ± 0.04	0.004 ±0.004	0.07 ± 0.06 **[19]**	0.35 ± 0.36 **[89]**	0.03 ± 0.02 **[8]**	0.085 ± 0.08 **[21]**	0.09 ± 0.02 **[22]**	0.4 ± 0.21 **[99]**	0.5 ± 0.43 **[144]**
AZT	0.006 ± 0.004	0.008 ±0.008	0.005 ± 0.0002 **[1]**	0.003 ± 0.002 **[0]**	0.002 ± 0.0007 **[0]**	0.003 ± 0.002 **[0]**	0.004 ± 0.0005 **[1]**	0.01 ± 0.007 **[1]**	0.005 ± 0.0005 **[1]**

[a] EC_{50}: 50% effective concentration, or the concentration needed to block replication of the virus in MT-4 cells.

[b] Fold change in EC_{50} compared to that of the parental AR177-resistant strain used to generate the ADS-J1-resistant strains. Data represent the means and standard deviations of results of at least two independent evaluations done in triplicate.

[c] res, HIV-1 strain resistant to the corresponding drug.

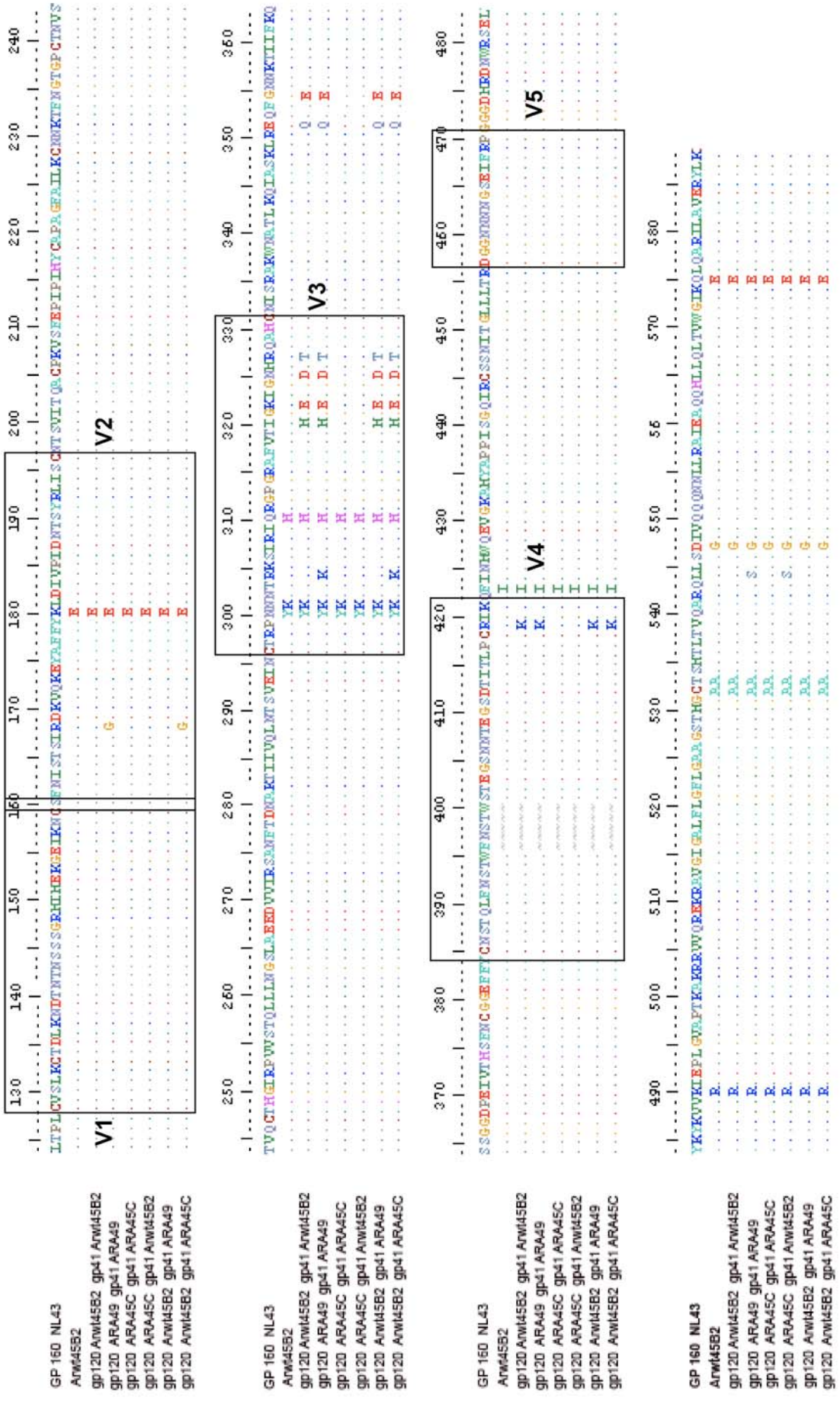

Figure 1. gp120 and gp41 sequences from the HIV-1 NL4-3 and Arwt45B2 and the recombinant strains generated. Arwt45B2 is the virus obtained after passages in parallel to ARA49 and ARA45C in the absence of ADS-J1. Arwt45B2 was used as the complementary moiety to the ADSJ-1-resistant portion in the generation of the recombinant strains. The numbering corresponds to the HXB2 HIV-1 strain. Variable loops of gp120 are indicated by boxes.

3.6 ADS-J1 does not prevent virus interaction with CD4 in cell-to-cell HIV transmission.

A flow cytometry-based assay was used to simultaneously quantify HIV-1 envelope (Env)-mediated cell death, endocytic cell-to-cell viral transfer (29, 41, 113), cell death (23), and cell-to-cell fusion, allowing the rapid identification of the mode of action of active compounds (24, 25). In this assay, agents that block virus entry, prevent Env-mediated cell death and agents with activity at any entry step after gp120-CD4 interaction, increase HIV-1 endocytosis. Thus, overnight cocultures of MOLT-Uninfected or MOLT-NL-43 cells with non-stimulated CD4+T cells were performed and intracellular p24 and cell death were evaluated. As expected, all HIV entry inhibitors, Leu3a (dilution 1/100), AMD3100 (12.05 μM), C34 (1.18 μM), BMS-155 (5.33 μM) and ADS-J1 (4.25 μM) blocked HIV-envelope induced cell death at the concentrations tested (cell death for all the compounds tested were roughly 4%, similar to that in the untreated coculture) (24). HIV-1 transfer from the infected MOLT-NL4-3 cells to CD4+ T cells was measured as percentage of CD4+ 120 T cells positively labeled with p24 antigen. The anti-CD4 mAb Leu3a (dilution 1/100) blocked the transfer of p24 antigen up to 97 ± 6% as compared to untreated samples. BMS-155 and ADS-J1 failed to block virus transfer at all the concentrations tested. Conversely, the gp41 fusion inhibitor C-34 or the CXCR4 coreceptor antagonist AMD3100 increased the amount of transferred NL4-3 antigen to CD4+T cells (2-fold and 2.4-fold respectively). These results suggest that ADS-J1, similarly to BMS-155, did not prevent virus- CD4 interaction; however, its mechanism of action is clearly distinct from agents affecting later steps in the HIV entry process.

The identification of the mechanism of action of antiviral compounds may be confounded by the experimental settings or the evaluation of agents in models that do not, or only partially represent the mechanism used by viruses (44, 80). Development of drug resistance in cell culture is a valuable tool to unravel the mechanism of action of anti-HIV agents (148, 156).

The resistance to AMD3100 is mapped in or near the gp120 V3 loop region (62), the putative site of interaction with CXCR4; resistance to polyanions such as dextran sulfate, AR177 or negatively charged albumins were also located in the gp120 coding sequence (30, 80, 83). Selection of drug-resistance to agents that block virus entry or fusion could be, however, confounded by the plasticity of the HIV-1 envelope that allows for the incorporation of mutations that do not necessarily hamper the replication capacity of the virus (148). Clearly,

recombination of gp120 of the ADS-J1 resistant virus was sufficient to recover the resistant phenotype. Conversely, recombination of the ADS-J1 resistant gp41 did not alter the virus sensitivity to the drug, pointing to gp120 as the target gene for resistance to ADS-J1.

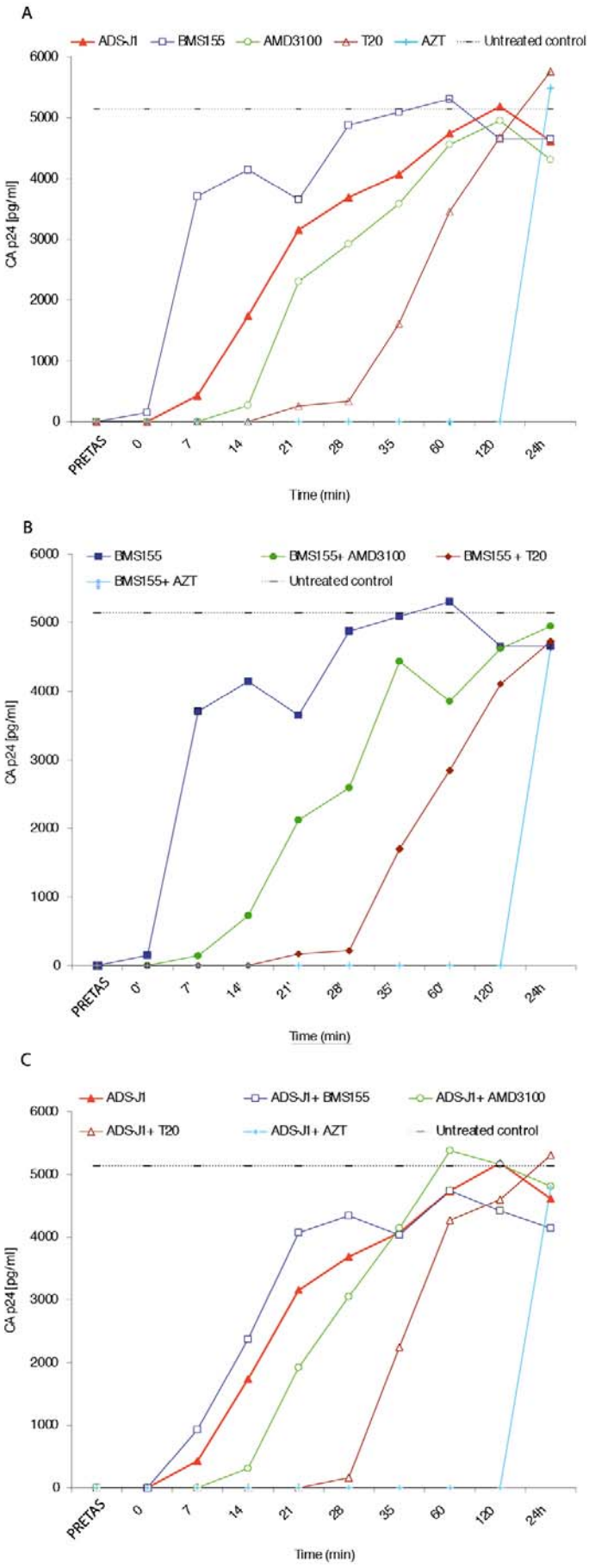

Figure 2. Effect of time of drug addition on the inhibition of HIV entry inhibitors. MT-4 cells infected with HIV-1 NL4-3 at a multiplicity of infection of 0.5 at 20°C for 1 h and 37°C thereafter were treated during virus infection and/or at various times post infection. Treatment with test compounds alone (A) or in combination with BMS-155 (B) or ADS-J1 (C) was performed at replication-blocking concentrations (roughly 100-fold the 50% effective concentration [EC_{50}]). Viral p24 levels in the culture medium were monitored at 30 h postinfection. The figure shows a representative result of three experiments.

In our previous work, we showed that ADS-J1 resistant strains may contain mutations in both gp120 and gp41; however, we had also shown that one of the selected viruses, ARA45C, containing mutations in the gp120 V3 loop but without mutations in gp41, was resistant to ADS-J1, a result that was overlooked by Shibo Jiang *et al*., when discussing the role of gp120 in ADS-J1 resistance (214). Distinct patterns of mutations may emerge when selecting for resistance to entry inhibitors (14, 21, 64, 79, 125, 128, 156, 164). Resistance often requires acquisition of multiple mutations that may induce further variation not necessarily representing a direct site of interaction of the drug but a compensatory mechanism.

Therefore, mutations arise in the gp41 of CCR5-resistant agents (8) or the gp120 of gp41- targeting agents (110) or as we reported, in gp41 of ADS-J1 resistant virus (12). However, taken together, our results strongly suggest that ADS-J1 is not a virus fusion inhibitor through interaction with gp41, but a gp-120 interacting compound.

It is puzzling that ADS-J1 remained relatively active against the polyanion AR177-resistant virus when first tested. However, we have shown that a DS-resistant virus, generated after passage of infected cells with DS molecular weight 5000 remained sensitive to DS molecules of higher molecular weight (MW >40000) (83). The number and position of negative charges in polyanions may affect their anti-HIV potency explaining the discrepant results with ADS-J1.

We clearly show that ADS-J1 lost its anti-HIV activity at a time/site before the corresponding to a fusion inhibitor. When the combination of T20 and ADS-J1 was evaluated, only the activity of T20 could be detected, confirming that ADS-J1 did not behave as a fusion inhibitor. In a TOA experiment, Jiang and colleagues (214) compared the activity of ADS-J1 to that of the RTI AZT and therefore, lacking the appropriate controls that could resolve the time of action of ADS-J1. To confirm their results, they used a, so called, time-of-removal coculture assay in which they compared the activity of ADS-J1 alone or in combination with soluble CD4 (sCD4). However, they failed to include a relevant control, that is, the activity of sCD4 alone. sCD4 binds to gp120-expressing cells, independently blocking gp120 binding to cell surface CD4 but also all subsequent downstream effects of Env leading to virus entry (5, 85, 118). Therefore, the alleged increased potency of ADS-J1 in the presence of sCD4, interpreted as an increased exposure of the drug-binding site in gp41, may be the consequence of the additive effect of two active compounds: ADS-J1 and sCD4.

Since peptidic fusion inhibitors are not orally bioavailable and must be administered via injection, the development of small molecule inhibitors of gp41–mediated fusion remains as a challenging and relevant objective in drug development (169). Unbiased identification of the mechanism of action of potential lead structures is a pre-requisite for successful drug development. Here, we demonstrate that mutations in gp120 conferred resistant to ADS-J1 and that early gp120-dependent entry was the functional site of interaction of ADS-J1, therefore suggesting that this compound might not be considered as a gp41 fusion inhibitor.

3.7 Acknowledgements.

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Chapter 4. Development of Resistance to VIR-353 with cross-resistance to the natural HIV-1 Entry Virus Inhibitory Peptide (VIRIP).

4.1 Objectives of this Study.

The generation of a HIV-1 strain resistant to VIRIP, a circulating natural peptide with antiviral activity, has been elusive until now. The development of strains resistant to new inhibitory compounds is an essential step for its characterization. Additionally, the generation of resistant strains give relevant information of the target site of the compound and could point to previously unknown therapeutic molecules.

In this study, our principal objective was,

A. To generate a HIV-1 strain resistant to VIR-353.

Additionally,

B. To evaluate the peptide VIR-353 against different HIV-1 strains resistant to entry inhibitors targeting either gp120 or gp41.
C. To determine the target/site of activity of VIR-353
D. To determine the viral gene involved in the resistance to VIR-353 and the amino acids that are relevant to the resistant phenotype.

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4.2 Resumen.

Objetivo. VIRIP fue identificado como un componente del hemofiltrado humano que inhibe la fusión dependiente de gp41 del VIH al interactuar con el péptido de fusión. Un análogo de VIRIP (VIR-576) ha mostrado su efectividad en un ensayo clínico en fase I/II. Nosotros hemos evaluado la actividad y mecanismo de resistencia del VIH-1 a VIRIP y a su análogo, VIR-353.

Métodos. Se evaluó la actividad anti HIV-1 de VIRIP y de VIR-353. Se generaron virus recombinantes con las mutaciones más relevantes para la resistencia y éstos fueron caracterizados.

Resultados. VIRIP y VIR-353 mostraron actividad anti HIV-1 de EC_{50} 28 0.3μM, respectivamente y mostraron actividad contra virus resistentes a BMS-155, AMD3100, T20, TAK-779 o nevirapina. Experimentos de tiempo de adición mostraron que VIR-353 actúa en un tiempo o sitio correspondiente a la fusión dependiente de gp41. El virus resistente a VIR-353 fue generado después de 450 días en cultivo celular, lo que sugiere una elevada barrera genética para generar resistencia. El virus resistente a VIR-353 presenta resistencia cruzada a VIRIP, sin embargo mantuvo la sensibilidad a T20, AMD3100 o AZT. La recombinación de gp41 en un virus silvestre, recuperó parcialmente el fenotipo resistente, pero tanto gp120 y gp41 del virus resistente son necesarios para restablecer la resistencia a VIRIP o VIR-353. Mutagénesis dirigida confirmó el papel de mutaciones específicas y se identificó una combinación de tres mutaciones (A433T/V489I/V570I) como las más relevantes en la resistencia a VIRIP.

Conclusiones. VIRIP debe interactuar con una region de gp41 que es esencial para la fusión pero no el peptide de fusión. Nuestros resultados resaltan las interacciones entre gp41 y gp120 que deben requerirse durante el proceso de fusión.

4.2 Abstract.

Objective. VIRIP has been identified as a component of human hemofiltrate that blocks HIV-1 gp41-dependent fusion by interacting with the fusion peptide. A VIRIP analogue (VIR-576) has been shown effective in a phase I/II clinical trial. We have evaluated the activity and mechanism of HIV-1 resistance to VIRIP and its analogue, VIR-353.

Methods. Anti-HIV activity and passage of HIV-1 strains in cell culture were used to generate and identify mutations that confer resistance to VIRIP and VIR-353. Recombinant virus harbouring the most relevant mutations were generated and characterized.

Results. VIRIP and VIR-353 showed anti-HIV-1 activity with EC_{50} of 28 and 0.3μM respectively and were active against virus resistant to BMS-155, AMD3100, T20, TAK-779 or nevirapine. Time of addition experiments showed that VIR-353 targets a time/site of action corresponding to gp41-dependent fusion. VIR-353-resistant virus was generated after 450 days in cell culture, suggesting a high genetic barrier for resistance. The VIR-353 resistant virus was cross-resistant to VIRIP but remained sensitive to T20, AMD3100 or AZT. Recombination of gp41 into a wild-type backbone partially recovered the resistant phenotype but both gp120 and gp41 from the resistant virus were necessary to restore resistance to VIRIP or VIR-353. Site directed mutagenesis confirmed the role of specific mutations and identified a combination of three mutations (A433T/V489I/V570I) as the most relevant to VIRIP resistance.

Conclusions. VIRIP may interact with region of gp41 that is essential for fusion but not the fusion peptide. Our results highlight interactions between gp41 and gp120 that may be required during the fusion process

4.3 Introduction.

HIV entry is a validated target for anti-HIV intervention (85, 137). At least two agents targeting HIV entry and fusion, enfuvirtide (T20)(134) and maraviroc (106, 206) have been approved for their use in patients and have played an important role in antiretroviral drug combinations(35, 82). T20 is a synthetic peptide that mimics the C-terminal heptad repeat (C-HR) of HIV-1 gp41, binding to the N terminal HR (N-HR) and prevents the conformational change required for membrane fusion. The N-HR region of gp41 is highly conserved; therefore, most HIV- 1 isolates are susceptible to T20. However, the genetic barrier for HIV resistance to T20 is generally low and restricted to the amino acid motif between residues 36 and 45 in gp41 that form part of the T20 binding site(104, 156). The efficacy of T20 has prompted the study of gp41 as a target for drug-development. Several peptidic (108, 109, 206, 219) and non-peptidic (39) agents have been designed with potent anti-HIV activity. Importantly, gp41 appears to be a natural target by both the innate and acquired immune response. Neutralizing antibodies against gp41 are potent and broad inhibitors of multiple clades of HIV-1 (36, 228).

Recently, an inhibitory peptide of HIV-1 replication was discovered as a circulating natural compound (172). The 20-residue peptide, named virus-inhibitory peptide (VIRIP), corresponds to a human hemofiltrate-purified fragment of the serine protease inhibitor α1-antitrypsin. VIRIP inhibited a wide variety of HIV-1 strains including those resistant to T20 and other antiretroviral drugs. Results showed that VIRIP, and other peptidic analogues with greater anti-HIV potency, blocked HIV Env dependent fusion through their interaction with gp41. The fusion peptide (FP) of gp41 was shown as the putative site of interaction of VIRIP and its derivatives, VIR-353 and VIR-576 (Fig. 1). A clinical trial phase I/II with VIR-576 showed reductions in plasma viral load of more than one order of magnitude after short-term monotherapy and without significant adverse events in patients (89). Thus, the specificity and potency of VIRIP and its derivatives could lead to the development of a new class of antiretroviral agents.

A relevant aspect in the characterization of the mode of action of an antiviral compound is the development of HIV drug resistant mutant strains. Generation of resistance *in vitro* to an antiretroviral drug may point to functionally relevant data such as the specificity of the drug, the site of interaction and the genetic barrier that HIV needs to overcome the inhibitory effect (79, 155). Until now, the generation of VIRIP-resistant virus has been elusive. Resistant virus did not emerge after two months of weekly passage in the presence of VIRIP. Moreover, site-directed mutagenesis study of the FP region of gp41 did not lead to the generation of resistant HIV (172). Here, we show the long-term passage of cells acutely

infected with wild type NL4–3 virus that lead to the generation of VIR-353 and VIRIP cross-resistance through the emergence of mutations in gp120 and gp41.

4.4 Materials and Methods.

Cells, viruses and compounds.

CD4 lymphoid cell lines MT-4 were obtained through the Medical Research Council (MRC) Centre for AIDS Reagents. London. UK. Cells were grown in RPMI 1640 (Invitrogen. Barcelona. Spain) and supplemented with 10% fetal calf serum (FCS. Cambrex. Barcelona, Spain) and antibiotics. 2 U/ml penicillin and 2 mg/ml of streptomycin (Invitrogen. Barcelona. Spain). MT-4/ CCR5 (expressing both CCR5 and CXCR4) were generated as described before (14). The HIV-1 strains BaL. HXB2 and NL4–3 were obtained from the MRC Centre for AIDS Reagents (London. UK). The HIV-1 strains, resistant to T20/C34, AMD3100 and Tak-779 have been described elsewhere (14, 80, 159). The BMS-155 resistant virus (146) was selected in cell culture as described below.

VIRIP. VIR-353. a scramble version of VIRIP (VIR-SRC) and sifuvirtide were synthesized by New England Peptide (Gardner. MA. U.S.A.) according to the peptide sequences described by Münch et al (172) and He et al (108, 109). AMD3100, T-20 and C34 were obtained from the NIH AIDS Reagent Program. BMS-155 (146) was synthesized by the Organic Chemistry department of Universitat de Barcelona. Spain.

The RT inhibitor 3-azido-3-deoxythymidine (zidovudine; AZT) was purchased from Sigma-Aldrich (Madrid. Spain).

Anti-HIV and cytotoxicity assays.

All anti-HIV activity and cytotoxicity measurements in MT-4/CCR5+ cells were based on viability of cells that had been infected or not with the corresponding HIV-1 strain at multiplicity of infection (moi) of 0.003 (100 $CCID_{50}$ in 30.000 cells) and quantified by a tetrazolium based colorimetric method (MTT method), commonly used in the evaluation of anti-HIV active drugs (164, 165, 184). The cut-off value in which a virus was considered resistant was a 4-fold increase of the EC50 when compared to the wild type virus and depended on the variance of the drug-screening assay in MT-4 cells (data not shown).

Anti-HIV activity determinations were performed in triplicates and data calculated from three independent experiments.

Time of drug addition and mode of action.

The time/site of drug action (TOA) assays were done as described (100). In brief, MT-4 cells were infected with NL4–3 virus at a multiplicity of infection of 0.5 and incubated for 1 h at 20ºC in the presence or absence of test compounds (AZT. AMD3100. T-20. C34. BMS- 155, and VIR-353). Then, cells were washed twice with cold PBS and the compounds were added at various times post-infection or as control cells were cultured in the absence of drug. The concentration of the different compounds used was high enough to block completely HIV replication (roughly 100-fold their EC_{50}). Virus production was measured by p24 antigen ELISA test (Innogenetics. Barcelona, Spain) in cells supernatants at 30 hours postinfection.

Development of resistant HIV-1 to VIR-353.

MT-4 cells were seeded at $3x10^5$/ml in RPMI 1640 medium containing 10% fetal bovine serum on a 96-well plate and infected with 100 $CCID_{50}$ of HIV-1 NL4–3.

Passages started with a VIR-353 concentration of 0.6 μM (the EC_{50} of VIR-353) and as controls 0.02 μM of BMS- 155 (146) and 0.01 μM of sifuvirtide (108, 109). After 5 days of incubation at 37ºC with 5% CO_2, when extensive cytopathic effect was observed, the supernatants from the cultures were collected and used to infect fresh MT-4 cells. The concentration of the compounds in the culture was progressively increased, finishing the passages when the concentration of the compounds reached 11 μm for BMS-155. 7 μM for sifuvirtide and 43.8 μM for VIR-353 (roughly>100-fold of the calculated EC_{50}).

Sequence analysis of HIV strains.

Genomic DNA from infected cells was extracted using the QIAamp DNA Blood Mini Kit (Qiagen. Barcelona. Spain). Extracted DNA was used to PCR amplify HIV-1 env gene using Expand High Fidelity PCR System (Roche) and the primers (Forward 50-aagggccacagagggagccat-30 and Reverse 50-gcgtcccagaagttccacaa-30). Before sequencing, the amplified DNA was purified by enzymatic cleanup that eliminates unincorporated primers and dNTPs (Exosap-ITTM, GE Healthcare).

Sequencing of amplified DNA was carried out with different primers to ensure obtaining the complete env sequence with the BIGDYE Terminator 3.1 kit (Applied Biosystems. Madrid, Spain) as described before (12, 18, 100, 164). Data was collected with the ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems. Madrid. Spain). Sequences were analyzed

with the Sequencher 4.5 software and edited with the BioEdit software. Amino acid positions were numbered according to HXB2 (Los Alamos database).

Recombination of HIV envelope.

Recombination of VIR-353-resistant gp120, gp41 or both, were done as described before (14). Briefly, the gp120 and extracellular region of gp41 of the NL4–3 wild type HIV-1 and the selected VIR-353 resistant virus (nucleotides from 5665 to 8486, relative to HXB2) were PCR amplified separately with the primer combination 5'-GATAAAGCCACCTTTGCCTAGT-3' and 5'- TCTTTGCCTTGGTGGGTGCTA-3' for gp120 and 5'-GGAGGAGGCGATATGAGGGACAATTGG-3' and 5'-TTCTAGGTCTCGAGATACTG-3' for gp41. The PCR product was then re-amplified with the primers 5'-GGATTTGGCTCCATGGCTTAGGG-3' and 5'-TGCTAAGGATCCGTTCACTAATCG-3', which contain the NcoI and BamH1 restriction sites (underlined) in order to amplify the HIV-1 env gene with the designed combination of gp120 and gp41 regions to generate the chimeric env gene. The resulting env products were sequenced and recombined into the pJJ5-(delta env HXB2 backbone) (62). HXB2-env clones were transfected into MT-4/CCR5+ cells by electroporation. Viral stocks from these cultures were generated and env sequences were confirmed in proviral DNA.

Site-directed mutagenesis.

Specific point mutations that reflected amino acid changes found in VIR-353 strain were introduced in the pNL4–3 wild type plasmid by site directed mutagenesis using the Quikchange II Site-directed Mutagenesis Kit (Stratagene. La Jolla. CA. USA), following manufacturer's recommendations. Briefly, specific oligonucleotide primers were designed complementary for each of the point mutations. For T244S: Sense: 5'-ACCATGTACAAATGTCAGCTCAGTACAATGTACACATGG-3' and Antisense: 5'-CCATGTGTACATTGTACTGAGCTGACATTTGTACATGGT-3'. For A433T: Sense: 5'-GGCAGGAAGTAGGAAAAACAATGTATGCCCCTCC-3' and Antisense: 5'-GGAGGGGCATACATTGTTTTTCCTACTTCCTGCC-3'. For V489I: Sense: 5'-TTGGAGAAGTGAATTATATAAATATAAAGTAATAAAAATTGAACCATTAGGAGTAGCA-3' and Antisense: 5'-TGCTACTCCTAATGGTTCAATTTTTATTACTTTATATTTATATAATTCACTTCTCCAA-3'. For L545M: Sense: 5'-CTGACGGTACAGGCCAGACAATTAATGTCTGATATAGTG-3' and Antisense: 5'-CACTATATCAGACATTAATTGTCTGGCCTGTACCGTCAG-3'. For V570I: Sense: 5'-CAGCATCTGTTGCAACTCACAATCTGGGGCATC-3' and Antisense: 5'-GATGCCCCAGATTGTGAGTTGCAACAGATGCTG-3'. For A612T: Sense: 5'-CCACTGCTGTGCCTTGGAATACTAGTTGGAGTAATAAATC-3' and Antisense: 5'-

GATTTATTACTCCAACTAGTATTCCAAGGCACAGCAGTGG-3'. For N625K: Sense: 5'-ATAAATCTCTGGAACAGATTTGGAATAAAATGACCTGGATGGAG-3' and Antisense: 5'-CTCCATCCAGGTCATTTTATTCCAAATCTGTTCCAGAGATTTAT-3'

The extension of specific oligonucleotides generates a mutation-containing newly synthesized plasmid that after digestion of parental DNA template is used to transform XL1-Blue super-competent cells (Stratagene, La Jolla, CA, USA). Plasmid DNA was then purified from liquid bacterial cultures by Qiaprep Spin Miniprep Kit (Qiagen Barcelona, Spain).

Once single mutants were obtained, the same procedure was used to obtain double and triple mutant plasmids, using the corresponding single or double mutant plasmids as templates.

The resulting plasmids were transfected into MT-4/CCR5+ cells by electroporation. Viral stocks from these cultures were generated and proviral env sequences were confirmed.

4.5 Results.

4.5.1 Activity and mode of action of VIRIP and VIR-353.

To confirm the anti-HIV activities of VIRIP and VIR- 353 (Fig. 1), the peptides were tested in a standard anti- HIV screening assay in lymphoid MT-4 cells. VIRIP and VIR-353 were active against wild type HIV-1 NL4–3 or BaL and to several HIV-1 strains resistant to BMS-155, AMD3100, TAK-779, T20 and nevirapine (Table 1). As shown, VIRIP and VIR-353 were similarly active against wild type and drug-resistant HIV strains including those viruses resistant to agents targeting gp41. A time of addition experiment also showed that VIR-353 blocked HIV replication at a similar step to fusion inhibitors such as T20 or C34 (Fig. 2). These results confirm the activity and potency of VIRIP and VIR-353 as previously described (89, 172) and suggest that mutations affecting the sensitivity to T20 and other HIV-1 inhibitors do not affect the activity of VIRIP and analogues thereof.

VIRIP: LEAIPMSIPPEVKFNKPFVF
VIR-353: LEAIP**C**SIPp**CFL**FNKPFVF
VIR-576: LEAIP**C**SIPP**EFL**F**G**KPFVFx2

Figure 1. Amino acid sequence of VIRIP and its derivatives VIR- 353 and VIR-576**.** p: D-proline; cysteine residues are linked via a disulfide bridge. The sequence of the inactive scramble VIRIP peptide (VIR-SCR) is also shown.

Table 1. Anti-HIV activity of VIRIP and VIR-353 against drug resistant HIV-1 strains.

Compound	EC_{50} [a] (μM) [FC][b]							CC_{50} [c] (μM)
	NL4-3	BaL	BMS-155-resistant	AMD3100-resistant	T20-Resistant	Tak779-resistant[d]	NVP-resistant (Y188L)[e]	
VIRIP	21.9	27.03	46.2 [2]	71.65 [3]	55.22 [2]	22.85 [1]	13.8 [1]	>86.81
VIR-353	0.6	3.15	0.14 [1]	0.66 [1]	0.37 [1]	1.88 [1]	1.48 [2]	>43.8
VIR-SCR[f]	>17.36	>17.36	>17.36	>17.36	>17.36	>17.36	>17.36	>17.36
AMD3100	0.003	>6.02	0.001 [1]	0.18 [60]	0.002 [1]	>6.02 [1]	0.008 [2]	>6.02
AZT	0.003	0.01	0.002 [1]	0.004 [1]	0.002 [1]	0.01 [3]	0.01 [3]	>7.47
BMS-155	0.02	0.20	>13.31 [>650]	0.009 [1]	0.03 [1]	0.18 [1]	0.05 [1]	>13.31

[a] Effective concentration required to block HIV-1 replication by 50% as measured by the MTT method in MT-4/CCR5 cells (expressing both CCR5 and CXCR4 receptors).
[b] FC: fold change or ratio of the corresponding EC50 and the EC50 value of the corresponding wild type HIV-1 strain.
[c] Concentration required to induce cell death by 50% as measured by the MTT method in MT-4/CCR5 cells.
[d] Tak-779-resistant was derived from the R5 HIV-1 strain BaL.
[e] The nevirapine-resistant virus contains the Y188L mutation in the reverse transcriptase coding gene.
[f] VIR-SCR: scrambled form of VIRIP devoid of anti-HIV activity [17].
EC_{50} values represent the mean of three independent experiments.

4.5.2 Generation of a VIR-353 resistant HIV-1 virus.

HIV-1 resistant virus to different inhibitors (VIR-353. BMS-155 and sifuvirtide) were generated after serial passages of HIV-1 NL4–3 in MT-4 cells. VIR-353 was chosen because of its greater potency than VIRIP or other analogues. VIR-353 and VIR-576 have similar anti-HIV activity in cell culture. However. VIR-576 is a dipeptide. Infected cells were cultured in the presence of the corresponding drug until the breakthrough concentration reached roughly 100-fold the calculated EC_{50} for the corresponding drug. VIR-353 was used to generate resistant HIV instead of the original VIRIP peptide sequence because of the weak potency of the parental compound. Passage in the presence of BMS-155 or sifuvirtide took 16 and 46 passages respectively to generate a resistant virus (data not shown). while it took 90 passages for VIR-353 until significant cytopathic effect was observed at a relatively high concentration (Fig. 3B).

The resulting VIR-353-resistant virus (49-fold increase in the EC_{50}) was cross-resistant to VIRIP (>4-fold. or the highest concentration tested: 87μM) but remained sensitive to the fusion inhibitor T20. the CXCR4 antagonist AMD3100 or the reverse transcriptase inhibitor AZT (Table 2). The VIR-353-resistant virus became hyper-susceptible to T20 (50-fold). most probably due to two amino acid changes in gp41 heptad repeat HR1, positions D36G and T37I, that occurred after passage in cell culture in the VIR-353 resistant virus compared to the NL4–3 virus. Mutants 36D and 37T which are present in the virus passed without drug are associated to resistance to T20 and C34 (156) (Fig. 3A).

4.5.3 Both gp120 and gp41 were required to rescue the VIR-353 resistant phenotype.

To identify the gene responsible for the resistant phenotype, the coding sequences of gp120, gp41 or both from the VIR-353-resistant virus were recombined into a wild type backbone. As shown in Table 2 recombination of the resistant gp120 partially restored the resistant phenotype (13-fold change to VIR-353) while the resistant gp41 induced a slight change in sensitivity that is confounded by the variability of the assay. Conversely, recombination of both gp120 and gp41 from the VIR-353-resistant strain fully restored the resistant phenotype (>73-fold change in EC50).

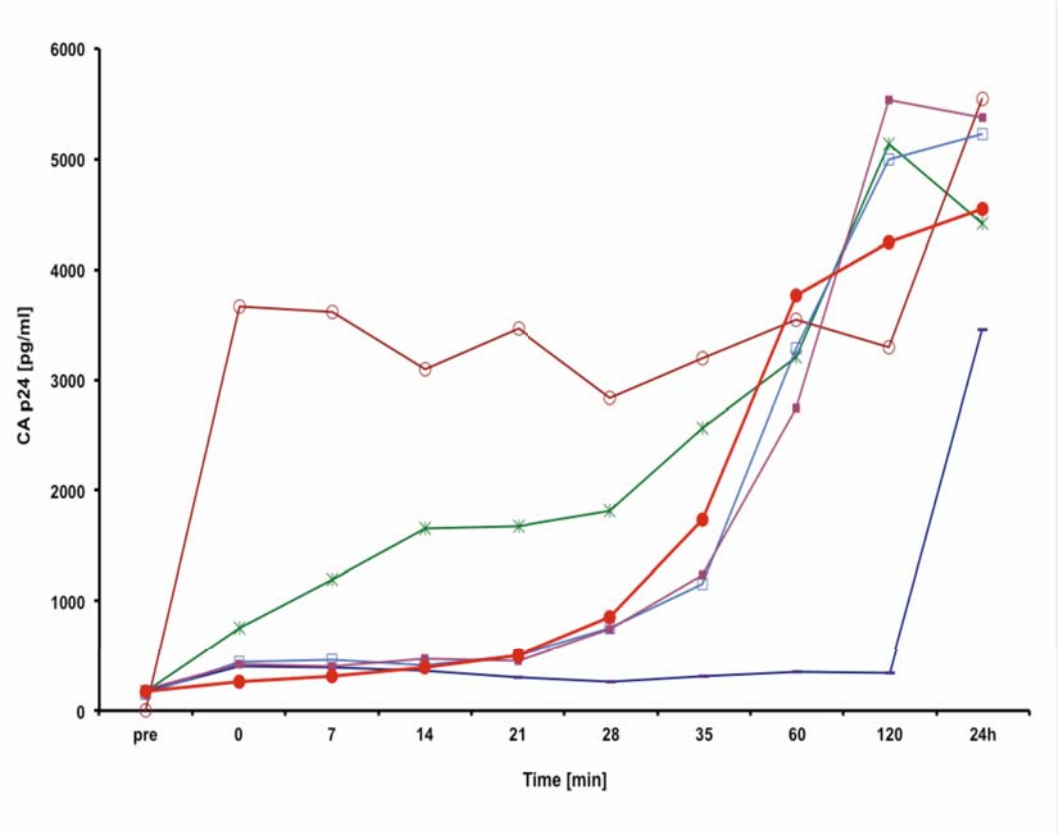
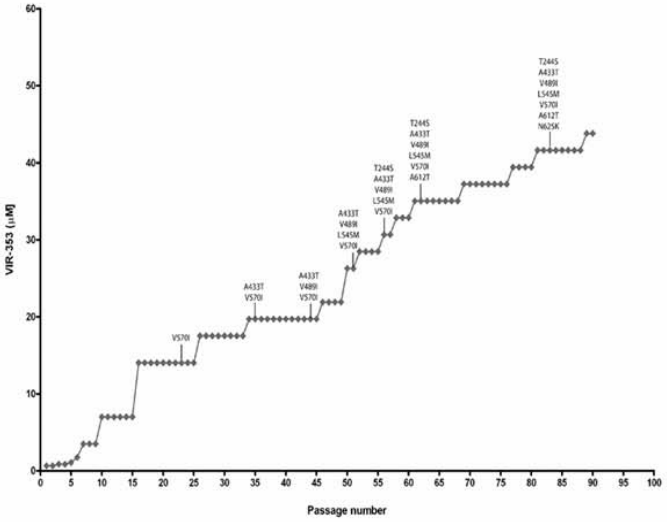


Figure 2. VIR-353 targets a time/site compatible with gp41-dependent fusion. MT-4 cells infected with HIV-1 NL4-3 at a MOI of 0.5 at 20º C for 1 h and 37º C thereafter were treated during virus infection and/or at various times postinfection. Treatment with test compounds BMS-155 (○), AMD3100 (*), T-20 (□), C34 (■), VIR-353 (●) was performed at replication-blocking concentrations (roughly 100-fold the EC_{50}). Viral p24 levels in the culture medium were monitored at 30 h postinfection. The figure shows a representative experiment of 3.

(a)



(b)

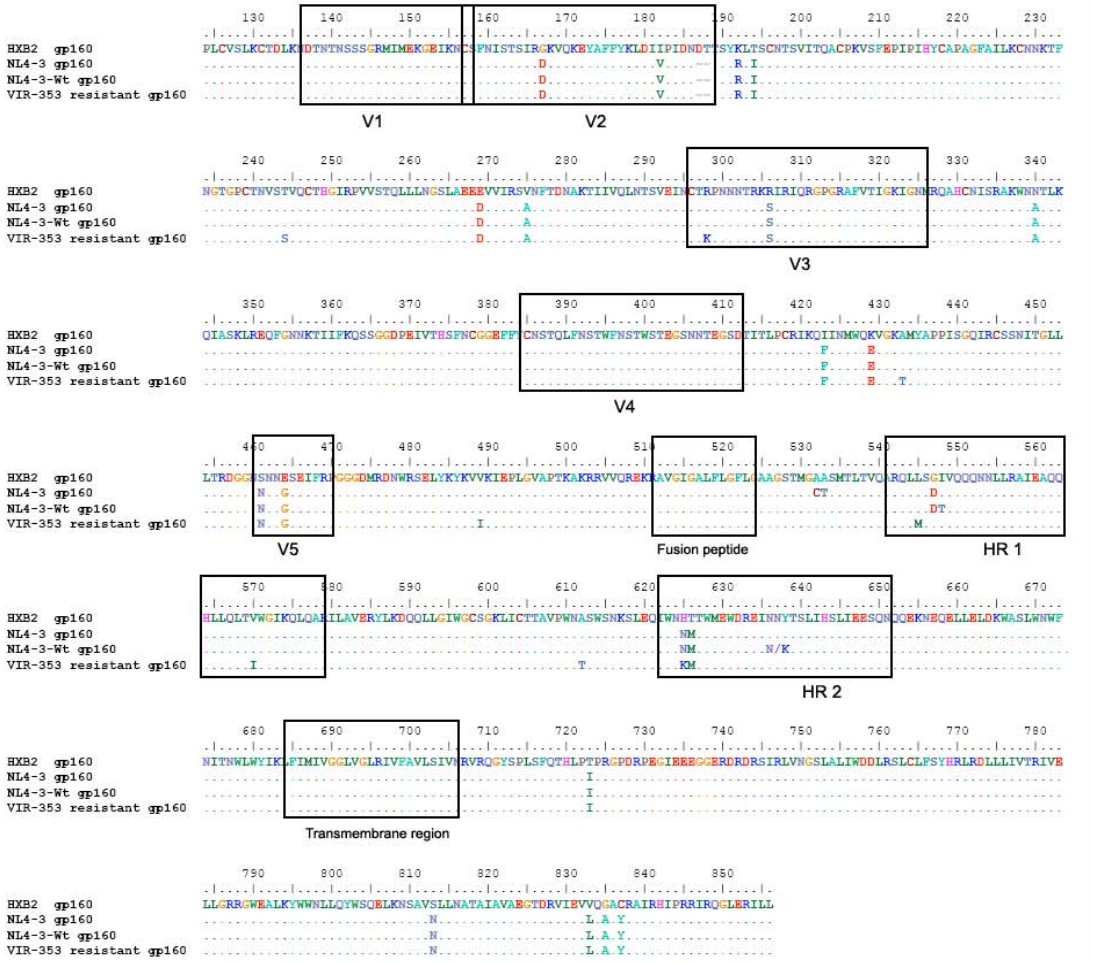


Figure 3. Generation of VIR-353 resistant HIV (a) Time course of the generation of VIR-353-resistant resistant HIV-1. Drug concentration versus passage number is depicted. The passage number were each of the mutations appeared is indicated. (b) gp160 sequence of wild-type and VIR-353 resistant viruses. Sequences were aligned together with the HXB2 and NL4–3 consensus sequence (NL4–3 gp160) and numbering are displayed starting equally to HXB2. Viral sequences are compared to its control strain (NL4–3 wt gp160; virus passed in parallel without VIR-353). Variable loops and conserved regions are indicated. Dots indicate residues equal to the control strain. Dashes (~) indicate absent residues.

Table 2. Activity of VIRIP and VIR-353 against VIR-353 resistant and recombinant strains.

Compound	EC_{50} [a] (μM) [FC] [b]						CC_{50} [c] (μM)
	Recombinant virus						
	NL4-3	VIR-353 Resistant	gp120 Wt – gp41 Wt	gp120 Wt - gp41 VIR-353 Resistant	gp120 VIR-353 Resistant- gp41 VIR-353 Resistant	gp120 VIR-353 Resistant - gp41 Wt	
VIRIP	21.9	>86.81 [>4]	30.14 [1]	84.96 [4]	>86.81 [>4]	>86.81 [>4]	>86.81
VIR-353	0.6	29.18 [49]	1.35 [2]	1.54 [3]	>43.81 [>73]	7.42 [13]	>43.81
AMD3100	0.003	0.001 [1]	0.002 [1]	0.002 [1]	0.002 [1]	0.002 [1]	>6
AZT	0.003	0.007 [2]	0.003 [1]	0.005 [1]	0.005 [1]	0.007 [2]	>7.48
T20	0.1	0.002 [<1]	1.17 [11]	0.002 [1]	0.006 [1]	>2.43 [>24]	>2.43

[a] Effective concentration required to block HIV-1 replication by 50% as measured by the MTT method in MT-4/CCR5 b cells.

[b] FC: fold change or ratio of the corresponding EC50 and the EC50 value of the corresponding wild type HIV-1 strain.

[c] Concentration required to induce cell death by 50% as measured by the MTT method in MT-4/CCR5 þ cells.

EC_{50} values represent the mean of three independent experiments done in triplicates.

4.5.4 Mutations in both gp120 and gp41 were required for the VIR-353 resistant phenotype.

Together with mutations conferring hyper-susceptibility to T20, DNA sequencing of the VIR-353-resistant virus allowed the identification of seven additional non synonymous mutations throughout the env gene that induced amino acid changes in both gp120 and gp41 (Fig. 3A). Surprisingly, none of these amino acid changes occurred within the gp41 fusion peptide. the putative site of interaction of VIRIP. The evolution of genotypic changes accumulated during 450 days in cell culture in the presence of VIR-353 is shown in Fig. 3. Accumulating mutations appeared in the following order: V570I. A433T. V489I. L545 M. T244S. A612T and N625K (Fig. 3).

To evaluate the role of individual mutations, virus carrying single, double or triple mutation combinations were constructed by site directed mutagenesis and tested for sensitivity to VIR-353 and control agents (Table 3). Introduction of single mutants A433T, V489I, L545M and V570I induced a slight reduction in sensitivity (5 to 7-fold change. FC) while T244S. A612Tand N625K did not affect the activity of VIR-353. Incorporation of single mutations did not affect the sensitivity to other agents targeting entry or RT (Table 3). The double mutant A433T/V570I, the first combination that emerged after passage 35 (Fig. 3), reduced the sensitivity to VIR-353 by 8-fold, whereas combinations of the A433T mutation with V489I. L545M or N625K did not have an effect beyond that shown by the A433T mutation alone. However, incorporation of a third mutation (V489I) to generate a virus similar to that emerging after passage 44, fully recovered the VIR-353-resistant phenotype (>80 FC, Table 3). Other double mutations had a mild or no effect in resistance to VIR-353. Although we do not exclude that other combinations could affect resistance, acquisition of mutations A433T/V489I/V570I clearly appeared to play a relevant role in resistance to VIR-353/VIRIP, providing further evidence of a coordinated effect of both gp120 and gp41 on the mechanism of resistance to VIRIP and VIR-353.

Table 3. Activity of VIRIP and Vir-353 against HIV-1 strains containing site directed mutations.

Compound	EC50[a] (μM) [FC][b]														
	HIV-1 NL43	VIR-353 Resistant	T244S	A433T	V489I	L545M	V570I	A612T	N625K	A433T V570I	A433T V489I	A433T L545M	A433T N625K	T244S N625K	V489I N625K	A433T V489I V570I
VIR-353	0,55	29,18 [49]	0,75 [1]	2,99 [6]	3,69 [7]	3,86 [7]	2,75 [5]	1,43 [3]	0,27 [1]	4,45 [8]	3,86 [7]	3,22 [6]	0,52 [1]	0,57 [1]	3,69 [7]	>43,8 [>80]
VIRIP	21,9	>86,81 [>4]	45,62 [2]	28,71 [1]	8,96 [1]	31,84 [1]	46,3 [2]	40,89 [2]	43,58 [2]	37,66 [1]	31,84 [1]	27,8 [1]	39,25 [1]	45,3 [2]	61,6 [3]	>86.81 [>80]
AMD3100	0,003	0,001 [1]	0,001 [1]	0,002 [1]	0,002 [1]	0,002 [1]	0,001 [1]	0,002 [1]	0,001 [1]	0,002 [1]	0,002 [1]	0,003 [1]	0,001 [1]	0,001 [1]	0,003 [1]	0,001 [1]
AZT	0,003	0,007 [2]	0,005 [1]	0,007 [2]	0,009 [3]	0,01 [3]	0,003 [1]	0,004 [1]	0,001 [1]	0,01 [3]	0,009 [3]	0,008 [2]	0,004 [1]	0,003 [1]	0,004 [1]	0,006 [2]
T-20	0,1	0,002 [1]	0,17 [1]	0,09 [1]	0,05 [1]	0,11 [1]	0,12 [1]	0,05 [1]	0,02 [1]	0,1 [1]	0,1 [1]	0,02 [1]	0,21 [2]	0,07 [1]	0,12 [1]	0,02 [1]

[a] Effective concentration required to block HIV-1 replication by 50% as measured by the MTT method in MT-4/CCR5 b cells.
[b] FC: fold change or ratio of the corresponding EC_{50} and the EC_{50} value of the corresponding wild type HIV-1 strain.
EC_{50} values represent the mean of three independent experiments done in triplicates.

4.6 Discussion.

Prior efforts on the selection of VIRIP-resistant virus or introducing mutations in the gp41 fusion peptide (FP) by site directed mutagenesis did not lead to the identification of mutations conferring a VIRIP-resistant phenotype (172). Previous conclusions on HIV escape were based on the recombination of the fusion peptide of polymorphic SIVmac239 strains leading to an average but weak 4.6- fold change in sensitivity that also altered that of T20 (5.6-fold gain). a peptide that targets the HR1 region but not the FP.

Here, virus resistance was generated against VIR-353 instead of VIRIP, as a more potent agent allows for sufficient drug pressure in cell culture that lead to the selection of resistant virus at relatively low drug concentrations, without compromising cell viability. The VIR-353-resistant virus was cross-resistant to VIRIP suggesting that both agents target gp41 in a similar fashion. Analysis of recombinant virus or virus harbouring single mutations or the double and triple combinations that conferred resistance to VIR-353, did not alter the resistance to inhibitors targeting HIV-1 entry or the reverse transcriptase, highlighting the unique mode of action of VIRIP. However, increased susceptibility to T20 was noted; most probably due to the reversion of polymorphism in the NL43 backbone that are associated to resistance to T20.

VIR-353 resistant virus did not have mutations in or near the FP. suggesting an alternative site of VIRIP-gp41 interaction. In fact, the site of interaction of VIRIP remains elusive and cannot be extrapolated from the resistance pattern shown herein. We have found that mutations in gp120 and gp41 generate a combinatorial effect on resistance to VIRIP that was neither achieved by gp120 or gp41 alone, but the combination of both. A time of addition experiment (Fig. 2) clearly pointed to gp41-dependent fusion as the functional step blocked by VIRIP. Thus, we speculate that VIRIP may be inducing a general conformational change that impedes the correct folding of gp41 and prevents fusion. Alternatively, VIRIP may be interacting with a specific site in gp41, different to the FP. In accordance to this, a series of potent D-peptides HIV-1 fusion inhibitors (PIE) have been shown to bind to the N-terminal trimer of gp41 and specifically to a ‘‘pocket’’ formed by the N-peptide’s 17 C-terminal residues (218, 219), a highly conserved motif among different HIV-1 strains that appears to play an essential role in viral entry (71). Similarly to VIR-353, development of resistance to PIE7 has been shown to select for HIV-1 resistance mutations E560K and V570I (71). These mutations weaken the binding of D-peptides to the gp41 pocket but not the C-peptide inhibitor C37

(218). Interestingly, V570I was the first mutation selected by VIR-353 (Fig. 3) that also retained sensitivity to T20. Other mutations conferring resistance to VIRIP were found in close proximity to the pocket-binding domain in the HR2 region (mutations A612T and N625K). Thus, VIRIP and analogue peptides may bind at a site close to, or that includes, the pocket-forming region, impeding appropriate folding of gp41 into a fusion competent conformation but without compromising the binding/ activity of peptides such as T20. Over 95% of HIV strains listed in the Los Alamos Database (http://www.hiv.lanl.gov/) contain a valine (V) residue at position 570 (160) (position 59 of gp41) suggesting that this residue is highly conserved. However, the replicative capacity of the VIR-353-resistant virus was not lower than that of the wild type strain (data not shown). We do not exclude that intermediate virus mutants may have lower fitness and that additional mutations, including those located in gp120 may be required to compensate the incorporation of mutations that would otherwise limit the fitness of the resulting virus. This hypothesis is further emphasized by the long term needed to generate PIE (218) and VIRIP resistant HIV.

The relatively weak potency of the natural VIRIP peptide argues against its relevance as a natural inhibitory agent. Significantly high concentrations would be required to exert an effect on HIV viral load. Nevertheless, chemical modification has led to the development of potent analogues that are active in cell culture and in vivo. VIR- 576 induced a greater that 1.5 log reduction in viral load in short-term monotherapy [18]. However, sequence variations in the HIV-1 gp41 fusion peptide were not responsible for the different virological responses to VIR- 576 treatment (89). VIR-576 and VIR-353 share a high degree of homology (despite VIR-576 being a dipeptide) and appear to share a similar mode of action (172). Thus, our results may highlight relevant amino acid positions that would serve as markers of VIR-576 treatment failure through the generation of resistant virus.

Previous evidence showing inhibition of FP-mediated haemolytic activity and direct binding of VIRIP to a synthetic FP peptide has been shown to hypothesize on the mode of action of VIRIP. However, T20 and other gp41 targeting peptides have also been found to display anti-haemolytic activity (163, 172). Assays evaluating synthetic peptide-peptide gp41 may not truly mimic the complex interaction of gp41 in its natural conformations. A number of polyanionic compounds including XTT and ADS-J1 were active in such synthetic peptide-peptide assays when in fact they are non-specific inhibitors of HIV-1 attachment (12, 100, 148).

Tools to elucidate the mode of action of HIV entry inhibitors (25, 44) and the generation of drug resistance to identify the molecular determinants of viral escape are

relevant proof of the mode of action of an active compound (155). Our results clearly identify VIRIP as a peptide targeting gp41-dependent fusion with a resistance profile that suggests that regions downstream in the HR1 and HR2 but not the FP may be the site of interaction of VIRIP. Primary resistance mutations are often found in or in close proximity to the inhibitor binding or catalytic sites for most anti-HIV agents described to date (156). The lack of mutations found in the FP of VIRIP-resistant HIV-1, the failure to identify mutations in the FP in virus with reduced sensitivity to VIRIP (172) and the observation that sequence variations in the HIV-1 gp41 FP are not responsible for the different virological responses to VIR-576 treatment (89), strongly indicate a relevant aspect of the mode of action of VIRIP: the fusion peptide appears not to be the site of interaction with gp41. Our results may help to identify new peptidic or non-peptidic agents with similar mode of action to VIRIP with a unique resistance profile. Importantly, next generation fusion inhibitors may be screened for activity against VIRIP-resistant HIV.

4.7 Acknowledgements.

Cells, drugs and viruses were received from the EU Programme EVA Centralised Facility for AIDS Reagents, NIBSC. UK (AVIP Contract Number LSHP-CT-2004– 503487) or the National Institutes of Health (AIDS Research and Reference Reagent Program). The authors declare no conflict of interest.

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Chapter 5. Compensatory mutations rescue the virus replicative capacity of VIRIP-resistant HIV-1.

5.1 Objectives of this Study.

The resistance to VIRIP/VIR-353 took a long time to emerge, which indicated a high genetic barrier for the virus to overcome the imposed selective pressure.

In this study, we had as main objective

A. To determine the role of the mutations responsible for the resistant phenotype to VIR-353 in the replicative capacity of the virus.

Additionally,

B. To evaluate the sensitivity to VIRIP/VIR-353 of several strains resistant to the fusion inhibitor T20.

C. To analyse the effect in the sensitivity to VIR-353 of the emerging mutations that lead to a fully VIR-353-resistant virus.

D. To evaluate the antiviral activity of combinations of VIR-353 and T20 over the VIR-353 and T20 resistant strains.

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5.2 Resumen.

VIRIP fue identificado como un inhibidor natural del VIH altamente específico que bloquea la fusión dependiente de gp41 del VIH al interactuar con el péptido de fusión de gp41. Dos análogos de VIRIP (VIR-353 and VIR-576) con algunos cambios en aminoácidos, incrementan su potencia anti retroviral en dos órdenes de magnitud en cultivo cellular y VIR-576 ha mostrado su eficiacia en un ensayo clínico en fase I/II. La resistencia a VIRIP y su análogo VIR-353 fue generada después de un largo periodo de pases en cultivo celular, sugiriendo una elevada barrera genética a la resistencia. Las mutaciones que confieren la resistencia a VIRIP y a VIR-353 redujeron significativamente la capacidad replicativa del virus, pero mantuvieron la resistencia a VIR-353 y completa sensibilidad a T20. Combinaciones de VIR-353 y T20 tuvieron un efecto aditivo en la inhibición de la replicación del VIH-1 silvestre, pero únicamente uno de los agentes fue activo cuando fueron evaluados contra VIH-1 resistente a T20, lo que sugiere que ambos péptidos de gp41 no interfieren mutuamente en su unión a gp41. Nuestros resultados aportan información adicional para el desarrollo de una nueva clase de agentes anti retrovirales que tengan como diana la fusión dependiente de gp41.

5.2 Abstract.

VIRIP has been identified as a highly specific natural inhibitor of HIV-1 that blocks HIV-1 gp41-dependent fusion by interacting with the gp41 fusion peptide. Two analogues of VIRIP (VIR-353 and VIR-576) with a few amino acid changes increase its antiretroviral potency by two orders of magnitude in cell culture and VIR-576 has been shown effective in a phase I/II clinical trial. Resistance to VIRIP and its analogue VIR- 353 were generated after long-term passage in cell culture suggesting a high genetic barrier to resistance. Mutations conferring resistance to VIRIP and VIR353 significantly reduced virus fitness. However, accumulation of additional mutations rescued the replication capacity of the virus while retaining resistance to VIR-353 and full sensitivity to T20. Combinations of VIR-353 and T20 had an additive effect on the inhibition of wild type HIV-1 replication, but only a single agent was active when combinations were tested against T20-resistant HIV-1, suggesting that both gp41 peptides do not interfere with each other in their binding to gp41. Our results provide additional support to the development of a new class of antiretroviral agents targeting gp41-dependent fusion.

5.3 Introduction.

The process of HIV entry is a validated and relevant target for anti-HIV intervention (85). Many agents targeting HIV-1 entry have been tested as putative antiretrovirals, two of them being approved (35) for use in HIV+ individuals: maraviroc (MVC), a CCR5 antagonist, and enfuvirtide (T20), a gp41- derived peptide that binds to the gp41 N-terminal heptad repeat (HR1) and blocks virus- cell fusion (106, 206). The approval of T20 as an antiretroviral therapy has led to the development of second generation HIV fusion inhibitors with similar or improved potency. Importantly, second generation fusion inhibitors are active against T20 resistant viral strains and resistance to these new fusion inhibitors may not confer cross-resistance to T20, suggesting that new agents may be designed as alternative fusion inhibitors (206).

A natural circulating 20-residue peptide, named virus-inhibitory peptide (VIRIP) was discovered as a potent inhibitor of HIV-1 replication (172). VIRIP was identified as a human hemofiltrate-purified fragment of the serine protease inhibitor α1- antitrypsin. VIRIP inhibited a wide variety of HIV-1 strains including those resistant to T20 and other antiretroviral drugs (101, 172). Results showed that either VIRIP or other peptidic analogues with a few amino acid changes that conferred greater anti-HIV potency (VIR-353 and VIR-576, Figure 1), blocked HIV Env-dependent fusion through their interaction with gp41. A clinical trial phase I/II with VIR-576 showed reductions in plasma viral load of more than one order of magnitude after short-term monotherapy and without significant adverse events in patients (89).

The genetic barrier for HIV resistance to T20 is low, generally restricted to the amino acid motif between gp41 residues 36 and 45, that are part of the T20 binding site (104, 156) and are commonly associated with a significant decrease in the replicative capacity of the virus (i.e. virus fitness).

Conversely, the genetic barrier for resistance to VIRIP and its analogues appeared to be relatively high. Initially, a VIRIP-resistant virus did not emerge after two months of weekly passages in the presence of VIRIP (172). Long-term passage of virus in cell culture (>450 days) in the presence of a potent VIRIP analogue (VIR-353) was necessary to generate a VIRIP-resistant strain (101). VIR-353- resistant HIV-1 accumulated up to 7 mutations located throughout the env gene (Table 1), indicating a complex mechanism requiring a coordinated action of both gp41 and gp120. Site-directed mutagenesis confirmed the role of specific

mutations and identified a combination of three mutations (A433T/V489I/V570I) as the most relevant to VIRIP resistance.

Here, we investigate the role of VIRIP-resistance mutations in the replicative capacity of HIV-1.

We have found that mutations required for resistance significantly reduced virus fitness and compensatory mutations rescued virus replicative capacity of VIRIP-resistant HIV-1, explaining the long term required for the resistant virus to emerge.

5.4 Materials and Methods.

Cells, viruses and compounds.

CD4+ lymphoid cell lines MT-4 were obtained through the Medical Research Council (MRC) Centre for AIDS Reagents, London, UK. Lymphoid cells or TZM-bl cells were grown in RPMI 1640 or DMEM (Invitrogen, Barcelona, Spain) and supplemented with 10% fetal calf serum (FCS, Cambrex, Barcelona, Spain) and antibiotics, 2 U/ml penicillin and 2 mg/ml of streptomycin (Invitrogen, Barcelona, Spain). MT-4/CCR5+ (expressing both CCR5 and CXCR4) were generated as described before (14).

The HIV-1 strains BaL, HXB2 and NL4-3 were obtained from the MRC Centre for AIDS Reagents (London, UK). The HIV-1 NL4-3 strain, resistant to T20 has been described elsewhere (13, 159). The AMD3100-resistant HIV-1 has been reported elsewhere (14, 80, 159). The VIR-353, VIRIP-cross resistant virus was selected in cell culture as described before (Gonzalez et al., 2011). VIRIP, VIR-353, and a scrambled version of VIRIP (VIR-SCR) were synthesized by New England Peptide (Gardner, MA) according to the peptide sequences described by Münch et al (172) and He et al (108, 109) (Figure 1). AMD3100, T20 and the broadly neutralizing monoclonal antibodies 2F5 and 4E10 were obtained from the NIH AIDS Reagent Program. The RT inhibitor 3-azido-3-deoxythymidine (zidovudine; AZT) was purchased from Sigma-Aldrich (Madrid, Spain).

Anti-HIV and cytotoxicity assays.

Anti-HIV activity and cytotoxicity measurements in MT-4 cells were based on viability of cells that had been infected or not infected with the corresponding HIV-1 strain at a multiplicity of infection (moi) of 0.003 and exposed to various concentrations of the test compound. After 5 days of incubation at 37ºC with 5% CO2, the number of viable cells was

quantified by a tetrazolium-based colorimetric method (MTT method) commonly used in the evaluation of anti-HIV active drugs (17, 18, 164, 165, 184). Cut- off value in which a virus was considered resistant was a 5-fold increase of the EC50 when compared to the wild type virus due to the variability of the MTT assay in MT-4 cells. Anti-HIV activity determinations were performed in triplicates and data represents the mean of three independent experiments. For drug combinations, checkerboard 1:5 drug dilutions were prepared and evaluated as described above. Combination indexes (CI) were calculated according to the isobologram analysis of drug combinations, following the equation CI=[(D)1/(Dm)1] + [(D)2/(Dm)2] where (D)1 and (D)2 are the doses of compounds 1 and 2 that in combination produce some specified effect and (Dm)1 and (Dm)2 are the doses of the chemicals that when applied singly also have the same effect (Fernandez-Piñas et al., 2010). CI values below 0.9 are synergistic, between 0.9-1.1 are additive and >1.1 are antagonistic (87).

Sequence analysis of HIV strains.

Genomic DNA from infected cells was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Barcelona, Spain). Extracted DNA was used to PCR amplify HIV-1 env gene using Expand High Fidelity PCR System (Roche) and the primers (Forward 5'- aagggccacagagggagccat-3' and Reverse 5'-gcgtcccagaagttccacaa-3'). Before sequencing, the amplified DNA was purified by enzymatic cleanup that eliminates unincorporated primers and dNTPs (Exosap-ITTM, GE Healthcare). Sequencing of amplified DNA was carried out with different primers to ensure obtaining the complete env sequence with the BIGDYE Terminator 3.1 kit (Applied Biosystems, Madrid, Spain) as described before (12, 164). Data were collected with the ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems, Madrid, Spain). Sequences were analyzed with the Sequencher 4.5 software and edited with the BioEdit software. Amino acid positions were numbered according to HXB2 (Los Alamos database).

Virus replicative capacity.

Virus stocks were titrated in CD4+ TZM-bl cells expressing the LacZ gene driven by the HIV-1 long terminal repeat (LTR) and virus infection was monitored by determination of β-galactosidase (β-gal) production as previously described (18). The dilution of virus stocks necessary to induce 0.5 β-gal absorbance units were used to infect lymphoid MT-4 cells as described above. Virus growth kinetics were followed by the MTT colorimetric method and

plotted to the wild type HIV-1 NL4-3 strain. The Student's t test was used to determine statistical significance between values. Values were considered significant when * $p<0.01$, ** $p<0.001$.

5.5 Results.

5.5.1 Anti-HIV activity of VIR-353.

We have previously shown that VIR-353 was active against virus resistant to BMS-155, AMD3100, TAK-779 or nevirapine and the VIR-353-resistant virus was cross-resistant to VIRIP but remained sensitive to T20, AMD3100 or AZT (101). VIRIP and VIR-353 were also active against two different T20-resistant HIV-1 strains, one derived from the HIV-1 HxB2 strain (HC43) and one derived from the HIV-1 NL4- 3 strain (NT38), which is also 5-fold resistant as compared to HxB2, due to the G36V mutation in gp41 that has been associated with resistance to T20. Two monoclonal antibodies targeting gp41 (2F5 and 4E10) were similarly active against the VIR-353- resistant virus (Table 2), highlighting differences in the mode of interaction of these agents with gp41 as compared to VIRIP. We observed that the VIRIP-resistant virus was 50-fold and 10-fold hypersensitive to T20 when compared to the NL4-3 and HxB2 strains, respectively. This effect was dependent on two mutations, D36G and T37I, found in the gp41 of the VIRIP-resistant virus that appeared after passage in cell culture for more than 400 days. Mutants D36G and T37I have been previously associated with resistance to T20 (156).

VIRIP: LEAIPMSIPPEVKFNKPFVF
VIR-353: LEAIP**C**SIPp**CFL**FNKPFVF
VIR-576: LEAIP**C**SIPP**EFL**F**G**KPFVFx2
VIR-SCR: KVINPEPIVEPFMSKPFALF

Figure 1. Sequence of VIRIP and its analogues VIR-353 and VIR-576. p: D-proline. Cysteine residues are linked via a disulfide bridge. Bold letters indicate changes to the VIRIP sequence.

5.5.2 Reduced replicative capacity of VIR-353/VIRIP-resistant virus.

To determine the effect of mutations conferring resistance to VIRIP in virus replicative capacity (fitness), the concentration of virus required to induce 0.5 absorbance units of β-galactosidase activity after infection of HeLa TZM-bl cells was used to infect lymphoid MT-4 cells. Virus growth kinetics were followed by the MTT colorimetric method and plotted relative to the wild type HIV-1 NL4-3 strain (Figure 2). Virus passages selected were those in which emerging mutations were first identified by sequencing of the proviral DNA of infected cells (Table 1, Table 3).

Table 1. Amino acid changes identified in the VIR-353 resistant virus.

Order of appearance	Mutation	Gene
1	V570I	gp41
2	A433T	gp120
3	V489I	gp120
4	L545M	gp41
5	T244S	gp120
6	A612T	gp41
7	N625K	gp41

Table 2. Antiviral activity of VIRIP and VIR-353 against T20 resistant strains.

Compound	EC_{50} [a] (μM) [FC] [b]					CC_{50} [c] (μM)
	HIV-1 NL4-3	HXB2	T20-Resistant (NT38)	T20-Resistant (HC43)	VIR353/VIRIP-Resistant	No virus
VIRIP	22	38.14 [1]	55.22 [2]	51.44 [2]	>86.81 [N.A.]	>86.81
VIR-353	0.6	0.55 [1]	0.37 [1]	1.08 [2]	29.18 [49]	>43.8
VIR-SCR	>17.36	>17.36 [N.A.]	>17.36 [N.A.]	>17.36 [N.A.]	>17.36 [N.A.]	>17.36
T-20	0.1	0.02 [0]	1.41 [14]	>2.42 [N.A.]	0.002 [0]	>2.42
2F5	0.2	ND	ND	ND	0.2	>10
4E10	1	ND	ND	ND	1	>10

[a] EC_{50} Effective concentration required to block HIV-1 replication by 50% as measured by the MTT method in MT-4 cells.
[b] FC: fold change or ratio of the corresponding EC_{50} and the EC_{50} value of the corresponding wild-type HIV-1 strain.
[c] CC_{50} Concentration required to induce cell death by 50% as measured by the MTT method in MT-4 cells.
Values represent the mean of three independent experiments each one done in triplicates.
ND: not determined

The V570I mutant had a significantly lower fitness that the parental wild-type strain. Further passages maintained a relative low fitness but increased drug-resistance without a change in T20 susceptibility (Table 3). Further passage of virus led to a significant increase in virus fitness while retaining the VIR-353/VIRIP resistant phenotype (Figure 2). However, virus isolated after passage 62 showed a marked increase (50-fold) in susceptibility to T20 (Table 3). Taken together, these results suggest that mutations conferring resistance to VIRIP had a significant cost in virus fitness, but complementary mutations in both gp120 and gp41 allowed the recovery of the virus replicative capacity.

Table 3. Antiviral activity VIRIP and VIR-353 against viral strains that emerged during the generation of resistance to VIRIP.

	EC_{50}[a] (μM) [FC][b]							
Virus passage	**0**	**25**	**44**	**51**	**56**	**62**	**83**	**90**
Compound	**Wild-type**	**V570I**	**A433T/V570I**	**A433T/V489I /V570I**	**A433T/V489I/ L545M/V570I**	**T244S/A433T/ V489I/L545M/V570I**	**T244S/A433T/ V489I/L545M/V570I/A612T**	**VIR353 Resistant**
VIR-353	0.6	4.77 [8]	3.33 [5]	11.14 [18]	>13.14 [>22]	>13.14 [>22]	>13.14 [>22]	>13.14 [>22]
VIRIP	22	42.47 [2]	29.92 [1]	>86.81 [>4]	>86.81 [>4]	>86.81 [>4]	>86.81 [>4]	>86.81 [>4]
T20	0.1	0.04 [1]	0.03 [1]	0.02 [1]	0.03 [1]	0.002 [0]	0.002 [0]	0.006 [0]
AZT	0.002	0.003 [2]	0.002 [1]	0.001 [1]	0.002 [1]	0.004 [2]	0.002 [1]	0.001 [1]
AMD3100	0.002	0.004 [2]	0.002 [1]	0.001 [1]	0.004 [2]	0.002 [1]	0.002 [1]	0.002 [1]

[a] Effective concentration required to block HIV-1 replication by 50% as measured by the MTT method in MT-4 cells.

[b] FC: fold change or ratio of the corresponding EC_{50} and the EC_{50} value of the corresponding wild-type HIV-1 strain.

Values represent the mean of three independent experiments each one done in triplicates.

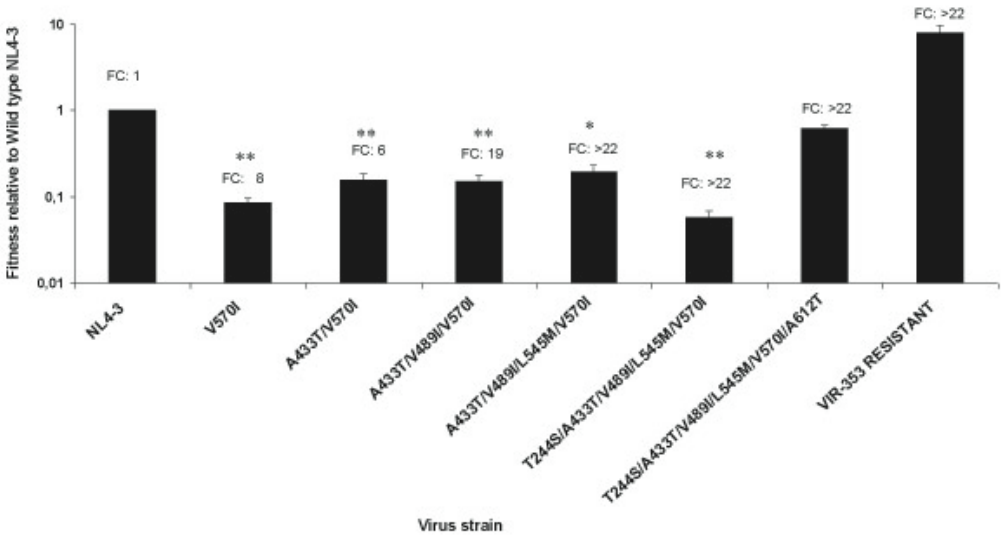

Figure 2. Replicative capacity of the viral strains isolated at different time points during the selection of the VIRIP resistant virus. The concentration of virus required to induce 0.5 absorbance units of b-galactosidase activity after infection of HeLa TZM- bl cells was used to infect lymphoid MT-4 cells. Virus growth kinetics were followed by the MTT colorimetric method and plotted relative to the wild type HIV-1 NL4-3 strain. Bars represent the mean and SD of three independent experiments. FC: Fold-change in EC50 of the corresponding virus relative to the wild type NL4-3 strain. Statistical significance (Student's t test) compared to the parental HIV-1 NL4-3 strain is show as $p<0.01$ (*), $p<0.001$(**).

5.5.3 Combinatorial effect of VIRIP and T20.

Since resistance to VIRIP may be affecting the sensitivity to T20 we evaluated the effect of combinations of both agents and AZT as a control unrelated drug (Figure 3). Checkerboard combinations of VIR-353 with T20 or VIR-353 with AZT demonstrated an additive effect when tested against wild-type NL4-3 (Figure 3A and 3B) with a mean CI of 0.99±0.52 and 0.97±0.27, respectively. Only VIR-353 was active when evaluating its antiviral activity in combination with T20 against a T20-resistant virus (Figure 3D), whereas the combination of VIR-353 and AZT showed an additive effect (mean CI 1.09±0.26) as with the wild-type virus (Figure 3C). These results suggest that T20 and VIR-353 do not interfere with each other in their binding to gp41.

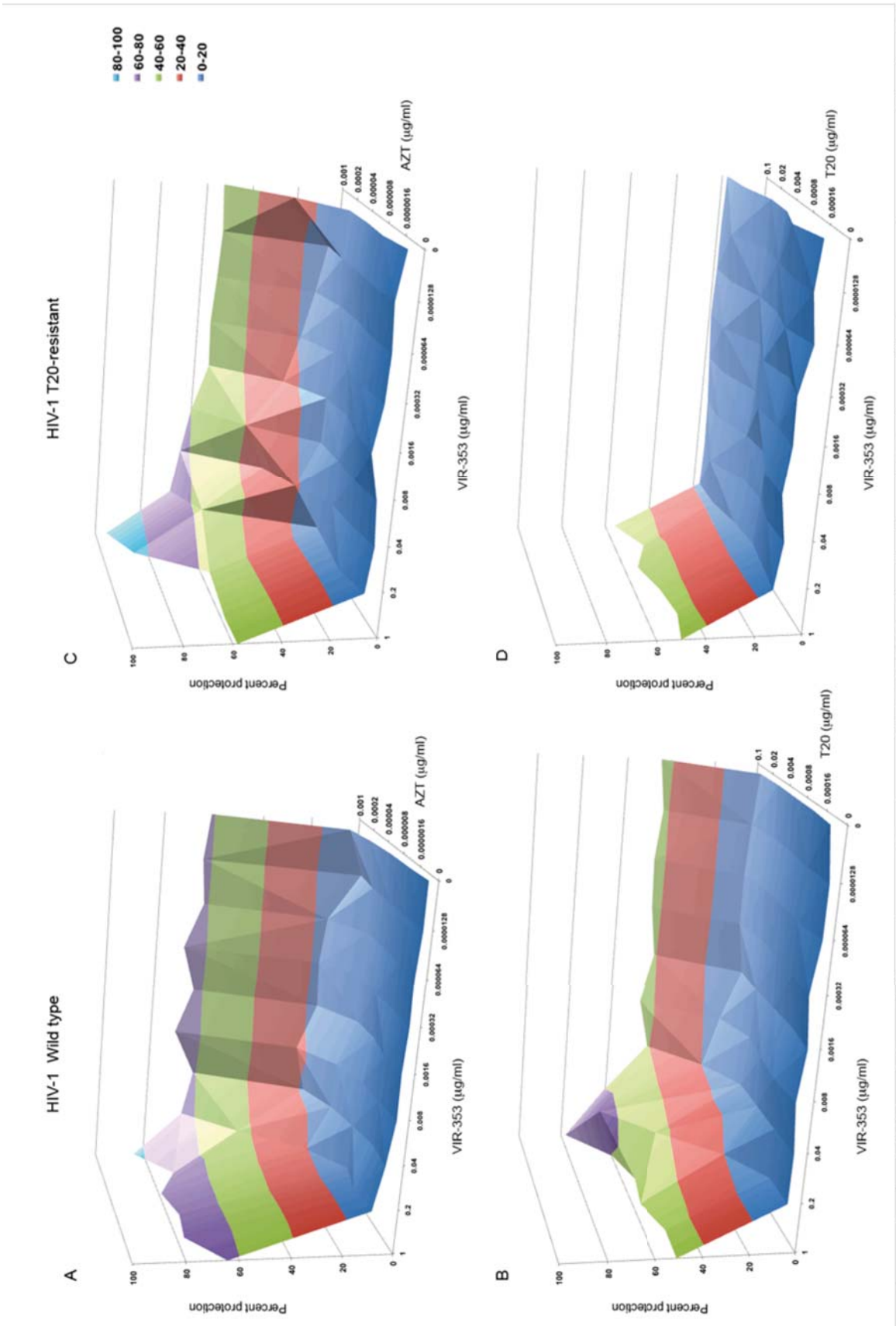

Figure 3. Anti-HIV activity of drugs in combination with VIR-353. Checkerboard 1:5 drug dilutions starting at roughly the 50% effective concentration or below, were prepared and evaluated for anti-HIV activity in lymphoid MT-4 cells by the MTT colorimetric method. Graphs show the activity of combinations of VIR-353 with AZT (upper panels) or T20 (lower panels) in cells infected with wild type (A, B) or T20-resistant (C, D) HIV-1 NL4-3 strains. Colors indicate the percentage of protection within a range. The Figures show only one representative experiment out of three done in triplicate.

5.6 Discussion.

The envelope gp120 has been selected by nature as a major target for neutralization of HIV. However, through its high mutation rate, the virus fights back by easily generating neutralization-resistant gp120 variants and decoys for the immune system. Although gp41 is also a target for neutralization, raising antibodies that target the fusion peptide (FP) of gp41 is a difficult process (27) because it may not be readily available. Thus, the identification of natural inhibitors targeting gp41 such as VIRIP is a remarkable discovery in need of further evaluation. Importantly, VIRIP is being used as a template to generate new, more potent anti-HIV agents (89).

Here, we further explored the anti-HIV effect of VIRIP and its derivatives by evaluating the effect of VIRIP-resistant mutations in viral fitness. Generation of drug resistance took a long time to develop, indicating a high genetic barrier to resistance. Indeed, the first mutation selected by VIR-353 (V570I) induced a marked decrease in virus fitness. Two other mutations were also necessary to increase resistance and further 90 passages were needed to rescue virus replicative capacity. Notably, HIV-1 was able to overcome the cost of viral fitness by the incorporation of 4 additional mutations, leading to a fully competent VIRIP-resistant virus.

Unfortunately, our results do not allow to clearly delineate the mode of action of VIRIP, previously thought to bind to gp41 FP. We could not discard a putative interaction between VIRIP and gp41 FP but the identified mutations are clearly located outside the fusion peptide. Moreover the resistant mutations significantly affect virus sensitivity to both VIRIP and VIR-353, together with a decrease in the replicative capacity of the virus, a hallmark of virus-drug resistance. The emergence of mutations throughout gp120 and gp41 suggests a general rearrangement of the virus envelope to compensate for the reduced replicative capacity.

Notably, virus passage in the presence of VIR-353 became hyper-susceptible to T20 as compared to the wild type NL4-3 virus that is naturally resistant to T20 when compared to other wild type HIV-1 strains such as HxB2. HIV-1 NL4-3 contains two polymorphisms (G36V and G36D) that are commonly associated to reduced sensitivity to T20 (Menendez-Arias, 2010). It has been shown that following discontinuation of T20, HIV-1 T20-resistant NL4-3 virus (D36) revert to the G36 sequence and therefore restoring replicative capacity associated with normal drug susceptibility (104). The HIV-1 NL4-3 (D36 / I37) and T20-resistant NL4-3 revert to the G36 / I37 sequence within two weeks of growth in the absence of inhibitor. Thus, VIR-353/VIRIP appears not to exert selective pressure on the same amino acid changes that induce

T20-resistance, suggesting a distinct mode of action for VIRIP and its more potent analogue. Combinations of VIR-353 and T20 showed an additive affect in blocking the replication of wild type HIV-1 but only the activity of a VIR-353 when evaluating the replication T20-resistant HIV. Taken together, these results suggest that VIRIP and its analogues may bind to gp41 in the presence of T20 and do not interfere with its anti-HIV activity. Despite its efficacy, treatment with VIR-576 has drawbacks that include high treatment doses and intravenous injection or infusion.

Furthermore, the large-scale production of peptides under GMP conditions is still relatively expensive. Nevertheless, the development of small-molecule inhibitors with an analogous mode of action that can be administered orally is ongoing (89). Our results shed light on the mechanism of resistance of VIRIP and its analogues and may help to identify new peptidic or non-peptidic agents with similar mode of action to VIRIP with a unique resistance profile.

5.7 Acknowledgements.

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Chapter 6. General discussion.

Chapter 6. General discussion.

Recently, the twenty-fifth anniversary of the discovery of AZT -the first antiretroviral drug- was celebrated. After these years, many antiviral drugs targeting several steps of the virus replication cycle have been included to the arsenal for the fight against HIV (35).

This have resulted at least in high-income countries, in drastic increases in the life expectancy of the HIV-infected individuals, a delay in the time to diagnosis with AIDS, and a decrease in the appearance of opportunistic infections and HIV-associated morbidity. Although relevant steps have been achieved in the prevention of new infections and the access to treatment for patients worldwide (by 1999 the number of new infections worldwide has fallen by 15%, www.unaids.org), significant challenges remain to be faced. Some of these include, among others: the emergence of resistance to drugs, the transmission of drug-resistant strains, the adverse effects of the administered drugs, the avoidance of the interaction between drugs and the search for new and more potent antiviral compounds.

ADS-J1, a low molecular compound targeting gp120.

The discovery and usage in the clinic of the HIV-1 entry inhibitor maraviroc and fusion inhibitor enfuvirtide (T20) are two hallmarks of the latest advances in the management of HIV-1 infection.

The understanding of the entry process of HIV-1 into the host cell has been based in molecular and structural strategies, and has pointed this essential step of the HIV replication as a relevant pharmacological target. Additionally, mutagenic studies have been effective to map regions of the HIV-1 envelope genes and to collect information about the complex gp120-gp41 interaction, which has been used in the design of antiviral compounds with high specificity and high genetic barrier for HIV resistance.

Structural studies driven to shed light into the viral and cell membrane fusion process, lead to the description of the first fusion inhibitor (T20), an analogue of the HR2 region of gp41 that prevents the formation of the six helix bundle by competing for binding to the HR1-HR2 domains of gp41 (127); and it marked the starting point for a series of breakthrough discoveries that confirmed the processes of HIV entry and membrane fusion represent an open road to explore new targets for therapeutic intervention in the HIV infection treatment.

However, T20 displayed a low genetic barrier to resistance (133, 136, 193) and the reengineering of peptidic fusion inhibitors derived from T20 was evidently required. A second and third generation of fusion inhibitors that were active against T20-resistant strains and with increased inhibitory activity, such as T-1249, Sifuvirtide or T-865 was developed (75, 133, 181). Unfortunately, its application in the clinic was been halted due to formulation issues.

Since most of the peptidic fusion inhibitors now discovered were modelled with the structure of gp41 as reference, they present some disadvantages, i.e. they must be administered via intravenous to bypass degradation by cellular proteases; additionally the cost of production is elevated. These arguments support the need to develop non-peptidic small-molecule gp41 fusion inhibitors.

The generation of *in vitro* HIV-1 resistant strains is a valuable tool to characterize the mode of action of an antiviral drug (157). This procedure provides relevant information such as the specificity and site of interaction of the drug, and the genetic barrier imposed to the virus to overcome the inhibitory effect of the compound. Additionally, the rational modelling of new compounds, and the *in silico* molecular docking techniques along with immunological assays, has powered the manner in which new antiviral compounds are designed and/or screened.

ADS-J1 was selected by these procedures. This non-peptidic compound was originally selected by computational docking analysis to interact with the hydrophobic cavities of the coiled coil region of gp41 (Annex I) (64, 214). Nevertheless, the generation of several HIV-1 strains resistant to ADS-J1 by Armand-Ugón *et al*, pointed the gp120 gene, specifically the V3 loop region, as the target of ADS-J1 (12). Using molecular dynamics and docking models, Manetti and collaborators confirmed that ADS-J1 disturbs the interactions between gp120 and the cell coreceptor, confirming gp120 as the target of the polyanionic compound (148).

Later, Wang *et al* suggested that ADS-J1 binds to a peptide that imitate gp41, and suggested that this small compound could serve as model for designing new HIV-1 inhibitors (214). We considered important to study in a more detailed manner the mode of action of ADS-J1.

The identification of the mechanism of action of the antiviral compounds may be confusing due to the experimental design or by the assays utilized, which sometimes do not, or only partially model the mechanisms or events that take place in a cell culture or *in vivo*. A case that illustrates this situation is the study of AR177 (zintevir), which was originally identified as

an integrase inhibitor, later it was demonstrated that in cell culture conditions, AR177 acts by blocking the binding and entry of the virus to the host cell (55, 76).

We confirmed that ADS-J1 does not target gp41. The generation of chimeric virus having gp120 or gp41 coding sequences from an ADS-J1 resistant virus showed that the recombinant virus having gp120 from the ADS-J1 into a wild-type backbone restored the resistant phenotype. On the contrary, the recombination of gp41 from the resistant strain did not affect the sensitivity to ADS-J1.

The time of drug addition (TOA) is a valuable and relative simple assay, widely used in our laboratory to dissect, in a highly sensitive manner, the time/site in which an antiviral compound acts (59). We have demonstrated that ADS-J1 does not show the same behaviour as compared to the other fusion inhibitor tested, T20. Moreover, it appeared that ADS-J1 acted in a previous step of the virus entry process.

The ADS-J1 resistant virus introduced several mutations, mainly in the gp120 region. Although the role of each individual mutation was not dissected in our study, it has been previously shown that the generation of resistance to entry inhibitors may produce complex patterns of mutations that in general point to the gene to which the drug targets (21, 84, 169).

In HIV-1, the emergence of resistance to entry inhibitors is due to multiple rearrangements of the gp120/coreceptor/gp41 complex. These rearrangements may induce mutations of the gp120-gp41 entry *machinery* of the virus in order to overcome the genetic barrier imposed by the drug.

The development of small-molecule inhibitors of gp41-mediated fusion is still a missing goal in the search for new antiviral drugs nevertheless, relevant steps have been taken to direct the constantly updated information of the structure, and biology of the gp41 mediated fusion process, to the design of non-peptidic fusion inhibitors.

High genetic barrier to resistance to VIRIP/VIR-353. The selective pressure imposed by the peptide may induce an altered conformation of the gp120-gp41 complex in the VIRIP resistance virus.

Several components in the human tissues have been pointed to show inhibitory activity of HIV-1 replication in infected individuals. There are remarkable examples that illustrate this characteristic, i.e. the entry of HIV can be blocked by chemokines, which bind to the receptors used by the virus to entry the cells, another example is the interferon inducible protein Tetherin (BST-2) which impedes the release of nascent HIV-1 particles from infected cells (10, 107, 142).

A 20-residue peptidic fragment derived from the most abundant blood-circulating serine protease inhibitor, alpha–1 antitrypsin (AAT) was isolated from a library generated from human hemofiltrate. This peptide, named virus inhibitory peptide (VIRIP), showed antiviral activity by blocking the membrane fusion mediated by gp41 (172). A previous study by Shapiro *et al,* showed the antiviral properties of alpha–1 antitrypsin, including an increase in the levels of the protease inhibitor under inflammatory conditions. At physiological concentrations, AAT blocked the replication of HIV in different cell types including PBMCs (201).

In a study aimed to explore the earliest immune responses that are activated after the exposure to HIV-1 and that have influence in the virus spread containment and disease progression, among others, the alpha–1 antitrypsin (from which the peptide VIRIP is derived) was detected in the plasma samples of donors. The plasma was collected over a time period that spanned the period before and immediately after detection of the infection with HIV-1 (129).

The levels of alpha–1 antitrypsin were increased in plasma during the acute infection phase, accounting for a concentration in μg/ml, which is similar to the range in which VIRIP blocks HIV-1 entry, as reported by Münch *et al* (172)*, and* by our own studies. The study by Kramer *et al* demonstrated for the first time that some acute phase proteins that are systemically induced, exhibit antiviral activity even before the detection of viremia (129).

We generated a HIV-1 virus resistant to VIR-353, a peptidic analogue of the originally isolated VIRIP. VIR-353 was engineered to enhance its antiviral activity and differs in 5 amino acid residues from the original alpha–1 antitrypsin derivate; also, VIR-353 contains a D-Proline at position 10.

The VIR-353 resistant strain is cross-resistant to VIRIP, suggesting that both peptides target gp41 in a similar fashion. Resistance to VIR-353 instead of VIRIP was developed because of the higher antiviral activity of VIR-353, allowing us to impose sufficient selective pressure in the cell culture conditions forcing a resistant virus to emerge.

Mutations conferring the resistant phenotype were found in both Env glycoproteins gp120 and gp41. The VIR-353 resistant strains introduced three mutations in different conserved regions of gp120. In the conserved region 2 (C2), a threonine (T) is substituted by a serine (S) at position 244 (numbering according to HXB2 HIV-1 strain). In the C4 region of the VIR-353-resistant strain at position 433 an alanine (A) was substituted by a threonine (T), and in the C5 region of the resistant strain, at position 489, a valine (V) was substituted by an isoleucine (I).

The emergence of mutations in the conserved regions of the gp120 glycoprotein may suggest that VIR-353/VIRIP alter the normal conformation of the gp120-gp41 complex in order to overcome the pressure imposed by the peptide.

Helseth *et al* found that the conserved regions C1 and C5 are relevant for the association between gp120 and gp41. Additionally, an alanine at position 433 in the highly conserved region 4 is relevant for the interaction between gp120 and gp41 (111).

The VIR-353 resistant virus introduced mutations in regions of gp41 different than the fusion protein, suggesting an alternative site of interaction between gp41 and VIR353. The pocket-forming domain (PFD) in the HR1 and the pocket-binding domain (PBD) in HR2 of gp41 have been shown to interact during the fusion process. Mutations within these regions of gp41 appear to severely affect the replicative capacity of the virus, suggesting that compounds targeting these domains would impose a high genetic barrier (199). The substitution V570I, in the pocket-forming domain of the VIR-353 resistant virus was detected. Sen *et al* reported that the mutation of this residue produced a virus defective in the fusion process (199).

Additionally, VIRIP/VIR-353 appears to have a target site different than the membrane proximal external region (MPER). The VIR-353 resistant virus did not introduce any mutation in the MPER (aa 660 to 683). Moreover, we evaluated the neutralizing capacity of the broadly neutralizing antibodies 2F5 and 4E10 against the VIR-353-resistant virus. We did not observe changes in the EC_{50} values of any of the bNtMAbs tested against the VIR-353-resistant virus,

suggesting that VIR-353 does not interact with the same region in which the two bNtMAbs tested bind gp41.

Our results showed that in the VIR-353 resistant virus, the mutations in gp120 and gp41 produce a combinatorial effect on the resistance to VIRIP, the fully resistant phenotype is not only given by mutations in gp120 or by the mutations in gp41. Additionally, we performed site directed mutagenesis in order to evaluate the role of the individual mutations.

A virus with the combination of three mutations, two in gp120 and one in gp41: A433T/V489I/V5701 recovered the resistant phenotype to VIR-353. These mutations are the same as the ones that were introduced at the passage number 44, during the generation of the virus resistant to VIR-353. The change A433T is located in the C4 region of gp120; the mutation V489I is located in the C5 region of gp120 and V5701 is located in the pocket-forming domain of gp41.

The residue valine 570 (position 59 of gp41) is listed over 95% of HIV strains in the Los Alamos Database (http://www.hiv.lanl.gov/), suggesting that this residue is highly conserved among HIV strains. These findings confirmed the combinatorial effect of the mutations in gp120 and gp41 that result in the resistant phenotype to VIRIP/VIR-353.

The residue V570 has been recently confirmed as essential for the entry process. A series of D-peptides with highly specific inhibitory activity, named D-peptides HIV-1 fusion inhibitors (PIE) have been reported (218, 219). It was shown that the D-peptides bind to the pocket-binding domain in gp41, preventing the entry of the virus into the cells. Development of resistance to one of these peptides, PIE7, selected for the mutations E560K and V570I, these mutations weakened the binding of D-peptides to the pocket in gp41 (71). Interestingly, the VIR-535 resistant strain introduced the V570I mutation and two other mutations, A612T and N625K, that are in close proximity to the pocket-binding domain of gp41, suggesting that VIR-353 may bind to the PBD or a region near to it, impeding the correct folding of gp41, leading to a defective entry of the virus.

The activity of a VIRIP analogue, VIR-576 has been tested in a phase I/II clinical trial. VIR-576 share a high degree of homology with VIR-353 despite VIR-576 is a dipeptide (89, 172). In the clinical trial, under monotherapy with VIR-576, the viral load was reduced 1.5 log and without severe adverse effects.

We evaluated the antiviral activity of VIR-576 against the VIR-353 resistant strain. Results showed cross-resistance to the VIR-576, confirming that VIR-353 and VIR-576 share a similar mode of action (Supplementary Table 1 of Annex).

Additionally to the intrinsic characteristics of the viral strain, the human host imposes different types of selective pressure over the HIV-1 in a variety of micro environments, these conditions ultimately define the ability of the virus to replicate and to cause damage to the immune system, as reflected by the onset of AIDS. Moreover, the degree of potential genetic changes drives the diversity of HIV, as a response to selective pressure or antiviral therapy (158). Due to the biology of HIV-1, most of the selective pressure is directed against the *env* gene, which plays an essential role in the fitness of the virus.

Fitness is defined as a variant's ability to produce successive generations (67) and is a descriptor the replicative adaptability of an organism to its environment (158). HIV-1 variants with high levels of fitness should have a selective advantage over the variants with lower fitness. The introduction of resistance mutations due to a selective pressure over the virus usually implies a decrease in the replicative capacity of the resistant virus, as compared to that of the wild-type strain (158, 187).

Resistance to VIR-353 took a long time to emerge, suggesting that VIR-353 imposed a high genetic barrier for the virus to overcome. We evaluated the replicative capacity of the mutations that lead to the VIR-353 resistant virus.

We found that the first mutation selected by VIR-353 (V570I) provoked a drastic reduction of the viral fitness while the EC_{50} of the peptide changed by 8-fold. As mentioned before, three mutations (A433T/V489I/V5701) yielded the resistant phenotype to VIRIP/VIR-353. At this point, the replicative capacity of the virus slightly recovered as compared with the one measured when the first mutation emerged.

The incorporation of four additional mutations recovered the virus fitness and reflected the phenotype of the VIR-353 resistant virus. The selection of mutations in the env gene of the VIR-353 resistant suggested a rearrangement of the envelope to overcome the selective pressure, which is reflected in the replicative capacity of the resistant virus.

We observed that the VIR-353 resistant virus is hyper-susceptible to T20, comparing to the wild-type NL4-3 virus, which is naturally resistant to T20, compared to other HIV-1 strains

(i.e. HxB2). Two polymorphisms in NL4-3 (G36V and G36D) have been associated with resistance to T20 (155, 156). Nevertheless, after discontinuation of treatment with T20, the T20 resistant strain D36 residue reverts to G36 recovering the sensitivity to T20 with a recovery of the viral fitness (104, 151).

The combination of VIR-353 and T20 showed an additive effect in blocking the replication of the wild-type virus but when the combination was tested against a virus resistant to T20, only the activity of VIR-353 was observed. These results suggest that VIR-353 bind to gp41 in the presence of T20 and may exert selective pressure not in the same residues than the ones that are changed in gp41 of the T20- resistant virus.

The gp120-gp41 double play: from coreceptor binding to the fusion process.

There is a continuously growing amount of information that confirms that gp41 has an essential role in entry steps that previously were only associated as gp120-dependent, as well as the cooperative role of gp120 in the membrane fusion process. This is reflected in the ability of HIV-1 to mutate in both gp120 and gp41 glycoproteins in order to subvert the selective pressure imposed by the environment, i.e. inducing a reduction in the association of the subunits in the envelope trimer, leading to a decreased binding to the chemokine receptor (224). It has been proposed that the entry of HIV follow a kinetic model. The binding of gp120 to CD4 and to the coreceptor trigger conformational changes of the Env glycoprotein, accumulating energy that is later required to proceed to the re arrangements that end with the membrane fusion (95).

Pancera and collaborators dissected the previously unknown interacting regions of gp120 and gp41 in the CD4-bound state. They performed more than 5000 crystallization trials of 20 different gp120-ligand combinations, including those with the gp41-interactive region.

These findings could be used for a better understanding of the complex mechanisms of viral entry involving the Env glycoproteins and the cell receptors and may offer new targets for drug design (183).

Huang *et al* showed that regions of the transmembrane domain of gp41 (TM) could influence the viral entry in env clones containing identical V3 regions having either CCR5 or CXCR4 as coreceptor (116). Additionally, it has been described HIV-1 variants that use CCR5 with mutations in gp41, mainly in HR2 and TM. These alterations, including several in the

cytoplasmic domain of gp41, provoked a reduced usage of CCR5 coreceptor by the virus to enter the cells. Moreover, the sensitivity to the inhibition by T20 can be modulated by differences in the V3 loop of gp120 (65, 205, 224).

In a study aimed to explore the communication between mutations in gp120 and gp41, Wang and collaborators generated resistant HIV strains to an HR1 peptide inhibitor. Several mutations appeared in both gp120 and gp41. They defined two principal genetic pathways in the emergence of resistance to peptide fusion inhibitors. Although the mutations that give the resistant phenotype were in gp41; they suggested that mutations in gp120 (some of them found in the V3 loop) may affect the usage of coreceptor (215).

Our results offer additional information of the plasticity of the HIV-1 Env glycoproteins which becomes evident when the virus replicates under drug selective pressure. The generation of resistance to fusion inhibitors may reveal that the emergence of compensatory mutations restore the function of the envelope glycoproteins that may end in conformational changes of the viral entry *apparatus* that permit the fusion of the viral and cell membranes.

Additionally, the results shown and discussed here may be useful in future studies aimed to design new peptidic and non-peptidic compounds with similar mode of action to VIRIP and its derivatives.

Chapter 7. Conclusions.

The most remarkable conclusions of the studies presented here can be summarized as follow:

- The HIV envelope glycoproteins are characterized for showing a high level of plasticity in order to ensure the survival of the virus. This characteristic becomes more evident when the virus replicates under drug selective pressure. In response to the selective pressure generated by an antiviral compound, the diversification and preservation of HIV are driven by genetic changes.

- The generation of *in vitro* resistance to anti-HIV agents is a widely utilized laboratory assay. It may provide relevant information such as the putative site of interaction of a drug and the genetic barrier that HIV needs to overcome the inhibitory effect. The identification of the mechanism of action of antiviral compounds is essential for successful drug design.

- The generation of resistance to agents that block the gp41-mediated membrane fusion, i.e VIRIP/VIR-353, implied the incorporation of additional mutations throughout the envelope gene which may represent a compensatory strategy to preserve the viability of the virus, reflected, i.e. the introduction of mutations that restore the replicative capacity of the virus.

- The HIV glycoproteins gp120 and gp41 interact in a highly orchestrated manner during the viral entry. Specific regions in both gp41 and gp120 glycoproteins interact with each other in order to maintain the association of the subunits in the native trimeric structure of the HIV envelope spike. The emergence of mutations during the generation of a resistant strain to an entry inhibitor suggest that both gp120 and gp41 glycoproteins adopt altered conformations in order to overcome the selective pressure imposed by the antiviral agent.

- Additional studies with VIRIP and its derivatives are needed. The resistance profiles studied here may be helpful in the design of new antiviral agents with enhanced potency and higher genetic barrier to HIV resistance.

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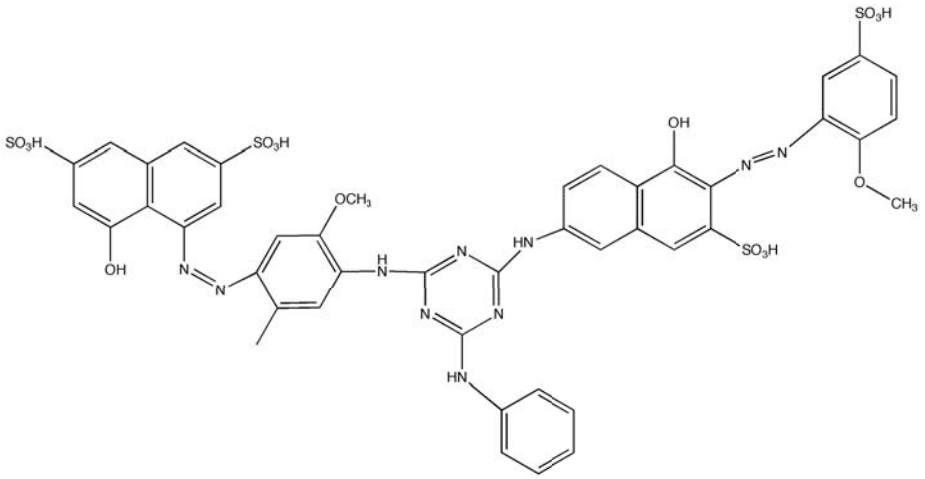
ANNEX.

Annex I

Molecular structure of ADS-J1.



Annex II

Supplementary Table 1

Anti-HIV activity of VIR-576 against the VIR-353 resistant strain and resistant strains to other antiviral compounds

Compound	EC_{50} (μM)		EC_{50} (μM) [a] [F-C][b]				CC_{50} (μM)
	NL43	**HXB2**	**BMS-155-Resistant**	**T20-Resistant**	**A433T V489I V570I**	**VIR-353 Resistant**	
VIR-576	0.26	0.25 [1]	0.43 [2]	0.21 [1]	>5.45 [>20]	>5.45 [>20]	>5,45
VIR-353	0.53	0.71 [1]	0.92 [2]	0.75 [1]	>11 [>20]	>11 [>20]	>11
T-20	0.11	0.04 [0]	0.07 [1]	>0.5 [>5]	0.01 [0]	0.005 [0]	>0,5
C34	0.0003	0.0001 [0]	0.0003 [0]	0.03 [100]	0.0006 [2]	0.0002 [1]	>0,23
BMS-155	0.06	0.03 [1]	>13.3 [>220]	0.03 [1]	0.11 [2]	0.06 [1]	>13,3
AZT	0.005	0.003 [1]	0.01 [2]	0.002 [0]	0.003 [1]	0.005 [1]	>3,7
AMD3100	0.002	0.003 [2]	0.003 [2]	0.001 [1]	0.001 [1]	0.001 [1]	>1,2

[a] EC_{50} Effective concentration required to block HIV-1 replication by 50% as measured by the MTT method in MT-4 cells.
[b] FC: fold change or ratio of the corresponding EC_{50} and the EC_{50} value of the corresponding wild-type HIV-1 strain.
[c] CC_{50} Concentration required to induce cell death by 50% as measured by the MTT method in MT-4 cells.
Values represent the mean of three independent experiments each one done in triplicates.

Annex III

Publications

Compensatory mutations rescue the virus replicative capacity of VIRIP-resistant HIV-1.

González-Ortega E, Ballana E, Badia R, Clotet B, Esté JA
Antiviral Res. 2011 Dec;92(3):479-83. Epub 2011 Oct 18.

Toward the discovery of novel anti-HIV drugs. Second-generation inhibitors of the cellular ATPase DDX3 with improved anti-HIV activity: synthesis, structure-activity relationship analysis, cytotoxicity studies, and target validation.

Maga G, Falchi F, Radi M, Botta L, Casaluce G, Bernardini M, Irannejad H, Manetti F, Garbelli A, Samuele A, Zanoli S, Esté JA, **González E**, Zucca E, Paolucci S, Baldanti F, De Rijck J, Debyser Z, Botta M.
ChemMedChem. 2011 Aug 1;6(8):1371-89. doi: 10.1002/cmdc.201100166. Epub 2011 Jun 22

Development of resistance to VIR-353 with cross-resistance to the natural HIV-1 entry virus inhibitory peptide (VIRIP)

González E, Ballana E, Clotet B, Esté JA.AIDS. 2011 Aug 24;25(13):1557-83.

Diarylpyrimidine-dihydrobenzyloxopyrimidine hybrids: new, wide-spectrum anti-HIV-1 agents active at (sub)-nanomolar level.

Rotili D, Tarantino D, Artico M, Nawrozkij MB, **Gonzalez-Ortega E**, Clotet B, Samuele A, Esté JA, Maga G, Mai A.
J Med Chem. 2011 Apr 28;54(8):3091-6. Epub 2011 Mar 25.

ADS-J1 inhibits HIV-1 entry by interacting with gp120 and does not block fusion-active gp41 core formation.

González-Ortega E, Mena MP, Permanyer M, Ballana E, Clotet B, Esté JA
Antimicrob Agents Chemother. 2010 Oct;54(10):4487-92. Epub 2010 Jul 19.

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Caporuscio F, Tafi A, **González E**, Manetti F, Esté JA, Botta M.
Bioorg Med Chem Lett. 2009 Nov 1;19(21):6087-91. Epub 2009 Sep 13.

Discovery of chiral cyclopropyl dihydro-alkylthio-benzyl-oxopyrimidine (S-DABO) derivatives as potent HIV-1 reverse transcriptase inhibitors with high activity against clinically relevant mutants.

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J Med Chem. 2009 Feb 12;52(3):840-51.

Annex IV

Methods

A considerable amount of the results obtained in this work where obtained mainly by three techniques: The *in vitro* generation of HIV-1 resistant strains, the screening of HIV-1 replication inhibitors by the tetrazolium-based colorimetric assay, and the time-of-drug-addition (TOA) for the identification of the target/site of antiviral compounds. In this manner, it is convenient to explain them in a brief manner.

***In vitro* generation of HIV-1 resistant strains.**

The high variability of the RNA viruses, including HIV-1, imposes complications for its clinical control. During the time-course of the infection by HIV-1, there are many genetic variants, instead a unique genetic virus genotype (185). These genetic variants, named quasispecies, are genetically related and its emergence is regulated by several conditions: the HIV-1 error-prone reverse transcriptase, a high rate of virus replication, the selection pressure imposed by the immune system and the presence of antiretroviral drugs. These factors impose a constant selective pressure over the HIV strains, ultimately leading to the emergence of escape mutants (68, 135) .

The *in vitro* development of drug resistant strains is a valuable tool for identifying the precise target of a determined antiviral compound, the mechanism of interaction between the compound and the viral protein and the genetic barrier needed by HIV to overcome the inhibitory effect of the compound (157).

The selection of in vitro resistant HIV strains is performed by continuously passaging of previously infected cells with a parental virus strain in the presence of the compound of interest. The test is usually initiated with low multiplicity of infection (moi) and a drug concentration between 1-to 5-fold its effective concentration 50 (EC_{50}), previously determined by an antiviral activity assay. After 5 days of culture or after considerable cytopathic effect is observed, fresh uninfected cells are infected with the supernatant recovered from the previous infection and in the same or higher concentration of the testing compound is added. This procedure is known as a passage.

Usually, two other cell cultures are set as comparative controls: one is infected with a determined volume of the supernatant from the previous passage and another having only

seeded cells. It is recommended to save the infected cells and the resting supernatant from previous passages in order to sequence the portion of the HIV genome of interest to find mutations induced by the selective pressure imposed by the drug.

After subsequent passages the concentration of the tested drug in the culture may be significantly increased. The cell supernatant is titrated for infectious virus and the sensitivity to the drug is evaluated (157).

Determination of the anti retroviral activity and toxicity of compounds.

The assay is based in the determination of the viability of cells in culture by a colorimetric technique. The HIV-1 cytopathic effect induced over the MT-4 cells is measured as the degree of killing due to the infection by HIV-1. The multiplicity of infection used in the assay allows only a few or none of the cells to be alive after five days of infection so, the effect of a biologically active antiretroviral compound will be reflected in the survival of the cells.

The viability of the cells is measured by the reduction of the 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan product (207).

The following is a resumed protocol of the technique which is further explained in the reference (184)

i) Fill flat-bottomed 96-well microtiter plates with 100 μl of complete RPMI medium.

ii) Add stock (10X highest final test concentration) solution of compounds in 25 μl volume to the six middle wells of the second column of the microtiter plate (2G-B)

iii) Make nine serial, fivefold dilutions of the compound directly to the microtiter plates using a multichannel pipette. Proceeding as follows: 25 μl out of column 2 and add it to column 3, mix and take 25 μl out of column 3. Repeat the dilution up to column 10. Column 11 is the control.

iv) Add 50 μl of the dilution of the previously tittered HIV-1stock at 100-300 $CCID_{50}$ (184) to the upper rows (rows B-D), and medium to the lower rows (rows E-G) of the microtiter plate.

v) Centrifuge exponentially growing MT-4 cells (220g/5 min). Discard the supernantant.

vi) Resuspend the MT-4 cells at 6 $X10^5$ cells per ml in complete RPMI and dispense 50 μl of cell suspension to the microtiter plate wells except for the outer rows (column 1 and rows A and H). Fill column 12 with cells as well with the multichannel pipette.

vii) Incubate the plates for in a 37 ºC with 5% CO_2 incubator.

viii) Five days after infection, add 20 μl of tempered MTT to each well of the microtiter plates using a multichannel pipette.

ix) Incubate the microtiter plates in an incubator at 37 ºC and 5% CO_2.

x) Remove 150 μl from each well of the microtiter plate using the multichannel pipette without aspiring the cells away from the well

xi) Add 150 μl of the cell lysis buffer: acidified Triton X-100 isopropanol solution, which is also used to inactivate the virus. Solubilize the formazan crystals.

xii) Read the absorbance of the wells at two wavelengths (540 and 690 nm). Substract the absorbance measured at 690 nm from the absorbance at 540 nm to eliminate the effect of scattering.

xiii) Calculate the EC_{50} and CC_{50}

EC_{50} is the concentration of compound needed to inhibit 50% of the HIV-induced cell death

$$EC_{50} = ((ODT)_{HIV} - (ODC)_{HIV}) / ((ODC)_{mock} - (ODC)_{HIV}) * 100$$

where

$(ODT)_{HIV}$ is the OD measured with a given concentration of the test compound in the HIV-infected cells; $(ODC)_{HIV}$ is the OD measured for the control untreated, HIV-infected cells (column 11), which stands for 100% infection-related CPE; and $(ODC)_{mock}$ is the OD measured for the control untreated, mock-infected cells (column 11), which stands for 0% infection-related CPE

CC_{50} is the concentration of compound needed to induce 50% of death in the mock-infected control sample non-infected cells, observed by the reduction in the absorbance (OD540) of the by 50%.

Identification of the target/site of antiviral compounds.

This *in vitro* assay is based in the determination of how long the addition of a compound can be postponed before it loses its antiviral activity.

In the time of addition assay (TOA), the knowledge of the chronological order of the major steps in the HIV replication cycle was taken into account for design of the experiment. Considering that a single round of HIV-1 replication cycle takes approximately 24 h, after a synchronized bulk HIV-1 infection of MT-4 cells, the test compound is added at 0 min, 7 min, 14 min, 21 min, 28, min, 35 min, 1 hour, 2 hours and 24 hour after the virus infection.

The target of the compound can be inferred by its behaviour in the time-course experiment when compared to that of the control drugs

The following is a resumed protocol of the assay, which is more detailed explained in the reference (59).

i) Prepare a solution of 1000-fold the EC_{50} of the test compound and the drugs used as controls, considering 11 time points (PreTAS, 0 min, 7 min, 14 min, 21 min, 28 min, 35 min, 60 min, 120 min, 24 h, 31 h) at 20 μl per well per time point, in a 96 well microtiter plate. In the experiment, the final concentration of the compounds will be 100-fold its EC_{50}.

ii) Time point 0 h. Add 20 μl of each of the previously prepared compound solution to a 1.5 ml tube.

iii) Count and place the required number of MT-4 cells for the experiment (10×10^4 cells are needed for a 96-well plate). Pellet the cell suspension and discard the supernatant. Resuspend the cells in the required volume of complete medium to have a final concentration of 5×10^5 cells per ml.

iv) In strictly order, add 175 μl of cells, then add 5μl of the solution with the virus to the previously prepared 1.5 ml tubes

v) Bulk infection. Considering 5μl of virus stock solution, add the required amount of virus to the cell suspension for having a multiplicity of infection of 0.5

vi) In order to synchronize the bulk infection, incubate the infected cells and the 1.5 ml tubes for 1 h at 19-21°C. This is the temperature arrested state (TAS).

vii) After 1 h of incubation, wash the bulk infection and the 1.5 ml tubes with PBS1X to eliminate the unbound virus.

viii) Resuspend the infected cells from the bulk infection and the PreTAS points considering 180 μl per compound per time point. Seed the resuspended cells in the 96-well plate.

ix) The time-course starts when the compounds are added in time 0 min.

x) Add the compounds according to each pre established time-point.

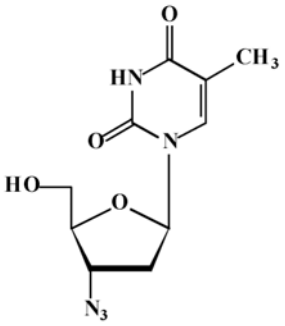
xi) At the time point 31 hour, collect the supernatant of each time point of the experiment. Quantify the CA p24 antigen in the supernatant by ELISA

xii) Plot the amount of p24 for each compound at each time point versus the time points.

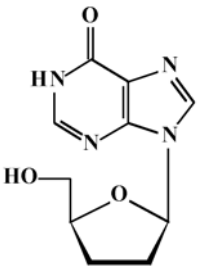
Annex V

Molecular structure of the anti-HIV drugs currently approved by the FDA

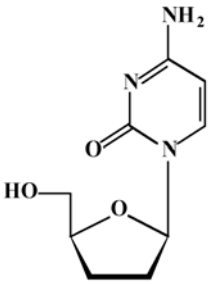
Nucleoside reverse transcriptase inhibitors (NRTI)



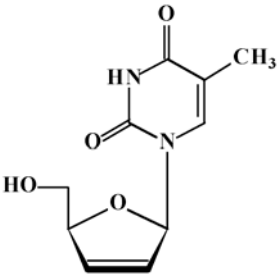
Retrovir ®, 3'-Azido-2',3'-dideoxythymidine azidothymidine (AZT)



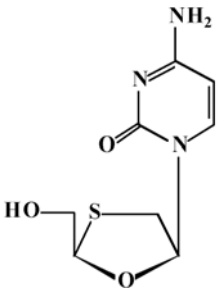
Videx ®, Didanosine (ddI)



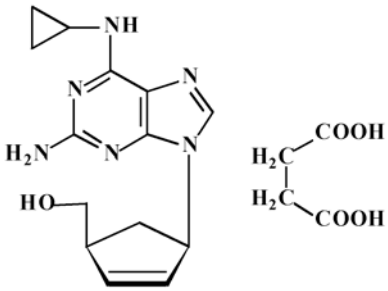
Hivid ®, Zalcitabine



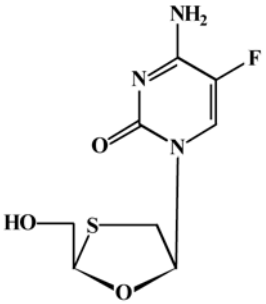
Zerit®, Stavudine



Epivir ®, Zeffix ® ,Lamivudine

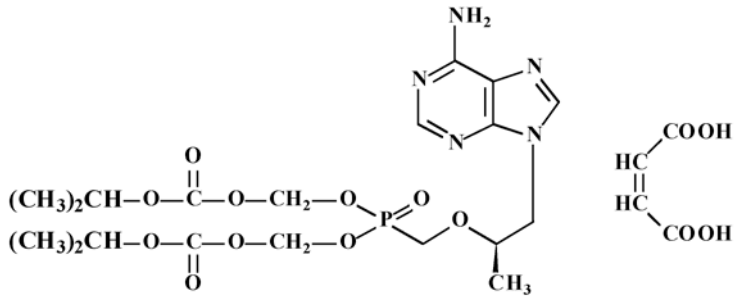


Ziagen ®, Abacavir (ABC)



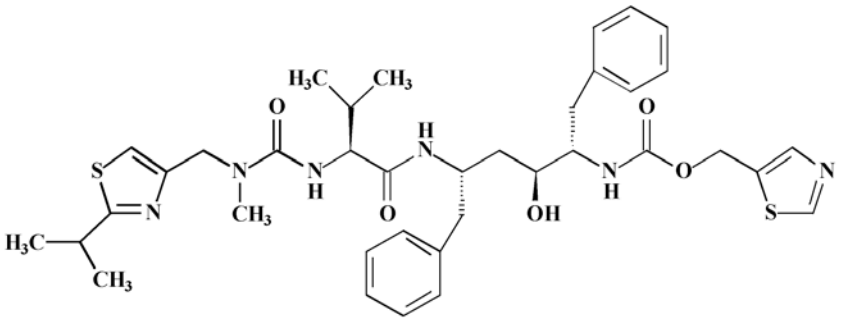
Emtriva®, Emtricitabine ((-)FTC)

Nucleotide reverse transcriptase inhibitor (NtRTI)

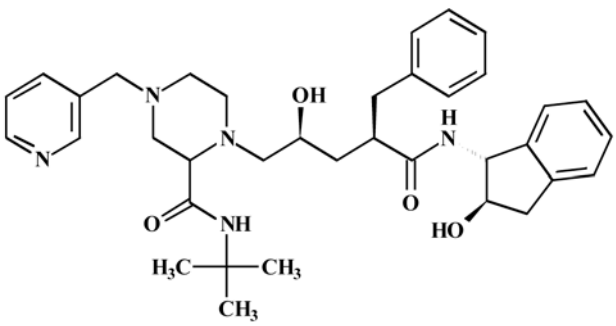


Viread®, Tenofovir disoproxil

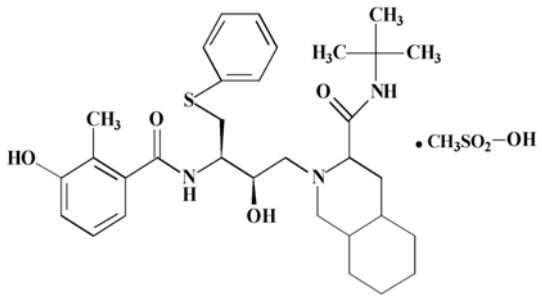
Protease inhibitor (PI)



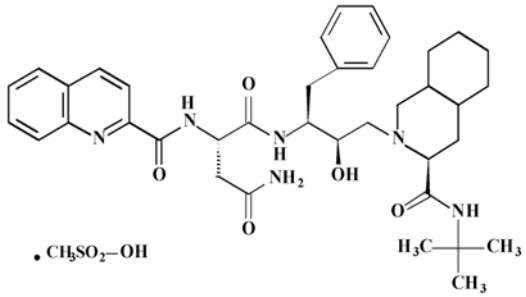
Norvir®, Ritonavir



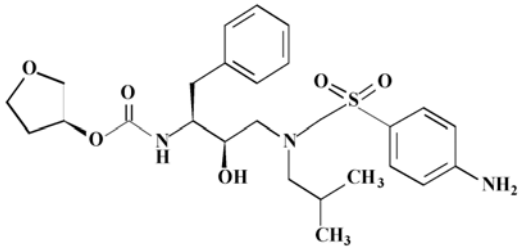
Crixivan®, Indinavir



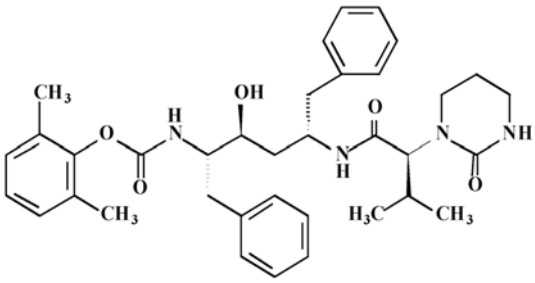
Viracept®, Nelfinavir



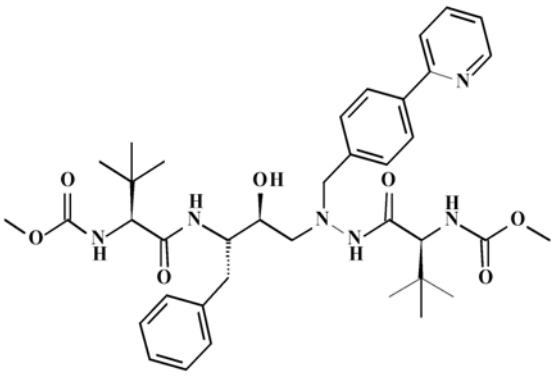
Fortovase®, Saquinavir Soft Gel Capsules



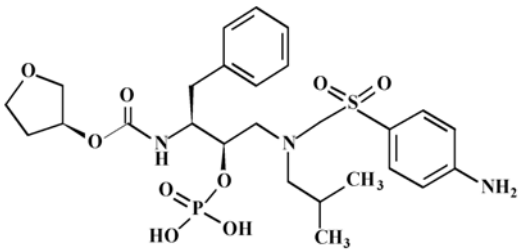
Agenerase ®, Prozei ®
Amprenavir



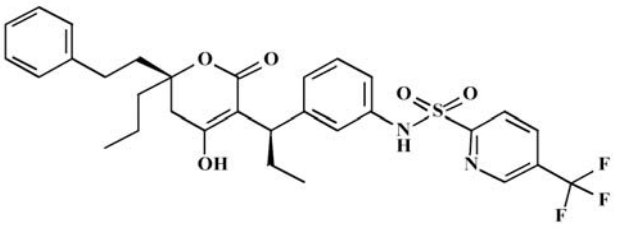
Kaletra®, Lopinavir + Ritonavir



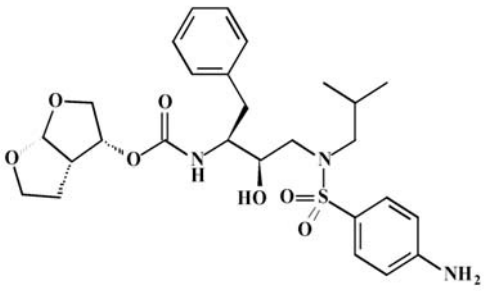
Reyataz®, Atazanavir



Lexiva ®, Fosamprenavir

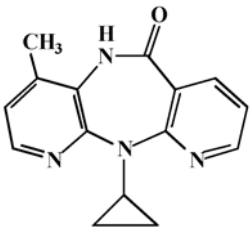


Aptivus ®, Tipranavir

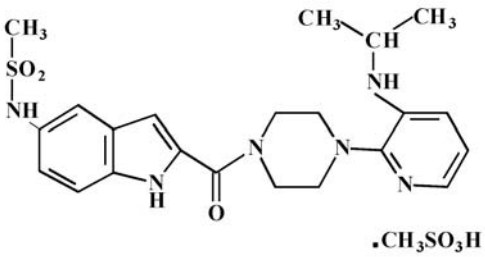


Prezista®, Darunavir

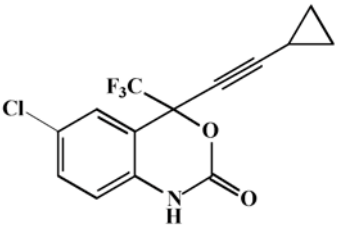
Non-nucleotide reverse transcriptase inhibitor (NNRTI)



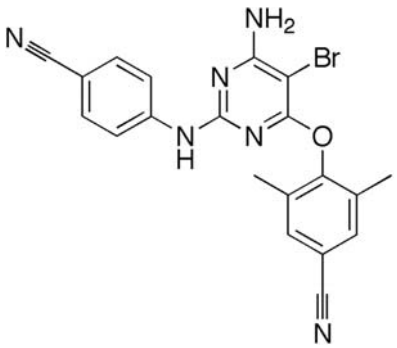
Viramune ®, Nevirapine



Rescriptor ®, Delavirdine



Sustiva ®, Stocrin ®, Efavirenz



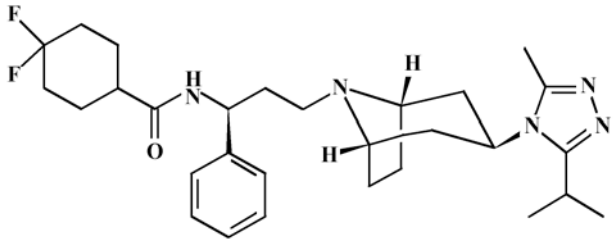
Intelence®, Etravirine

Fusion inhibitor

YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF

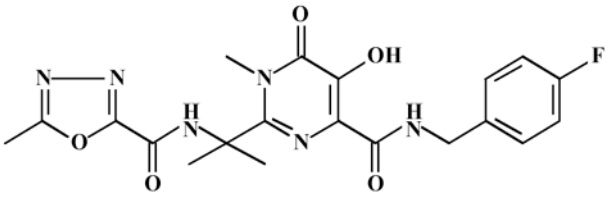
Fuzeon®, Enfuvirtide (T20)

Co-receptor inhibitor (CRI)



Selzentry®, Maraviroc

Integrase inhibitor (INI)



Isentress ®,Raltegravir

Annex VI

Acronyms

AIDS: Acquired Immunodeficiency Syndrome

bNtAb: Broadly neutralizing antibody

CA: HIV capsid or P24 protein

CD4: Transmembrane glycoprotein. Member of the immunoglobulin super family.

CI: Coreceptor inhibitor.

CT: Cytoplasmic tail region of the HIV gp41 protein

DNA: Deoxyribonucleic Acid

DS: Dextran Sulfate

ELISA: Enzyme Linked ImmunoSorbent Assay

FDA: United States of America Food and Drug Administration

gp120: HIV Glycoprotein 41

gp41: HIV Glycoprotein 120

GPCR: G-coupled receptor protein

HAART: Highly active antiretroviral therapy

HIV: Human Immunodeficiency Virus

HR: Heptad repeat

IN: HIV integrase

INI: HIV Integrase inhibitor

kb: Kilobase

MA: HIV matrix or P17 protein

MHC-II: Major histocompatibility complex class II

MPER: Membrane-proximal external región of the HIV gp41 protein

MOI: Multiplicity of infection

MTT: 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NC: HIV nucleocapsid or P7 protein

nm: Nanometer

NNRTI: Non-nucleoside reverse transcriptase inhibitor

NRTI: Nucleoside reverse transcriptase inhibitor

PBMC: Peripheral Blood Mononuclear Cells

PCR: Polymerase Chain Reaction

PI: Protease Inhibitor

PR: HIV protease

RANTES: Regulated on activation normal T-cell expressed and secreted

RNA: Ribonucleic Acid

RNase H: Ribonuclease H

RT: Reverse Transcriptase

SFV: Sifuvirtide. Peptidic fusion inhibitor.

SPR: Surface plasmon resonance assay

SU: HIV surface protein or gp120

TAS: Temperature arrested state

TCR: T-cell antigen receptor

TOA: Time of drug addition assay

TM: HIV transmembrane protein or gp41

UNAIDS: Joint United Nations Programme on HIV/AIDS