AIDS Rev 2001; 3: 121-132

# HIV Resistance to Entry Inhibitors

*José Andrés Esté*

*Retrovirology Laboratory IrsiCaixa, Hospital Universitari Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, Spain*

**Abstract**

**The replicative cycle of HIV can be interrupted at several stages. The reverse transcriptase and protease are the enzymes currently targeted by approved antiretroviral agents. However, a number of compounds are being developed that are targeted at earlier stages of infection, namely HIV adsorption (binding) to the host cells and virus-cell fusion. The discovery of chemokine receptors as coreceptors for HIV entry has also prompted the development of chemokines and chemokine analogues as anti-HIV agents. HIV will escape the inhibitory action of entry inhibitors by different mechanisms depending on the mode of action of the specific agent or how the selection of resistant variants was designed. As expected, development of resistance to entry inhibitors is followed by the emergence of mutations in the corresponding gene. Resistance to agents that block virus attachment, virus binding to CD4 and agents that block coreceptor interaction generate HIV strains with mutations in the gp120 coding region. Agents that target gp41-dependent fusion select for HIV variants with mutations in the gp41 gene. Since HIV may use more than one coreceptor for entry, there are HIV strains that are naturally resistant to agents that block a specific coreceptor. Under selection pressure and with an alternative coreceptor available, HIV should switch coreceptor use. Alternatively, HIV may develop resistance to a coreceptor antagonist in the absence of coreceptor switch. Notably, the fact that envelope glycoproteins are key determinants of virus induced pathogenicity, tropism, replicative capacity and viral fitness suggests that mutations that confer resistance to entry inhibitors will modify these parameters. Thus, therapeutic strategies that aim at blocking virus entry may also be used to alter the natural evolution of HIV in an unprecedented way. By altering HIV envelope-dependent pathogenicity, we could envision new ways to manage a chronic virus infection. Results from recent clinical trials with the fusion inhibitor T-20 are encouraging. HIV entry appears to be the next target for therapeutic intervention as new agents that block the early steps of HIV replication are being evaluated in the clinic. New technology for phenotypic and genotypic assessment of drug resistance needs to be in place for the coming of a new generation of antiretroviral agents.**

**Key words**

**HIV. Inhibitor. Entry. Fusion. Resistance. Coreceptor.**

***Correspondence to:***
José Andrés Esté
Fundació IrsiCaixa, Retrovirology Laboratory
Hospital Universitari Germans Trias i Pujol
08916 Badalona, Spain
E-mail: jaeste@ns.hugtip.scs.es

## General introduction to HIV entry

The need for new classes of antiretroviral drugs has become apparent from the increasing concern on the long term toxicity[1] and the spreading of HIV-1 variants that are resistant to current treatment options[2,3]. All available regimes consist of combinations of inhibitors of two viral enzymes, the reverse transcriptase (RT) and protease (P). Nevertheless, there are several steps of the human immunedeficiency virus (HIV) replication cycle that may be a target for intervention. These can be divided into entry steps, which involve viral envelope glycoproteins and their receptors, and the post-entry steps involving viral accessory gene products and the cellular proteins with which they interact[4]. Viral entry may be dissected into four interrelated steps, namely, virus attachment to the cell surface, virus binding to the CD4 receptor[5,6], the interaction of the CD4-envelope glycoprotein complex to entry coreceptors and virus-cell fusion. Despite several indications that HIV variants may enter cells in a CD4-independent manner[7,8] or use alternative surface antigens such as CD8, it is well established that gp120 is essential for all steps of HIV entry and that replication competent viral entry is driven by protein-dependent membrane interactions that are mediated by gp41.

The interactions of enveloped viruses with susceptible cells are mediated by glycoprotein oligomers that provide both binding to cellular receptors and post-binding events in virus entry, including membrane fusion. HIV possesses two glycoproteins that are associated with one another and derived by endoproteolysis from a single precursor (gp160)[10]. The larger of these (gp120) is derived from the amino-terminal portion of the precursor, lies entirely outside of the envelope lipid bilayer, mediates receptor binding and drives the fusion process. The smaller, derived from the carboxyl-terminal portion of gp160, is the transmembrane protein (gp41) and mediates both oligomerization of the complex into multimers as well as membrane fusion[11,12]. A model for gp120, demonstrating five variable domains interspersed with conserved regions is shown in figure 1. The predicted sequence of gp120 shows 18 cysteine residues which are highly conserved in diverse HIV-1 strains, disulfide bonds are presumed to play a critical role in the structure and function of these viral proteins: the disulfide bonding pattern of gp120 delineates the protein into several functional regions, which include a conformational-dependent domain for recognition of the CD4 receptor[13].

Regions of HIV-1 gp120 interacting with the CD4 receptor have been deduced from site-specific mutations in the *env* gene which demonstrate that a limited number of conserved amino acids in different regions of gp120 are required for efficient binding to CD4[14]. Both primary sequence and conformational features of the envelope (Env) glycoprotein gp120 produce a configuration that recognizes the CD4 receptor in a selective fashion and with high affinity. Many of the points of contact between gp120 and CD4 are made using the peptide backbone of gp120 amino acids. This allows HIV-1 to alter the residues that form the CD4 binding domain and changing the antigenic structure of the site (that normally involves the amino acid side chains) while retaining the capacity to bind CD4.

The region of gp120 that binds the coreceptor has been revealed by the crystal structure of gp120[15-18]. The residues involved are located within the highly conserved stem of the V1/V2 structure, near the base of the V3 loop, and in other regions folded into proximity.

The V3 sequence, one of the five variable domains in the gp120 subunit of HIV-1, usually contains 34 to 36 amino acids arranged in a disulfide loop involving

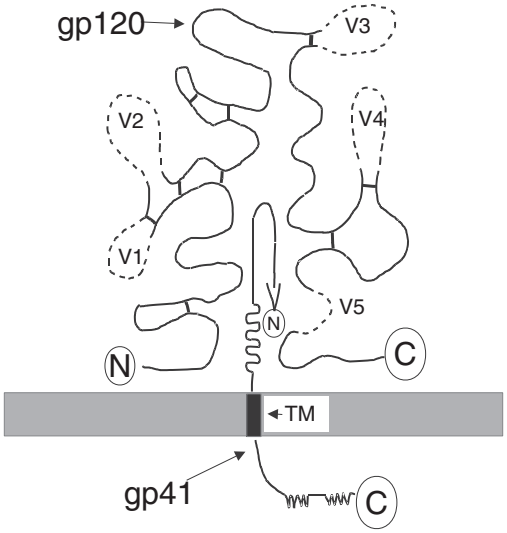


**Figure 1.** *Model of the envelope glycoprotein complex of HIV-1. The predicted folding pattern of gp120 and the positions of disulfide bonds that form the hypervariable loops (V1 through V5). The folding pattern of gp41 is also shown. TM, transmembrane domain. Amino and carboxyl termini are labeled N and C, respectively.*

Cys 296 and Cys 331 (Fig. 2)[10]. This domain plays an important role in governing several biological properties of the virus (i.e. cell tropism, cytopathicity, fusogenicity and coreceptor use)[19, 20]. Deletions in the V3 loop abrogate viral infectivity[21] and deletion of the two bordering cysteines of the V3 region forming the disulfide bridge giving it a loop structure appeared to be detrimental in the processing of gp160 into gp120 and gp41[22]. Although the actual sequence between the two cysteines seems not to be of importance for processing and binding to CD4, it is important for the syncytium-inducing property of some virus strains (see legend of figure 2)[23,24].

The V3 loop has been identified as the principal determinant of the cell tropism of different HIV strains[24]. The first report demonstrating that only changes in the V3 loop determines the tropism for macrophages came from Hwang, et al.[19]. They showed that the exchange of a V3 fragment containing 20 amino acids of the non-macrophage tropic strain HIV-1IIIB for that of the strain BaL lost its ability to replicate in the T-cell lines H9 and CEM. In contrast to these results, Cheng-Mayer, et al.[25] and Groenik, et al.[26] showed that differences in T-cell tropism and macrophage tropism could also be ascribed to regions outside of the V3 in the C-terminus of gp120.

Recent experiments with viruses containing chimeric V3 loops of gp120, highlight the functional importance of V3 in the use of chemokine receptors as cofactor for HIV-cell fusion. Sequences in the V3 determine the fusogenic activity of Env with cells expressing different chemokine receptors[27] and the V3 loop appears to be required in assays measuring physical interaction between gp120 and CCR5[28,29]. It is therefore likely that the V3 loop contains determinants involved in coreceptor (chemokine receptor) binding.

The simplest model of coreceptor use suggests that CCR5 and CXCR4 are the main coreceptors used by HIV. In general terms, HIV strains that use CCR5 (R5 strains) are macrophage-tropic and do not infect stable T-cell lines. CXCR4-using HIV strains (X4 strains) are able to infect both primary cells and lymphoid cells that express CXCR4[30]. Interaction of Env with the chemokine receptors appears to follow the interaction of gp120 with the CD4 receptor that mediates binding. Probably, conformational changes in both CD4 and gp120 lead to folding, bringing the effector and target membranes in close apposition and exposure of the epitopes required for chemokine receptor-gp120 interaction (Fig. 3). Soluble forms of gp120 have been shown to inhibit chemokine binding to cells expressing their receptors[28,29]. This in turn, appears to be enhanced by soluble CD4 providing evidence for the conformational change induced by CD4 to promote gp120 interaction with chemokine receptors. The recent reports on the crystal structure of gp120, complexed with CD4 and a monoclonal antibody that binds to the chemokine receptor interaction support the idea that conformational changes following CD4-gp120 interaction are required to uncover the chemokine receptor-binding region in gp120.

The role of the chemokine receptor after its initial contact with gp120 is far from clear. The normal physiological activity of these receptors is to signal through G-proteins[31,32]. However, pertussis toxin, a known inhibitor of intracellular signaling by G-protein coupled receptors (GPCR) had no effect on the ability of RANTES to inhibit fusion and entry of HIV[24,33]. Furthermore, fusion mediated by a signaling deficient truncated CCR5 or CXCR4 is still sensitive to chemokine inhibition of HIV replication, supporting the idea that intracellular signaling through the chemokine receptors is not required for HIV entry or is it needed for anti-HIV activity[32,34].

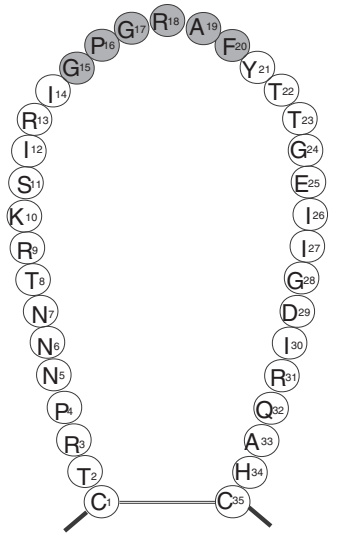


**Figure 2.** *V3 loop of HIV-1 envelope gp120. Consensus amino acid sequence of the V3 loop. The shaded amino acids represent a relatively conserved sequence. Syncytium-inducing (SI) viruses generally have a basic amino acid at one or more of the following positions: 11, 24, 25, 32. Non-SI viruses have either an acidic amino acid or alanine at positions 25.*

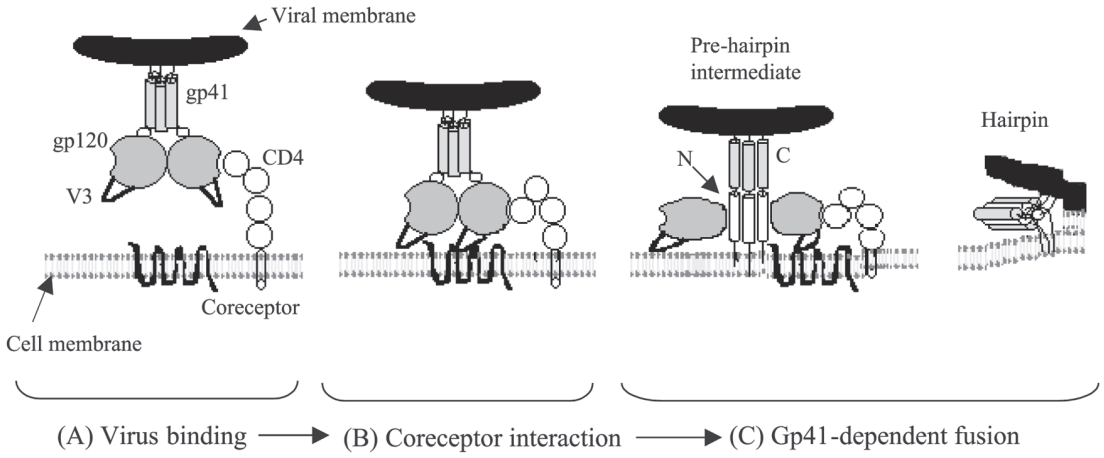


**Figure 3.** *Early steps of HIV life cycle. (A) The viral particles binds to the target cells though interaction with components of the cell surface (not shown). Binding of gp120 to the CD4 receptor induces a conformational change in the gp120/CD4 complex that leads to (B) the interaction of gp120, with involvement of the V3 loop, with the appropriate coreceptor (usually chemokine receptors CCR5 or CXCR4). (C) Further conformational changes lead to the exposure of the fusion peptide and the formation of a pre-hairpin intermediate in which the fusion peptide is inserted into the cell membrane. The pre-hairpin resolves to the fusion-active hairpin structure when the C region binds the N region of gp41 bringing the viral and cell membranes together and leading to membrane fusion.*

After coreceptor interaction, membrane fusion is driven by gp41. The gp41 molecule is a transmembrane protein in which the amino terminus contains a hydrophobic, glycine-rich fusion peptide that is essential for membrane fusion[35]. There are two regions (N-terminal and C-terminal) with a heptad repeat each that is characteristic of coiled coils[36]. Gp41 exists in a nonfusogenic conformation on the surface of free virions. However, upon gp120 binding to target receptors, gp41 undergoes a conformational change to a fusion-active state by the formation of a pre-hairpin intermediate that leads to insertion of the fusion peptide (The N-terminal region) into the target membrane. The C region associates to the N region to form a hairpin structure that brings the viral and cellular membranes to contact and fuse (Fig. 3c).

## Inhibition of viral entry

A potentially powerful alternative to reverse transcriptase and protease inhibitors would be to stop HIV replication before it actually infects the CD4 positive cells. A large number of compounds that inhibit early stages of virus replication have already been studied. Recent discoveries have prompted the development of new therapeutic strategies targeted at early stages of the HIV replicative cycle[37]: i) chemokine receptors act as coreceptors for HIV infection; ii) some chemokines have HIV suppressive activity; iii) natural mutations in the genes that encode chemokine receptors that serve as coreceptor for R5 strains of HIV protect from HIV infection, and iv) synthetic peptides to the C region of gp41 inhibit HIV infection and syncytium formation at nanomolar concentrations[38,39].

Historically, inhibitors of virus-cell attachment were described as potent inhibitors of HIV-1 replication. The use of soluble CD4 (sCD4) was one of the first attempts to block infection[40,41]. However, sCD4 proved inactive against primary isolates. Polyanionic compounds of widely diverging structure and size can block the replication of HIV in cell culture

**Table I.** *Number of passages (days) required to develop HIV-1 NL4-3 resistance to different anti-HIV agents*

| | Number of passages (days) | Fold-resistance | Reference |
|---|---|---|---|
| Wild type NL4-3 | Parental strain | - | - |
| AMD3100-res. | 63 (315) | 300 | [71] |
| AMD2763-res. | 23 (115) | >172 | [71] |
| DS-res. | 17 (100) | >900 | [67] |
| AR177-res. | 33 (200) | >200 | [68] |
| SDF-1a-res. | 24 (100) | >20 | [72] |
| Aco-HSA-res. | 24 (126) | 27 | [69] |
| Suc-HSA-res. | 24 (126) | 37 | [69] |

[a]Fold increase in EC50 as compared to the EC50 of wild type NL4-3.

**Table II.** *Mutations found in the gp120 gene of HIV-1 strains that were made resistant to different HIV-1 entry inhibitors*

| | Amino acid position | gp120 region | | Amino acid position | gp120 region |
|---|---|---|---|---|---|
| Dextran sulfate | S144N | V1 | Zintevir | K178E | V2 |
| (DS) | S164N | V2 | | Q310aH | V3 |
| Ref. [67] | K302E | V3 | Ref. [68] | K322Q | V3 |
| | Q310aH | V3 | | F423I | CD4 binding domain |
| | N325D | V3 | | | |
| | N355S | C3 | | 396-400d (FNSTW) | V4 |
| | R419I | CD4 binding domain | | | |
| | 396-400d (FNSTW) | V4 | | | |
| Siamycin I | N186K | V2 | Suc-HSA | S142I | V1 |
| | G321E | V3 | | I165T | V2 |
| Ref. [70] | N340D | C3 | Ref. [69] | D279N | C2 |
| | | | | S306R | V3 |
| | Other mutations in gp41 | | A316S | V3 | |
| | | | | I329M | C4 |
| | | | | 396-400d (FNSTW) | V4 |
| | | | | Other mutations in gp41 | |
| AMD2763 | S306R | V3 | Aco-HSA | S164G | V2 |
| | Q310aH | V3 | | D279N | C2 |
| Ref. [71] | I320V | V3 | Ref. [69] | T409A | V4 |
| | A329T | V3 | | | |
| | P417L | V4 | | Other mutations in gp41 | |
| | 396-400d (FNSTW) | V4 | | | |
| AMD3100 | F175L | V2 | SDF-1α | N136K | V1 |
| | N303S | V3 | | S164N | V2 |
| Ref. [71] | R304T | V3 | Ref. [72] | F175L | V2 |
| | S306R | V3 | | F277I | C2 |
| | Q310aH | V3 | | N302E | V3 |
| | I320V | V3 | | Q310aH | V3 |
| | N325H | V3 | | I320V | V3 |
| | A329T | V3 | | N325D | V3 |
| | P417L | V4 | | 396-400d (FNSTW) | V4 |
| | Q442E | C4 | | | |
| | S465P | V5 | | | |
| | V489I | C5 | | | |
| | 396-400d (FNSTW) | V4 | | | |
| AOP-RANTES | A316T | V3 | MIP-1α | V170M | V2 |
| Ref. [74] | | | Ref. [73] | S303G* | V3 |

For consistency, the numbering of amino acids shown have been modified according to the HIV gene and protein numbering scheme described in the Los Alamos database web site (http://hiv-web.lanl.gov) for HIV-1 HXB2 gp160. The numbering shown here may not coincide with the numbering shown in the original publications. "a" and "d" represent an insertion or deletion to the HXB2 amino acid sequence respectively.
*This mutation was reported to appear also in the wild type virus that was passaged without MIP-1α73.

by interfering with the binding of virus to CD4. The only common structural denominator appears to be the presence of a sufficient number and adequate density of negative charges[42]. Heparin and dextran sulfate were extensively studied as lead compounds for the development of new polyanions as anti-HIV agents. Oligonucleotides such as AR177 (zintevir)[43] or negatively charged albumins (NCA) have been evaluated *in vitro* and *in vivo* for anti-HIV activity. Irrespective of their therapeutic potential, polyanionic compounds may be considered as important tools in attempts to resolve the molecular determinants of gp120 for HIV infectivity.

The identification of chemokine receptors as cofactors for HIV entry has paved the way for the development of new targets for inhibition of HIV entry. Natural chemokines have been shown to block HIV entry and infection that are mediated by the corres-

ponding chemokine receptor, either by direct blockade or by downregulation of the receptor. These include the ligands for CCR5[44,45] and CXCR4[32,46,47], or minor coreceptors: CCR3[27,48], CCR4[49] and CCR8[50]. Alternatively, chemokine-based synthetic peptides such as Aminooxypentane (AOP)-RANTES and N-nonanoyl (NNY)-RANTES have also been developed[48,51]. Similarly, small molecule inhibitors of chemokine receptors have already been identified. Agents that block CXCR4 include small peptides (Allelix-40-4C, T22 and its analogues)[52,53], peptoids (CGP64222 and arginine conjugates such as R3G and NeoR)[54-56] and the bicyclams[57-60]. A number of compounds, Tak-779[61], the new spirodiketopiperazine derivative E913[62], the monoclonal antibodies 2D7[27] and Pro140[63] and the small molecular weight compounds SCH-C and SCH-D have been shown to block CCR5 function and HIV replication[64]. The good oral bioavailability and pharmacokinetic profile of SCH-C and SCH-D has prompted their evaluation in phaseI/II clinical trials.

Several peptides from gp41 have been reported to inhibit the replication of HIV[35]. Two of these agents C34 and DP178 (T-20) are derived from the C region of gp41, bind to the N region during preharpin intermediate formation and block gp41 hairpin formation that is necessary for HIV fusion[65]. T-20 blocks HIV replication at nanomolar concentrations, has been shown effective *in vivo*[66] and is currently in phase III clinical trials for the treatment of advance HIV-infected patients.

## HIV resistance to inhibitors of viral entry

The driving force behind the numerous HIV variants is the combination of an error-prone reverse transcriptase, the viral enzyme transcribing the viral RNA genome into DNA on the one hand and the human immune system on the other hand. This puts a constant selection pressure on the HIV population leading to the emergence of escape mutants. Development of resistance to antiretroviral agents poses an additional challenge on the discovery and development of HIV inhibitors. However, the *in vitro* selection of drug-resistant strains may help in identifying the genes responsible for resistance and may shed light on the mechanism of action of the compound(s) being studied.

In general terms, selection of resistant strains of HIV is achieved by continuously passaging the cells, originally infected with a parental (wild type) strain, in the presence of the test compound. The experiment is usually initiated with a low multiplicity of infection (moi) and a drug concentration that is 1- to 5-fold the 50% effective concentration ($EC_{50}$) of the compound in replication inhibition assays. After 4 to 5 days postinfection or after maximum cytopathic effect is observed, the cleared culture supernatant is used to re-infect fresh, uninfected cells in the presence of equal or higher concentrations of the compound. Gradually, after subsequent passages, the drug concentration is significantly increased. Cell supernatant is titrated for infectious virus and its sensitivity to the drug at low moi is re-tested. Table I shows the number of passages or days that it took for the development of resistance to different anti-HIV compounds targeted at virus-cell binding or virus-cell fusion.

As it can be seen, resistance to the bicyclam AMD3100 took considerably longer time to evolve than resistance to the bicyclam AMD2763, to the polyanions or to the chemokine SDF-1? This is reflected in the number of mutations required to generate a resistant phenotype for each of these compounds.

Table II shows the mutations found in the gp120 of HIV-1 strains that have been made resistant to the sulfated polysaccharide dextran sulfate (DS)[67]; the G-quartet forming oligonucleotide zintevir[68], the negatively charged albumins suc-HSA and aco-HSA[69], the binding inhibitor siamycin I[70], the bicyclams AMD3100 and AMD2763[71], the chemokines SDF-1$\alpha$[72] and MIP-1$\alpha$[73], and the modified chemokine AOP-RANTES[74].

## Development of resistance to CXCR4 antagonists

An AMD3100-resistant strain derived from HIV-1 NL4-3 was obtained after long-term passaging (63 passages or 315 days) in MT-4 cells in the presence of progressively increasing concentrations of compound[71]. The NL4-3 AMD3100-resistant strain proved 300-fold resistant to AMD3100 and cross-resistant to other bicyclam analogues with similar or lower anti-HIV activity[57] suggesting that all bicyclam analogues share a common mode of action. However, the AMD3100-resistant strain also proved cross-resistant to compounds that inhibit virus binding or the gp120-CD4 interaction, such as heparin and DS[75,76]. While, at first glance, these results may suggest that sulfated polyssacharides could intervene in a post binding effect similarly to the bicyclams, the cross-resistance could most likely be explained by modifications in the gp120 three-dimensional structure that not only alter gp120-chemokine receptor interaction but also gp120-CD4 binding.

The $EC_{50}$ of SDF-1$\alpha$ for the AMD3100-resistant strain was at least 10-fold higher ($EC_{50}$ > 1 µg/ml) than for the parental NL4-3 strain ($EC_{50}$: 0.1µg/ml). Furthermore, SDF-1$\alpha$ did not inhibit virus binding and has been shown to specifically inhibit HIV entry by blocking CXCR4. Thus, the cross-resistance observed to SDF-1$\alpha$ with the AMD3100-resistant virus supports the idea that the mode of anti-HIV activity of AMD3100 is through selective antagonism of CXCR4.

Surprisingly, Arakaki et al.[77] have found that T134, an analogue of the CXCR4 antagonist T22, was still active against an AMD3100-resistant strain. The AMD3100-resistant strain that was selected from the HIV-1 NL4-3 virus is cross-resistant to CXCR4 agents such as SDF-1$\alpha$ [Schols, 1998 #9], Allelix-40-4C, R3G and NeoR[54,55], and to T22 (unpublished observation). T134 is a polycationic peptide with a similar mechanism of action as AMD3100. In fact, a T134-resistant strain that is

cross-resistant to AMD3100 has been recently reported[78]. Thus, it is unclear how T134 could remain active, without any loss of potency against a virus that is resistant to multiple CXCR4 antagonists.

The AMD3100-resistant phenotype was rescued by transferring the envelope gp120 gene of the AMD3100-resistant virus into the NL4-3 parental genetic background. Several mutations in the gp120 were identified leading to amino acid substitutions in the C4, V5, C5 regions and at least 7 mutations in the V3 region[71] (Table 2). The mutations found in the V3 loop of the AMD3100-resistant NL4-3, could be responsible for the resistant phenotype against AMD3100 and SDF-1α since the V3 loop appears to be part of the putative gp120 binding site with the chemokine receptor[79].

If AMD3100 can effectively block CXCR4 for use as HIV coreceptor, then HIV resistance may emerge only by two possible alternatives: HIV-1 may change coreceptor use or continue to use CXCR4 in a manner that is not blocked by AMD3100. In the first case, we envision that quasispecies that contain mutations in the gp120 or V3 loop, that confer the CCR5 or other receptor phenotype, would emerge in the presence of AMD3100[80]. However, the selection of the NL4-3, AMD3100-resistant strain was done in the lymphoid cell MT-4. This cell line can be easily infected by X4, laboratory adapted strains of HIV and express CXCR4; but, MT-4 cells cannot be effectively infected with R5 strains such as HIV-1 BaL and do not express CCR5. Therefore, unless an unidentified coreceptor is expressed in MT-4 cells that could be used by AMD3100-resistant NL4-3, the second hypothesis appears more plausible. That is, HIV-1 NL4-3 in the absence of coreceptors other than CXCR4 and under selective pressure by AMD3100 will develop into a strain that continues to use CXCR4 differently, and retains the X4 phenotype. Alterations in the interaction with the coreceptor may have important implications in the pathogenesis of HIV. Changes in envelope glycoprotein have been associated with the replication capacity and the pathogenicity of the resulting virus[81-83]. For example, The CXCR4-using HIV-1 IIIB-envelope glycoproteins may induce apoptosis[84] that can be inhibited by AMD3100[85]. Then, it could be expected that virus that are resistant to CXCR4 antagonists may have altered viral fitness and replication capacity when compared to their parental virus (Armand-Ugón, et al. Manuscript in preparation[86]).

## Resistance to SDF-1α

Since the discovery of the chemokines and of the chemokine receptors as cell entry cofactors for HIV, significant progress has been made in the understanding of HIV pathogenesis. It has been suggested that elevated levels of SDF-1, as a result of a variant form of the SDF-1 gene, may delay the onset of AIDS in HIV-infected individuals[87]. In fact, our group has found that SDF-1 plasma levels are associated to the maintenance of the non-syncytium inducing phenotype *in vivo*[88]. Furthermore, Glushakova, et al.[89] reported that the HIV phenotype switch from R5, to X4 phenotype was associated with disease progression. Thus, the phenotypic changes related to chemokine receptor use could have profound implications in the outcome of HIV infection.

An SDF-1α-resistant NL4-3 strain showed mutations in the gp120 that were also present in the AMD3100-resistant virus and this strain also showed 10-fold resistance to AMD3100[72]. It remains to be resolved which of the mutations found in the AMD3100- and SDF-1α-resistant virus are relevant for the resistant phenotype and which are coincidental.

Similarly to the AMD3100-resistant virus, the SDF-1α-resistant strain did not change coreceptor use and was dependent on CXCR4 to enter the cells. These results might be interpreted to suggest that blockade of CXCR4, as a treatment strategy, would not drive HIV strains with the X4 phenotype into R5 phenotype. Nevertheless, primary isolates are heterogeneous in nature, that is, they are composed of quasispecies of both CCR5-using and CXCR4-using phenotypes[90]. In PBMC, blockade of CXCR4 selects for those quasispecies that use CCR5 (or another receptor distinct from CXCR4) and generate a switch in phenotype towards R5 strains of HIV[80].

SDF-1α-dependent internalization of the chemokine receptor CXCR4 contributes to the inhibition of HIV replication[32]. That is, its anti-HIV activity is composed of two modes of action: blockade of the receptor and downregulation. Therefore, it is also puzzling that the SDF-1α-resistant virus could use a receptor that is downregulated by the chemokine. A virus strain regardless of phenotype cannot use a coreceptor that is not expressed on the cell surface or that has been downregulated. Therefore, the SDF-1α resistant virus must be resistant to the blocking component of SDF-1α but not to the downregulating component. It is expected that the concentration of SDF-1 < that is required to inhibit HIV replication by solely blocking the receptor would be higher than that required to inhibit virus replication by both blocking and downregulating. This hypothesis could explain why the resistant virus is still sensitive to the effect of SDF-1α, albeit at a higher concentration than the wild type strain.

## Resistance to agents that block CCR5-virus interaction

AOP-RANTES efficiently and specifically blocks entry of non-syncytium-inducing (NSI), R5 HIV-1 into host cells. Inhibition appears to be mediated by increased intracellular retention of the CCR5 coreceptor and/or competitive binding of AOP-RANTES with NSI R5 HIV-1 isolates for CCR5. Different sensitivity to AOP-RANTES has been observed depending on the virus isolate use. This difference in the inhibitory potency of AOP-RANTES was linked to a single amino acid change in the V3 lop region (A316T) without change in coreceptor use of the less sensitive virus[74]. A virus escape mutant to the chemokine MIP-1α has also been developed with rather similar results. That is, the emerging resistant

virus was up to 6-fold less sensitive to MIP-1α, had a least two mutations; one in the V2 loop (V170M) and one in the V3 loop (S303G) that was also present in the wild type virus passaged without MIP-1α, and resistance did not induce coreceptor switch to CXCR4[73]. A resistant virus to the CCR5-specific entry inhibitor SCH-C was recently reported without loss of CCR5 or gain in CXCR4 use (Moore, et al. Data presented at the 1st International Congress on HIV Pathogenesis. Buenos Aires, 2001).

These results contrast the report of Mosier, et al.[91]. They showed that in the human peripheral blood lymphocyte-SCID mouse model, NNY-RANTES rapidly selected for resistant virus with mutations in the V3 loop that altered coreceptor usage although the X4 viruses reverted to the R5 phenotype in the absence of the inhibitor.

It is difficult to understand the lack of coreceptor switch in the development of resistance to CCR5 inhibitors above mentioned. Cultivation of a heterogeneous population of HIV, composed of laboratory adapted, CXCR4-using (NL4-3) strain and a CCR5-using (BaL) strain, in the presence of AMD3100, leads to the selection of the CCR5-using strain, even when the initial virus population consisted of only 1% BaL[80]. If the minor population that uses a different coreceptor is present (ej. CXCR4), then blocking the alternative coreceptor (i.e. CCR5) will allow the minor population to expand. Therefore, the results on the development of resistance to CCR5 inhibitors without coreceptor switch suggests that insufficient time has been given for the emergence of the CXCR4-using variant to appear or that pharmacological blockade of CCR5 is altering the capacity of HIV to mutate into a CXCR4-using virus to the point that mutations required for drug-resistance without coreceptor use emerge faster that those required for coreceptor switch. The later hypothesis seems unlikely because the emergence of mutations is a stochastic event, minimal changes are required for a virus to gain CXCR4 use and this normally confers higher replication capacity and expanded tropism to HIV.

## Resistance to soluble CD4 (sCD4)

sCD4 was one of the first agents tested as anti-HIV agent *in vivo*. Despite its potent activity against laboratory adapted (LA) HIV strains, clinical isolates (CI) are considerably less sensitive to sCD4, presumably because the escape variants have a decreased affinity for CD4 and do not attach to CD4 as firmly as wild type virus. HIV variants have been obtained that are 100-fod resistant to sCD4[93]. Other factors affecting the resistance of primary isolates to sCD4 are the number of Env molecules on the viral surface and the number of Env molecules required for attachment and fusion. These factors appear to be different between LA strains and CI and when combined, may create a 1000-fold CI/LA neutralization resistance ratio[94]. The naturally resistant phenotype of CI of HIV halted the development of sCD4 as an anti-HIV agent.

## Resistance to polyanions dextran sulfate

The notion that DS may give rise to resistance came as a consequence of the results obtained with the AMD3100-resistant NL4-3. The later contained 12 different mutations that were not present in the wild type NL4-3 and showed cross-resistance to DS, heparin and other polyanions. This observation indicated that if interaction with DS with its molecular target was specific, the virus should be able to overcome the inhibitory effect of DS on infectivity through mutations of the specific amino acids. Furthermore, the development of a DS-resistant strain could elucidate which amino acids in the AMD3100-resistant strain were responsible for DS resistance and, in turn, which amino acids could be involved in virus-cell binding and which were involved in the post-binding step. Indeed, the DS-resistant strain showed mutations that also appeared in the AMD3100-resistant strain[67]. However, the DS-resistant virus was still as sensitive to AMD3100 as the parental wild-type strain. The DS-resistant virus was able to bind to MT-4 cells even in the presence of 125 µg/ml of DS, whereas the binding of the wild type strain was inhibited by DS at an $EC_{50}$ of 1-5 µg/ml, suggesting that the mutated amino acids found in the DS-resistant strain confer specific resistance at the level of virus-cell binding rather than virus-cell fusion (i.e. CXCR4 use).

## Zintevir

AR177 (zintevir) was developed as an inhibitor of integrase. It was first suggested that its antiviral activity was due to its capacity to inhibit the integrase *in vitro.* Zintevir was the subject of a clinical phase I/II trial, however, its mechanism of action had not been unequivocally identified. To precisely characterize the site of intervention by zintevir, we selected an HIV-1 strain resistant to this compound[68]. Such strain could not be inhibited by zintevir at concentrations up to 125 µg/ml, and, as in the case of the DS-resistant strain, the binding of the zintevir-resistant virus to MT-4 cells could no longer be inhibited by zintevir or DS. Furthermore, zintevir inhibited the binding of recombinant gp120 to soluble CD4, and no mutations were found in the integrase gene of the resistant virus[95], adding further proof to the notion that the compound inhibited an early step of HIV replication rather than the HIV integrase.

The development of resistance to zintevir brought our attention to a particular observation: the resistant mutant as in the case of the AMD3100-, AMD2763-, suc-HSA and DS-resistant strains, showed a mutation at position 310α (Q to H in the HIV-1 NL4-3 strain) and a deletion of five amino acids in the V4 loop region (FNSTW). These mutations are not sufficient to generate significant resistance to any of these compounds. However, they appear in all of the resistant viruses but they do not appear in the parental strains that have been passaged in parallel, for the same number of passages as any of the resistant viruses, without drug. These

changes could be required for the correct folding of mutant gp120 without a direct involvement in the resistant phenotype. Such dramatic changes in the genotype as a five amino acid deletion could be the base of molecular marker diagnostic tests for resistance to compounds directed to virus binding or virus-cell fusion.

## Negatively charged albumins (NCA)

Following 24 passages (126 days) HIV-1 strains that were resistant to NCA were isolated. These virus strains were resistant to succinilated human serum albumin (suc-HSA) ($EC_{50}$ > 125 µg/ml) and accotinilated-HSA (aco-HAS) ($EC_{50}$ 56 µg/ml) whilst the wild type strain remained sensitive to both compounds (suc-HSA, $EC_{50}$: 4.7 µg/ml; aco-HSA, $EC_{50}$: 1.5 µg/ml). The greater potency of aco-HSA as an anti-HIV agent could explain the slower emergence of resistance to this compound than to suc-HSA[69].

DNA sequence analysis showed the emergence of mutations in the gp120 molecule in the resistant strains but not in the wild-type strain. Despite the close similarities between these two proteins (they only differ from one another in that suc-HSA contains one, and aco-HSA two carboxylic acid groups per lysine residue), the pattern of mutations for the suc-HSA-resistant virus was different from that for the aco-HSA-resistant strain, that is, the suc-HSA-resistant virus had mutations that were not present in the aco-HSA-resistant virus and *vice versa*. The suc-HSA-resistant virus was 100-fold cross-resistant to the G-quartet containing oligonucleotide zintevir but was not resistant to DS, the bicyclam AMD3100 and the chemokine SDF-1α. However, the strains that were resistant to NCAs clearly differed in the mutation patterns from the DS-, zintevir- and AMD3100-resistant strains (Table 2), suggesting that resistance to polyanions, inhibitors of virus-cell binding and virus-cell fusion may be governed primarily by the overall change in the conformation of the gp120 molecule, following substitution of one or more amino acid in this molecule.

## Resistance to gp41-dependent fusion inhibitors

The first description of resistance to a gp41-specific inhibitor came from Rimsky, et al.[96]. They showed that changes in a 3-amino acid sequence (GIV) within the N region of gp41 (positions 36 to 38) were associated with resistance to the peptide DP178 (T-20). Resistance to T-20 was 100-fold of the wild type (HXB2) strain if mutations G36S and V38M were present and only 10-fold by the G36S alone. Other combinations of the tripeptide motif (DIM and DTV) may also confer resistance to T-20. Furthermore, sequence analysis of HIV isolates from patients participating in T-20 clinical trials have revealed additional mutations to those identified *in vitro*, namely, mutations V38A, Q39H, Q40H and N43D may modify the affinity for its target and the antiviral activity of T-20 up to 28-fold[97]. It is important to note that in heavily experience patients to antiretroviral agents, T-20 and its analogue T-1249 provided dose-related suppression of HIV viral load[98]. Taken together, these results suggest that I) T-20 targets the N-terminal heptad repeat of gp41 and II) the antiviral activity of T-20 is independent of resistance to any of the classes of currently approved antiretrovirals.

Interestingly, it has been suggested that virus tropism and coreceptor preference, defined by V3 loop sequences, modulate virus sensitivity to T-20[99]. It is unclear how coreceptor specificity would influence susceptibility of a viral isolate to inhibition by T-20. In theory, greater sensitivity of CXCR4-using viruses to T-20 inhibition could be explained by more efficient dissociation of gp120 from gp41 upon contact with CD4/CXCR4 than with CD4/CCR5, leading to conformational changes that promote T-20 interaction with its target site. Nevertheless, confronting evidence was recently presented. Greenberg, et al.[97] showed that I) a panel of SI and NSI isolates displayed no differences in susceptibility to T-20, II) CXCR4-using and CCR5-using isolates from a T-20 clinical study exhibited similar sensitivities to T-20 and III) different clones from a single HIV-1 isolate but with different coreceptor-use showed similar sensitivities to T-20. It is now generally believed that coreceptor preference does not affect virus sensitivity to T-20.

## Conclusions and perspectives

A helpful strategy towards the understanding of the mechanism of action of new anti-HIV agents is the development of drug-resistant HIV strains. Through the study of resistant mutants the genes responsible for resistance can be identified. Furthermore, the molecular determinants of drug-resistance, that is, the amino acid changes in the target molecule, that are responsible for resistance, can be evaluated *in vitro*. This strategy has been intensively exploited in the study of RT inhibitors and protease inhibitors and has yielded a good understanding of the determinants of drug-enzyme interaction and virus sensitivity to RT and protease inhibitors. Moreover, it has open the door to the development of diagnostic assays to determine treatment-failure in HIV+ patients.

In order to further investigate the mechanism of action of binding and fusion inhibitors the same strategy has been followed. For example, the development of resistance to compounds such as DS one of the first binding/fusion inhibitors that were discovered, became of interest as means to unravel its mode of action. Furthermore, the clinical efficacy of a potential antiviral agent could depend on the rate of resistance development and on the resulting phenotype of the resistant strain.

It is also still unclear whether any of the mutations found in the resistant mutants could serve as a marker for the detection of *in vivo* resistance to binding/fusion inhibitors. As expected the resistant phenotype correlates with the emergence of mutations in the gp120 (for binding inhibitors and coreceptor inhibitors) and gp41 for fusion inhibitors. In the case of CXCR4 and CCR5 antagonists, drug-re-

sistance is clearly associated with the V3 loop, a putative site of interaction with the chemokine receptors. Mutations in gp120 appear to modify the replication capacity, pathogenicity, and tropism of HIV and this, in turn, may have important implications in the use of these agents in the management of HIV infection. As seem for the selection of AMD3100-resistant virus, targeting cellular receptors instead of a viral target may imply that the virus can overcome the anti-HIV activity by different mechanisms (coreceptor switch or differential use of the same coreceptor) and through different, and probably unrelated, patterns of mutations. Other groups have shown the development of resistance to binding/fusion inhibitors[70,100] that have mutations in the gp120 but are distinct from the ones presented here. Moreover, at least 4 AMD3100-resistant variants have been described[71,77,80,101]. In the contrary, by specifically targeting a viral component i.e. gp41, selective pressure will lead to specific mutations that confer the resistant phenotype[66,96].

Diagnostic test for genotypic resistance to approved therapies have become an important tool to assess treatment failure. As fusion inhibitors gain prominence as therapeutic agents, both genotypic and phenotypic assays designed to evaluate drug-resistance need to be in place. Recombination of gp160 derived from plasma viral RNA or cellular proviral DNA amplification have already been described for the evaluation of coreceptor use[102], drug susceptibility[103] or virus pathogenicity[104]. Nevertheless, chimeric virus technology that can be applied to the throughput screening of drug-resistance requires further validation and implementing. The advent of fusion inhibitors will speed forward this new technology. In turn, a better understanding of HIV drug resistance to entry inhibitors will help to develop novel, more effective anti-HIV agents.

## Acknowledgments

Our own studies were supported in part by the Spanish *Fondo de Investigación Sanitaria* (*FIS)* projects 98/0868, 00/0893 and 01/1116, *Fundación para la Investigación y Prevención del SIDA en España* (*FIPSE*) project 00/3111, *Ministerio de Ciencia y Tecnología* project 00/1382 and the *Fundación irsiCaixa.*

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