Chapter 11

Coreceptor Usage in HIV Infection

Kerina Duri

Additional information is available at the end of the chapter

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1. Introduction

Chemokines are small low molecular weight proteins or cytokines secreted by cells that function as chemical messengers. They were originally found to attract leukocytes to site(s) of inflammation. As ligands they activate and signal through their respective chemokine receptors triggering an influx of intracellular calcium (Ca^{2+}) ions causing a process known as chemotaxis. Chemokine receptors are integral membrane proteins that specifically bind and respond to chemokines. They are members of the class A subfamily of G-protein coupled receptor (GPCRs) superfamily, a name derived from the characteristic cysteine motif of the group of chemokines they interact with. Despite their pivotal roles in the immune system and angiogenesis, chemokines as well as their receptors have been associated with a number of pathologies including autoimmune disorders, pulmonary diseases, transplant rejection, cancers, vascular diseases and human immunodeficiency virus (HIV) infection. Scientists have noted that while the CD4 receptor is necessary for the successful infection of host immune cells by all naturally occurring HIV-1 strains it is not sufficient. Thus, another specific cell surface molecule called chemokine receptor is required. The recent introduction of entry inhibitors in the clinic as components of antiretroviral treatment has increased the research interest of coreceptor usage in HIV infection. Chemokine receptors are subjects of significant medical importance which not only provide new insights into the mechanisms of viral entry, tropism and pathogenesis, but have also culminated into new control strategies from the host's perspective influencing HIV transmission along with disease progression. Identification of the different phenotypes of HIV-1 strains with different prevalence during various stages of disease progression and the role of these phenotypes in treatment outcome has further revolutionalised research in this field. This chapter seeks to depict a simple and clear understanding of the basics of HIV phenotypes or genotypes and the respective current as well as prospective diagnostic tools. Milestones and challenges in this relatively new class of antiretroviral therapy of coreceptor antagonists will also be highlighted.

2. Structure of chemokine receptors

Chemokine receptors consist of about 350 amino acids that are divided into a short and acidic N-terminal end, seven helical transmembrane domains since they span the cell membrane seven times with three intracellular and three extracellular hydrophilic loops, and an intracellular C-terminus containing serine and threonine residues that act as phosphorylation sites during receptor regulation as shown in **Figure 1** below. Chemokine receptors are usually linked to a G-protein through which they signal. The N-terminus of the chemokine receptor is an extracellular domain that binds the chemokine(s) and has been shown to influence infection tropism. The first two extracellular loops of chemokine receptors are linked together by disulphide bonding between two conserved cysteine residues. The disulfide bonds keep the extracellular loops in place thereby maintaining the structural integrity necessary for ligand binding and signal transduction. In spite of the high amino acid similarity of their primary sequences, chemokine receptors bind a limited number of ligands.

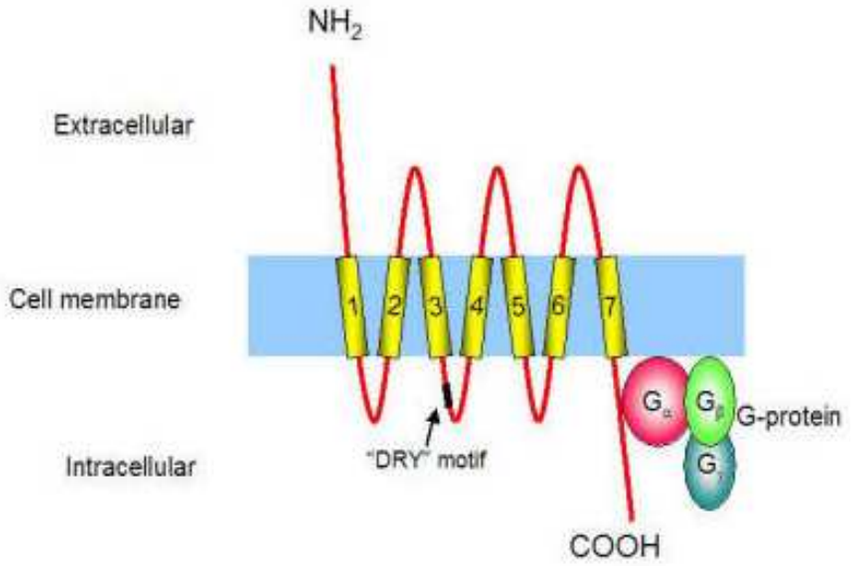

Figure 1. Typical structure of a chemokine receptor

3. Classification of chemokine receptors

To date, approximately 50 human chemokines and 20 receptors have been discovered. Basically chemokines and their receptors are divided into four subfamilies; CXC, CC, CX3C and XC depending on the position of the two pairs of the highly conserved cysteine residues on the ligands, where C denotes the cysteine amino acid residue whilst X represents non-cysteine amino acids. For the main subfamilies, the first two cysteines are either contiguous (CC) or intercalated by one non cysteine amino acid residue (CXC). A system of nomenclature has been introduced where each ligand and receptor is identified by its subfamily and an identifying number. For example, CCL2 refers to a chemokine ligand of the CC subfamily, number 2. Similarly, the **r**eceptors are referred to by subfamily **R** and

similarly an identifying number. Thus, for instance one of the receptors of CCL5 is called CCR5. Despite the numerous chemokine receptors that are prospective coreceptors for HIV *in vitro*, only CCR5 and CXCR4 have attracted substantial interest because they form portals of cellular entry for both HIV-1 and HIV-2 inclusive of other related simian or feline retroviruses. The lack of crystallography for these two highly hydrophobic coreceptors makes them difficult to isolate.

4. CXC chemokine receptors

The first HIV coreceptor to be identified was CXCR4 which was originally an orphan receptor called leukocyte-derived seven-transmembrane domain receptor (LESTR). By then it did not receive much attention until it was recognized as a coreceptor for HIV-1. Thus, in the mid 1990s the second receptor, CXCR4, needed for successful entry of HIV into cells was discovered. At that time this coreceptor was termed "fusin" as it facilitated certain HIV strains to fuse with and enter immune cells called T cells. A detailed analysis of the structure of fusin revealed that it was a receptor for chemokines which were previously shown to suppress HIV activity. CXCR4 has a wide cellular distribution. It is commonly expressed on most immature and mature hematopoietic cell types, cells of the central nervous system, neutrophils, monocytes, T and B cells, dendritic cells (DCs), Langerhans cells and macrophages. To date there are seven CXC chemokine receptors in mammals, named CXCR1 through CXCR7. The CXC chemokines include stromal cell-derived factor-1 alpha and beta now officially designated CXCL12a and CXCL12b respectively. CXCL12 is often induced by pro-inflammatory stimuli such as lipopolysaccharide, interleukin-1 (IL-1) or tumour necrosis factor-alpha (TNF-α) and has been shown to be strongly chemotactic for lymphocytes. Its high level of expression in the genital mucosa may help to explain the inefficient transmission of CXCR4-tropic HIV isolates.

5. CC chemokine receptors

Another chemokine receptor necessary for the entry of HIV into macrophages called CCR5 that was subsequently identified. CCR5 receptors have been shown to be involved in leukocyte activation and mobilization. CCR5 is expressed on several cell types including peripheral blood-derived DCs, CD34+ hematopoietic progenitor cells and some activated/memory Th1 lymphocyte. Chemokines that bind these receptors are RANTES (regulated-upon-activation normal T expressed and secreted); MIP-1α and MIP-1β (macrophage inflammatory protein-1 alpha and beta) previously observed to suppress HIV infection but now officially called CCL5, CCL3 and CCL4 respectively. I*n vitro* studies have demonstrated several CC chemokines bind CCR5. However, CCL3, CCL4, CCL5, and CCL8 have shown the most suppressive effects in HIV-1 infection assays. To date ten members of the CC chemokine receptor subfamily have been described, namely CCR1 through to CCR10 according to the IUIS/WHO Subcommittee on Chemokine Nomenclature. CCR5 is one of the major coreceptor implicated in susceptibility to HIV-1 infection and disease progression. The lack of CCR5 gene expression has been associated with resistant to HIV-1 infection as will be discussed later on.

6. CX3CR1

CX3C chemokine receptor 1 (CX3CR1) also known as the fractalkine receptor or G-protein coupled receptor 13 (GPR13) has been shown to bind chemokine CX3CL1, also called fractalkine. Fractalkine is a transmembrane chemokine involved in the adhesion and migration of leukocytes. CX3CR1 is expressed on monocytes and plays a major role in the survival of monocytes. It has been shown to interact with human respiratory syncytial virus protein G consequently, modulating the host immune response. It also interacts with HIV-1 envelope (env) polyprotein glycoprotein (gp) 160. Thus CX3CR1 is also a minor coreceptor for HIV-1. Certain variations in the expression this gene has been associated with increased susceptibility to HIV-1 infection and rapid progression to AIDS.

7. XCR1

The "C" sub-family of chemokine receptors contains only one member: XCR1, also known as GPR5. It is the receptor for chemokines XCL1 and XCL2 formerly, lymphotactin-1 and-2, respectively. This receptor is closely related to the MIP-1 α and RANTES yet to date its expression and the function of its ligand XCL1 remain elusive. However, at least in murine models, XCR1 has been shown to be expressed on $CD8^+$ DCs and that its ligand XCL1 has shown to be a potent and highly specific chemo-attractant for this subset of DCs.

8. Tropism

HIV-1 was initially isolated from peripheral blood cells and consequently characterised as a virus that infects the CD4+ T-lymphocyte population, T tropic isolates. However, subsequent isolation of HIV-1 from non-lymphoid organs demonstrated that HIV-1 could also infect cells of the monocyte-macrophage lineage; macrophage tropic isolates. Studies have shown that T-cell and macrophage isolates display significant different biological properties with respect to cellular tropism, genetic diversity and relative replication rates including their inherent ability to induce syncytia.

9. MT-2 cell tropism

Biological differences of HIV-1 isolates and depletion of CD4 positive lymphocytes have been shown to correlate with the pathogenesis of AIDS. Direct cytopathic effects of HIV can be studied *in vitro* in T cell lines. An MT-2 tumour cell line assay is generally used for the phenotypic characterisation of HIV-1 isolates. The ability of HIV-1 isolates to replicate in MT-2 cell lines is a prototype where viruses that do not infect MT-2 cells are designated non-syncytium inducing (NSI), while those that infect cells are termed syncytium inducing (SI). Studies have shown that HIV-1 isolates from patients with low CD4 counts have been shown to replicate rapidly to high titres in peripheral blood mononuclear cells (PBMCs) with the infected cells forming syncytia and such isolates are called rapid/high replicating or syncytium inducing (SI). HIV-1 isolates from asymptomatic individuals replicate much slowly with low titres and such isolates are termed slow/low or NSI. Consequently, HIV-1 isolates can be classified into two main groups; those that replicate in T–cell lines, grow

rapidly in cultures forming syncytia in target cells and the other group that replicate in macrophages, grow relatively slowly in culture but are not able to induce syncytia, SI and NSI, respectively. The formation of syncytia does not always happen in HIV infected people. However, autopsies have found syncytia in the spleens of some patients. More frequently CD4 syncytia have been observed in the brains of patients who would have died from serious AIDS related neurological complications.

10. HIV strains classification based on tropism

Following the identification of the coreceptors, HIV-1 isolates have also been characterized based on their ability to infect and induce syncytia in $CD4^+$ T-cell lines that express CXCR4 but not CCR5.While all the HIV-1 strains require CD4 to enter and infect cells, some isolates utilize the chemokine receptors X4 or R5 while other variants use both receptors, dual tropic (R5X4) strains for binding and entry. Coreceptor usages correspond to the phenotypes previously defined by the MT-2 assay with SI and NSI viruses using CXCR4 and CCR5, respectively. Dual tropic isolates exhibit both the M and T tropic characteristics. They are further classified as dual-R; R5X4 variants with more efficient use of CCR5 than of CXCR4 or dual-X; R5X4 with more efficient use of CXCR4 than of CCR5. *In vitro* studies have shown that some HIV-1 strains can use a variety of other chemokine receptors. Interestingly, this does not appear to have major relevance to HIV infection nor pathogenesis *in vivo*. MT-2 positive variants are defined as either X4 or R5X4. Absence of viral growth in this assay may be either due to the exclusive presence of R5 variants or failure to isolate HIV.

Frequencies of R5 HIV-1 variants vary among different populations, being 80% and 50% in drug naïve individuals and patients receiving antiretroviral therapy, respectively. Tropism shifts have been associated with long term use of antiretroviral therapy. Some studies have shown that drug selection pressure may gradually select for pre-existing X4 virus from cellular reservoirs during sustained highly active antiretroviral therapy. Thus, the use of CCR5 coreceptor antagonists is associated with a selective pressure that promotes the emergence of CXCR4-using variants.

11. Evolution of X4 viruses

There are different theories regarding the origin of X4 viruses. One theory postulates that X4 viruses emerge directly from the pre-existing R5 pool as a result of mutations within the HIV V3 loop *env* gene. At least for HIV-1 subtype C, X4 variants have been associated with an amino acid substitution mutation from the conserved GPGQ crown motif to a GPGR. Still some authors argue that X4 viruses are already in the existing pool of viruses but somehow the X4 viruses and R5/X4 viruses remain suppressed by varied host mechanisms and only to be detected in the late phases of infection suggestive that the immune system exerts a selective pressure that hinders the emergence of CXCR4-using variants. However, when the host immune competence begins to deteriorate during the HIV disease progression, it paves way for the emergence of CXCR4-using variants. Another hypothesis proposes that chemokines and the C2-V3-C3 region of HIV gp120 have a common origin such that the HIV

ancestor incorporated a chemokine gene into its *env* gene such that this captured chemokine gene rapidly diverge by frequent mutations thereby attaining the ability to effectively interact with various chemokine receptors in a short period of time. Another possible explanation is based on cell division rate where it has been observed that X4 and R5 viruses show preferential tropism for naive and memory T cells, respectively. Since memory T cells divide 10 times more frequently than naive cells, it would be an advantage to have a tropism for CCR5 coreceptor during the first stages of infection.

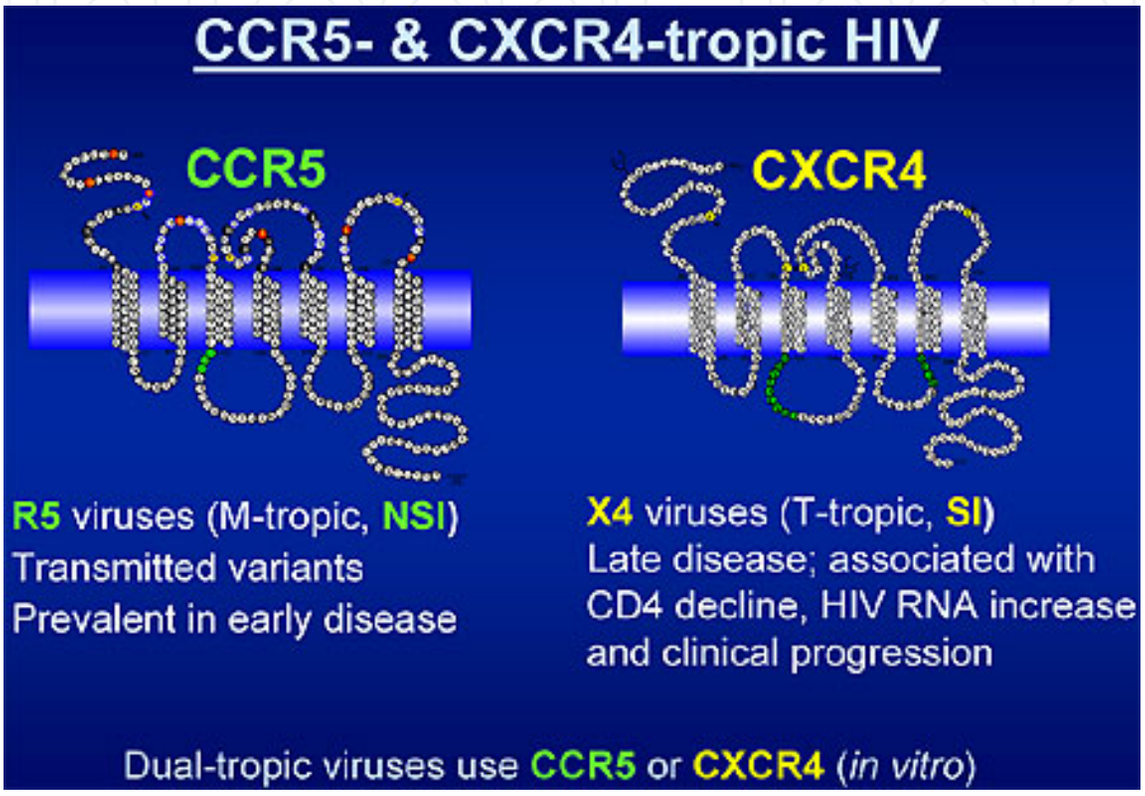

Figure 2. HIV classification adopted from Moyle G, 2008.

12. Coreceptor usage in HIV-2

Studies have shown that the use of coreceptors is much broader in HIV-2 infections relative to HIV-1. In addition to CCR5 and CXCR4 HIV-2 is able to utilise alternative coreceptors such as GPR15, or CXCR6 and to a lesser extent CCR1, CCR2b or CCR3 in aviremic patients whilst CXCR4 is only used in viremic individuals. It still needs to be explored whether this wide use of coreceptors is the underlying reason behind the less virulent phenotype associated with HIV-2, compared to HIV-1.

13. Clinical application of tropism

One property that has been closely correlated with clinical prognosis is the ability to induce syncytia formation in susceptible cells with clinical course of AIDS being associated with a shift from NSI to SI. HIV isolates obtained during early acute infection are generally M-

tropic/NSI whilst T-tropic/ SI are associated with disease progression. The emergence of CXCR4-using HIV-1 variants in a patient is almost invariably associated with a subsequent increase in the rate of decline of circulating $CD4^+$ T cells, an accelerated disease progression and a poor prognosis for survival. While CXCR4-using variants can emerge at any stage of infection, untreated individuals who develop such variants progress to AIDS within an average of two years after their first detection. However, the presence of CXCR4-using variants is not an obligatory prerequisite for disease progression as a significant proportion of individuals' progress to AIDS whilst harbouring exclusive R5 HIV-1 variants. The emergence of SI HIV-1 in a sero-positive individual is now generally regarded as a negative prognostic indicator and thus considerable interest has been focused on the HIV-1 genetic determinants of the SI phenotype. Tropism assays are important for determining HIV phenotype before administering coreceptor antagonists in combination antiretroviral therapy. In view of this, tropism assays must taken by HIV infected people with drug resistance who are considering taking coreceptor antagonists. This new class of anti-retroviral targets CCR5-specific HIV during entry into the cell. Thus, knowing the patient's viral tropism can assist and guide the clinician make an informed and effective HIV treatment plan. Consequently, the importance of tropism screening to detect the presence of R5-using HIV may not be over emphasized.

14. Methods for determination of coreceptor usage

With the recent introduction of HIV-1 chemokine receptor antagonists on the market as components of antiretroviral therapy, it is increasingly important to screen HIV patients' coreceptor phenotype or genotype prior to therapy. Hence simple and efficient methods for routinely characterising and monitoring HIV-1 coreceptor phenotypes or genotypes are of paramount importance. Studies have demonstrated that the ability to induce syncytia is determined by regions of env outside the V3 loop that encompass residues that contribute to the binding of CD4 by gp120. Such observations suggest that areas of the HIV-1 *env* gene contributing to the CD4 binding site may also contribute to the determination of SI and NSI genotypes. HIV-1 tropism can be determined by phenotypic or genotypic based methods.

15. Phenotypic methods

HIV-1 tropism can be assessed using phenotypic assays which are currently the most accurate method based on recombinant viruses. Determination of coreceptor use of HIV-1 isolates is done in cell lines such as U87 and GHOST transfected with CCR5 or CXCR4. Patients' plasma is used to generate pseudoviruses or infectious recombinant viruses of full-length or partial viral envelopes derived from the patient's viral population. The recombinants are subsequently tested on indicator cell lines expressing CD4 and either CCR5 or CXCR4. The first commercially available tropism assay became available on the market almost at the same time of approval of the CCR5 antagonist, maraviroc. Its brand name of phenotypic assay is Trofile® manufactured by Monogram Biosciences South San

Francisco, USA. The test can detect 10% of X4 variants with 100% sensitivity. More recently, an enhanced Trofile® assay with better sensitivity to improve detection of low level X4-using variants has been developed that can detect 0.3% of these variants with 100% sensitivity. Trofile® phenotypic assay continues to be the only clinically validated assay to identify coreceptor tropism and is considered the gold standard for tropism testing capable of distinguishing pure R5, D/M and pure X4 populations.

Another phenotypic tropism assay, Phenoscript-tropism; Eurofins is also on the market. It was specifically developed to cater for phenotypic test for the evaluation of viral tropism in HIV-1 non-B subtypes. It is worthwhile to appreciate that the HIV-1 env V3 loop is implicated in the determination of phenotypic tropisms

16. Challenges of phenotypic methods

Despite having high sensitivity phenotypic assays are laborious, expensive, and time-consuming and can only be done in sophisticated laboratories by highly qualified personnel. More so the presence of CXCR4 viruses, which frequently constitute less than 5% of viral population is a challenge as phenotypic assays' sensitivity does not seem to permit this range of detection threshold. Consequently, individuals may be misdiagnosed as harboring only CCR5 coreceptor using virus, when in actual fact they may also be harbouring R4 virus in very low quantities such that once initiated on CCR5 coreceptor antagonists their CXCR4 tropic viruses may emerge. Due to the foregoing bottlenecks there is need for the development of simple, more sensitive, accurate and less expensive tests with a shorter turnaround time to replace slow and resource intensive phenotypic assays.

17. Genotypic coreceptor analysis methods

HIV env gp120 is composed of about 400 amino acids which consists five relatively conserved constant (C1-C5) and five hyper-variable regions (V1-V5). The genetic determinants of HIV-1 coreceptor usage are localized in the V3 loop of gp120 which has a highly conserved crown motif and glycosylation sites. The third variable region also called the V3 loop is composed of 31-39 amino acids. . The V3 loop is closed by a disulfide bridge formed by two cysteines. Functionally it is critical in maintaining the right conformation to facilitate coreceptor interaction with the virus. This region has been shown to be the major determinant of viral tropism and accordingly, prediction of coreceptor usage based on the interpretation of V3 sequences using bioinformatics tools could be a good alternative to infer tropism in the clinical routine Sequences in the N-terminus of V3 loop have been shown to modulate the levels of infection through CCR5 coreceptor. Minor or a few sequence alterations or mutations in V3 are sufficient to switch coreceptor use from CCR5 to CXCR4 or from dual-tropic to X4-tropic virus. Additional mutations within the V1/V2 have also been observed during coreceptor switching. Studies have shown that such

mutations seem to compensate for the harmful V3 mutations. Functional studies have demonstrated that the V3 loop interacts with the N-terminal extra-cellular domain of CCR5 and the extracellular loop 2. Bioinformatics tools based on V3 sequences can be used to predict HIV-1 tropism. The identification of viral genotypic changes associated with different coreceptor usage has led to the development of sequence-based algorithms to predict coreceptor usage. Different rules have been published based on the amino acid sequence of the env V3 region of HIV-gp120, which is known to be the major determinant of coreceptor usage.

18. Predictive algorithms of HIV-1 coreceptor usage

At least eight different bioinformatics tools have been used to predict viral tropism in different HIV-1 subtypes which uses phenotypic data to predict the corresponding viral genotype. Studies of genotypic predictors have been retrospective with patient samples selected based on availability of phenotypic tropism determinations. Three of the interpretation systems namely, WetCat, WebPSSM, geno2pheno [coreceptor] are freely available on internet. All three focus on the env-V3 region and only take the amino acid sequence into account. Such genotypic systems provide the possibility for rapid screening of patients who may be administered with CCR5 blockers like maraviroc. On cloned viruses belonging to genetic subtype B, the specificity and sensitivity of most predictive methods exceed 90% and 80%, respectively. While genotypic assays may have lower specificity and sensitivity, retrospective analyses have found that they are comparable to phenotypic tropism assays for prediction of response to treatment with CCR5 antagonists in populations pre-screened with a phenotypic assay. The first genotypic algorithm designed to predict HIV-1 tropism takes into account only the net charge of amino acids at two key residues located within the V3 loop, amino acids at positions 11 and 25. The most widely used is the 11/25 rule which focuses on identifying sequence patterns within the V3 loop. Predictions using the “11/25 charge rule” are relatively satisfactory.

19. Correlation between phenotypic and bioinformatics tools in determining HIV coreceptor use

Evaluation of the performances of genotypic tools to predict HIV-1 tropism has been investigated. Paired genotypic and phenotypic determination of HIV-1 coreceptor usage has been performed to assess several genotypic approaches for detecting CXCR4-using and CCR5-using viruses in a clinical setting. Excellent correlations between HIV-1 V3 genotype and phenotype have been observed. Overall, the accuracy of the bioinformatics tools to detect CXCR4-using virus was similar for ES Trofile and Trofile. However, the negative predictive values for genotypic tools with ES Trofile were slightly higher than they were with Trofile. The accuracy of genotypic algorithms for detecting CXCR4-using viruses is high when using Trofile as the reference. The concordance with ES Trofile is better with

higher CD4 cell counts and non-exposure to antiretroviral therapy. The global concordance between genotypic and phenotypic data is 91% with the rule combining the amino-acid residues at positions 11/25 and V3 net charge. Gaining a better understanding of the output of these assays and correlating them with clinical progression and therapy response will provide some indication on how both genotype-based and phenotypic assays for determining HIV coreceptor usage can be improved. Deep V3 sequencing is a promising tool for identifying treatment-experienced individuals who could benefit from CCR5-antagonist-containing regimens.

20. Challenges of genotypic methods

Genotypic predictions are relatively satisfactory but because not all determinants of coreceptor usage lie within the V3 loop, the region employed by most current predictors, causing occasional disagreements. Accurate prediction is also complicated by the fact that the V3-C4 region of the *env* gene, which has the greatest influence on tropism, also has a relatively high rate of diversity such that the sensitivity drops with uncloned sequences and HIV-1 non-B subtypes. Since non-B subtypes show a wide genetic variability in the V3 region and taking cognisance that X4 viruses might be more prevalent in some subtypes than others, there is an urgent need to know the reliability of genotypic tools for inferring HIV-1 tropism in non-B subtypes, especially in regions where these HIV-1 variants are quite prevalent and may soon have access to CCR5 antagonists. Moreover, technical limitations to the generation of unambiguous DNA sequences from the HIV-1 env region that has insertions and deletions may interfere with the generation of clean and clear electropherograms thereby interfering with a predictive determination of tropism in a significant portion of patients' samples. Prospective studies are needed to firmly establish the clinical usefulness of genotypic tropism determination. Further research is also warranted regarding the need for specific genetic characteristics of dual/mixed-tropic HIV-1 strains which also exist in a significant proportion of patients.

21. HIV-1 coreceptor usage and genetic subtypes

There are remarkable differences in the prevalence of CXCR4-using variants among different HIV-1 genetic subtypes and circulating recombinant forms (CRFs). Since CXCR4-using variants emerge after an accumulation of mutations, the different prevalence observed may reflect the same phenomenon at the population level. Infection with subtype-C accounts for over half of the worldwide HIV-1 epidemics and is rapidly expanding in Southern Africa, South East Asia and India. Studies have shown this rapidly expanding subtype C isolates almost exclusively use the CCR5 coreceptor, with CXCR4 usage being rarely observed. Some authors argue that the predominant usage of CCR5 by HIV-1 subtype C isolates is more due to sampling artifacts rather than any fundamental biological properties of these viruses. Unique to subtype C is the determinant of coreceptor usage, the V3 regions which has been shown to be highly conserved and have a low overall positive

charge, which is consistent with the NSI phenotypes compared to other subtypes. Moreover, this atypical property of the subtype C envelope glycoproteins might be the reason behind the rapid expansion of the virus being currently observed. In view of this, there is need for intervention strategies that are subtype specific to curb this pandemic tailor designed for use in areas where subtype C viruses predominate. R5-using viruses have also been found to be more common in subtype A than subtype D HIV-1 infections. The emergence of X4 viruses occurs very early among subtype D-infected individuals. More so, a high proportion of subtype D infections have been shown to display D/M tropism throughout the course of disease. An inverse skewing in coreceptor usage, with an increased presence of CXCR4-using strains, has instead been reported for subtype-D HIV-1. This observation is consistent with the faster pace of disease progression reported for subtype-D infection both in Africa and abroad. An increased rate of CXCR4 usage has also been reported for CRF AE isolates common in South East Asia. There are proposals to the effect that the increase in prevalence of CXCR4-using HIV-1 variants increases with the age of the subtype epidemic. Indeed recent phylogenetic studies suggest that the proportion of patients with detectable CXCR4 using HIV-1 variants varies with subtype D having the highest CXCR4 switch rate being the oldest whilst subtype C with the lowest CXCR4 switch rate being the youngest. Subtype B predominant in North America and Europe has demonstrated that CXCR4 coreceptor usage increases with time following infection with or without concurrent use of R5 in 50% of HIV-1 infected individuals. The HIV-1 subtype-B epidemic has an intermediate pattern, both in terms of age and prevalence of CXCR4-using HIV-1 variants. This assumption is highly speculative and not supported by all the data available at present. However, if confirmed it would imply that all the subtype epidemics are evolving towards a higher prevalence of CXCR4-using HIV-1 variants although it is plausible that each epidemic would reach a point of equilibrium beyond which such prevalence will not further increase.

22. HIV coreceptor usage and compartmentalization

Compartmentalization is the occurrence of distinct yet phylogenetically related HIV-1 phenotypes or genotypes within different anatomic sites, an observation common amongst both treated and untreated individuals. Differences in selective pressures may shape the distinct viral populations in different compartments. Anatomic compartmentalization of HIV coreceptor usage variants has been described in diverse tissues including in blood, lungs, brain, central nervous system, breast milk and genital tract. Studies have shown that the distribution of R5 and CXCR4-using variants differ in different blood compartments. Higher prevalence of predicted CXCR4-using variants in PBMC than in plasma has been reported. The limited compartmentalization and the clonal amplification of evolving functional viruses in milk indicate continual seeding of the mammary gland by blood virus variants, followed by transient local replication of these variants in the breast compartment.

Gender studies have demonstrated different viral variants between genital tract and blood for both women and men. Differences in genetic strains between blood- and semen-derived HIV isolates within the same individual have been documented. Male genital tract tissues such as the prostate, seminal vesicles, and epididymis which serve as sites of viral replication have been found to develop distinct, compartment-specific HIV strains in response to these local selective pressures. Studies seeking to determine chemokine receptor preference for all sequences derived from patients with compartmentalized virus to determine if seminal tropism correlated with altered coreceptor usage have shown a trend towards reduced CXCR4 usage in the male genital tract.

Other environmental factors such as sexually transmitted infections (STIs) have been also shown to exert selective pressures. Genital inflammation can stimulate the expression of R5 receptors dramatically, conferring a selective advantage on R5 virus in the genital tract of women with STIs. A history of intravenous drug use (IDU) among women has been shown to correlate with larger proportions of X4 strains in plasma relative to those without an IDU record. Quantifying the proportion of R5 and X4 viruses in each compartment has been found to vary significantly between them. Thus, the proportion of X4 strains in one compartment does not necessarily reflect coreceptor usage in the other suggesting that measuring coreceptor usage in say the genital tract and blood may aid in effective monitoring of disease progression and response to therapy as efficiencies of antiretroviral drug penetration has also been found to differ with compartments. Hence, there is need for compartment specific treatment outcome monitoring of patients and also the most appropriate choice of patient material for the determination of HIV-1 coreceptor usage remains to be established.

23. Chemokine receptors and viral entry into host cells

The entry of HIV into cells is critically dependent on the sequential interaction of the viral envelope with two cell-surface receptors, the CD4 glycoprotein and CCR5 or CXCR4. The evolutionary choice of HIV of exploiting chemokine receptors as entry gateways has established a tight biological bond between HIV and the chemokine system, making the respective natural ligands of these receptors potent viral inhibitors. Entry into target cells by HIV occurs by a multi-step process that culminates with the fusion of viral and cellular membrane as shown in **figure 3** below. Many enveloped viruses including HIV possess a fusion protein in their envelopes which confers the ability of the virion to fuse with the host cell membrane and thus allowing entry of the infectious genomic material into the cell cytoplasm. Receptor interactions then trigger gp41 to promote membrane fusion. This reaction is thought to involve extension of the gp41 subunit to allow insertion of its N-terminal 'fusion peptide' into the target cell membrane, followed by refolding the prefusion intermediate into an energetically favorable six-helix bundle that brings the two membranes together so that fusion can occur. During replication of the virus, expression of the fusion protein on the cell membrane can result in the fusion of neighbouring cells forming multi-nucleate cells or syncytia.

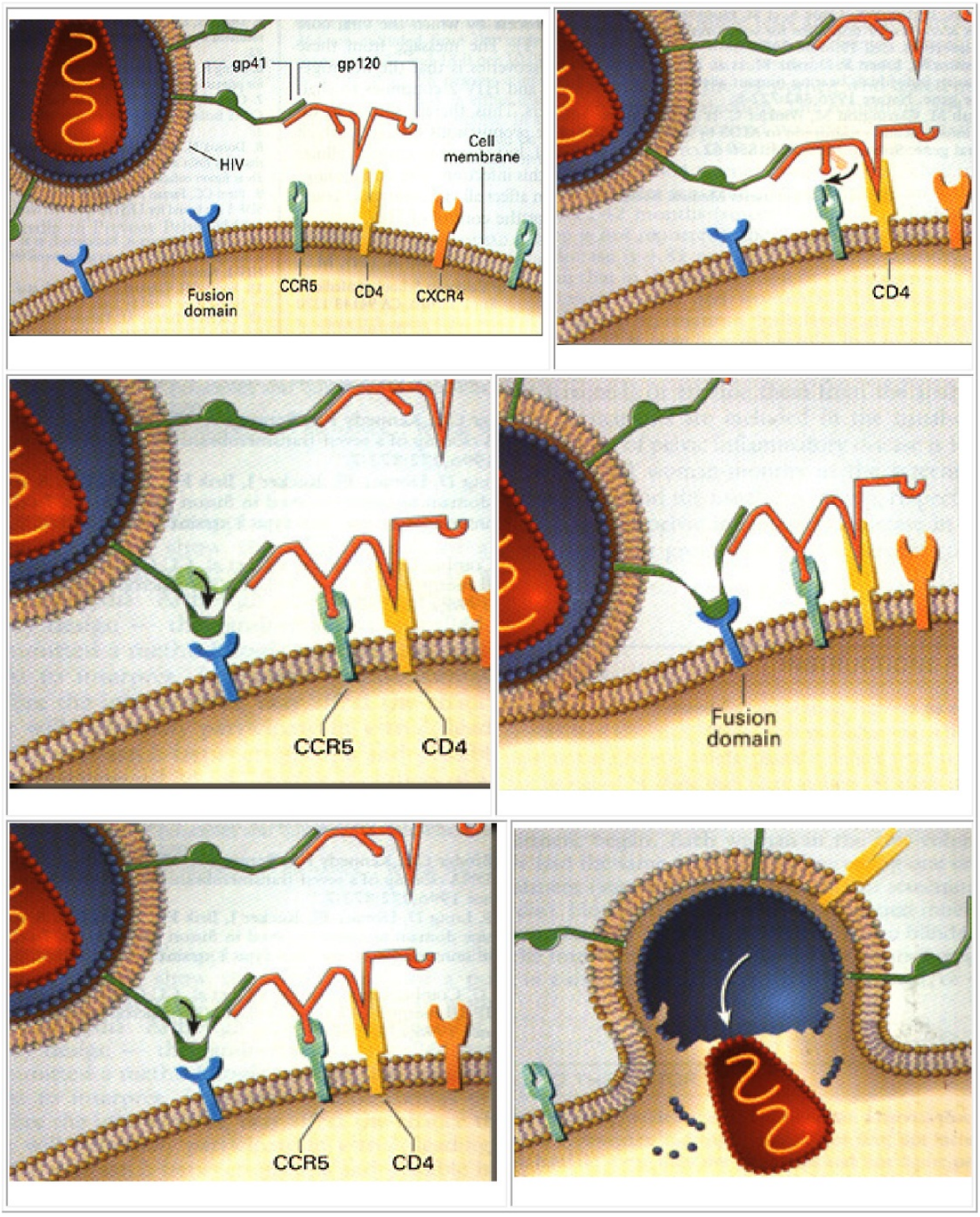

Figure 3. Interaction between HIV-1 and the cell surface Molecules, adopted from Levy J, 1996

24. Coreceptor usage and HIV-1 transmission

Irrespective of the transmission route or HIV-1 subtype, R5 viruses are preferentially transmitted in both horizontal and vertical transmission events except for subtype D. HIV transmission via breastfeeding accounts for a considerable proportion of infant HIV acquisition. However, no conclusive evidence has been provided to indicate that CXCR4-using strains are less able or unable to sustain mucosal transmission. For example simian chimeric immunodeficiency viruses (SHIV) bearing an X4 HIV-1 envelope can be readily transmitted via the mucosal route in macaques, and have widely been used as a reference model. Another important element that is rarely taken into consideration in the HIV-1 transmission equation is the transmitter bias which suggests that individuals with replicating CXCR4- using viruses are more likely to be in a more advanced stage of their disease progression and hence are more likely to be too ill to engage in risky sexual behavior. Majority of transmissions occur from asymptomatic individuals who generally harbor R5 variants. Consequently, the transmission of CXCR4-using variants may in fact be more frequent than it appears, albeit underestimated due to late sampling. Although primary infection with CXCR4-using HIV-1 strains is believed to be a rare event, mixed R5/X4 primary infections have been documented. Vertical transmission of dual tropic HIV-1 has also been demonstrated. Genotypic characteristics of HIV-1 V3 loop that are preferentially vertically transmitted for different subtypes remain unclear yet this information is critical for the development of effective transmission preventive strategies. At least for HIV-1 subtype B maternal viral phenotype can be predictive of the newborn's viral phenotype whilst the dual R5X4 phenotype is predominantly lost during vertical transmission. Antenatal HIV-1 subtype C coreceptor usage is generally preserved in vertical transmission and can be predictive of the newborn's viral genotype.

25. Co receptor usage and HIV Disease progression

Coreceptor usage is a marker for disease progression. There is a continued evolution in viral coreceptor usage in vivo, resulting in a broad range of coreceptor affinities within the HIV-1 quasi-species. Discriminating between these and other alternatives is central to increasing our understanding of the fundamental pathogenic processes involved in HIV-1 infection.

26. CCR5 HIV-1 variants

During the asymptomatic phase of HIV-1 infection a homogeneous R5 virus population is commonly present that can replicate efficiently in both T cells and macrophages. The early stages of HIV infection and the latency phase are characterised by CCR5 coreceptor using viral variants which are less virulent, non-syncytium-inducing and are associated with reduced progression to AIDS. A significant proportion of patients progresses to full-blown AIDS without experiencing an overt switch to CXCR4 usage, indicating that CCR5 usage remains a critical coreceptor throughout the course of HIV infection. This has been supported by the observation that the ability of R5 isolates to replicate in macrophages is progressively reduced during the course of infection, resulting in a predominantly T-cell

tropic R5 HIV-1 quasi-species even before the progression to AIDS. However, studies have shown that these late stage homogeneous CCR5 isolates are more pathogenic than the earlier isolates. In line with this observation is the ability of late-stage CCR5-restricted HIV-1 variants to use chimeric coreceptors where some parts of CCR5 would have been replaced with segments of CXCR4 (R5 broad), whilst early CCR5-using HIV-1 variants are restricted to the use of wild-type CCR5 (R5 narrow). This *in vivo* evolution of CCR5-restricted HIV-1 in humans is similar to that observed in non-human primates infected with SIV, which never acquires CXCR4 usage even though its pathogenicity increases during the late disease stages. This evolution is accompanied by improved coreceptor-binding affinity, which in turn is reflected in decreasing sensitivities of R5 variants to inhibition by CCR5-binding chemokines and small- molecule CCR5 antagonists.

27. Co-existence of R5 and CXCR4- HIV-1 variants

Some HIV-1 variants can use either coreceptor hence they are termed dual/mixed (DM)-tropic. These have been detected in all stages of infection although they are more common in infections of longer duration, with lower $CD4^+$ cell counts and higher viral loads. Such strains produce gp120 molecules capable of recognizing the CXCR4 protein on CD4-bearing T-cells. During this phase HIV-1 may infect both macrophages and T-cells. Still later, the bulk of the viral population may switch it's preference to the CXCR4 receptor and become T-tropic. T-tropic viruses readily destroy infected T-cells, contributing to the collapse of the immune system and the onset of AIDS. Evidence suggests that the evolutionary changes in the V3 loop involved in the coreceptor-usage switch are gradual with dual coreceptor usage (R5X4) representing an intermediate transitional phase. Once established the DM–tropic viruses have been shown to develop the optimal fitness to predominate during the transition phase, although they may eventually be outcompeted by HIV-1 variants with a pure X4 phenotype.

28. X4 variants

Following years of chronic HIV infection X4 using strains emerge although this phenomenon is not consistently observed in all patients progressing to AIDS. This switch of coreceptor usage has been shown to be associated with accelerated decrease in CD4 cells and hence it could be an important determinant of HIV pathogenesis and disease progression. The emergence of X4 variants has also been shown to coincide with disease progression and has been associated with longer duration of antiretroviral treatment including higher risk of death. The mechanism by which X4 viruses are associated with accelerated disease progression has never been properly elucidated although one theory proposes that R5-viruses lose their fitness with time, showing an abrupt decline in their ability to use CCR5 coreceptors and to infect cell lines with low CCR5 expression, demonstrating an increased susceptibility to CCR5 inhibitors consequently creating a pro-X4-virus environment. It has also been postulated that the decline of the host immune system associated with clinical AIDS may allow X4 viruses to evolve and replicate freely in late-stage infection. Furthermore, it has also been reported that disease progression among

individuals infected with subtypes D and C is faster than in those infected with subtypes A and A/G in Africa and that subtype D infection leads to faster rates of CD4 cell decline and subsequent virological failure compared to infection with subtype B and other non-subtype B HIV strains in England.

29. Host coreceptor genes & insights on resistance to HIV infection

A disproportionate transmission and distribution of HIV epidemic in the world has been observed with alarming rates in Sub Saharan Africa (SSA). Currently there is very little explanation for this observation of differences in susceptibility to HIV infection which could among other factors be attributable to variation in host genetics. Host genetic factors, including some polymorphisms in chemokine receptors and chemokine genes have been identified as having an impact on both HIV-1 infection and disease progression to death. Scientists have observed that despite multiple or repeated unprotected sexual exposures to HIV-1, some individuals remain HIV sero-negative. More so the discovery of long-term non-progressors (LTNP) in HIV infection prompted further investigations to ascertain the role of host genetic factors in the progression of HIV infection to AIDS. Polymorphisms in these human chemokine receptor genes will therefore affect the evolution of HIV-1.

30. CCR5 gene mutation

Analysis of blood samples from infected persons and the repeatedly exposed but somehow uninfected have shown specific molecular differences within the coding and regulatory regions of chemokine receptors and coreceptors genes. There is a deletion of 32 base pairs from the coding region of CCR5 gene in the second ECL. In these individuals the 32-base deletion in the CCR5 gene results in a frame shift and truncation of the normal CCR5 protein which renders them uninfectable after exposure to CCR5 tropic HIV viruses. This aberrant protein has been associated with protection against HIV-1 infection in people who are homozygous for mutant genotypes. Thus individuals resistant to HIV infection inherit two mutated copies of the dysfunctional gene for CCR5 from either parent. Without functional coreceptors, HIV is not able to enter immune cells. Despite the strong protective effect conferred by congenital CCR5 deficiencies, a handful of infected *CCR5-Δ32* homozygotes have been reported, all invariably harboring CXCR4- dependent HIV-1 strains. The rare homozygous individuals that got infected by HIV have been shown to be through CXCR4 coreceptor mediated entry only. Interestingly it has been found that in such homozygous conditions there is no evidence of health/phenotypic impairment caused by the absence of functional CCR5 coreceptors. However, HIV-infected individuals who would have inherited a copy of the defective CCR5 gene from only one parent, heterozygotes for a Δ32 deletion (CCR5-wt/Δ32) are not protected against HIV-1 infection but are associated with a much slow progression to AIDS relative to those with two normal copies of the gene. Heterozygous individuals have lower plasma HIV RNA levels in the early years of infection, which gives them a medical advantage of delaying disease progression. Since these mutations do not account for all cases of resistance to HIV infection, scientists are

looking for other possible host factors, including genetic defects involving other coreceptors. It was this protective property of CCR5Δ32 against HIV infection that has prompted pharmaceutical companies to develop a CCR5 antagonist for clinical use in the treatment of HIV/AIDS. Studies have shown that inhibition of CCR5 coreceptor seems not to cause significant clinical harmful consequences yet surprising to date there has not been any description of natural genetic alteration in CXCR4 human gene, suggestive that mutations in this gene are incompatible with life.

31. Epidemiology of CCR5Δ32 gene

A general North to South downhill gradient in CCR5Δ32 gene frequency has been observed. The highest frequency has been found in Northern and North-eastern Europe especially amongst the Finnish and Swedish populations. Data confirm the high frequency of CCR5-Delta 32 among northern European Caucasians, a gene frequency declining across Europe and Asia reflecting recent population admixture. The virtual absence of CCR5-Delta 32 deletion among native Africans, East Asians, and American-Indians is suggestive that the mutation arose in northern Europe in response to selective pressures due to other factors including infection epidemics. The CCR5Δ32 has a prevalence rate of between 20-35% among the Jewish population. In Africa studies indicated that the allelic frequency of CCR5Δ32 mutation is 0.1% in the black South African population.

32. CCR2 –V641 mutation

A CCR2 –V641 mutation from a conservative valine to isoleucine substitution in the transmembrane region has also been associated with some protective effects against HIV infection and reduction in progression of HIV to AIDS. The protective effect of CCR2 –V641 is believed is to be through regulation linkage disequilibrium in the regulatory region or promoter region of this gene. CCR2-V64I is common among Africans, yet this race is one of the most affected populations by the HIV pandemic. Hence, the protective effect of CCR2 –V641 against HIV infection remains controversial. Despite the availability of evidence linking some host genetic factors to protection against HIV infection, very little information is available regarding the role of CCR5-Δ32 and CCR2-V64I polymorphism on HIV transmission.

33. Coreceptor antagonists and antiretroviral therapy

To successfully infect an immune cell HIV env gp 120 has to interact with the host cellular receptor CD4 and either a CCR5 or CXCR4. Both coreceptors are recognized as novel targets for anti-HIV-therapy consequently resulting in the development of a new class of antiretroviral drugs called coreceptor antagonists or blockers. Blocking these coreceptors would protect the host cell from viral entry and would reduce the viral transmission and pathogenesis. Interestingly coreceptor antagonists differ from the other antiretroviral agents that target HIV proteins in the sense that they bind and inhibit receptors encoded by the

host itself. Besides blocking replication of the virus in other cells, coreceptor antagonists may reduce the decline of immune system through other ways. It is known that HIV-1 env glycoproteins may trigger autophagy in uninfected CD4 positive T cells, leading to their apoptosis, and consequently increasing immune decline. These cells need to present, among other things, a CXCR4 coreceptor in their surface. The blockage of the interaction between glycoproteins and the coreceptor of uninfected cells could help to avoid immune impairment. CCR5 antagonists design and development are a step ahead relative to their CXCR4 counterparts. This unique new class of drugs not only increases the alternative therapeutic options for HIV treatment but also maximizes potency, minimizes toxicity and reduces the risk of drug resistance development. Range of coreceptor antagonists discovered to date includes modified chemokines monoclonal antibodies, peptides and small organic molecules.

34. Mechanism of action of coreceptor antagonists

Coreceptor antagonists' antiviral properties are related to their ability to internalize the receptor, preventing it from being expressed on the cell surface thereby avoiding viral replication by reducing coreceptors availability to bind to HIV glycoproteins.

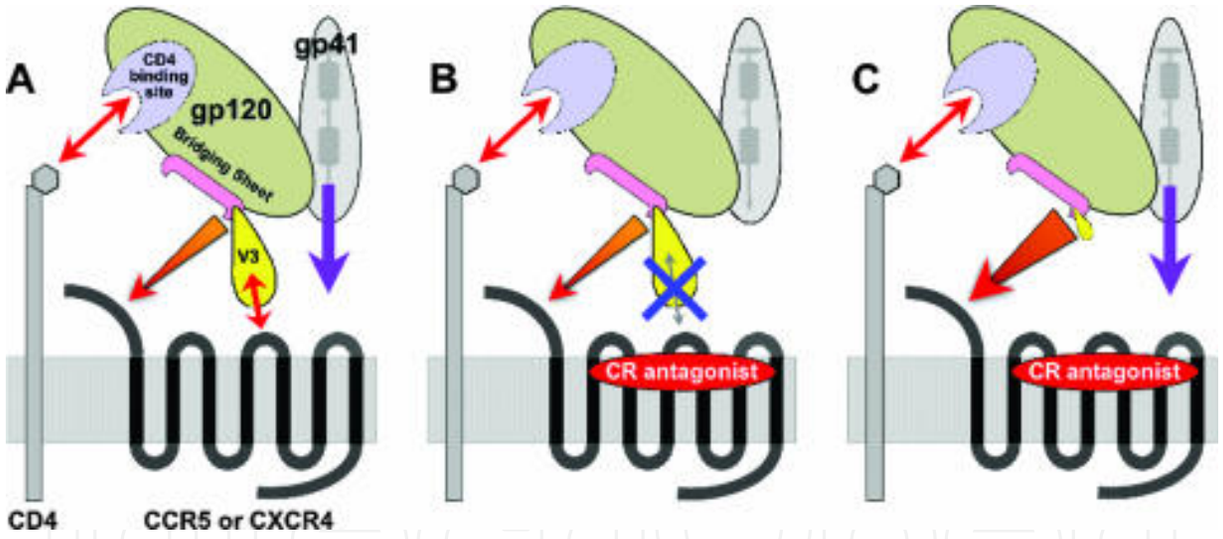

Figure 4. Mode of Action of Coreceptor antagonists

35. Coreceptor antagonists in development

35.1. Modified chemokines

Modified chemokines include RANTES, a natural chemokine ligand for CCR5 manufactured by RANTES engineering technology including other novel agents that have not been very successful and their future remains uncertain. Other possible antagonists are zinc finger nucleases that cause mutagenesis in the CCR5 gene by binding and cleaving the gene, producing abnormal proteins. Zinc fingers, along with RNA interference, ribozymes, intrakines, and intrabodies are HIV-1 vector-delivered genetic disruption mechanisms that target HIV-1

chemokine receptors. Although chemokines or derivative-molecules could be exploited as therapeutic agents against HIV, the risk of inducing inflammatory side-effects or of interfering with the physiology of the homeostatic chemokine system represents a potential limitation.

36. Monoclonal antibodies as coreceptor antagonists

Another type of coreceptor antagonists are monoclonal antibodies. Examples of such agents are CCR5mAb004 of Human Genome Sciences (Rockville, MD, USA) and PRO 140 of Progenics Pharmaceuticals (Tarrytown, NY, USA). They are both mouse-derived but humanized monoclonal antibodies capable of blocking CCR5 without activating the signal transduction pathways. In phase I clinical trials CCR5mAb004 has been shown to be safe and well tolerated.

37. Small molecule CXR4 chemokine receptor antagonists

AMD3100 is a small-molecule CXCR4 antagonist with activity against X4 viruses' in vitro and antiviral activity in vivo, although it is no longer being pursued clinically because of pharmacology and toxicology considerations. More examples of small molecule chemokine receptor antagonists of CXCR4 coreceptor include AMD11070/ AMD070, AMD3465, ALX40-4C, T22, T134 and T140. AMD11070 is known to specifically inhibit CXCR4 in a reversible way. Other CXCR4 selective inhibitor is AMD3100 from AnorMED. AMD3100 is highly specific for CXCR4 that blocks replication of X4 strains. However, clinical trials of AMD3100 have been stopped due to side effects of cardiotoxicity. A relatively safer derivative of AMD3100 known as AMD070 is currently undergoing clinical trials. Previously AMD3100 has proved to be a useful tool to probe interactions between env proteins and CXCR4 which is important to identify pathways by which HIV-1 may become resistant to this class of antiviral agents. To date none of CXCR4 blockers has successfully proceeded into clinical practice due to their severe side effects. Generally, CXCR4-based blocking agents are less attractive due to the crucial role of CXCR4 in many biological processes. However, agents that aim at down-modulating CXCR4 expression may provide some benefits to HIV-positive patients. Antagonism of CXCR4 has been shown to significantly improve survival from lethal infection through enhanced intraparenchymal migration of West Nile Virus-specific CD8+ T cells within the brain, leading to reduced viral loads and decreased immunopathology at affected sites.

38. Small-molecule CCR5 antagonists

The CCR5 antagonists act on a wide spectrum of viruses with affinity or tropism for this receptor. They are absorbed orally and have powerful antiviral activity through interaction with amino acids from the pocket formed by the transmembrane (TM) domains of CCR5. Mechanism of action is through allosteric effects, altering extracellular CCR5 conformation after binding to a hydrophobic pocket of CCR5, formed by transmembrane helices. Mutations within transmembrane domains are associated with impaired activity. Small molecule CCR5 antagonists such as maraviroc, vicriviroc, aplaviroc, TAK-779, and TAK-220) have been found to fit in the same binding pocket. Other small-molecule CCR5 antagonists include:

Tak-652, spirodiketopiperazine derivatives such as E913 and SCH-C/SCH-351125. Spirodiketopiperazine derivatives have been associated with severe hepatotoxicity, leading to the cancellation of their development. SCH351125 or SCH-C was the first CCR5 antagonist to enter clinical efficacy studies but has been dropped out due to variable antiviral effect and provocation of prolonged QTc interval. To counter these setbacks Vicriviroc was developed which was much more potent than SCH-C, safer and better oral bioavailability.

Two agents, maraviroc and vicriviroc, are the agents within this family that have passed the final stages of clinical development. These agents have their scientific names ending in "–viroc", to denote their action of "viral receptor occupancy". Since these drugs show activity against R5 viruses only, viral tropism testing should be made before their prescription and eventually during treatment in order to exclude the presence of X4 viruses. They are less expensive to produce and have good oral bioavailability. Their half maximal inhibitory concentrations (IC50) are in the nanomolar ranges.

39. Maraviroc

A success story has been the introduction CCR5 antagonist, Maraviroc, trade name Selzentry or Celsentri into clinical practice. Maraviroc is a non-competitive CCR5 antagonist that selectively binds to the human CCR5, present on the cell membrane, preventing the interaction with HIV-1 env gp 120. CCR5 is necessary for CCR5-tropic HIV-1 to enter cells. It is metabolized in the liver. Drug plasma concentrations are likely to be increased in patients with hepatic impairment. Route of metabolism is through Cytochrome P450 3A4 (CYP3A). The drug has shown potent activity against multidrug-resistance CCR5-tropic HIV-1 strains. It has antiviral activity *in vitro* against CCR5 tropic HIV-1 isolates from various subtypes. However, Maraviroc has no activity against CXCR4-tropic and dual tropic HIV-1. It has been found to be effective, efficient and safe to use in combination with other antiretroviral agents such as with lopinavir/ritonavir plus efavirenz or saquinavir/ritonavir plus efavirenz in adult patients with exclusive CCR5 tropic HIV-1 isolates. Maraviroc was developed by Pfizer Inc. (Kent, UK).

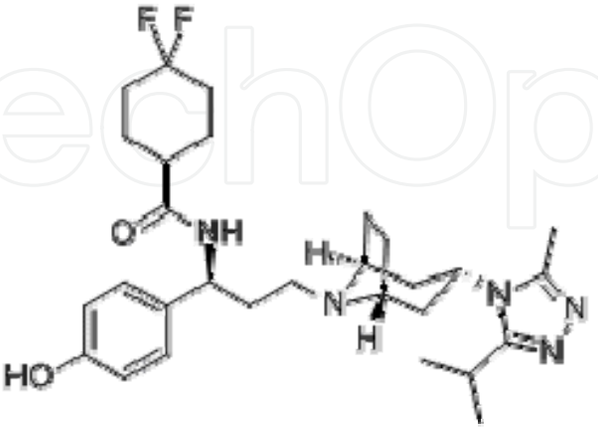

Figure 5. Structure of Maraviroc adopted from chemicalbook.com

Maraviroc inhibit CCR5 binding and signaling at nanomolar (nM) concentrations (IC_{90} of 2 nM). It is well tolerated with most common mild to moderate side-effects including

headache, asthenia, dizziness, gingivitis and nausea. It is known that before starting on a coreceptor antagonist, patients need to determine their viral tropism. The introduction of the CCR5 antagonist, maraviroc for HIV-1 therapy has increased the research interest in the epidemiology of tropism and its relationship with HIV-1 subtype. A greater understanding of the tropism of non-B subtypes is key for the optimal use of CCR5 antagonists in the treatment of these infections in the developing world and HIV-1 prevention strategies. There is no food effect as it can be taken with or without food and can be stored at room temperature. Hence it is very suitable for resource poor settings which also bear the brunt of the HIV scourge with predominant R5 variants.

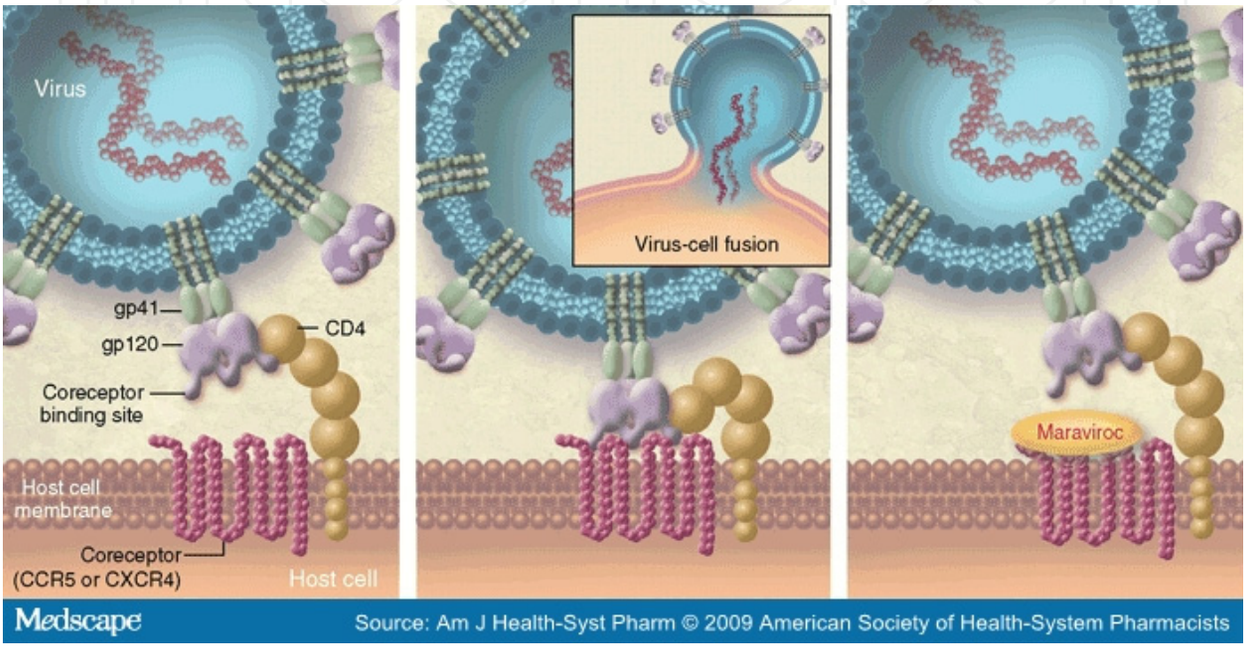

Figure 6. Mode of action of maraviroc, adopted from Qian K, 2008.

40. Challenges of CCR5 antagonists

The CCR5-to-CXCR4 switch represents a concern because while CCR5 inhibitors suppress R5 viruses they may allow the emergence of CXCR4-tropic viruses. Efforts to develop HIV entry inhibitors are hampered by problems associated with rapid evolution of the virus, leading to drug resistance. Blocking only one of the pathways for HIV entry into cells has resulted in the opening of the other pathways potentially accelerating disease progression by promoting the evolution of more virulent CXCR4-dependent variants. In this view, AnorMED Company has announced the discovery and development of a joint CXCR4/CCR5 antagonist although little is known about the binding mechanism at this time.

41. Future of coreceptor antagonists

There have been speculations that new, safe and effective chemokine receptor inhibitors should facilitate CCR5 and CXCR4 internalization independent of the cellular signaling. The most anticipated eventual combination of CXCR4 and CCR5 inhibitors would be beneficial,

since selective pressure could direct the virus to use less productive coreceptors, avoiding the progression of the disease. In addition leveraging new technologies capable of detecting low-level minority species may provide the most significant advances in ensuring that individuals with low levels of dual/mixed tropic virus are not inadvertently prescribed CCR5 antagonists.

Author details

Kerina Duri
University of Zimbabwe, College of Health Sciences, Department of Immunology, Harare, Zimbabwe

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