HIV Type 1 Tropism and Inhibitors of Viral Entry: Clinical Implications

Jan Weber[1], Helen Piontkivska[2] and Miguel E. Quiñones-Mateu[1,3]

[1]Department of Molecular Genetics, Section of Virology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio, USA; [2]Department of Biological Sciences, Kent State University, Kent, Ohio, USA; [3]Center for AIDS Research, Case Western Reserve University, Cleveland, Ohio, USA

Abstract

Since their discovery in 1996, the two main coreceptors used by human immunodeficiency virus type 1 (HIV-1) to enter human cells (CCR5 and CXCR4) have been the subject of numerous scientific articles. A recent search in PubMed (www.pubmed.gov) using "HIV coreceptor" as keywords led to more than 1100 original research publications and 90 review articles. This number skyrocketed to more than double if we used "HIV CCR5". Most of the reviews described in detail several aspects of HIV tropism, viral entry mechanism, coreceptor usage and its implication on disease progression, antiretroviral therapy, and vaccine development. A few others centered on the tools utilized to measure the ability of HIV to use these coreceptors to infect target cells. On the other hand, identification of the HIV coreceptors renewed the effort and expectation to block HIV replication by targeting viral entry into the target cells. As with HIV tropism, hundreds of articles have been published addressing this topic (more than 350 original publications and 50 review articles when using "HIV entry inhibitor" as a descriptive word). Therefore, in addition to providing a brief update of the most important aspects described above, we discuss here how an accurate quantification of HIV coreceptor usage is essential for the successful management of HIV-infected individuals in this new era of entry inhibitors, mainly CCR5- or CXCR4-antagonists. **(AIDS Reviews 2006;8:60-77)**

Corresponding author Miguel E. Quiñones-Mateu, quinonm@ccf.org

Key words

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Introduction

After the discovery of the CD4 molecule as the major cellular receptor for HIV entry[1,2], multiple studies suggested the presence of a secondary cellular receptor for HIV, e.g. the infection of cells lacking CD4[3,4]. The identification of the two major coreceptors was triggered by the initial finding that the natural ligands of CCR5 (i.e. RANTES, macrophage inflammatory protein-1α [MIP-1α], and MIP-1β) could block the infection of certain HIV-1 strains known as non-syncytium-inducing (NSI)[5]. Several elegant studies then lead to the conclusion that HIV requires a second (co-) receptor to enter target cells, mainly the chemokine receptors CCR5 and CXCR4[6-8]. This new information helped to establish a new nomenclature defining HIV-1 strains based on their coreceptor usage. Original HIV phenotypic classifications used the ability of the virus to cause or not syncytia in cell cultures, i.e. syncytium-inducing (SI) or NSI[9]. This characteristic was linked to the replication rate of the virus in peripheral blood mononuclear cells (PBMC), where usually a SI virus would replicate rapidly and highly while the replication of NSI virus would be slow and low[10]. Interestingly, these viral attributes correlated with the tropism of HIV for certain types of cells: SI/rapid/high viruses replicate in T-lymphoid cells and NSI/slow/low

Correspondence to:
Miguel E. Quiñones-Mateu
Cleveland Clinic Foundation
Lerner Research Institute
Department of Molecular Genetics, Section of Virology
9500 Euclid Avenue / NN10
Cleveland, OH 44195, USA
E-mail: quinonm@ccf.org

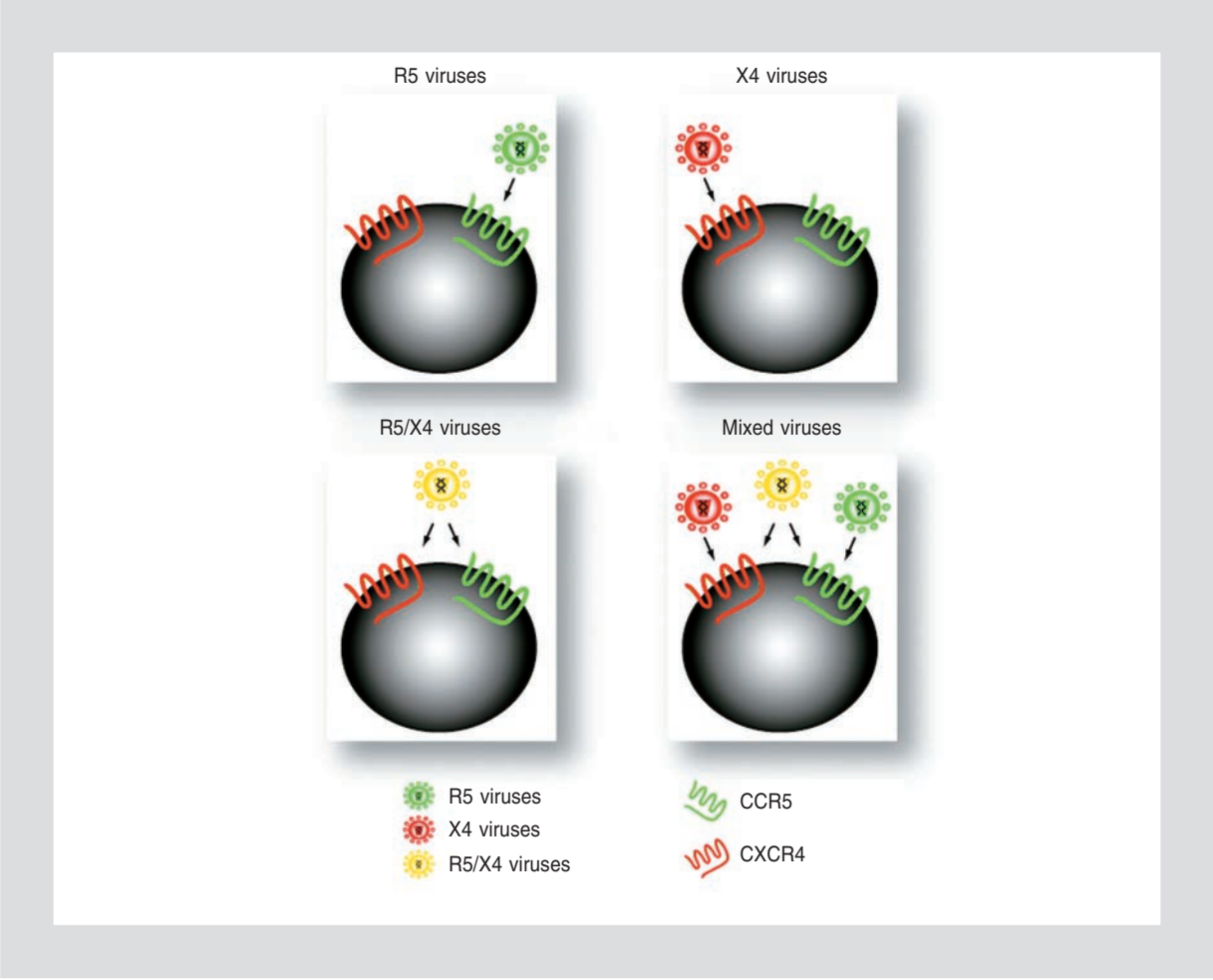

Figure 1. *Different possibilities for HIV-1 coreceptor usage (tropism)* in vivo.

strains preferentially infect monocyte-derived macrophages[11]. A new classification based on this tropism adopted the terms T-tropic (SI) and M-tropic (NSI)[11-13]. However, as described above, the current and more frequently used nomenclature was established based on coreceptor usage[14]. SI/T-tropic usually utilizes the CXCR4 coreceptor for entry and NSI/M-tropic strains use the CCR5 coreceptor[14-16]. Therefore, CCR5- and CXCR4-tropic viruses are denominated R5 and X4, respectively; while HIV strains able to use both coreceptors are termed dual tropic (R5/X4)[14] (Fig. 1).

In vivo findings suggest that R5 HIV isolates may out-compete X4 variants at the site of primary infection. For example, humans who are homozygous for a deletion in the CCR5 gene (i.e. lack CCR5 on any cell surface) are typically resistant to HIV infection[17,18]. However, preferential transmission of R5 HIV strains and predominance during the asymptomatic disease are not well defined. Langerhans cells (expressing CCR5 receptors) found embedded in the vaginal mucosa may be the first cell targets for primary heterosexual transmission[19;20]. In addition, R5 viruses have a higher affinity for dendritic cells, which transport the virus past the mucosal layer to the lymph nodes[21]. Although R5 viruses are typically predominant following HIV transmission, X4 viruses often dominate the viral quasispecies late in disease[12,16,22]. These changes seem to evolve in about 50% of HIV-infected individuals and are usually associated with an accelerated decline of $CD4^+$ T-cells, a burst in plasma viral load, and a rather rapid progression toward AIDS[15,21,23,24]. Interestingly, this switch in coreceptor usage within the HIV quasispecies may occur through intermediary R5/X4 viruses, capable of utilizing both CCR5 and CXCR4 coreceptors[25]. CXCR4-tropic viruses seem to be more virulent than the initial R5 variants, perhaps due to their ability to access a larger pool of target cells (CXCR4 is expressed on a higher number of naive

Table 1. Env protein domains associated with HIV coreceptor usage

Env domain(s)	Properties	References
V3	X4 phenotype is linked to positively charged residues (e.g. H, R, or K) in two different positions: 11 and 25 (i.e. 306 and 320, HIV-1_{HXB2}) R5 phenotype is characterized by the absence of positively charged residues (e.g. negatively charged E, D or uncharged A, Q, S, and G).	34-36,62,221-227
V2	Higher net positive charge in X4 than in R5 variants	41,43,44
V2 and V3	Changes in this region and interactions with V4 and V5 may play role in HIV tropism	39,44
V1	S141N amino acid substitution led to a different $CD4^+$ tropism	40
C1-V4	Changes in this region affect HIV tropism	42,64

H: histidine; R: arginine; K: lysine; E: glutamic acid; D: aspartic acid; A: alanine; Q: glutamine; S: serine; G: glycine; ASN: asparagine.

$CD4^+$ cells, while CCR5 is present on memory T-cells)[26]. However, conclusive differences in transmissibility, replication, and pathogenicity between R5 and X4 viruses will require further studies[27,28]. Finally, identification and even quantification of HIV coreceptor usage is essential in the clinical setting, particularly for the design and development of novel antiretroviral drugs targeting HIV entry (see below).

Regions within the *env* gene associated with R5 and X4 phenotypes

Numerous studies have associated different HIV genomic regions, mainly in the *env* gene, as determinants of CCR5 and CXCR4 tropism[29,30]. For example, the SI/X4 phenotype seems to be determined by the presence of a positively charged V3 region of the envelope protein gp120, particularly amino acids in positions 11 and/or 25 (i.e. positions 306 and/or 320 of *env*, based on HIV-1_{HBX2} number; http://hiv-web.lanl.gov/content/index)[31-38]. On the other hand, regions outside the V3 loop have been shown to be involved with R5, X4, or R5/X4 phenotypes. The other *env* hypervariable regions (V1, V2, V4, and V5) as well as the entire C1-V4 region may also play a role in HIV tropism[39-44]. Table 1 summarizes *env* protein domains which have been related with HIV coreceptor usage.

In vitro methodology used to determine HIV coreceptor usage

The determination of coreceptor usage of HIV is becoming a crucial step for optimal design and evaluation of clinical trials with drugs aimed to block the interaction of the virus with CCR5 or CXCR4 receptors. We now know that the HIV population in patients may contain a heterogeneous swarm (quasispecies) composed of any combination of X4-, R5- and dual X4/R5-tropic species. Therefore, HIV-infected individuals exposed to entry inhibitors need to be monitored during whole course of therapy for a possible shift in viral coreceptor usage. It is evident then that a rapid and cost-effective assay for HIV coreceptor usage is needed to detect even minor variants within the patient viral population.

Thus far, *in vitro* assessment of HIV coreceptor usage can be divided into methods based on (i) HIV isolation and use of cell lines expressing different receptors, and (ii) *env* recombinant viruses (Table 2). Sequence-based HIV coreceptor usage predictions are described below. Differences in viral phenotype were recognized long before the discovery of HIV coreceptors[13,45]. Infection of MT-2 cells was originally used to differentiate between NSI and SI HIV variants, which had been associated with slow and rapid decline of $CD4^+$ T-cells, respectively[46-48]. More recently, several methods that use HIV-1 isolates to infect reporter cell lines expressing specific sets of receptors on their surface have been developed[48,49]. Most of these assays use human glioma cell lines (e.g. U87, U373, NP-2) which were stably transduced with vectors expressing CD4 and one or more coreceptors[16,50,51]. Another frequently used is the indicator GHOST cell line, which is derived from human osteosarcoma cells and carries an HIV-1 tat-inducible GFP gene, CD4 and a variety of coreceptors[52-54]. Specific inhibitors of CCR5 and CXCR4 chemokine receptors, such as TAK-779 or AMD-3100, respectively, are often used to detect the susceptibility of HIV-1 variants to inhibition by these

Table 2. *In vitro* methods used to determine HIV coreceptor usage

Assay	Coreceptor usage	Detection	References
MT-2 cells	Positive: CXCR4 Negative: CCR5	CPE, p24	46,49,228
Infection of PBMC from CCR5 Δ32 homozygous donor	Positive: CXCR4 Negative: CCR5	p24	49
Inhibition with CCR5 and CXCR4 specific antagonists	CCR5, CXCR4	p24	55
GHOST cell line	CCR1, CCR2b, CCR3, CCR4, CCR5, CCR8, CXCR4, V28/CX3CR1, BOB/GPR15, Bonzo/STRL33	GFP	52
U87 cell lines	CCR1, CCR2b, CCR3, CCR5, CXCR4	p24, RT	16
U373-MAGI cell lines	CCR5, CXCR4	β-galactosidase	50
NP-2 cell lines	CCR1, CCR3, CCR5, CCR8, CXCR4	RT	51
PHENOSCRIPT™ HIV-1 Entry Inhibitor	CCR5, CXCR4	β-galactosidase	57,60
PhenoSense™ HIV Coreceptor Tropism	CCR5, CXCR4	luciferase	48,229

CPE: cytopathic effect; p24: p24 antigen EIA; GFP: green fluorescent protein; RT: reverse transcriptase assay.

compounds, thereby showing their dependence on a particular coreceptor[55].

Although widely used, all these methods have a few important caveats. Most of them need viral isolates from HIV-infected patients. Standard viral isolation procedures require co-culturing of the patient's HIV-infected PBMC with PBMC from an HIV-seronegative donor stimulated with phytohemagglutinin or anti-CD3/CD28 antibodies in the presence of interleukin-2. However, long virus culturing may lead to adaptation of HIV to the new *ex vivo* environment, which may not represent the original *in vivo* HIV population[56,57]. In addition, small differences in HIV isolation protocols (e.g. stimulation of PBMC) may alter the level of coreceptor expression on the cell surface[58]. More importantly, the levels of receptor and coreceptor expression on reporter cell lines may also differ from the natural host cells for HIV. Indeed, coreceptor utilization may be influenced by the level of receptor expression and by the ratio of the coreceptors[59].

Two HIV tropism assays based on *env* recombinant viruses are currently commercially available. The PHENOSCRIPT™ HIV-1 entry inhibitor assay (VIRalliance, Paris, France) permits both tropism determination and assessment of viral susceptibility to HIV-1 entry inhibitors[57,60]. In this assay, *env* recombinant viruses are produced by co-transfection of 293T cells with (i) a linear pNL4-3 plasmid lacking the *env* gene (gp120 and the ectodomain of gp41, positions 6480 to 8263), and (ii) RT-PCR *env* amplicons from HIV-infected plasma samples. Replication-competent *env* recombinant viruses are produced by homologous recombination in the target cells[60]. This virus is used to infect indicator U373MG-CD4 cells expressing either CCR5 or CXCR4 carrying an HIV-1 LTR-*lacZ* cassette, which allows the quantification of single cycle infectivity by a colorimetric assay based on HIV-1 Tat-induced expression of ß-galactosidase[57,60]. The second assay (PhenoSense™ HIV-coreceptor tropism assay, Monogram Biosciences, Inc., South San Francisco, California, USA, formerly ViroLogic Inc.) uses a similar methodology. The entire envelope coding region (approximately 2500 bp) is amplified from plasma samples of HIV-infected individuals and cloned into an envelope expression vector. This vector is co-transfected into 293 cells with an HIV genomic vector carrying a luciferase reporter gene. These pseudo-typed, replication-defective viruses are used to infect U87 cells expressing CD4 and either CCR5 or CXCR4. Coreceptor usage is quantified by measuring luciferase activity after a single round of infection, while luciferase production must be inhibited by specific coreceptor antagonists[48]. Both HIV recombinant tropism assays presume to provide a rapid and accurate representation of the patient's plasma virus population. However, the final result de-

pends on the reliability of the RT-PCR reactions to sample the *in vivo* HIV quasispecies. The limit of detection for minor members of the viral population in these assays has been reported to be in the 10-20% range[48]. Further studies are necessary to demonstrate that this level of detection is sufficient to provide clinically relevant information about HIV coreceptor usage in patients, particularly in this new era of entry inhibitors.

In silico approaches to estimate HIV coreceptor usage

Cell-based *in vitro* methods designed to determine HIV tropism can be costly and somewhat difficult to carry out outside research laboratories. Therefore, numerous studies have devoted considerable effort to the possibility of inferring HIV coreceptor usage based on *env* sequences, an attractive, faster, and less expensive alternative. While certainly the final determination of HIV tropism will remain with the biological assays, computational approaches are becoming more popular as more molecular sequence data become available. Table 3 summarizes different bioinformatics systems used to infer HIV coreceptor usage. Early attempts to distinguish between R5 and X4 phenotypes considered amino acid sequence variability in the V3 region, with emphasis on positions 11 and/or 25: positively charged amino acids (i.e. arginine, lysine, or histidine) in these positions usually indicate X4 tropism, while other amino acid residues are associated with R5 phenotype[35,61-63]. Although a rather simplistic approach, this analysis frequently matches *in vitro* assays. The problems begin when V3 sequence-based predictions fail to correlate with biological tests. As described above, other regions within the *env* gene may play a similar role in determining HIV coreceptor usage.

Sequence analyses can be extended beyond the borders of the V3 region to pinpoint other sites that may determine HIV coreceptor usage. For example, when the V3 sequence variability profile was correlated with the reminder of the envelope protein, a potential linkage was found with sites in the more conserved C4 region[64]. This same region physically interacts with V3[65,66]. However, it is important to remember that recombination between the myriad of members in the viral quasispecies may potentially influence these results[67]. A co-variation approach has been used to try to identify pairs of sites that display co-varying (interdependent) mutations[68,69]. The so-called "mutual" information is used as a measure of co-variation of mutations at different amino acid sites. One of the potential caveats of this approach is the possibility of shared ancestry, where statistical co-variation is due to shared evolution of a group of sequences that are descendants of a single ancestral virus. This effect may be reduced by taking into account the evolutionary history of the sequences and by partitioning the dataset into clades. Uneven sampling of certain groups, sparsity of the data, and having to select arbitrary prune-out thresholds may be another source of potential bias[69]. Similarly, analyses of amino acid sequence variability profiles have been used as phenotype classifiers[62,64]; in particular, a set of amino acid substitutions at sites other than 11 and 25 was identified that appeared predominantly in X4 phenotypes[64].

To analyze the variability of V3 sequences, position-specific scoring matrices (PSSM) were constructed to detect nonrandom distribution of amino acids at the adjacent sites[29,70]. Frequently used for motif finding (e.g. to identify potential epitopes in MHC sequences[71]), PSSM has the potential to detect even relatively minor sequence changes that nonetheless may have biological consequences on HIV coreceptor usage. This is because each sequence can be assigned a score that describes its likelihood of having the target properties[70]. For example, Jensen, et al.[70] found that when applied to V3 sequences, PSSM is able to differentiate between three different phenotypes: R5 exhibited low scores, X4 had high scores, and intermediate R5/X4 variants had intermediate scores. Thus, they concluded that R5 to X4 conversion is a gradual process that involves multiple amino acid changes. Some changes, such as those at sites 11 and 25, may have a large impact on the phenotype, albeit not being necessary provided that enough smaller changes have already been accumulated[70].

Recently, several machine-learning approaches, including support vector machines (SVM) have been successfully implemented[72-74], allowing the incorporation of many HIV subtypes as well as recombinant forms (Table 3). Overall, SVM and PSSM approaches appear to perform quite well in correctly identifying R5 and X4 phenotypes[29,72]; however, the performance of these methods partly depends on the data set used for training[29]. With the accumulation of more HIV sequence data, further improvement and development of such predictive tools will be necessary as they have a great potential to contribute toward the choice of effective therapy strategies and selection of the drug regimens based on the viral sequences from individual patients.

Table 3. *In silico* approaches to estimate HIV coreceptor usage.

Approach	Analysis steps and selected major findings	References
Information theoretic analysis (co-variation approach)	Compute mutual information (measure of co-variation of mutations at different sites) to identify interdependent sites and mutations as coreceptor usage predictors. Identified multiple pairs of sites that have the highest most significant mutual information score	68
Phenetic analysis	Phenetic sorting (clustering) of protein sequences with differential use of amino acid substitution matrices, to maximize the pair-wise comparison score among sequences	230
Codon usage analysis	Presence of positively charged, uncharged or negatively charged codons in certain positions in the V3 region is taken as indicator of R5 or X4 phenotype	63,223
Information theoretic analysis (co-variation approach)	Similar to the co-variation approach of Korber, et al. (1993), improved to adjust for the possibility of "founder effect"	69
Evolutionary genetics/phylogenetic approach	Estimate rates of synonymous and non-synonymous substitutions. Substitution rate was found to be higher in X4 than in R5 variants. Positive selection, possibly due to interactions with the immune system, is operating at V3 sites within a single host	231
Sequence variability analysis	Analyze distribution of amino acid substitutions by creating sequence profiles of each phenotype. Uses presence of basic amino acids as indicators of HIV coreceptor usage. X4 variants tend to have higher proportion of basic amino acids (reviewed in[232])	62,64
Regression analysis	Use multiple linear regression to estimate the positive, negative, and net charge of the V3 loop.	233
Neural networks	Use of neural networks to discriminate between X4 and R5 phenotypes of subtype B sequences. Trained neural network (in Matlab) is available from http://cancer.med.unc.edu/swanstromlab/resources.html	234
Bioinformatics I	Uses multiple measures of differential sequence composition to discriminate between HIV phenotypes: (i) diversity of amino acid (D)[235], (ii) Shannon entropy (E)[236], and (iii) binomial Z score, Zij, generated by the permutation procedure. Only part of the V3 variability can be associated with differential HIV coreceptor usage. An increased positive charge in the V2 region is also a contributing factor	44
Bioinformatics II	Use of position-specific scoring matrices (PSSM) to analyze amino acid variability. Conversion from R5 to X4 viruses seems to be a gradual process that involves accumulation of multiple amino acid changes in V3	29,70
Machine learning I	Includes support vector machines (SVM). Available online at http://genomiac2.ucsd.edu:8080/wetcat/index.html	74
Machine learning II	Based on mixture of localized rules[73]. Geno2pheno tool is available online at http://www.genafor.org/	72,73

Antiretroviral strategies targeting HIV entry

HIV entry into target cells is a complex, multistage process involving the gp120 and gp41 subunits of the envelope glycoprotein, primary cellular receptor CD4, and at least two main cellular coreceptors, CCR5 and CXCR4[75]. This crucial step in the viral life-cycle was one of the first targeted after the discovery of the virus[3]. Later on, the identification in the mid 1990s of two HIV coreceptors led to the rapid development of several drugs that blocked this interaction. Since then, the search for inhibitors of HIV entry has focused on three major stages: (i) attachment of the virus to the target cells through gp120-CD4 interaction; (ii) interaction of gp120 with cellular coreceptors (mainly CCR5 or CXCR4); and (iii) fusion of the virus and host cell membranes.

Inhibitors of gp120-CD4 interaction

As CD4 is essential for HIV entry, many strategies initially focused on identifying CD4-based anti-HIV compounds. The first obvious candidate, soluble CD4, failed to demonstrate strong effect against primary isolates[76]. Better results were obtained using CD4-immunoglobulin G2 (PRO 542), which is a recombinant, antibody-like, tetrameric, fusion protein compromised of human IgG2, where the heavy and the light-chain variable domains of human IgG2 have been replaced by the D1 and D2 domains of human CD4[77]. PRO 542 showed potent inhibition of a large panel of primary HIV-1 isolates including all major clades[78] as well as un-passaged viral isolates from plasma[79]. Negative-stain immunoelectron microscopy demonstrated the ability of PRO 542 to bind four gp120s, indicating that PRO 542 has considerable potential to cross-link envelope trimers on the virion surface[80]. More important, PRO 542 was well tolerated in phase I clinical trials and decreased viral load after a single intravenous dose[81]. Additional phase I/II clinical trials are currently underway. A second example of this approach is CD4M33, designed using structural information on a CD4-gp120-17b antibody complex, 27-amino acid peptide mimicking the gp120-binding CD4 domain D1[82]. Unlike soluble CD4, CD4M33 inhibited HIV infection by both primary and laboratory HIV-1 isolates *in vitro* at nanomolar concentrations[82]. The recently developed TNX 355 exploits the same strategy. TNX 355 is a humanized IgG4 anti-CD4 monoclonal antibody recognizing epitope in D2 domain of CD4[83]. Results from the phase I clinical trial showed that single doses of TNX 355 reduced plasma HIV-1 RNA loads and increased $CD4^+$ T-cell counts in HIV-positive subjects[83].

The first interaction between the virus and the host cell target involves the binding of positively charged regions of the V3 loop (gp120) to the negatively charged cell-surface molecules, such as heparan sulfate proteoglycans[84,85], galactosyl ceramides[86], mannose receptors[87] and/or interaction between gp120 and C-type lectins (i.e., DC-SIGN [ICAM-3][88], ICAM-1[89] and LFA-1[89,90]). Strongly cationic peptides, such as SPC3, have been shown to disrupt attachment of the virus to the cell by binding preferentially to cell surface glycosphingolipids[91]. Further studies revealed that SPC3 inhibits HIV infection likely through its interaction with CXCR4[92]. Moderate effects of free SPC3 on viral load in phase II clinical trials led to liposomal encapsulation of this peptide, which resulted in a tenfold improvement of antiviral efficacy *in vitro*[93]. Alternative strategies to cationic peptides include polyanionic compounds that act by shielding off the positively charged regions of V3 loop, preventing contact between HIV and the target cells[85]. The leading compound of this class was dextran sulfate, which inhibits adsorption of virus to the host cells (i.e. IC_{50} value of 9.1 μg/ml[94]). Unfortunately, despite good absorption after oral administration in HIV-positive subjects[95], dextran sulfate was not very effective in clinical trials[96].

Numerous compounds have been shown to inhibit gp120-CD4 interaction, including polyacid compounds such as polyphosphates, polyphosphonates, polycarboxylates, polysulfates, and polysulfonates[97,98], cosalane analogs[99], resobene[100], and a bisazo dye FP-21399[101]. A few of them merit a brief description. The 17-base G-quartet oligonucleotide Zintevir (AR177, T30177) stabilized with single phosphorothioate internucleoside linkages at its 5' and 3' ends prevents V3 loop-CD4 interaction[102]. Initially reported to act as integrase inhibitor, Zintevir potently inhibits laboratory and clinical HIV strains with sub-micromolar IC_{50} values[102], while resistance mutations have been mapped in the V3 loop[103]. Unfortunately, Zintevir was discontinued in phase II/III clinical trials. BMS-378806 is a recently discovered small-molecule inhibitor with potent activity against HIV laboratory strains and clinical isolates[104,105]. It targets a specific region within the CD4 binding pocket of gp120, where two mutations conferring resistance to this inhibitor were found[106]. BMS-488043 is an orally available compound related to BMS-378806, but with superior pharmacokinetic properties that exhibit potent and selective antiviral activity[107]. Cyanovirin-N (CV-N), a monomeric 11 kDa protein isolated from cyanobacterium *Nostoc ellipsosporum*, is a highly potent inhibitor of HIV replication *in vitro*[108]. CV-N contains two binding sites for N-linked high mannose oligosaccharides[109] and at least two mutations abolishing glycosylation sites are necessary to confer resistance to CV-N[110]. Finally, cyclotriazadisulfonamide (CADA) and its analogs inhibit HIV replication by downregulating CD4[111-113]. Table 4 summarizes the information of the most promising compounds developed to inhibit gp120-CD4 interaction.

Inhibitors of gp120-HIV coreceptor interactions

The binding of gp120 to CD4 leads to a conformational change, which results in high and low affinity interactions of the virus with HIV coreceptors[114]. So far, 17 potential coreceptors for HIV have been iden-

Table 4. Selected compounds inhibiting gp120-CD4 interaction

Entry inhibitor	Target	Status	Developer	Reference
PRO 542	gp120	Phase II	Progenics	77-81,237,238
CD4M33	gp120	Preclinical	Academic	82
BMS-378806	gp120	Preclinical	Bristol-Myers-Squibb	104-106
BMS-488043	gp120/CD4	Phase II	Bristol-Myers-Squibb	239
SPC3	Cell surface glycosphingo-lipids/CXCR4	Phase I	Columbia Research Laboratories	91-93
TNX-355	CD4	Phase II	Tanox/Biogen Idec	83
Dextran sulfate	gp120	Discontinued	Academic	94,95
Dextrin-2-sulfate	gp120	Phase II/III	UK Medical Research Council	240,241
Cyanovirin-N	gp120	Preclinical	Biosyn, Inc.	108-110,213
PRO-2000	gp120	Phase III	Indevus Pharmaceuticals, Inc.	216,217
Cyclotriazadisulfonamide (CADA)	CD4	Preclinical	Academic	111-113
Zintevir (AR177, T30177)	gp120	Discontinued	Antigenics (Aronex Pharmaceuticals)	102,103,242
Cosalane analogs	gp120	n.a.	n.a.	99
FP-21399	gp120	Discontinued	Fuji Pharmaceuticals	101

n.a.: information not available.

tified[115] but, as described above, CCR5 and CXCR4 are considered the major HIV-1 coreceptors. The search for agents blocking the interaction of HIV with its coreceptors aims at the discovery of drugs that do not affect the signaling function or induce internalization of these receptors[116]. This is particularly important for CXCR4. Knocking out CXCR4 results in abnormal cerebral development and can be the cause of embryonic lethality in mice[117], while deletion of stromal cell-derived factor-1α (SDF-1α, the only ligand of CXCR4) leads to defects in B-cell lymphopoiesis and bone marrow myelopoiesis in mice[118]. On the other hand, deletion of 32 bp in the CCR5 gene has little impact on health and Δ32 allele homozygous individuals are highly resistant to HIV infection[119]. According to this, and the fact that R5 variants dominate early in HIV infection, it would seem more important to develop CCR5 antagonists against HIV. However, there is always the possibility that treating patients with these drugs would favor the selection of X4 variants ("coreceptor switch"), which has been associated with rapid progression to AIDS (see above). Here we list a series of strategies to develop drugs blocking HIV coreceptor interactions. A summary of all these approaches is included in table 5.

Inhibitors of gp120-CXCR4 interactions

Initial strategies to block coreceptor engagement were based on the capacity of the CXCR4-natural ligand (SDF-1α) to inhibit X4 viruses[120]. A 10-residue peptide from the N-terminus of SDF-1α showed antiviral activity (low micromolar range) without interfering with CXCR4 signaling function[121]. This independence between transduction and antiviral led to further development of chemokine-derived peptides with a highly polar cationic character[122-125]. T22, an 18-mer peptide[126,127], and its shorter analogs T134 and T140[128-130], inhibit selectively the replication of X4 viruses through their specific binding to the CXCR4 coreceptor. Further development of T140 and its bio-stable analogs, such as TE14011, resulted in a novel, more potent, less cytotoxic and more bio-stable CXCR4 antagonist, 4F-benzoyl-TE14011-Me[131]. Another strong cationic oligopeptide consisting of 9 arginine residues (ALX40-4C) inhibits X4 viruses by interacting with the second ex-

Table 5. Selected compounds inhibiting gp120-coreceptor interaction

Entry inhibitor	Target	Status	Developer	Reference
AMD3100	CXCR4	Discontinued	AnorMED	97,98,140,141,143,144
AMD3465	CXCR4	Preclinical	AnorMED	145
AMD070	CXCR4	Phase Ib/IIa	AnorMED	107
KRH-1636	CXCR4	Preclinical	Kureha	147
KRH-2731	CXCR4	Preclinical	Kureha	243
KRH-3955	CXCR4	Preclinical	Kureha	244
KRH-3140	CXCR4	Preclinical	Kureha	244
CGP64222	CXCR4		Novartis	138,139
ALX40-4C	CXCR4	Discontinued	NPS Pharmaceuticals (Allelix Pharmaceuticals)	132-135
T-22	CXCR4	n.a.	Academic	126,127
T-134	CXCR4	n.a.	Academic	128,129
T-140	CXCR4	n.a.	Academic	130,131
TF14013 (4F-benzoyl-TE14011-Me)	CXCR4	n.a.	Academic	131
p3bv	CXCR4	n.a.	Academic	137
PRO 140	CCR5	Phase I	Progenics	180
NNY-RANTES	CCR5	n.a.	Academic	154
MET-RANTES	CCR5	n.a.	Academic	155
AOP-RANTES	CCR5	n.a.	Academic	154,245
PSC-RANTES	CCR5	Preclinical	Academic	160
TAK-779	CCR5	Discontinued	Takeda	162,163
TAK-220	CCR5	Preclinical	Takeda	164,166,167
TAK-652	CCR5	Preclinical	Takeda	165
SCH-C (SCH-351125)	CCR5	Discontinued	Schering-Plough	168-170
SCH-350581 (AD101)	CCR5	Preclinical	Schering-Plough	173
Vicriviroc (SCH-417690, SCH-D)	CCR5	Phase II	Schering-Plough	172
AMD 887	CCR5	Preclinical	AnorMed	107
Maraviroc (UK-427857)	CCR5	Phase II/III	Pfizer	175-177
Aplaviroc (GSK873140, GW873140, ONO4128, AK602)	CCR5	Discontinued phase II/III	GlaxoSmithKline	178,179
CMPD167	CCR5	Preclinical	Merck	184,185,246
NSC651016	CCR5, CXCR4, CCR1, CCR3	Discontinued	Pfizer (Pharmacia & Upjohn)	182,183

n.a.: information not available.

tracellular loop of CXCR4[132,133]. Interestingly, ALX40-4C directly binds to and prevents the use of APJ receptor as HIV-1 coreceptor[134]. Because APJ receptor is abundantly expressed in central nervous system-based cells, ALX40-4C may help to elucidate HIV-1 infection and pathogenesis in the brain[134,135]. Several secreted phospholipases A_2 from bee and snake venoms have potent anti-HIV-1 activity[136]. Interestingly, p3bv, a peptide derived from bee venom phospholipases A_2 (amino acids 21-35) was able to inhibit X4, but not R5 virus strains[137]. Finally, the peptoid CGP64222 inhibits the HIV Tat/transactivation response element complex formation[138] and blocks HIV-1 replication through a selective interaction with the CXCR4 coreceptor[139].

Perhaps some of the best known, more potent and specific CXCR4 antagonists are the bicyclam derivatives AMD3100, AMD3465 and AMD070[97,98,140,141]. AMD3100 is a low molecular weight bicyclam analog that has an IC_{50} value in the low nanomolar concentrations (~1.4 nM) and does not induce receptor signaling[141,142]. AMD3100 was the first chemokine receptor antagonist to enter clinical trials as a therapeutic candidate for the treatment of HIV-1 infection[143]. In phase II clinical trials, AMD3100 suppressed infection by X4 strains in most subjects; however its development as an anti-HIV-1 drug was discontinued due to pharmacologic properties, negligible oral bioavailability, and cardiac side effects[144]. Compared to AMD3100, the N-pyridinylmethylene cyclam AMD3465 is tenfold more effective as a CXCR4 antagonist[145]. However, despite a decreased molecular charge when compared to the bicyclams, AMD3465 still lacks oral bioavailability. Further decreasing of molecular weight led to the first orally bioavailable AMD derivative, AMD070 (also known as AMD11070). AMD070 is highly potent and specific for CXCR4, with binding site similar to AMD3100[146]. AMD070 is currently in phase I/II clinical trials.

Finally, the screening of a chemical library and further optimization of a lead compound resulted in the discovery of a very potent arginine-base CXCR4 antagonist KRH-1636[147]. This compound completely inhibited the replication of X4 viruses at a concentration as low as 0.06 μM. This antiviral effect was reproduced in the SCID-hu PBL/PBMC animal model[147]. Absorption into the blood after intraduodenal administration in rats indicates that this promising compound may be orally bioavailable[147].

Inhibitors of gp120-CCR5 interactions

As described above, the dispensability of CCR5 coreceptor and the predominance of R5 viruses in HIV transmission and at early stages of infection established CCR5 coreceptor as a prime target for new therapeutic strategies. Initial strategies focused on the evaluation of the inhibitory potency of natural β-chemokines ligands (both full-length and small-peptide chemokine derivatives[148,149]). The natural β-chemokines ligand RANTES (regulated on activation, normal T-cell expressed and secreted), MIP-1α and MIP-1β inhibit HIV-1 replication[5,150,151]. These ligands block CCR5-mediated HIV-1 infection directly by competing with gp120 for coreceptor binding, by downregulating the coreceptor expression, and/or by inducing signaling events that result in changes in cell differentiation and susceptibility to HIV-1[152]. Since the latter two inhibition mechanisms may have significant undesirable effects in patients, non-agonistic CCR5 chemokine derivatives, small molecule inhibitors, and CCR5-binding antibodies are the most valuable strategies for new therapeutics.

Amino-terminal modifications of RANTES such as aminooxypentane (AOP)-RANTES, N-nonanoyl (NNY)-RANTES, and methionylated (Met)-RANTES are potent inhibitors of R5 viruses, with IC_{50} in the nanomolar to sub-nanomolar values[153-156], but maintaining the ability to trigger CCR5-mediated signaling[157]. C1·C5-RANTES was the first RANTES derivative without agonistic functions on both CCR3 and CCR5, maintaining high anti-HIV potency[158]. Two RANTES mutants, P1 and P2, are potent inhibitors of R5 viruses while retaining only trace levels of signaling activity via CCR1 and CCR3[159]; however, the most promising RANTES derivative, PSC-RANTES, is being developed as a potential microbicide[160]. A more recent approach to block HIV infection includes bifunctional inhibitors to simultaneously target the receptor (CD4) and coreceptor (CCR5) of HIV R5 strains. A molecule consisting of RANTES/CCL5 and a single-chain Fv Ab fragment against CD4, bound to both receptors, competed with RANTES/CCL5 binding, and induced down-modulation of CCR5[161].

As mentioned before, the most promising inhibitors of R5 viruses are low molecular weight compounds that bind directly to CCR5 without triggering downregulation and signaling. The first small molecule reported as a CCR5 inhibitor was TAK-779[162], a non-peptide organic compound that binds within a cavity formed by the transmembrane helices 1, 2, 3, and 7 of CCR5[163]. TAK-779 is a potent CCR5 antagonist preventing chemokine-induced signaling at nanomolar concentrations without down-modulating CCR5 expression[162,163]. However, TAK-779 has poor oral bioavailability, and its development was discontinued because of unfavorable effects at the injection sites[164]. TAK-652, an orally bio-

available derivative of TAK-779, has been recently proposed as a novel entry inhibitor of HIV-1[165]. It is active against a multitude of R5 viruses from different subtypes and clinical isolates containing reverse transcriptase and protease inhibitor-resistant mutations[165]. A single oral administration of TAK-652 up to 100 mg was safe and well tolerated in humans and the compound displayed favorable pharmacokinetics[165]. Chemically unrelated to TAK-779, TAK-220 is another CCR5 inhibitor that has shown some promise *in vitro*[164,166,167].

A few years ago, high-throughput screening techniques led to the discovery of a large series of piperidine and piperazine derivatives with potent CCR5 antagonistic activity[168,169]. One of these compounds, SCH-C (SCH 351125), has broad and potent antiviral activity *in vitro* against primary HIV-1 isolates (mean IC_{50} range 0.4-9 nM) and strongly inhibits the replication of R5 viruses in SCID-hu Thy/Liv mice[170]. The compound is highly bioavailable, but further development has been halted, in part due to prolonged heart-rate rhythm at the higher doses[171]. Vicriviroc (formerly SCH-D or SCH-417690) is a second-generation compound with improved antiviral activity and pharmacokinetic properties compared to those of SCH-C[172]. Vicriviroc showed potent, broad-spectrum activity against genetically diverse and drug-resistant HIV-1 isolates[172] and is anticipated to start phase III clinical trials shortly. Interestingly, viruses resistant to SCH-C, SCH-D, and SCH-350581 (AD101) retained the R5 phenotype[173,174]. In the case of SCH-D, resistance mapped outside of the V3 region, particularly in the V2, C3 and V4 regions[174]. Maraviroc (UK-427857) is a selective CCR5 antagonist with potent antiviral activity (mean 90% IC_{50} of 2.0 nM) against R5 viruses of different subtypes[175,176]. Ten days monotherapy in HIV-infected individuals harboring R5 viruses reduced viral load up to 1.6 $\log_{10}$ with maximum reduction in viral load at a median of 10-15 days[177]. These promising results justify further phase II/III clinical trials of Maraviroc as a potential therapeutic for HIV-1 infection. A spirodiketopiperazine derivative, Aplaviroc (GW873140, formerly ONO4128, AK602) represents another promising group of anti-HIV compounds that specifically binds CCR5[178]. Aplaviroc exerted potent activity (IC_{50} values of 0.1-0.6 nM) against a wide spectrum of laboratory and primary R5 HIV-1 isolates, including multidrug-resistant viruses *in vitro*[179]. Unfortunately, recently phase II/III clinical trials of Aplaviroc were terminated due to cases of severe hepatotoxicity.

Several other promising compounds are at different stages of development. PRO 140 (PA14) is a murine anti-CCR5 monoclonal antibody that potently inhibits HIV-1 entry at concentrations that do not affect CCR5-induced signaling[180]. Certain analogs of the antibiotic distamycin have been described to antagonize several chemokine receptors[181]. NSC651016 inhibits chemokine binding, Ca^{2+}-signaling and chemotaxis mediated by CCR5, CXCR4, CCR1 and CCR3, but does not affect the function of CXCR2 or CCR2b[182]. As described below, NSC651016 and related compounds could be used as topical microbicides to prevent the sexual transmission of HIV-1[183]. Finally, a CCR5-specific small molecule, CMPD 167, has shown potent antiviral activity *in vitro* and has been evaluated in SIV-infected rhesus macaques as a potential microbicide[184,185].

Inhibitors of viral fusion

To date, the most successful class of entry inhibitors was designed to block virus-cell fusion and target conserved fusion domains in gp41 (Table 6). The binding of gp120 to CD4 and coreceptor triggers conformational changes in gp41, which lead to the fusion of the viral and the host cell membrane[114]. Crystal structures revealed that the gp41 is characterized by the formation of a 6-helix bundle, consisting of N- and C-terminal helical heptad repeats[186,187]. The 36-amino acid peptide T-20 (formerly DP178) is identical to amino acid residues 127 to 162 of the HR2 in C-terminal helices of gp41, and by binding to the N-terminal helices prevents the transition into 6-helix bundle conformation[188]. Although T-20 effectively blocked both laboratory-adapted and primary HIV-1 isolates[188], inhibitor-naive primary HIV-1 isolates exhibited *a* wide range of susceptibilities to this peptide[60]. Nevertheless, T-20 was safe and showed potent antiviral effects in early clinical trials[189]. Further phase II/III clinical trials proved its long-term safety and antiviral potency[190-192]. Thus, the FDA approved T-20 in 2003 for therapeutic use in the treatment of AIDS under the name enfuvirtide (Fuzeon®).

In addition to enfuvirtide, included in this class of inhibitors are T-1249, C34, 5-helix, and IQN-17[186,193,194]. T-1249 is a second-generation fusion inhibitor shown to preserve antiretroviral activity *in vitro* against HIV-1 isolates that have decreased susceptibility to enfuvirtide[195]. T-1249 is a 39-amino acid peptide derived from HR2 region and is ten times more potent *in vitro* than enfuvirtide[196]. However, the clinical development of T-1249 was put on hold in 2004 due to challenges in achieving the desired technical profile of the current formulation[197]. Recent data on two new fusion inhibitors, TRI-999 and TRI-1144, indicate that these compounds have potent antiviral activity and durable control of HIV

Table 6. Selected compounds inhibiting viral-cellular membrane fusion

Entry inhibitor	Target	Status	Developer	Reference
Enfuvirtide (Fuzeon®, T-20)	gp41	Commercially available	Trimeris/Roche	60,188-191,247,248
T-1249	gp41	Discontinued phase I/II	Trimeris/Roche	195,196
TRI-999 (TR-290999)	gp41	Preclinical	Trimeris/Roche	198
TRI-1144 (TR-291144)	gp41	Preclinical	Trimeris/Roche	198
Sifuvirtide	gp41	Phase I (China)	FusoGen Pharmaceuticals	205
5-helix	gp41	Preclinical	Howard Hughes Medical Institute	187,199
ADS-J1	gp41/gp120	Preclinical	Academic	206,208
RPR103611	gp41	n.a.	n.a.	201,202

n.a.: information not available.

replication *in vitro*, with desirable pharmacokinetic properties *in vivo*[198].

Other molecules designed as fusion inhibitors have been described. 5-helix, a chimeric gp41 molecule consisting of three N and two C-terminal helices of gp41 connected with a linker, strongly binds the C-peptide region of gp41 and displays potent inhibitory activity against various HIV-1 isolates[187]. Optimization in binding affinity may lead to the development of an improved drug[199]. Other small compounds acting as fusion inhibitors include compounds targeting protein disulfide isomerase (PDI)[200], betulinic acid derivatives (RPR103611 and IC9564)[201-203], Tannin[204], sifuvirtide[205], ADS-J1[206] and its analogs[207]. A recent study, however, suggested that ADS-J1 acts prior to gp41-dependent fusion, probably through binding of the HIV-1 coreceptor site[208].

Microbicides

HIV transmission through unprotected sex is the predominant mode of the AIDS pandemic spread. Therefore, topical microbicides are, in the absence of an effective prophylactic anti-HIV therapy or vaccine, the most attractive therapeutic approach for preventing HIV infection[209-212]. According to the Alliance for Microbicide Development (Siver Spring, MD, USA), 20 clinical trials are currently being conducted with 15 unique compounds, although many more products are in the preclinical stage of development (Table 7, http://www.microbicide.org/publications/digest/Microbicide.Clinical.Trials.Summary.Table.December.2005.pdf). Microbicidal strategies against HIV can be divided according to the mechanisms of action: (i) products maintaining and/or enhancing normal vaginal defense (e.g. acidic pH, local immune response); (ii) direct inactivation by nonspecific, surface-acting agents; (iii) products inhibiting entry/fusion; and (iv) inhibitors of post-entry viral life steps. In keeping with the scope of this review we will discuss only compounds directly disrupting viral envelope or inhibiting entry/fusion.

Specific topical microbicides include cyclodextrins, polyanionic dendrimers SPL7013, CV-N, CCR5-inhibitors (e.g., AOP-RANTES and PSC-RANTES), and gp41-fusion inhibitors[209,212,213]. Recombinant CV-N effectively blocked infection of human ectocervical explants by the R5 virus HIV-1_{Ba-L}. Interestingly, vaginal application of a CV-N gel protected 15 of 18 $SHIV_{89.6P}$-challenged macaques while showing no evidence of cytotoxic or clinical adverse effects[214]. Another promising CCR5-inhibitor, PSC-RANTES, blocked viral replication after intravaginal challenge with SHIV-SF162 in 12 of 15 macaques that had been pretreated with progesterone to thin the genital epithelium[160]. Similarly, a SPL7013 gel prevented vaginal transmission of $SHIV_{89.6P}$ in macaques in a dose-dependent manner[215].

Sulfated/sulfonated polyanionic polymers represent most of nonspecific entry microbicides, including carrageenan, cellulose sulfate, dextrin-2 sulfate and naphthalene sulfonate polymer PRO-2000[209]. These compounds bind to X4 and R5 monomeric gp120 with similar high binding affinities, inhibiting R5, X4 and X4/R5 viruses[216]. A PRO-2000 double-blind, placebo-controlled trial demonstrated sufficient bioavailability and anti-HIV activity in cervicovaginal lavage samples after intravaginal application of 0.5% PRO-2000 gel[217]. Sodium do-

Table 7. Topical microbicide blocking entry/fusion of HIV-1

Microbicide	Developer	Phase of development
Cyanovirin-N	Biosyn, Inc.	Preclinical
Antibodies and fusion proteins (HIV, HSV, HPV), tobacco-derived	Mapp Biopharmaceutical, Inc.	Preclinical
Cellulose acetate 1,2-benzenedicarboxylate (cellavefate/CAP)	Lindsey F. Kimball Research Institute, Dow Pharmac	Phase I clinical trial
Anti-ICAM-1 antibody	Johns Hopkins University	Preclinical
Mandelic acid condensation polymer (SAMMA)	Mount Sinai Medical School	Preclinical
Novaflux proprietary product	Pennsylvania State University College of Medicine	Preclinical
Porphyrins	Emory University	Preclinical
PRO-2000	Indevus Pharmaceuticals, Inc.	Phase III clinical trial
C85FL	Weill Medical College of Cornell University	Preclinical
VivaGel™ (SPL7013 gel)	Starpharma Ltd.	Phase I clinical trial
Invisible Condom	Laval University (Division of Microbiology)	Phase I/II clinical trial
K5-N, OS(H)	San Raffaele Scientific Institute Lab	Preclinical
Cellulose sulfate gel	Global Microbicide Project	Phase III clinical trial
Carraguard®	Population Council	Phase III clinical trial
Betacyclodextrin	Johns Hopkins University School of Medicine	Preclinical

Adapted from the Alliance for Microbicide Development database, March 2006 (http://www.microbicide.org/).

decyl sulfate (SDS) is a prototype of alkyl sulfates (another example of surface-acting agents), which is effective against both enveloped and non-enveloped viruses[211]. Low concentrations of SDS (i.e. 0.1-1%) effectively inactivated high concentrations of purified HIV-1 in breast milk[218]. However, the major problem with using surfactants is their usually detergent-type effect on epithelial cells and normal vaginal flora, which could cause vaginal infection, irritation or ulceration[210]. For example, despite showing anti-HIV-1 activity *in vitro*, the spermicidal antimicrobial compound nonoxynol-9 (N-9) did not show protective effect on HIV-1 transmission in high-risk women[219]. More importantly, frequent use of nonoxynol-9 may cause toxic effects enhancing HIV-1 infection[219]. On the other side of the spectrum, an amphoteric compound containing myristamine oxide and cetyl betaine (C31G)[220] is the most advance surfactant currently entering phase III clinical trials[212].

Conclusions

The success story of enfuvirtide helped the establishment of entry inhibitors as a valid new class of antiretroviral drugs. With more entry inhibitors in different stages of clinical trials, the expectations are that the clinicians will have a significantly higher number of possible combinations of drugs for therapy of both treatment-naive and treatment-experienced patients. Several promising compounds are in various stages of preclinical and clinical development for every step in HIV entry process. Agents targeting gp120/CD4 binding or the fusion of the viral and cellular membranes have been shown to be effective against HIV-1 strains with different tropism (i.e. R5, X4, and R5/X4 variants). On the other hand, the intrinsic nature of potential drugs directed to block the interaction of the virus with CCR5 or CXCR4 receptors make them "phenotype specific", i.e. CCR5 and CXCR4 coreceptor antagonists block the replication of R5 and X4 viruses, respectively. However, HIV-infected individuals can harbor R5, X4, R5/X4 or a mixture of R5 and X4 variants within the viral quasispecies. Therefore, it is evident that HIV coreceptor usage of the predominant viral population (i.e. tropism, phenotype) needs to be analyzed before initiating, and closely monitored during, treatment with CCR5 or CXCR4 antagonists. Nevertheless, the main obstacle for the successful management of patients treated with these entry inhibitors is the lack

of an assay to accurately quantify HIV coreceptor usage. The methods described here, although the most advanced up to date, (i) are not fully quantitative, (ii) do not provide a relative ratio of R5 or X4 viruses in the viral quasispecies, and (iii) are not able to differentiate between dual tropic (R5/X4) or a mixture of R5 and X4 viruses. Further studies aimed to the development of novel methods able to address these problems are essential for the success of entry inhibitors, particularly CCR5 or CXCR4 antagonists.

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