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Chemokine Receptors as Therapeutic Targets in HIV Infection

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1. Introduction

Acquired immunodeficiency syndrome (AIDS) evolved into a pandemic in less than a decade since the first reports of a new set of symptoms that included severe opportunistic infections and unusual neoplasms, particularly Kaposi's sarcoma, in homosexual men [1]. Nearly 30 years since the discovery of the causative agent [2, 3], human immunodeficiency virus (HIV) cannot still be eradicated, nor is there sight of a safe and effective prophylactic vaccine in the horizon. Infection with either virus type invariably leads to AIDS; however, the less pathogenic HIV-2 strains have been geographically restricted mainly to West Africa, whilst the more virulent HIV-1 strains have spread around the globe causing the AIDS pandemic [4]. The worst afflicted region is sub-Saharan Africa, where in a few countries more than one in five adults is infected with the virus; at the same time, the epidemic is spreading most rapidly in Eastern Europe and Central Asia, where the number of people living with HIV increased by 250% between 2001 and 2010 [5]. Worldwide, an estimated 34 million people, including 3.4 million children, were living with HIV at the end of 2010, while the related deaths and new infections were 1.8 and 2.7 million, respectively [5].

Despite these frightening numbers, which are indicative of a growing epidemic far from the UNAIDS vision of zero AIDS-related deaths and zero new infections, considerable progress has undoubtedly been noted in basic and clinical research in the field of HIV/AIDS [6]; we now understand most aspects of HIV pathogenesis and have identified targets suitable for therapeutic intervention. The introduction of combination antiretroviral therapy (ART) in 1996 revolutionized patient care and brought about a significant reduction in AIDS-related morbidity and mortality; HIV infection has been transformed into a chronic condition that is generally controllable with lifelong treatment, at least in the developed world where treatment is available. At the forefront of current anti-HIV research, CCR5 inhibitors represent a novel drug class that broadens the therapeutic options of patients, which are

currently limited by chronic toxicities or by the presence of resistance to conventional antiretrovirals targeting ever mutating virus-encoded structures.

This chapter focuses on describing the scientific rationale that led to the development of coreceptor inhibitors as a new class of host-targeted antiretroviral agents. This description entails a revision of the biology of coreceptor usage during HIV entry into target cells and viral tropism during the course of infection. The various approaches undertaken to pharmaceutically target the most commonly used coreceptor, CCR5, will then be described, with a special focus on small molecule CCR5 inhibitors (also termed “CCR5 antagonists”) and their mechanism of action. The current status of the small molecule CCR5 inhibitor pipeline, with emphasis on the generation of resistance, including *in vitro* and *in vivo* HIV escape pathways and mechanisms, will be presented, while implications as well as future perspectives for the clinical use of CCR5 inhibitors will be discussed.

2. The HIV entry process and the coreceptors

2.1. Discovery of the HIV coreceptors

CD4 had been known to be the principal cell surface receptor for HIV-1, and also for HIV-2 and simian immunodeficiency virus (SIV), since 1984 [7, 8]. It had also been known for almost as long, however, that the presence of the CD4 antigen alone was not sufficient to allow for HIV entry. To infect CD4⁺ cells, HIV-1 strains of different biological phenotypes, namely macrophage (M)-tropic and T-cell line (T)-tropic, were supposed to utilize different auxiliary co-factors [9], the identity of which remained elusive until early 1996. At that time, a member of the seven-transmembrane (7-TM) spanning family of chemokine receptors initially termed “fusin”, was identified as the coreceptor for T-tropic HIV-1 variants [10]; the term was changed to C-X-C chemokine receptor type 4 (CXCR4) once its natural ligand was discovered to be stromal derived factor-1 (SDF-1)/CXCL12 [11, 12]. CXCR4 had been previously identified as an orphan receptor called leukocyte-derived seven-transmembrane domain receptor (LESTR), but it only received attention after its isolation as an HIV-1 coreceptor [13].

The knowledge that the β -chemokines macrophage inflammatory protein (MIP)-1 α /CCL3, MIP-1 β /CCL4 and regulated upon activation normal T-cell expressed and secreted (RANTES)/CCL5 exhibited antiviral activity against M-tropic HIV-1 isolates *in vitro* [14], provided the basis for the discovery of C-C chemokine receptor type 5 (CCR5) as the entry co-factor for these strains by five groups simultaneously [15-19]. The virus thus uses chemokine receptors, mainly CCR5 and CXCR4, to enter susceptible cells, while the cognate chemokine ligands act as natural entry inhibitors. Other chemokine receptors may also function as HIV coreceptors; nonetheless, infection of primary cells via alternative coreceptors is rare and the process can normally be fully blocked by CCR5 or CXCR4 inhibitors [20-22]. Hence, at present, CCR5 and CXCR4 are considered as the only HIV coreceptors of physiological significance.

2.2. Physiological roles of the main coreceptors

CCR5 and CXCR4 are two structurally related, heptahelical chemokine receptors that belong to different classes [C-C and CXC, respectively, based on the position of the two conserved cysteine (Cys) residues in their N-termini (Nt)] of the superfamily of G protein-coupled receptors (GPCRs) (reviewed in [13, 23-25]). About half of the drug targets in the pharmaceutical industry are GPCRs, which also, not coincidentally, comprise the largest family of cell-surface receptors [26]. GPCRs become activated by chemokines, small (8-10 kDa) soluble protein ligands that are either promiscuous or specific for a given receptor. Once activated by such extracellular signals, GPCRs undergo conformational changes that trigger the intracellular signal transduction cascade; these series of events begin with the rapid phosphorylation by G protein-coupled receptor kinases (GRKs) predominantly on serine (Ser) and threonine (Thr) residues within the C-tail and third intracellular loop and continue with the activation of heterotrimeric G proteins [27]. In this mode, chemokines and their receptors control cell migration associated with routine immune surveillance, inflammation and development [28].

CCR5, in particular, appears to play a role in the initiation of adaptive immune responses and the trafficking of effector cells to sites of infection and inflammation as indicated by its expression profile on several effector T cell subsets and antigen-presenting cells, including macrophages, immature dendritic cells and Langerhans cells (reviewed in [22, 29, 30]). Nonetheless, the lack of expression of this gene due to a deletion polymorphism known as “delta32” does not appear to have any deleterious effects; in fact, heterozygosity for the CCR5 Δ 32 allele is generally associated with delayed disease progression, and homozygosity with incomplete protection from HIV transmission (reviewed in [31]). The observation that its congenital absence does not lead to any overt pathology suggested that CCR5 might be a valid target for pharmacological blockade.

CXCR4, on the other hand, and its single known ligand SDF-1/CXCL12 are both highly conserved [13]; they play an essential role during embryonic development and in several major processes in the adult, including hematopoiesis, leukocyte trafficking in the adaptive immune system, and vascularization [22]. CXCR4 knockout mice show hematopoietic and cardiovascular defects during embryogenesis and die *in utero*; mice lacking SDF-1/CXCL12 are characterized by deficient B-lymphopoiesis and myelopoiesis and abnormal neuronal and cardiovascular development [13]. Clinically tested CXCR4 inhibitors AMD3100, which lacked oral bioavailability, and AMD070 caused significant leukocytosis (reviewed in [22]). The non-cyclam antagonist AMD070 (or AMD11070) is currently in Phase II clinical trials as an HIV cell-entry inhibitor [32]. The development of AMD3100 (by AnorMED) for use in the treatment of HIV infection was terminated, but further studies led to a new indication for this drug; AMD3100 (now Plerixafor, Genzyme) has been approved by the US Food and Drug Administration (FDA) for autologous transplantation in patients with non-Hodgkin's lymphoma and multiple myeloma [32]. The fact that Plerixafor is well tolerated in the developed organism minimizes the concerns of antagonizing such a crucial receptor/ligand axis in mature individuals. CXCR4 antagonists could be used in combination with

antiretrovirals targeting other steps of the viral life cycle; they could also be administered simultaneously with CCR5 antagonists, even as one compound with high affinity for both receptors, as suggested recently [32]. Down-modulating the expression of CXCR4 may certainly be beneficial for some HIV patients [13]. The pharmacologic targeting of CXCR4 may additionally have therapeutic utility for the treatment of acute viral infections of the central nervous system (CNS) [33]. The rest of this chapter focuses on issues related to the development of CCR5 inhibitors.

2.3. The coreceptors in HIV entry

Conformationally masked by carbohydrate structures and variable amino acid loops that enable it to evade the humoral immune response, the envelope glycoprotein (Env) on virions is organized into trimers of noncovalently associated surface gp120 and transmembrane gp41 heterodimers (reviewed in [34]). The interaction of HIV with host cell surface receptors trigger the fusogenic potential of Env and allow the virus to enter into its target cells by fusion, presumably at the plasma membrane [35]. The process has long been assumed to occur at the cell surface for such pH-independent viruses as HIV [36], although recent studies support the notion that HIV enters disparate cell types through fusion with endosomes [37, 38]. A schematic of the first steps of the HIV entry process, according to the presently accepted molecular mechanism, is shown in Figure 1.

Figure 1. Schematic diagram of the first steps of the HIV entry process. (A) HIV entry into target cells is mediated through the interaction of the viral envelope glycoprotein (Env) with host cell surface receptors. (B) Surface gp120 first contacts CD4 and then (C) a chemokine receptor, typically CCR5 or CXCR4, triggering molecular rearrangements in the gp41 that result in membrane fusion and the initiation of infection.

HIV entry begins with the high affinity binding of gp120 to the host cell CD4, which induces a major conformational change in Env that exposes or creates a binding site on gp120 for the coreceptor, typically either CCR5 or CXCR4. Current structural models suggest that the gp120 “bridging sheet”, formed between the constant C1, C2, and C4 domains of gp120 after CD4 binding, interacts with the tyrosine (Tyr)-sulfated coreceptor N-terminus (Nt), while the V3 crown interacts principally with the second extracellular loop (ECL2) region of the

coreceptor [39-42]. Coreceptor engagement of CD4-bound gp120 induces additional reconfigurations, leading to the insertion of the gp41 fusion peptide (FP) into the host cell membrane and the formation of a pre-fusion complex. This pre-fusion intermediate is then refolded into an energetically favorable six-helix bundle that brings the two membranes in close proximity so that fusion can occur; the viral core is thereby released into the cytoplasm and a new cycle of infection is initiated.

Our understanding of HIV entry has provided invaluable insights into viral tropism and pathogenesis and led to the development of novel classes of antiretroviral agents that inhibit specific stages of the process. CCR5 inhibitors interrupt the viral replication cycle by preventing CCR5 binding and aborting fusion.

3. Coreceptor use and HIV tropism

3.1. Classification systems of HIV biological phenotype

Before the identification of the coreceptors, three biological properties were the criteria used to classify the phenotype of HIV variants: (i) the preference for specific target cells (cellular tropism) that distinguished between macrophage (M)-tropic and laboratory T-cell line (T)-tropic viruses; (ii) cytopathology, which distinguished between syncytium-inducing (SI) and non-syncytium-inducing (NSI) strains based on the capacity or not to form syncytia (i.e., giant multinucleated cells) through cell fusion in the MT-2 cell line; and (iii) replicative capacity, which mainly considered the *in vitro* growth kinetics of viral strains in culture and distinguished between slow/low (S/L) and rapid/high viruses (R/H) (reviewed in [43]).

The above dichotomies may be explained by the more precise, newly adopted classification system that is based on coreceptor usage; accordingly, viruses that use CCR5 alone or CXCR4 alone for cell entry are currently termed R5 and X4, correspondingly, while variants that can use either coreceptor with comparable efficiency are termed R5X4 [44]. A more appropriate designation for the latter is R5+X4 or D/M (Dual/Mixed), particularly when tropism has not been determined at the clonal level, since many R5X4 isolates are in fact mixtures of R5 plus X4 or, less commonly, either R5 plus dual (R5X4), or even R5 plus dual (R5X4) plus X4, clones [22]. The term R5X4 should thus be reserved for clones that are genuinely capable of using both coreceptors.

3.2. Determinants of HIV biological phenotype and coreceptor tropism assessment

The determinants of coreceptor choice, and thus of biological phenotype, have been mapped to the surface gp120 subunit of Env and primarily to the V3 domain. Swapping the V3 region alone often suffices to switch the coreceptor tropism of a viral clone from R5 to X4 and vice versa [45]. Furthermore, the charge of the V3 region appears to be an important parameter affecting biological phenotype and, tropism, although it alone cannot be used as a marker for phenotype prediction; in general, however, higher net charges ($\geq +5$) characterize X4 variants compared to the lower V3 region net charges ($\leq +4$) of R5 variants typically

associated with acute infection (reviewed in [43]). The increase of the overall positive charge of the V3 region that may switch the viral phenotype results from the introduction of basic amino acid residues at one of the two positions 11 and/or 25 of the V3 [46, 47], a property that may not be shared by the uncommon subtype C CXCR4-using strains (reviewed in [48]).

Simple genotypic approaches undertaken for predicting coreceptor usage include the detection of positively charged amino acids at positions 11 and 25 (often referred to as the “11/25 rule”) and the determination of the total charge of V3, with the cut-off set at +5; heteroduplex tracking assays and various bioinformatics approaches are additional genotypic methods for determining HIV tropism (reviewed in [49-51]). Predictive algorithms of coreceptor usage have been developed based on genetic sequences in the V3 region, but also taking into account genotypic correlates outside the V3, structural information and clinical data [48]. Apart from these genotypic tools, different phenotypic assays have been developed to assess HIV tropism. These phenotypic methods, which are reviewed elsewhere [49-52], include the original MT-2 assay or coreceptor usage assays using reporter cell lines, and cell-based assays that use different methodologies to generate *env*-recombinant or pseudotyped viruses with distinct detection systems. The Trofile assay (Monogram Biosciences, Inc., South San Francisco, CA; now Laboratory Corporation of America [LabCorp]) is the most widely used phenotypic test in clinical practice. In the new era of CCR5 inhibitors, an accurate determination, and perhaps quantification, of coreceptor usage is necessary for the successful clinical management of HIV-infected individuals.

3.3. HIV phenotypes, tropism and disease course

Regardless of the route of HIV transmission, R5 viruses seem to prevail in the vast majority of primary and early stage HIV infection cases, whilst the X4 phenotype evolves *in vivo* in approximately 40-50% of (subtype B or D) infected subjects, usually 5–7 years after infection (reviewed in [43, 53]). Recent transmitted/founder virus genetic studies confirmed that only R5 (and typically a single R5 virus isolate) and in a few instances R5X4, but not X4 HIV-1, are initially transmitted [54, 55]. Consequently, a “gatekeeper” that nearly always selects transmission of R5 over X4 HIV may well exist. The suppression of X4 transmission has been suggested to result not from one powerful gatekeeper, but from an aggregate of multiple functional gatekeepers, the localization and precise molecular mechanisms of which remain to be determined (reviewed in [56]). CCR5 thus has a pivotal role in viral transmission, but infection by X4 (or R5X4) viruses is not impossible as indicated by the few known cases of HIV acquisition by CCR5-Δ32 homozygotes; the lower pre-AIDS viral loads and slower disease progression of CCR5-Δ32 heterozygotes, in whom the CCR5 expression levels on target cells are lower, underscore the importance of the CCR5 coreceptor in the early asymptomatic stages of the infection [31, 53].

The emergence of X4 viruses in a proportion of cases correlates with accelerated disease progression [43, 53]. The virological or immunological basis of the selection mechanism is still debated; in other words, it is still unclear whether disease progression is accelerated as a direct consequence of the emergence or predominance of X4 isolates, or, conversely,

whether the declining competence of the host immune system permits the outgrowth of viral strains with increased replicative capacity [43]. The pathogenicity of X4 viruses seems to be greater than that displayed by R5 strains both *in vitro* and probably *in vivo*, as suggested by experiments in macaques [22]. R5 viruses alone can also cause severe damage to the immune system; X4 viruses are never detected in about half of HIV-infected subjects who do progress to AIDS [22, 53]. These late-emerging, R5 viral strains, which have reduced sensitivity to entry inhibitors and increased ability to cause CD4⁺ T-lymphocyte loss, also possess an improved ability to utilize relatively low levels of CD4 and CCR5 expressed on macrophages (reviewed in [57]). Correlations between increased gp120 net charge, enhanced viral fitness, and augmented cell attachment were disclosed as well for these R5 HIV-1 variants that emerge in an opportunistic manner during severe immunodeficiency [58].

Different CD4⁺ T cell subpopulations are targeted by the phenotypic variants of HIV: replication of R5 viruses is favored in activated, memory/effector T cells, whereas X4 viruses may additionally infect intrathymic T progenitor cells and naïve T cells in the peripheral lymphoid system [39, 43]. Of note is the massive depletion in the gut-associated lymphoid tissue (GALT) of CCR5-expressing memory CD4⁺ T cells as a consequence of acute HIV infection (reviewed in [59]). The engagement by gp120 of integrin $\alpha_4\beta_7$, the gut homing receptor, facilitates infection of CD4⁺ T cells in the earliest stages of transmission [60]. HIV replicates intensively first in the GALT and then systemically in all lymphoid tissues, where it establishes stable viral reservoirs (in the form of latently infected resting CD4⁺ T cells at the cellular level) that constitute major impediments to virus eradication [59]. The switch in coreceptor usage at the peak of viral diversity expands virus accessibility to circulating naïve CD4⁺ T cells, thereby increasing viral burden and leading to rapid CD4⁺ T cell depletion [61, 62]. The regenerative capacity of the immune system is also affected by the X4 emergence or predominance since chronic immune activation is then exacerbated. T cell homeostasis is disrupted, eventually leading to the collapse of the immune system [43].

4. The development of CCR5 inhibitors as anti-HIV therapeutics – Focus on small molecules

As discussed above, the first clues regarding the potential therapeutic utility of targeting CCR5 came from the observation of the naturally occurring blockade of the coreceptor due to the CCR5 Δ 32 polymorphism. Recently, the first successful allogeneic stem cell transplantation has been reported in an HIV-positive, acute myeloid leukemia patient from Berlin with a CCR5 Δ 32 homozygous donor [63]. Investigators claimed to have “cured” this HIV-infected patient as indicated by the functional reconstitution of his T cell immunity without any signs of viral rebound after the discontinuation of ART [64]. The long-term effects of stem cell transplantation remain nevertheless unknown. Even though it would obviously be impractical to follow such a therapeutic approach in the millions of HIV-infected subjects worldwide, the proof-of-principle case of “the Berlin patient” demonstrates that a functional cure, i.e. a permanent suppression of viral replication in the absence of ART, may be within reach, under certain circumstances, at present; a true sterilizing cure with complete eradication of the virus continues to be the ultimate goal of therapy [6].

Towards this goal, there has been hope that CCR5 inhibitors will make an impact on controlling the epidemic by enhancing our therapeutic arsenal against HIV.

It should be noted, however, that the development of these host-targeting agents raises additional safety concerns that lie beyond the typical pharmacological concerns associated with the development of any antiretroviral drug, such as efficacy, safety and tolerability, bioavailability, drug-drug interactions, and the emergence of escape mutants (reviewed in [22, 65]). These safety concerns stem from the disruption of the physiological functions of the coreceptor as well as from the potential escape of R5 viruses by switching coreceptor use to CXCR4, or the breakthrough of X4 viruses following the suppression of R5 strains [22, 65]. The latter virological concern will be discussed in the next section on the generation of resistance to CCR5 inhibitors. The absence of any apparent immunological deficits of significance among individuals with naturally occurring loss-of-function mutations (i.e., CCR5 Δ 32 homozygotes) provides some reassurance that pharmacological blockade of CCR5 will be relatively benign [66]. Redundancy in the chemokine network presumably allows other chemokine receptors to subsume the function of CCR5 [67]. However, the abrupt pharmacological blockade of a receptor in mature individuals may have different consequences than the congenital absence of the receptor where the immune system has had years to adapt to its loss [22, 67]. Corroborating the results of previous studies in mice (reviewed in [67]), studies in humans have shown that CCR5 Δ 32 heterozygotes have a six-fold increased risk for severe West Nile virus (WNV) infection and a five-fold increased risk of mortality [68]. A meta-analysis of the four patient cohorts in the United States confirmed that CCR5 deficiency is a strong and consistent risk factor for symptomatic WNV infection [69]. An additional association between the lack of functional CCR5 due to the CCR5 Δ 32 deletion and tick-borne encephalitis has also been reported [70]. Overall, however, currently available data suggest that pharmacological blockade of CCR5 is likely to be largely well tolerated; stringent safety studies will nevertheless be required to monitor the long-term safety of CCR5 blockade, with particular attention paid to the consequences of infection by certain pathogens, effects on responses to immunization, and the emergence of opportunistic infections and malignancies [22, 65].

Several different approaches have been undertaken to pharmaceutically target CCR5, including the use of chemokine analogues, anti-CCR5 antibodies, gene knockdown and knockout strategies, and small molecule coreceptor inhibitors. The development of coreceptor inhibitors for use in prevention, including topical microbicides, is discussed elsewhere [22, 67].

4.1. Chemokine analogues

Chemokine analogues engineered to enhance their natural anti-HIV properties were the first generation of CCR5 inhibitors (reviewed in [22, 71, 72]). RANTES, an N-terminally truncated variant of RANTES/CCL5 was the first such engineered analogue, but it was found to exhibit only modest anti-HIV activity. Its identification nonetheless helped establish the concept that the agonist activity on coreceptors is not a component of the anti-

HIV mechanism of native chemokines. Further N-terminal modifications led to the generation of chemokine analogues such as AOP-RANTES [73] and PSC-RANTES [74], which have improved potency due to their mechanism of action; they induce receptor sequestration that results in the internalization and physical removal of the coreceptor from the cell surface, effects that are more profound and prolonged compared to those of native chemokines [22]. The clinical development of chemokine analogues as anti-HIV therapeutics is complicated by the fact that as proteins, chemokines would not be expected to be orally bioavailable and their production costs would be prohibitively high; moreover, chemokine analogues are unlikely to show suitable pharmacokinetics after injection because they readily bind to and form aggregates on cell surface proteoglycans [22]. The potential adverse effects due to the possible inflammatory activity mediated through CCR5 activation is another concern associated with their use.

4.2. Anti-CCR5 antibodies

Most anti-CCR5 monoclonal antibodies (MAbs) have been described as chemokine antagonists that inhibit the receptor via steric blockade (reviewed in [22]). Many rodent anti-CCR5 MAbs with anti-HIV activity have been identified and extensively characterized. PRO 140 (Progenics pharmaceuticals) is a humanized monoclonal antibody, currently available only for parenteral administration; PRO 140 received fast-track approval by the FDA. The efficacy, tolerability and toxicity profiles of PRO 140 have been assessed by several preclinical and clinical studies, which showed promising results (reviewed in [75]). Of note is the evidence of activity shown by PRO 140 against escape mutants with cross-class resistance to small molecule CCR5 inhibitors [76]; however, as a complex biological molecule requiring parenteral administration, PRO 140 faces a different set of development challenges compared to the orally bioavailable small molecule compounds [77]. Human CCR5mAb004 antibody also performed well in early clinical trials [22]. Currently available data further suggest that anti-CCR5 MAbs act synergistically with other CCR5 inhibitors and have different resistance profiles. Their typically long circulatory half-lives do not necessitate frequent dosing, suggesting that the lack of oral bioavailability may not be a serious limitation in their development as anti-HIV drugs.

4.3. Gene knockdown and knockout strategies

CCR5 expression in HIV-1 target cells can be suppressed by RNA-based gene modulation technologies such as RNA interference (RNAi), or completely eliminated by such techniques as zinc finger nuclease (ZFN)-mediated gene disruption. RNAi refers to the sequence-specific degradation of messenger RNA (mRNA) that follows the cellular introduction of homologous, short-interfering RNA (siRNA), a technique that has emerged as a powerful tool to probe the function of genes of known sequence *in vitro* and *in vivo* [78, 79]. RNAi has been shown to decrease the replication of HIV-1 in lymphocytic cells using siRNA targeting both viral (e.g., Tat, Gag and Rev) and host proteins, including CD4 and CCR5 (reviewed in [80]). The numerous challenges associated with converting RNAi from a laboratory technique to an anti-HIV therapeutic have been

reviewed elsewhere [22]. Recent advances in the development of RNAi-based therapeutics for HIV-1 are presented by Zhou and Rossi [81]. Zinc-finger nucleases (ZFNs) are artificial restriction enzymes engineered by fusing a zinc finger DNA-binding domain to a DNA-cleavage domain; the generated double-stranded DNA break is repaired by the endogenous DNA repair machinery of the cell, potentially leading to the permanent disruption of the gene's open reading frame [82]. CCR5 gene knockout in T cells or hematopoietic stem/progenitor cells (HSC) by ZFNs effectively suppresses the replication of CCR5-tropic strains of HIV-1 in animal models (reviewed in [83]). ZFNs are currently being evaluated in a phase I clinical trials using *ex vivo* expanded T cells; HSC targeted therapies are under development as well.

4.4. Small molecule CCR5 inhibitors

Small molecule CCR5 inhibitors (Table 1) prevent HIV-1 from entering its target cells, mainly CD4⁺ T cells and macrophages, by blocking the CD4-dependent binding of the viral surface envelope glycoprotein (gp120) to the most commonly used coreceptor CCR5 (reviewed in [22, 67, 72, 77]).

The identification of the first such molecule, TAK-779, in 1999 [84] was followed by several reports on additional CCR5 inhibitors with improved potency and more favorable pharmacological properties (Table 1). The development of these compounds has been principally pursued for the treatment of HIV-1 infection, although, theoretically, they could also be used as preventive agents [98]. Maraviroc (MVC, Selzentry; Pfizer, Inc., Figure 2A) was the first, and so far only, small molecule CCR5 inhibitor to be approved for treating HIV-1 infection by the US FDA and by the European Agency for the Evaluation of Medicinal Products (reviewed in [99]). MVC is licensed for administration in both treatment-naïve (i.e., yet to receive any ART) and in treatment-experienced patients with R5 HIV-1. Other investigational compounds have also entered clinical trials, but a number of them were discontinued due to observed side effects (Table 1). The development of Vicriviroc (VCV, Schering-Plough Research Institute; now Merck Research Laboratories, Figure 2B) was abandoned because it did not meet primary efficacy endpoints in late stage (Phase III in treatment-experienced subjects and Phase II in treatment-naïve individuals) clinical trials. Additional compounds are in preclinical or clinical development (reviewed in [77]). Previously designated as TBR-652 or TAK-652 (Figure 2C), cenicriviroc is a small-molecule CCR5 antagonist licensed by the Takeda Pharmaceutical Company to Tobira Pharmaceuticals; cenicriviroc has pharmacokinetic data supportive of once daily dosing and a favorable tolerability profile in single-dose studies in healthy volunteers. PF-232798 (Figure 2D), a second-generation small-molecule oral CCR5 antagonist with potency similar to MVC and the potential for once daily dosing, has demonstrated activity against a laboratory-generated MVC-resistant R5 virus. INCB9471 (Incyte, Wilmington, DE, USA) is yet another small-molecule oral CCR5 inhibitor that showed promising results in a Phase IIa trial in treatment-naïve and treatment-experienced patients with once daily dosing over 14 days [77].

Drug	Other names	Key features	Manufacturer	Current status	References
TAK-779	N/A	(Nonpeptidic) quaternary ammonium anilide that prevents gp120 and CC-chemokines, but not anti-CCR5 MAbs, from binding to CCR5	Takeda	Preclinical only	[84]
Cenicriviroc	TBR-652, TAK-652	Orally bioavailable, improved potency TAK-779-derivative; also blocks CCR2	Takeda; now Tobira	Phase II completed	[85, 86]
AD101	SCH-350581	Piperidino-piperazine-based compound structurally related to, but more potent than SCH-C; also blocks MAbs mainly against the ECL2 of CCR5	Schering-Plough	Preclinical only	[87, 88]
SCH-C	SCH-351125	Oximino-piperidino-piperidine amide that also blocks MAbs mainly against the ECL2 of CCR5; unable to exert its antiviral effects when residue 198 in TM helix 5 of CCR5 is methionine (Met) as in rhesus macaques [89]	Schering-Plough	Discontinued; hERG K ⁺ channel blockade & CNS side effects	[90-92]
Vicriviroc (VCV)	SCH-D, SCH-417690, VVC	Piperazino-piperidine-based compound; improved potency & pharmacokinetics, reduced tendency for hERG channel blockade compared to SCH-C	Schering-Plough; now Merck	Discontinued from Phase III; primary efficacy points unmet	[93, 94]
Aplaviroc (AVC)	AK602/ONO4128/873140, GW873140, APL	Spirodiketopiperazine-based compound; it preserves RANTES and MIP-1 β binding to CCR5 ⁺ cells and their functions	Glaxo SmithKline	Discontinued from Phase IIb/III; idiosyncratic hepatotoxicity	[95]
Maraviroc (MVC, Selzentry)	UK-427,857	Spirodiketopiperazine with favorable pharmacological properties	Pfizer	Approved for clinical use	[96, 97]

hERG K⁺, human ether-a-go-go related gene potassium channel; MAbs, monoclonal antibody; N/A, not applicable.

Table 1. Nomenclature, characteristics, and developmental status of selected small molecule CCR5 inhibitors

Figure 2. Chemical structures of selected small molecule CCR5 inhibitors. A. Maraviroc (MVC, Selzentry), B. Vicriviroc (VCV), C. Cenicriviroc (TBR-652), D. PF-232798.

Binding site. The binding site of small molecule CCR5 inhibitors does not overlap with the binding site for HIV gp120. In the absence of a high-resolution structure of a gp120-coreceptor complex, biochemical studies of CCR5 have revealed the importance of its N-terminus (Nt) and second extracellular loop (ECL2) in binding gp120 and mediating viral entry (reviewed in [34]). Small molecule CCR5 inhibitors, on the other hand, are believed to bind within a cavity between the transmembrane (TM) helices on the extracellular surface of the coreceptor, as suggested by site-directed mutagenesis experiments and molecular modeling of CCR5 on the basis of the available crystal structure of bovine rhodopsin that is characterized by a similar TM topology [100, 101]. More specifically, the binding “pocket” of such molecules as TAK-779, AD101, and SCH-C is thought to be located between TM helices 1-3, and 7; other TM helices, particularly helix 6, contribute to some compound-specific variations [102, 103]. Simultaneous administration of multiple antagonists showed that, indeed, they bind to a common site on CCR5 [104]. A key set of aromatic and aliphatic residues that serve as a hydrophobic core for this ligand binding pocket has also been identified, while a glutamic acid (Glu) in helix 7, E283, appears to be critical for high affinity interaction; this residue probably acts as the counterion for a positively charged nitrogen atom common to TAK-779, AD101, and SCH-C [105].

The binding of all small molecule CCR5 antagonists to a broadly similar site within the TM helices of the coreceptor renders cross-resistance within this drug class possible, but not necessarily inevitable since compound-specific variations with respect to properties such as structure, shape, and electrostatic potential, do exist [106]; hence, despite the insensitivity of AD101 escape mutants to all other tested CCR5 inhibitors, including SCH-C, aplaviroc (AVC), MVC and VCV [107-109], a MVC-resistant variant has been reported to retain its

sensitivity to SCH-C and AVC [110]. Susceptibility to other classes of licensed drugs that target different stages of the viral life cycle, or even different steps of the same stage of the viral life cycle such as viral entry, should be maintained. Accordingly, clinical resistance to Enfuvirtide (ENF, Fuzeon/T-20) was not found to affect viral sensitivity to the fusion inhibitor T-1249 or to coreceptor inhibitors [111].

Mechanism of action. Small molecule (molecular weight 500-600 D) CCR5 inhibitors act via a similar, allosteric (from the Greek “*allos*”+“*stereos*” meaning “other”+“shape,” respectively) mechanism; in other words, they induce or stabilize a conformation of the coreceptor that renders it unrecognizable by the wild-type virus. The results of recent biochemical studies support the noncompetitive and allosteric nature of the mechanism of action of small molecule antagonists of CCR5 [104]. The difference in binding sites between the two ligands also supports the allosteric modulation of the HIV-1 gp120 binding site by this class of drugs; as mentioned above, CCR5 inhibitors bind within the TM helices in the extracellular facet of the coreceptor, whereas HIV gp120 targets the extracellular domains of CCR5 and, more specifically, the negatively-charged Tyr-sulfated amino terminus (Nt) and the ECL2 between TM helices 4 and 5 [103].

Moreover, the action of some CCR5 inhibitors may be blocked by the introduction of certain mutations into CCR5 despite the binding of the inhibitor to the coreceptor; namely, the antiviral effects of SCH-C are blocked, although its antagonism of the signaling by RANTES/CCL5 persists, when residue 198 in the TM helix 5 of CCR5 is methionine (Met) as in rhesus macaques [89]. This observation led to the hypothesis that the I198M substitution may be involved in the induction of a CCR5 conformation that is incompatible with HIV-1 infection. It should be noted, however, that not all CCR5 inhibitors are modulated in the same manner by this amino acid change. I198M has been shown not to block the antiviral effects of AD101, whereas another mutation, F113A, may block the inhibitory action of AD101, but not of SCH-C, via a similar mechanism [89]. Further subtle differences exist in the stabilized or induced conformations by the binding of these small molecules to the TM pocket of CCR5 and, therefore, in the mechanism of action of these compounds. For instance, although most CCR5 inhibitors antagonize potently the three natural ligands of the coreceptor, AVC only antagonizes MIP-1 α /CCL3 efficiently and much less potently the other two ligands (MIP-1 β /CCL4, and RANTES/CCL5) [95].

Displacement binding assays and dissociation kinetics demonstrated that TAK779 and MVC inhibit CCL3 and gp120 binding to CCR5 by a noncompetitive and allosteric mechanism, supporting the view that these compounds bind to regions of CCR5 distinct from the gp120- and CCL3-binding sites [112]. MVC is predicted to insert deeply in the CCR5 TM cavity where it can occupy three different binding sites, whereas CCL3 and gp120 lie on distinct, yet overlapped regions of the CCR5 ECL2 [113]. TAK779 and MVC were found to be full and weak inverse agonists for CCR5, respectively, indicating that they stabilize distinct CCR5 conformations with impaired abilities to activate G-proteins [112]. The finding that the TM cavity remains accessible for MVC in CCL3-bound and gp120-bound CCR5 [113], provides an explanation for the enhancement by TAK779 and MVC of the dissociation of

preformed ligand-CCR5 complexes with an efficiency that correlates with their ability to act as inverse agonists [112]. The identification of residues mandatory for gp120 binding in the predicted CCR5 dimer interface suggests that receptor dimerization might represent a target for new CCR5 entry inhibitors [113].

AK530 and AK317, two CCR5 inhibitors containing spirodiketopiperazine scaffolds, lodge in a hydrophobic cavity located between the upper TM domain and the ECL2 of CCR5 [114]. The interaction profile of the inhibitors with ECL2 residues S180 and K191 was found to be an important determinant of antiviral potency; furthermore, amino acid residues in the beta-hairpin structural motif of ECL2 were critical for HIV-1-elicited fusion and binding of these spirodiketopiperazine-based inhibitors to CCR5. The direct ECL2-engaging property of the inhibitors likely produces an ECL2 conformation, which HIV-1 gp120 cannot bind to, but it also prohibits the virus from utilizing the “inhibitor-bound” CCR5 for cellular entry - a mechanism of HIV-1's resistance to CCR5 inhibitors, as discussed in the next section.

5. Generation of resistance to small molecule CCR5 inhibitors

The most parsimonious escape pathway from the selection pressure of a CCR5 inhibitor would be coreceptor switching to CXCR4 use, with its well documented devastating consequences on the rate of CD4⁺ T cell depletion and disease progression of patients [22, 53, 67]. Both *in vitro* and *in vivo* studies have nevertheless shown that this is not the preferred escape pathway by HIV-1 despite the short distance in sequence space (few mutations) that separate R5 from X4 variants. The reduced replication fitness or the suboptimal coreceptor use, as indicated by the increased sensitivity to the selecting CCR5 antagonist of viral intermediates in the R5 to X4 evolution process compared to baseline virus, may account for this apparently paradoxical phenomenon; the requirements of inserting charged amino acids at specific locations and the strong bias in favor of G-to-A substitutions rather than random mutations are thought to impose additional constraints on the transition [115, 116]. Whatever the reasons may be, when R5 viruses escape from the selection pressure of CCR5 inhibitors they tend to retain the R5 phenotype.

Several factors diversify the pathways of resistance development to CCR5 inhibitors compared to those to conventional antiretroviral drugs [22]. The seemingly facile, for HIV, escape pathway of mutating the active site of a viral enzyme in order to prevent the binding of an antiviral would simply not work in the case of CCR5 antagonists that target a host- rather than a virus-encoded gene. Instead, the virus must devise a strategy to recognize and use the coreceptor to gain access into its target cells despite the presence of the inhibitor. The highly variable nature of the *env* gene certainly facilitates the escape process; however, *env* is also under the constant selection pressure from neutralizing antibodies [34]. Thus, during the development of resistance to CCR5 inhibitors the HIV-1 Env complex must accommodate sequence changes that enable the virus to escape both from the selection pressure of the inhibitors as well as from the humoral immune response, without violating the structural constraints of the Env protein.

5.1. *In vitro* resistance: No coreceptor switch to CXCR4 and no uniform genotypic signature

To gain insights into what may happen *in vivo* if or when small molecule CCR5 inhibitors are used more extensively, resistance is first studied *in vitro*. To generate resistant variants, primary R5 isolates are cultured with physiological target cells that are permissive for replication of both R5 and X4 viruses in the presence of increasing concentrations of the inhibitor under study. Despite the intrinsic difficulty associated with the selection process in each case that proved to require prolonged periods of culture in the presence of these compounds, several such studies have been published (reviewed in [22, 72, 117]). An alternative *in vitro* system that allows for the selection of escape mutants to CCR5 inhibitors over a relatively short period of time is the construction of V3 loop libraries of R5 infectious clones (reviewed in [118]). The results of these studies collectively show that the escape mutants tend to retain usage of CCR5 despite the availability of CXCR4. The mechanism that allows resistant viruses to continue to use CCR5 in the presence of the inhibitor is discussed in the next section. X4 viruses have been observed in CCR5 inhibitor-selection experiments, either in the control culture alone [119] or in both the selection and the control cultures [110], but their emergence was probably due to adaptation to growth in culture rather than the inhibitor escape process. Conditional recognition of CXCR4 in specific cell lines has also been reported for one VCV-resistant virus [109]. HIV-1 can be “forced” to escape CCR5 drug pressure through coreceptor switch under certain experimental conditions, which may not be very relevant physiologically [120].

To date, no clear signature resistance mutations have been identified for CCR5 antagonists and different genetic pathways can lead to the same resistance phenotype (Table 2).

The determinants of resistance commonly map to the V3 region of gp120, a localization that is consistent with the highly variable nature of this region as well as with its role in coreceptor engagement [22, 45]. Four V3 substitutions (K305R, H308P, A316V and G321E) were, for example, responsible for the resistant phenotype of isolate CC101.19 when the CCR5-using, subtype B virus CC1/85 [62] was cultured with the small molecule CCR5 inhibitor AD101, a preclinical precursor of VCV [107, 108]. A much rarer route to resistance was undertaken by viruses derived from the same CC1/85 lineage in an AD101/VCV selection culture [109]; the resulting D101.12 escape mutants contained at least one substitution in V3 (H308P) as well as either of the following combinations of substitutions within and downstream of the gp41 fusion peptide (FP): G514V+V535M (Pattern I) or M518V+F519L+V535M (Pattern II) [127]. We have also described a unique VCV escape pathway for viruses from the D1/85.16 lineage that involved no V3 changes; instead, resistance mapped to three conservative substitutions in the FP: G516V, M518V and F519I [126]. As shown recently [131], the G516V change is critical to VCV resistance, although it must be accompanied by either M518V or F519I to have a substantial impact phenotypically. Nevertheless, introducing the three FP changes together into a heterologous virus, JR-FL, did not create a VCV-resistant variant [126]. CCR5 inhibitor resistance is usually dependent upon the Env genetic context and it is not transferred when introduced into other viruses [124, 126, 132], but that is not always the case [129].

Drug	Parental virus		Resistance mutations			References
	Name	Subtype	gp120		gp41	
			V3	Elsewhere		
TAK-779	JR-FL _{V3Lib}	B	I304V, H305N, I306M, F312L, E317D	None	None	[121]
TAK-652	KK	Unknown	ND	ND	ND	[119]
AD101	CC1/85	B	K305R, H308P, A316V, G321E	None	None	[107, 108]
VCV	Clinical isolate	C	K305R, T307I, F316I, T318R, G319E and S306P	None	None	[122]
VCV	Clinical isolate S91	D	Q315E, R321G	E328K, G429R (C4)	None	[123]
VCV	RU570	G	K305R, R315Q, K319T	P437S (C4)	None	[124, 125]
VCV	CC1/85	B	None	None	G516V, M518V, F519I	[109, 126]
VCV	CC101.6 (CC1/85 passage 6 AD101 selection)	B	H308P and one or more of: K305R, A316V, G321E	None	G514V, V535M (Pattern I) or M518V, F519L, V535M (Pattern II)	[109, 127]
AVC	Clinical isolates	Unknown	Several	Several	Several	[128]
MVC	Clinical isolate	B	P/T308H, T320H, I322aV	D407G, ΔN386 (V4)	None	[129]
MVC	CC1/85	B	A316T, I323V	ND	ND	[110]
MVC	RU570	G	ΔQAI (315-317)	ND	ND	[110]
MVC	JR-FL _{V3Lib}	B	I304V, F312W, T314A, E317D, I318V	T199K, T275M (C2)	None	[130]

ND, Not determined; **I322aV** occurs at an amino acid not present in HXB2 located between residues 322 and 323 and is designated 322a.

Table 2. Genetics of HIV-1 escape from the selection pressure of small molecule CCR5 inhibitors

The findings of these studies have important implications for the clinical use of small molecule inhibitors, particularly for the generation of genotypic algorithms and phenotypic assays to predict the emergence of resistant viruses and the likelihood of cross-resistance to other members of this drug class. Cross-resistance to other CCR5 antagonists may or may not arise with these molecules that retain sensitivity to all other drug classes (e.g. nucleoside and non-nucleoside reverse transcriptase inhibitors, protease and integrase inhibitors) as well as to other entry inhibitors acting at different stages of the entry process (e.g. the fusion

inhibitor enfuvirtide) [117]. Small molecule CCR5 inhibitors also retain sensitivity to anti-CCR5 MAbs that act by a dissimilar mechanism.

Viral fitness. When HIV-1 develops resistance to conventional antiretroviral drugs such as reverse transcriptase or protease inhibitors, its fitness is often impaired. To investigate whether the *in vitro* development of resistance to small molecule CCR5 inhibitors has an associated fitness cost, we developed a growth-competition assay involving dual infections with molecularly cloned viruses that are essentially isogenic outside the *env* genes under study. Real-time TaqMan quantitative PCR (QPCR) was used to quantify each competing virus individually via probes specific to different, phenotypically silent target sequences engineered within their *vif* genes. Head-to-head competition assays of *env* clones derived from the AD101 and VCV resistant isolate, the inhibitor-sensitive parental virus, and a passage control virus showed that CCR5 inhibitor resistance was not associated with a fitness loss [133]. This observation is consistent with the retention of the resistant phenotype when the escape mutant virus CC101.19 was cultured for a total of 20 passages in the absence of the selecting compound [133]. Amino acid substitutions in the V3 region of gp120 that confer complete resistance cause a fitness loss when introduced into an inhibitor-sensitive, parental clone; however, in the resistant isolate, changes elsewhere in *env* that occurred prior to the substitutions within V3, appear to compensate for the adverse effect of the V3 changes on replicative capacity. Hence, a decrease in fitness resulting from CCR5 inhibitor resistance may not be as common as with the fitness loss observed with resistance to almost all other antiretroviral drugs (reviewed in [72]).

The *in vivo* situation may differ, but further studies are needed to explore the fitness impact of HIV-1 with acquired resistance to CCR5 antagonists. In one case, pre-treatment V3 sequences reemerged in a subtype C-infected patient following VCV discontinuation, implying that VCV resistance has associated fitness costs [122]. Nonetheless, all the VCV-resistant isolates we have studied (CC101.19, D101.12 and D1/85.16) have highly stable phenotypes in peripheral blood mononuclear cells (PBMC), in that they do not rapidly revert to sensitivity when cultured without the inhibitor [108, 126, 127, 133]. During reversion experiments of individual resistant clones in the absence of the inhibitor, we discovered that M518V, which has little or no effect on VCV sensitivity on its own, appears to be under no pressure to revert either [131]. Val-518 is a naturally occurring FP polymorphism that becomes enriched during VCV or AD101 selection [126]. However, a resistant clone D1/85.16 cl.23 (= R) reverted to a sensitive phenotype by five weekly, VCV-free passages in PBMC culture. Interestingly, the complete cluster of the three FP changes persisted within the genetic context of the gp120 derived from the resistant clone R; however, the three FP changes were accompanied by a Thr-to-Ala substitution at position 244 of the second conserved region (C2) of gp120 that appears to counter the VCV-resistance phenotype created by the FP substitutions. This residue is located in strand β 7, part of a β -sandwich structure in the inner domain that is involved in CD4-induced conformational changes within gp120 and the interaction with gp41 [134-137]. Examining the interplay of these changes will enhance our understanding of Env complex interactions that influence both HIV-1 entry and resistance to CCR5 inhibitors.

Figure 3. Mapping residue 244 on the structure of the gp120 of the representative clone, CC1/85 cl.7, of the parental, inhibitor-sensitive virus CC1/85. Residue 244 (magenta) is located in strand $\beta 7$, part of a β -sandwich structure in the inner domain of gp120 that is thought to be involved in interactions with gp41. The CC1/85 cl.7 gp120 core structure was modeled based on the V3-containing structure of gp120 [138] with Swiss-Model [139] using 2B4C.pdb as template and visualized using PyMOL [140].

5.2. Mechanisms of resistance to small molecule CCR5 inhibitors

As portrayed graphically in Figure 4, resistance to small molecule CCR5 inhibitors may likely develop via two, not necessarily exclusive, mechanisms: competitive resistance and noncompetitive or allosteric resistance (reviewed in [22, 72, 117, 118, 141]), not to be confused with the competitive or not, properties of the inhibitors themselves [142]. Either mechanism may be effective at overcoming inhibition for some CCR5 inhibitors, while some HIV-1 escape mutants may undertake a combination of the two mechanisms. Understanding how resistance arises is relevant to how it is detected and quantified.

Competitive resistance. Competitive resistance (Figure 4A) is defined as resistance that results in a shift in the IC_{50} of an inhibitor to a higher concentration; complete inhibition can still be achieved at sufficient inhibitor concentrations [142]. Competitive resistance could arise from more efficient utilization of inhibitor-free CCR5 to gain access into target cells; in turn, this could arise either from a greater affinity of Env for the coreceptor, or from an increase in the kinetics of Env-mediated fusion after CCR5 engagement. Either scenario would allow the virus to better compete with the inhibitor for the coreceptor, reflecting its adaptation to scavenge low levels of inhibitor-free CCR5 [142]. A partially (~5-fold) AD101-resistant isolate had this ability [108]; however, this mechanism alone cannot count for complete resistance to CCR5 antagonists [117].

Figure 4. Resistance mechanisms for viral escape from CCR5 inhibitors. Model inhibition curves are displayed for sensitive and resistant viruses (solid grey and dashed black curves, respectively). The atypical dose-response curves of resistant viruses with gp41 fusion peptide (FP) mutations are shown in blue. **A.** Competitive resistance results in a shift in the IC_{50} of an inhibitor to a higher concentration, with complete inhibition potentially achievable at a high enough inhibitor concentration. **B.** In noncompetitive resistance, the IC_{50} values are typically equivalent to those for the sensitive virus, but the extent of inhibition is incomplete for the resistant virus. The maximum percent inhibition (MPI) value, at a saturating inhibitor concentration, reflects the efficiency with which the inhibitor-CCR5 complex is used relative to free CCR5, thereby providing a measure of the degree of resistance of the HIV-1 variant under study. MPI values vary by cell type for inhibition by VCV and related compounds. **C.** In the novel paradigm of noncompetitive resistance, resistant FP mutant viruses display reduced MPI and IC_{50} values. Adapted from [143].

Noncompetitive or “allosteric” resistance. Noncompetitive resistance (Figure 4B) is defined as resistance that results in saturable inhibition, such that increasing concentrations of the inhibitor have no effect on the virus; the EC_{50} required to reach this saturation level is the same as the IC_{50} for the fully sensitive virus [142]. Phenotypically, resistance manifests as a plateau in the maximum achievable suppression of viral replication. The implication of this mechanism is that the resistant Env has adapted to use the inhibitor bound form of CCR5 as a coreceptor as well as, but not instead of, the free coreceptor [110, 142]. The level of residual inhibition, termed the “plateau” or the maximum percent inhibition (MPI), once inhibition has reached saturation reflects the efficiency with which the inhibitor-CCR5 complex is used relative to free CCR5; the higher the MPI value, the less efficiently a resistant virus uses the inhibitor-CCR5 complex [110, 142]. This mechanism is consistent with the allosteric mode of action of small molecule CCR5 inhibitors; these compounds lock the extracellular domains of CCR5 in a conformation that can be recognized only by resistant variants -in addition to the natural coreceptor-, but not by wild-type HIV-1 [117]. In a few instances, the preferred use of the inhibitor-bound CCR5 over the unbound receptor by some CCR5 antagonist-resistant viruses leads to modestly increased replication of resistant viruses in the presence of the inhibitor *in vitro* [142]. The cell type influences the mode in which resistance to small molecule CCR5 inhibitors is manifested, particularly MPI values [110, 124, 126, 127, 131, 142]. Moreover, the differences between the MPI values of VCV-resistant and sensitive viruses we have studied are generally much smaller in cell lines engineered to express CCR5, such as TZM-bl or U87.CD4.CCR5 (that serves as the basis of the PhenoSense and Trofile tropism assays), than in PBMC [126, 127, 131].

Resistant FP mutants: A novel paradigm of noncompetitive resistance. The VCV-resistant viruses carrying FP changes represent a novel paradigm of noncompetitive resistance (Figure 4C); apart from the plateau effect, these FP mutants paradoxically have lower IC_{50} values than wild-type viruses, particularly in certain cell types (e.g. TZM-bl cells). Hence, although by one measure (MPI) the FP-mutant viruses display a modest level of resistance, by another (IC_{50}) they appear to be hypersensitive [126, 127, 131]. To explain this paradox, we created a theoretical model of resistance [126]. The mathematical formulation of the model is presented in detail in the Supporting Information (SI) section of [126]. The model is based on the assumption that distinct forms of CCR5, with varying affinities (ranging from low to high) for small molecule CCR5 inhibitors, are present in different proportions on disparate cell types; these coreceptor forms, which could be conformers (conformational isomers) or differentially Tyr-sulfated forms, are used selectively by resistant HIV-1 variants when ligated with an inhibitor. According to the model, wild-type virus can use both forms of unligated CCR5 for entry, possibly with a preference between them, but it cannot enter via the inhibitor-coreceptor complexes. The resistant virus, on the other hand, uses preferentially the unoccupied high-affinity coreceptor and the occupied low-affinity coreceptor. Applying this model led to the generation of theoretical inhibition curves that closely mimic the experimental data for resistant viruses in each case [126, 127, 131]. Using the model, we have recently identified the third FP mutation, F519I, (of the V3-independent or FP only- genetic pathway to resistance) as an independent determinant of preference for the unoccupied, high-VCV affinity form of CCR5 [131]. Modeling the inhibition data from two cell types, PBMC and TZM-bl cells, suggested that D101.12, which harbors both V3 and gp41 substitutions, discriminates between high- and low-affinity forms of CCR5 less than D1/85.16, the resistant virus with three FP substitutions [127].

Although derived from a common parent, CC1/85, two resistant viruses that we have described, CC101.19 and D1/85.16, followed different genetic pathways to reach the same phenotypic endpoint: CC101.19 acquired four substitutions (K305R, H308P, A316V and G321E) in the V3 region [107], while the key determinants of resistance in D1/85.16 were three changes in the gp41 FP (G516V, M518V and F519I) [126] (Table 2). Both resistant variants acquired the ability to use the VCV-CCR5 complex for entry [107, 108, 126, 142]. However, unlike a clone derived from CC101.19 (V3 mutations), the D1/85.16 cl.23 virus (FP mutations) did not have an increased dependency on the CCR5 Nt, and its CCR5 binding site was not obviously more exposed [144]. The FP-mutant was, however, atypically sensitive to a CCR5 MAb that stains discrete cell surface clusters of CCR5 that might correspond to lipid rafts [145]. The precise molecular mechanism by which the FP changes confer resistance remains to be determined.

The V3 changes that confer resistance to CCR5 inhibitors have been suggested to render the virus more dependent on the CCR5 Nt, to compensate for impaired interactions between the V3-crown and ECL2 [144]. Accordingly, substantial deletions in V3 from both HIV-1 and HIV-2 confer complete resistance to coreceptor antagonists, presumably by disrupting the interaction between V3 and ECL2 [146-150]. Any adverse effect the V3 sequence changes have at the CCR5-binding stage may be compensated for by increases in the affinity of resistant viruses for CD4 and/or in the kinetics of virus entry [147, 151]. The altered virus-

CCR5 interactions are nonetheless characterized by considerable complexity. Thus, a subtype D, VCV-resistant patient isolate recognizes the drug-bound form of CCR5 more efficiently but still uses both the Nt and ECL2 [123]. A recently proposed model suggests that broad cross-resistance to multiple inhibitors is associated with an increased dependence on the N-terminus, while a more specific pattern of resistance to individual compounds involves more subtle changes in how the virus interacts with both the Nt and ECL2 [129].

5.3. *In vivo* resistance: Potential expansion of pre-existing, CXCR4-using viruses

The limited information currently available from published results of clinical studies suggests that the escape process from small molecule CCR5 inhibitors *in vivo* bears similarities to that observed *in vitro*; clinical resistance is also likely to map to the V3 region of gp120, but the lack of consistency of observed mutations renders impossible the prediction of *in vivo* resistance by sequence analysis at present (reviewed in [72, 77, 117, 141]). As mentioned above, two pathways of virological escape from the selection pressure of CCR5 antagonists have been identified: continued use of CCR5 via selection of R5 virus that can use drug-bound CCR5 for entry in addition to free coreceptor and expansion of pre-existing minority populations of dual-tropic or X4 virus (collectively called “CXCR4-using” viruses). The first pathway of R5 virological failure with reduced susceptibility to CCR5 antagonists is not the most common mechanism of failure. Virologic failure most commonly involves expansion of pre-existing, CXCR4-using viruses that are insensitive to CCR5 inhibitors. This outcome underscores the importance of developing tropism assays with improved ability to detect minor or archived virus populations capable of using the CXCR4 coreceptor.

The Trofile assay has been the assay of choice for clinical trials of CCR5 inhibitors. However, the sensitivity of the original commercially available assay was compromised with respect to the detection of low abundance (minority) CXCR4-using variants. As a result, a number of subjects were misjudged to have R5 virus at screening, whereas they actually harbored Dual/Mixed (D/M) virus populations when they entered clinical trials of CCR5 inhibitors; these patients appeared to have a blunted virologic response and high rates of early virologic failure [152-154]. Application of an enhanced-sensitivity Trofile assay or “deep sequencing” and re-analysis of the data showed that virologic failure in a significant proportion of these patients stemmed from the expansion of pre-existing minority CXCR4-using variants that went undetected during the first round of testing [155-157]. Accurate determination of coreceptor usage is therefore necessary to optimize treatment strategies before the initiation of and during therapy with CCR5 inhibitors. Techniques such as deep V3 sequencing may be useful for identifying treatment-experienced individuals who could benefit from CCR5-antagonist-containing regimens [158]. Ongoing improvements in genotypic methodologies and algorithms may eventually render them a viable alternative to phenotypic assays, much like the genotypic resistance methods.

6. Conclusions and future directions

The identification of CCR5 as the principal HIV coreceptor for cellular entry over a decade ago combined with the observed effects of the CCR5Δ32 polymorphism (natural resistance

to HIV infection in homozygotes and delayed disease progression in heterozygotes) in the absence of any overt pathology, sparked great interest in its pharmaceutical blockade. The orally bioavailable small molecule CCR5 inhibitors are perhaps the most promising drugs that have been developed to block CCR5. In 2007, Maraviroc (MVC) became the first member of this new class of antiretrovirals, which target host- rather than virus-encoded structures, to receive regulatory approval for clinical use in the treatment of HIV infection. The theoretical virological concern that use of CCR5 antagonists in therapy may accelerate the evolution towards the more pathogenic CXCR4-using viruses has not been observed in clinical studies to date. MVC and other CCR5 antagonists have the potential for use in a variety of other clinical situations, such as the prevention of HIV transmission, intensification of HIV treatment and prevention of rejection in organ transplantation (reviewed in [159]). Moreover, CCR5 antagonists may be used in combination with other entry inhibitors or with agents such as rapamycin which downregulates CCR5 receptors thus decreasing CCR5 density [159]. New drugs that promote CCR5 and CXCR4 internalization, independent of cellular signaling, might also provide clinical benefits with minimum side effects [13].

Abbreviations

AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
CCR5	C-C chemokine receptor type 5
CNS	Central nervous system
CXCR4	C-X-C chemokine receptor type 4
ECL2	Second extracellular loop
Env	Envelope glycoprotein
FP	Fusion peptide
GALT	Gut-associated lymphoid tissue
GPCRs	G protein-coupled receptors
HIV	Human immunodeficiency virus
MAb	Monoclonal antibody
MVC	Maraviroc
MIP-1α	Macrophage inflammatory protein-1-alpha
MIP-1β	Macrophage inflammatory protein-1-beta
Nt	Amino terminus
PBMC	Peripheral blood mononuclear cells
RANTES	Regulated on activation, normal T cell expressed and secreted
R5 HIV	CCR5-tropic HIV
R5X4 HIV	Dual-tropic HIV
RNAi	RNA interference
SDF-1	Stromal derived factor-1
SIV	Simian immunodeficiency virus
7-TM	Seven-transmembrane

VCV	Vicriviroc
V3	Variable loop 3
X4 HIV	CXCR4-tropic HIV
ZFNs	Zinc-finger nucleases

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