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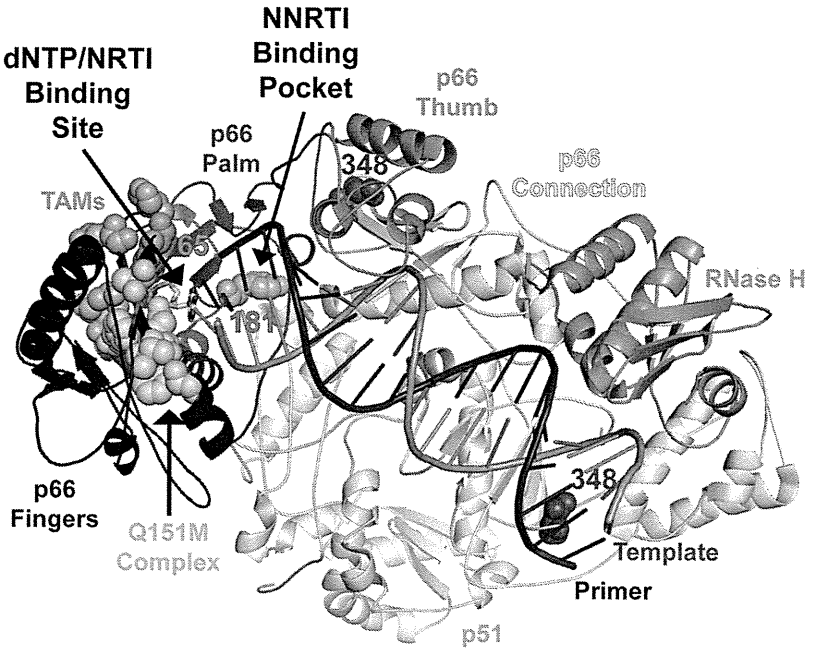


Figure 1. HIV-1 RT structure with highlighted residues of drug-resistance

The RT color scheme is as follows: fingers in blue, palm in red, thumb in green, connection in yellow, RNase H in orange, and p51 in gray. The Q151M complex is shown in cyan, TAMs in magenta, and 348 residue in purple. The RT coordinates are from PDB ID 1T05. The figure was made using PyMOL.

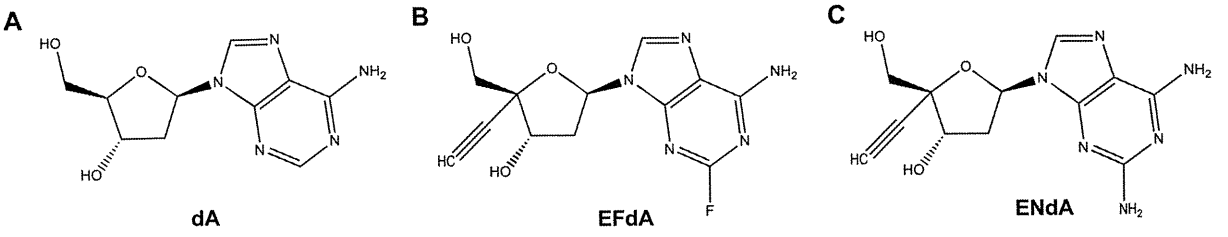


Figure 2.
Chemical structures of dA, EFdA and ENdA

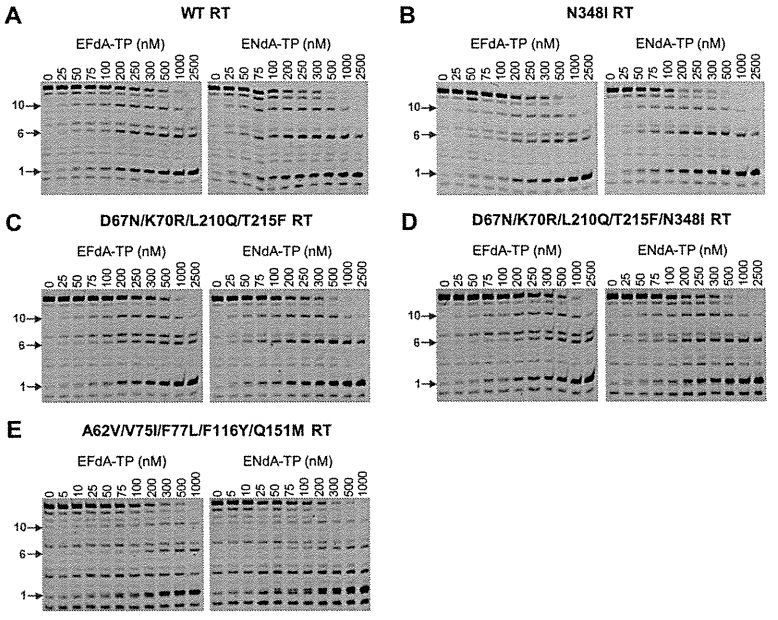


Figure 3. EFdA-TP and ENdA-TP inhibit WT and drug-resistant HIV-1 RTs

(A) T_{d31}/P_{d18} was incubated with various HIV-1 RTs for 15 minutes in the presence of 1μM dNTPs, $MgCl_2$ and increasing concentrations of EFdA-TP or ENdA-TP. The products synthesized by HIV-1 RT were quantified and plotted against increasing concentrations of the inhibitors. The IC_{50} values of the nucleotide analogs were determined by quantifying the percent of full extension and fitting the data points to GraphPad Prism 4 using one-site competition nonlinear regression (shown in Table 2). Arrows indicate the positions where dATP or dATP analogs are expected to be incorporated.

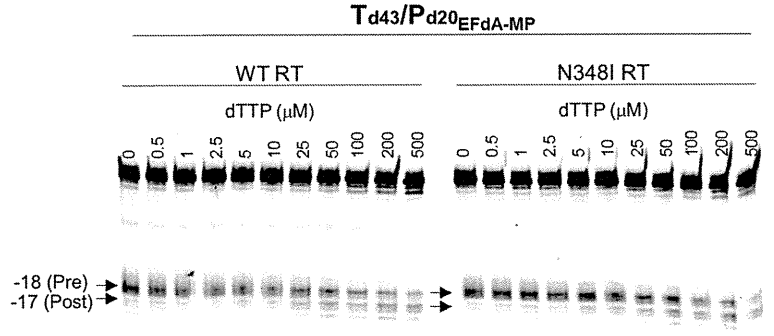


Figure 4. Determination of the translocation state of WT or N348I HIV-1 RT bound to T_{d43}/ $P_{20\text{-}EFdA\text{-}MP}$

The translocation state of RT after EFdA-MP incorporation was determined using site-specific Fe^{2+}footprinting. $T_{d43}/P_{20\text{-}EFdA\text{-}MP}$ (100 nM) with 5′-Cy3 label on the DNA template was incubated with HIV-1 RT (600 nM) and various concentrations of the next incoming nucleotide (dTTP). The complexes were treated for 5 min with ammonium iron sulfate (1 mM) and resolved on a polyacrylamide 7 M urea gel. An excision at position -18 indicates a pre-translocation complex, whereas the excision at position -17 represents a post-translocation complex. In both WT and N348I RT EFdA-MP prevents translocation with similar efficiency.

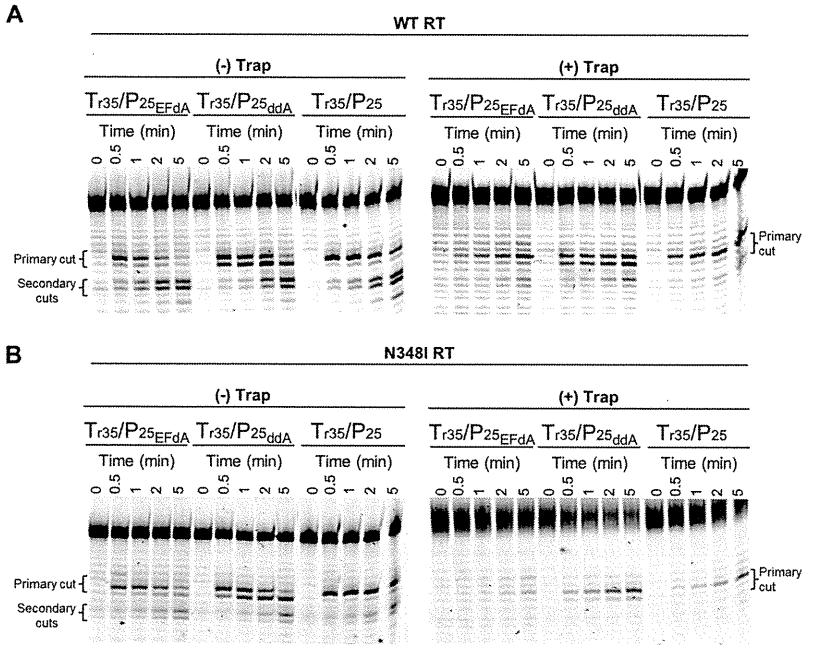
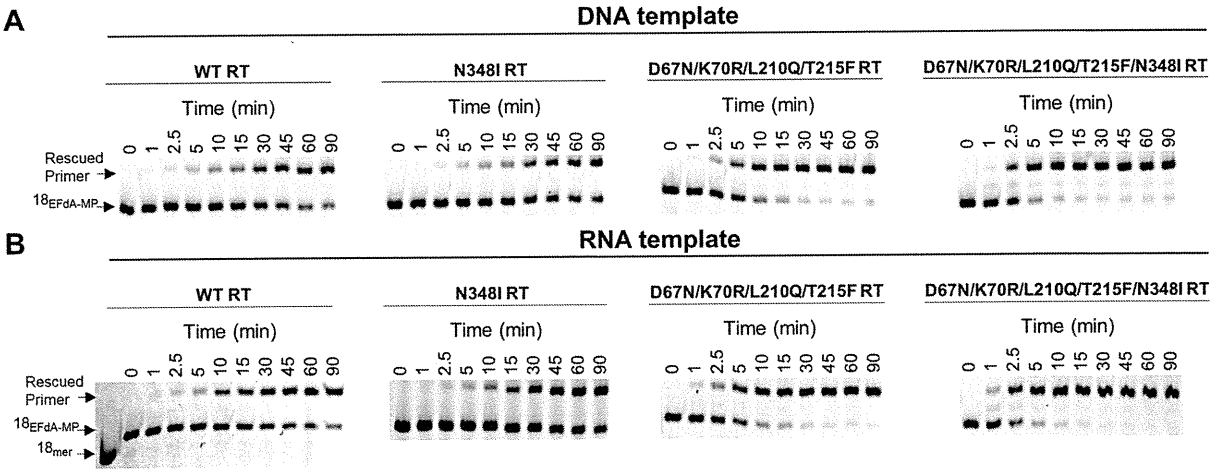


Figure 5. Effect of EFdA on RNase H activity of WT and N348I HIV-1 RTs

50 nM Cy3-$T_{r35}/P_{d25\ -\ EFdA\text{-}MP}$ or Cy3-$T_{r35}/P_{d25\text{-}ddAMP}$ or Cy3-T_{r35}/P_{d25} was incubated with 50 nM WT (A) or N348I (B) HIV-1 RT for varying times (0–5 minutes) at 37°C in RT buffer. The experiment was carried out in the presence or absence of non-labeled T_{d35}/P_{d25} trap (25 μM). Reactions were initiated with the addition of $MgCl_2$ and stopped with formamide. The primary and secondary cuts are indicated in the gel images.



C

Rates of ATP-mediated rescue (min^{-1})

	WT RT	N348I RT	D67N/K70R/L210Q/T215F RT	D67N/K70R/L210Q/T215F/N348I RT
DNA template	0.030 (1)	0.028 (0.9)	0.112 (3.7)	0.240 (8)
RNA template	0.037 (1)	0.025 (0.7)	0.154 (4.2)	0.369 (10)

Figure 6.

ATP-dependent unblocking of EFdA-MP terminated primers. ATP-dependent rescue of $T_{d31}/P_{d18\text{-}EFdA\text{-}MP}$ (A) and $T_{r31}/P_{d18\text{-}EFdA\text{-}MP}$ (B). Purified $T/P_{EFdA\text{-}MP}$ was incubated with WT, N348I, D67N/K70R/L210Q/T215F or D67N/K70R/L210Q/T215F/N348I HIV-1 RT in the presence of ATP (3.5 mM), dATP (100 μM), dTTP (0.5 μM), ddGTP (10 μM) and 10 mM $MgCl_2$ at 37 °C. Aliquots of the reaction were stopped at the indicated time points (0–90 min). (C) The rates of the ATP-dependent rescue of EFdA-MP terminated primers were calculated after quantifying the rescued products and plotting to the burst equation in GraphPad Prism 4.

Table 1

DNA and RNA sequences used in this study.

Polymerization experiments	
T_{d31}	5′-CCA TAG ATA GCA TTG GTG CTC GAA CAG TGA C
T_{r31}	5′-CCA UAG AUA GCA UUG GUG CUC GAA CAG UGA C
P_{d18}	5′-Cy3-GTC ACT GTT CGA GCA CCA
Footprinting experiments	
T_{d43}	5′-Cy3-CCA TAG ATA GCA TTG GTG CTC GAA CAG TGA CAA TCA GTG TAGA
P_{d30}	5′-TCT ACA CTG ATT GTC ACT GTT CGA GCA CCA
RNase H experiments	
T_{r35}	5′-Cy3-GGA AAU CUC UAG CAG UGG CGC CCG AAC AGG GAC CU
P_{d25}	5′-AGG TCC CTG TTC GGG CGC CAC TGC T

Table 2

IC_{50} values of EFdA-TP and ENdA-TP against WT and drug-resistant HIV-1 RTs

Inhibitor/Enzyme	WT	N348I	D67N/K70R/L210Q/T215F	D67N/K70R/L210Q/T215F/N348I	A62V/V75I/F77L/F116Y/Q151M
EFdA-TP (nM)	130	122	157	217	121
ENdA-TP (nM)	71	54	98	110	85

EXPERT OPINION

CCR5 inhibitors: emergence, success, and challenges

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Introduction: The discovery of CC-chemokine receptor 5 (CCR5) as a human immunodeficiency virus type 1 (HIV-1) coreceptor opened a new avenue to exploit CCR5 as a potential target for the intervention of HIV-1's cellular entry.
Areas covered: Various small-molecule CCR5 inhibitors were identified in the last decade; however, maraviroc (MVC) is the only CCR5 inhibitor currently used in the clinic. Concerns and challenges that exist for wider clinical use of CCR5 inhibitors are discussed.
Expert opinion: Although MVC-containing regimens have been recommended for treatment-naïve patients, MVC appears to have been used as one of drugs for salvage therapy rather than for treating drug-naïve patients. This is apparently due to MVC's twice-daily dosing schedule. Another significant disadvantage is that a costly tropism assay must be performed prior to MVC treatment. The access to inexpensive, sensitive, and rapid tropism tests should be made easily available. Only a few novel CCR5 inhibitors are presently in the pipeline. Development of potent and metabolically-stable novel CCR5 inhibitors allowing once-daily dosing regimens is needed. Development of CXCR4 inhibitors should greatly improve the treatment options available to patients infected with X4- and/or dual-tropic HIV-1 strains in combination with a CCR5 inhibitor.

Keywords: AIDS, antiretroviral therapy, CCR5, CCR5 inhibitor, chemokine, HIV

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1. Background

1.1 Therapy for HIV-1 infection and AIDS

After the development of the first AIDS (acquired immune deficiency syndrome) drug, zidovudine or AZT [1], a number of antiviral agents were added to our armamentarium in the fight with human immunodeficiency virus type 1 (HIV-1) infection. The antiretroviral therapy (ART) using such agents in combination has been shown to potently suppress HIV-1 replication and extend the life expectancy of HIV-infected individuals [2,3]. Recent analyses have revealed that life expectancy in HIV-infected patients treated with ART was significantly extended between 1996 and 2005, that mortality rates for HIV-infected persons have become close to general mortality rates since the introduction of ART, and that the advent of the first-line ART with boosted PI-based regimens made the development of HIV resistance relatively less likely within and across drug classes [4-7].

However, we have encountered a number of challenges in achieving the optimal benefits of the currently available therapeutics of AIDS and HIV-1 infection in individuals receiving ART. They include i) drug-related toxicities; ii) only partial restoration of immunologic functions achieved once individuals developed AIDS; iii) development of various cancers as a consequence of survival prolongation; iv) flame-up of inflammation in individuals receiving ART or immune reconstitution syndrome (IRS); and v) increased cost of antiviral therapy [8,9]. Importantly, HIV-1 is believed to ultimately develop resistance to any existing antiretroviral regimens. It is thus critical that efforts to develop more potent and safer therapeutics that are effective

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to wild-type isolates as well as existing drug-resistant HIV-1 strains and delay or prevent the emergence of HIV-1 variants resistant to those very therapeutics must be continued.

1.2 CCR5 as a target for developing therapeutic agents

After the identification of CD4 as the primary receptor for HIV entry into the cells of the immune system, it soon became evident that CD4 alone was not sufficient to establish a productive HIV-1 infection, but it took another 10 years until 1996 when the G-protein-coupled 7-transmembrane chemokine receptors CXCR4 (CXC-chemokine receptor 4) and CCR5 (CC-chemokine receptor 5) were finally identified as the coreceptors for HIV-1 entry [10-18]. HIV-1 infection is initiated by the attachment of the virus envelope glycoprotein, gp120, to CD4 on the target cell. The gp120 binding to CD4 triggers a conformational change in gp120, resulting in exposure of the otherwise concealed binding domain of gp120 for a chemokine receptor that acts as a coreceptor [19-21]. Interactions of the gp120/CD4 complex with CCR5 subsequently trigger rearrangement of the transmembrane subunit of the envelope glycoprotein, gp41, which leads to fusion of the virus and cell membranes [22-25]. The predominant chemokine receptors used as coreceptors for entry by primary HIV-1 isolates are CCR5 and CXCR4, although other chemokine receptors including CCR2 and CCR3 can be used by some virus isolates with much lower efficiency [26-30]. CCR5 is the most important coreceptor for the macrophage (M)-tropic (also designated as R5) strains that are commonly transmitted between individuals, and CXCR4 is the most relevant coreceptor for the T-cell-tropic (also referred to as X4) isolates that emerge after several years following initial HIV-1 infection [30-32]. Blocking the function of CCR5 may not significantly impact human health since approximately 1% of Caucasians naturally lack CCR5 due to a protein-disrupting mutation without any detectable consequences [33-36], although certain reports have demonstrated that CCR5△32 homozygosity is significantly associated with fatal outcome when CCR5△32-lacking individuals are infected with West Nile virus (WNV), and that CCR5 mediates resistance to symptomatic WNV infection [37]. In turn, a recent pooled analysis has shown that the CCR5△32 deletion reduces the risk of non-HIV-related non-Hodgkin lymphoma (NHL) in HIV-1-uninfected Caucasian men and NHL risk was also reduced in men with the CCR2/CCR5 haplotype [38]. Thus, although clinical trials have shown no clinically relevant differences in effects between individuals receiving MVC and those receiving the placebo, the long-term safety of blocking CCR5, a receptor whose function in healthy individuals is not fully understood, remains to be determined.

2. Medical need

As discussed above, although the appearance of combined antiretroviral therapy made a sizable impact on the prognosis of HIV-1 infection and AIDS, we have faced various challenges such as toxicities, the emergence of drug-resistant HIV-1 variants, etc. There are a substantial number of patients who do not tolerate ART, harbor multi-drug-resistant HIV-1 variants, and do not respond to any existing ART regimens. All small-molecule CCR5 inhibitors cause allosteric changes in CCR5's, conformation critical for the binding of gp120/CD4 complex and block the fusion between the cellular and viral membranes, inhibiting the entry of the virus. Entry inhibition is unique in that the virus entry *per se* is blocked, the feature totally differing from the mechanisms of other classes of HIV-1 inhibitors such as reverse transcriptase inhibitors, integrase inhibitors, and protease inhibitors, all of which block HIV-1 replication by inhibiting the replication cycle after HIV-1's cellular entry. Thus, it is expected that novel CCR5 inhibitors exert activity to wild-type HIV-1 species as well as HIV-1 variants resistant to the existing classes of antiretroviral drugs. If properly combined with other classes of antiretroviral drugs, CCR5 inhibitors are expected to work synergistically or at least in an additive manner.

3. Market review and existing treatment

HIV-1 infection is recognized as a global threat and is a major health problem in many countries. The UNAIDS reported in 2010 that there are 33.3 million people living with HIV-1/AIDS in the world, with 2.6 million new HIV-1 infections per year and 1.8 million annual deaths due to AIDS. Since the beginning of the epidemic, almost 60 million people have been infected with HIV-1 and 25 million people have died of HIV-1-related causes [39,40]. Funding for HIV-1/AIDS treatment has increased since 1990s; however, the global economic recession has led to declining financial commitment [41]. It is estimated that 700,000 people received treatment in high-income countries in 2008, and more than 4 million people in low- and middle-income countries had access to HIV-1 treatment at the end of 2008, that was 10-fold increase over five years. However, despite considerable progress in the development of AIDS therapeutics, global coverage remains low: in 2008, only 42% of those in need of treatment received antiviral therapy.

It is considered that the global market for HIV-1/AIDS drugs will continue to grow albeit at a slower rate after 2010 because a series of patent expiries during this period. Currently, more than two dozens of AIDS drugs are approved by the U.S. Food and Drug Administration (FDA), and they are categorized in four classes [reverse transcriptase inhibitors (RTIs), protease inhibitors (PIs), integrase inhibitors, and entry inhibitors including CCR5 inhibitors]. The most recent DHHS (Department of Health and Human Services) guideline suggests several preferred, alternative, and acceptable antiretroviral regimens for antiretroviral therapy-naïve patients, and the CCR5 antagonist-based regimens (MVC + zidovudine/lamivudine, MVC + tenofovir/emtricitabine, or MVC + abacavir/lamivudine) have been listed as acceptable regimens, but they are less satisfactory than preferred or alternative regimens [42].

When MVC was first approved by the U.S. FDA as the first-in-class CCR5 inhibitor in 2007, industry experts forecasted annual MVC sales of $500 million by 2011. However, MVC has been suffering several disadvantages. A significant limitation of MVC is that it is dosed twice daily, whereas one of key drugs, efavirenz, and the leading protease inhibitors in the market such as darunavir and atazanavir are administered once daily (QD). MVC's twice-daily (*bid*) dosing prevents coformulation with other once-daily fixed dose combination tablet such as Truvada® and Epzicom®. Thus, development of more potent novel CCR5 inhibitors with the possibility of once-daily (QD) regimens is urgently needed.

Another significant disadvantage of MVC is that an HIV-1 tropism test such as the costly and time-consuming enhanced sensitivity Trofile® assay developed by Monogram Biosciences of the United States must be performed prior to initiation of MVC treatment. The Trofile assay takes about 2 weeks to perform and requires a plasma HIV-1 RNA level ≥ 1,000 copies/ml, an inconvenience and limitation in its own right for both physicians and patients. In this regard, recent studies, in which V3 genotyping was performed on samples from patients screening for clinical trials of MVC, suggest that genotyping performed as well as phenotyping in predicting the response to MVC [43,44]. Based on such data, accessibility, and cost, European guidelines currently favor genotypic testing for determining coreceptor usage [45]. However, given that there is an uncertainty in interpreting the V3 geneotyping data and fewer logistical barriers to get access to the Trofile assay in the United States, the DHHS Panel presently recommends that the Trofile assay be used as the preferred coreceptor tropism screening test in the country [42]. The access to inexpensive, highly sensitive, and rapid HIV-1 tropism tests should be made available for wider use of CCR5 inhibitors.

4. Scientific rationale

In 1996, the chemokine receptors CXCR4 and CCR5 were identified as coreceptors for HIV-1 entry [30]. CCR5 is an important coreceptor for macrophage-tropic (R5) HIV-1 strains that are seen at the early stage of HIV infection, whereas CXCR4 is the most relevant coreceptor for T-cell-tropic (X4) isolates that emerge in the late stage of HIV-1 infection or AIDS [30]. Blocking CCR5 might not significantly impact human health, as ~1% of Caucasians naturally lack CCR5 with no apparent detectable consequences [33-36]. Thus, CCR5 represents a new target for the intervention of HIV-1 replication. MVC is the first and only CCR5 inhibitor, which has been used in the clinical settings since 2007; however, MVC suffers from several disadvantages as described above.

5. Competitive environment

As for the chemokines' activity to block HIV-1 replication by blocking chemokine receptors, Cocchi *et al.* made an initial demonstration of such activity in the beta-chemokines (RANTES [regulated on activation, normal T expressed and secreted], MIP-1α [macrophage inflammatory protein-1-α], and MIP-1β) [46]. Thus, for early studies to block CCR5, natural ligands (e.g., RANTES) to CCR5 were used with some modifications, but because of their poor oral bioavailability, the focus of the research moved to small-molecule antagonists. To date, various small-molecule CCR5 inhibitors have been reported [47-54], and they have been given generic names with the suffix "-viroc," an abbreviation standing for "viral receptor occupancy."

5.1 Maraviroc

Maraviroc (MVC, Selzentry®, UK-427, 857, Figure 1), the first-in-class CCR5 inhibitor, exerts potent *in-vitro* and *in-vivo* anti-HIV-1 activity. MVC blocks MIP-1α and RANTES-mediated signaling [47]. In a 10-day monotherapy trial conducted in HIV-1-infected patients with R5-HIV-1, the administration of MVC (300 mg twice-daily) resulted in 1.8-$\log_{10}$ reductions of plasma HIV-1 [55]. The safety and efficacy of MVC in ART-experienced patients was examined in MOTIVATE 1 and 2 Phase IIb/III clinical trials, and superior virological response was achieved in patients with lower baseline viral loads (<100,000 copies/ml) and higher CD4 cell counts [56,57]. In these trials, patients were confirmed to have R5-HIV-1 prior to treatment initiation by the Trofile assay (Monogram Biosciences, South San Francisco, California, USA) [58] and were randomized to receive one of two dosages of MVC (300 mg given once or twice daily) or placebo; all patients also received an optimized background regimen on the basis of drug resistance testing and treatment history. In these studies, patients in the MVC arms had plasma HIV-1 RNA reductions that were more than twice as great as those in the control arms (1.7 – 1.9-$\log_{10}$ copies/ml versus 0.8-$\log_{10}$ copies/ml, respectively). Increases in CD4 cell counts were 113 – 128 cells/mm^3 in the MVC groups *vs.* 54 – 69 cells/mm^3 in the placebo groups. Virologic failure was associated with the emergence of CXCR4-using virus in 57% of patients, in whom a repeated tropism test was conducted at the failure time point [56,57]. Recent report of the analysis for the MOTIVATE studies concluded that the MVC 150 mg QD appears to be as effective as *bid* when combined with a booster PI [59]. Safety profile was similar in the placebo and MVC arms in terms of adverse effect-related discontinuation. Analysis of hepatitis B- and C-coinfected patients indicated that there was no increase in hepatic abnormalities; suggesting that MVC can be used safely in coinfected individuals. No increase in malignancy was seen at 48 weeks [60,61].

The MERIT study was conducted to determine the role of MVC *vs.* efavirenz in combination with Combivir®. At 48 weeks, MVC was not noninferior to efavirenz (65.3% undetectable viral load with MVC *vs.* 69.3% with efavirenz) [62,63]. However, after the data from the MERIT trials were re-reviewed with a newer, more sensitive assay in order

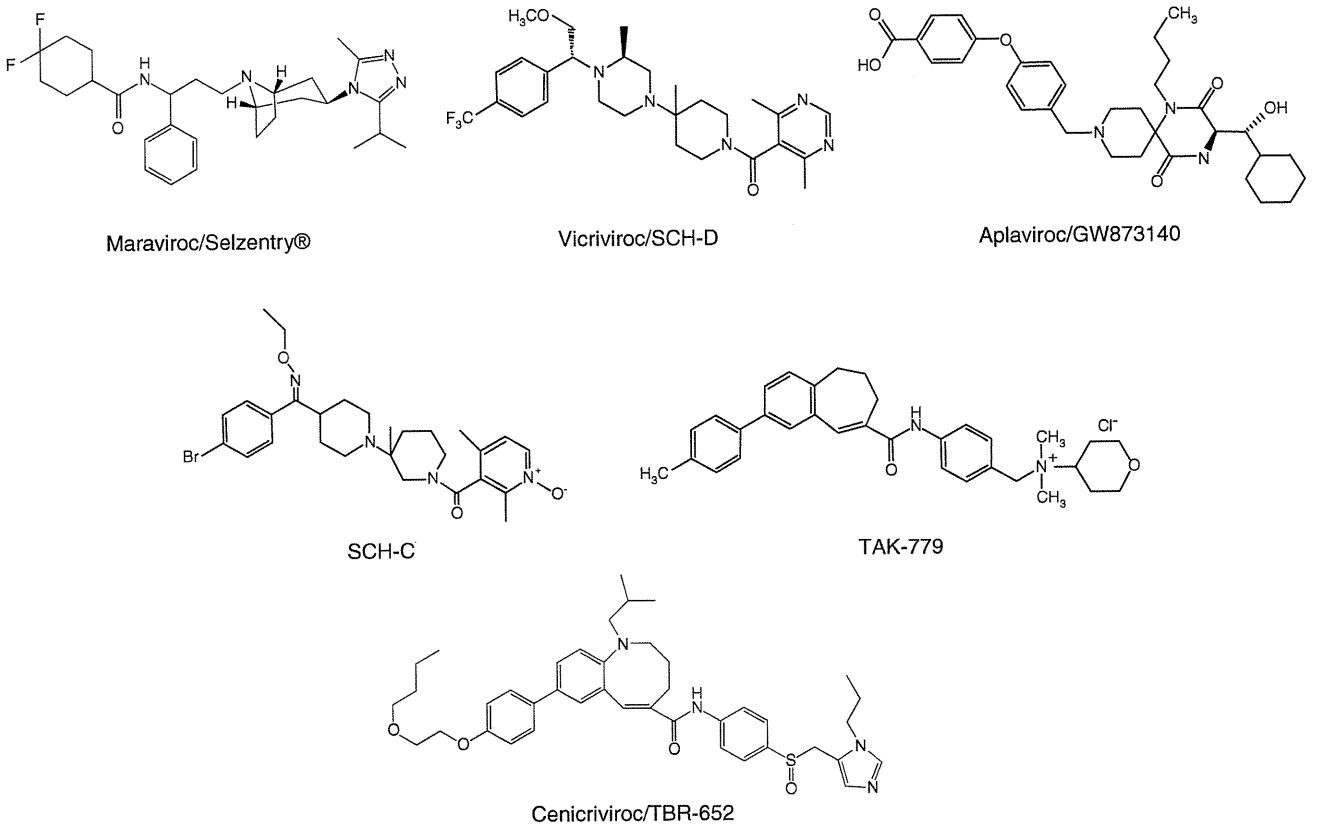

Figure 1. Structures of selected CCR5 inhibitors. MVC is the only clinically approved CCR5 inhibitor.

to exclude individuals harboring non-R5-HIV-1, 68.0% of the MVC-treated patients achieved a viral load less than 50 copies/ml similar to the efavirenz arm [63,64].

Hardy *et al.* reported the 2-year (96 weeks) follow-up of MOTIVATE 1 and 2. HIV-1 RNA was < 50 copies/ml at week 96 in 39% and 41% of patients receiving maraviroc every day and twice a day, respectively. Among patients with HIV-1 RNA < 50 copies/ml at week 48, 81% and 87% of patients receiving maraviroc every day and twice a day, respectively, maintained this response at week 96 [65]. The efficacy through 96 weeks of the MERIT study was also assessed. Proportions of subjects < 50 copies/ml (58.8% maraviroc, 62.7% efavirenz) was similar and maraviroc recipients had greater CD4 increases (+212 *vs.* +171 cells/mm^3) [66].

MVC was accordingly approved by the FDA in August 2007 for treatment-experienced people, who have HIV-1 strains that are resistant to multiple antiretroviral drugs. In November 2009, MVC was approved for individuals with drug-sensitive HIV-1 strains as a first-line drug in combination with other antiretroviral drugs.

5.2 Vicriviroc

SCH-351125 (SCH-C) and SCH-D (vicriviroc, Figure 1) are orally bioavailable CCR5 inhibitors with potent antiviral activity [48,49]. SCH-C was administered to HIV-1-infected subjects in the setting of monotherapy over 10 days. Mean viral load reductions when administered 25 – 100 mg *bid* were by up to -1.50 $\log_{10}$. However, the compound was associated with cardiac adverse effects (e.g., QTc elongation) and was subsequently dropped off the pipeline. Vicriviroc, which has greater *in vitro* potency than SCH-C, was forwarded to clinical trials. In the Phase IIb clinical study, in which treatment-experienced patients were enrolled, vicriviroc showed > 1.5 $\log_{10}$ decrease of plasma viral load in combination with an optimized background regimen that included a ritonavir-boosted protease inhibitor (PI). In this study, five instances of cancer were observed but the association between vicriviroc and the occurrence of malignancies was not confirmed [67,68]. In 2009, Phase III studies (VICTOR-E3 and VICTOR-E4) enrolling treatment-experienced individuals were launched. In these studies, more than 800 treatment-experienced participants with documented resistance to at least two antiretroviral drug classes were enrolled and assigned to take either 30 mg once-daily vicriviroc or placebo, in combination with an optimized background regimen. However, after 48 weeks, no statistically significant difference was observed between the vicriviroc arm and the placebo arm in achieving viral load < 50 copies/ml (64% *vs.* 62%), and it was concluded that vicriviroc did not reach the predefined threshold for non-inferiority to placebo. Thus,

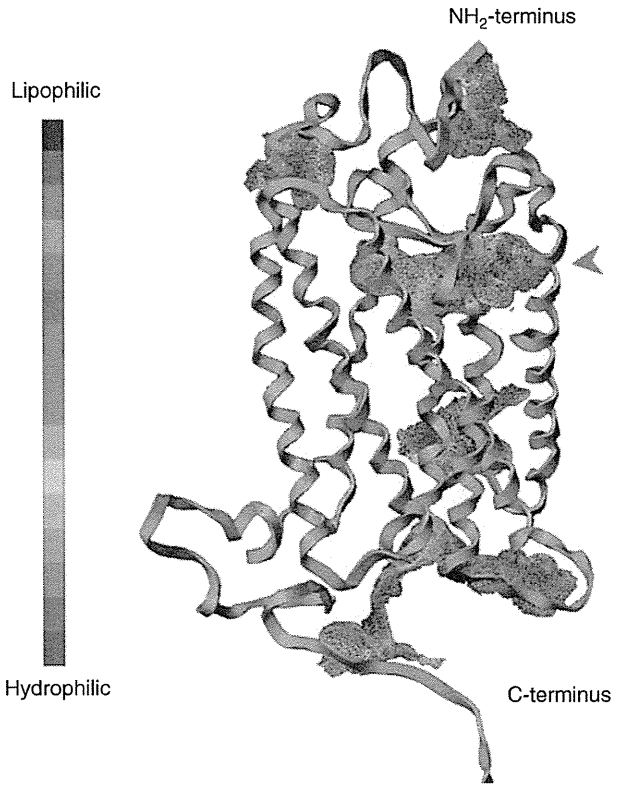

Figure 2. Hydrophobic cavities identified within CCR5. Six hydrophobic cavities are identified within human CCR5, defined using MOLCAD (Sybyl 7.0) [90]. Note the largest hydrophobic cavity (red arrowhead), which is likely to accommodate a molecule of the size of MVC [94], aplaviroc [90], and other CCR5 inhibitors.

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the development of vicriviroc for the treatment of HIV-1 infection was discontinued by Merck in 2010 [69].

5.3 Aplaviroc

Aplaviroc (APL or GSK873140, **Figure 1**), a spirodiketopiperazine derivatives, was developed and reported in 2004 [50]. APL had a high affinity to CCR5 (K_D values of ~3 nM), blocked HIV-1-gp120/CCR5 binding, and exerted potent activity against a wide spectrum of R5-HIV-1 isolates including multi-drug-resistant HIV-1 strains (IC_{50} values of 0.1 – 0.6 nM) *in vitro* [50]. In human peripheral blood mononuclear cell-transplanted R5 HIV-1_{JR-FL}-infected, non-obese diabetic-SCID interleukin-2 receptor γ-chain-knock out mice, in which massive and systemic HIV infection occurred, APL produced ~ 2 $\log_{10}$ reduction in viremia [70]. In Phase IIb clinical trials, patients receiving 600 mg of APL twice daily had a mean decrease in viral load of ~ 1.6 $\log_{10}$ from baseline. The Phase III clinical trials of APL involving ~ 2,000 drug-experienced patients with AIDS were implemented in the United States in the summer of 2005; however, Grade 4 idiosyncratic hepatotoxicity occurred in a few patients and all the trials were terminated [71-73].

5.4 INCB009471

INCB009471, whose structure is related to vicriviroc, exerts potent activity against R5-HIV-1 and exhibits long plasma half-life. Administration of a 200-mg once-daily dose in a 14-day Phase IIa study demonstrated ~ 1.8 $\log_{10}$ reduction in plasma HIV-1 RNA. INCB9471 was safe and well tolerated, and Phase II study was completed, but no further studies are planned at this time [74,75].

5.5 Cenicriviroc

TAK-779, a precursor of cenicriviroc, is the first small-molecule CCR5 inhibitor reported in 1999 [51], which was only moderately active against R5-HIV-1. Optimization of TAK-779 produced TAK-652 (Cenicriviroc or TBR-652, **Figure 1**). Cenicriviroc has good oral bioavailability and long plasma half-life, being expected to allow once-daily administration. In Phase IIa study, cenicriviroc demonstrated potent antiviral activity in treatment-experienced HIV patients. Cenicriviroc also interacts with CCR2, which is known to be associated with inflammation-related diseases, and it is expected to be a potential inflammation mediator as well. In 2011, a Phase IIb clinical trial, in which 150 patients were enrolled, was started to investigate its antiviral, immunologic and anti-inflammatory effects in HIV-1-infected patients [52,76,77].

6. Potential development issues

6.1 Resistance to CCR5 inhibitors

In vitro and *in vivo* studies have suggested the two possible mechanisms, by which CCR5 inhibitors acquire resistance: i) tropism change (X4-HIV-1 becomes predominant in the patient body), or ii) emerging R5-HIV-1 variants that can utilize "drug-bound" CCR5 for viral entry to the cell. *In vitro*, MVC-resistant virus was selected and one isolate had A316T and I323V mutations in the V3 loop of gp120. In the virus assay, such a resistant strain had a unique profile; it showed a decrease in the maximal inhibition with MVC, but its IC_{50} value did not shift substantially [78]. It is considered that this pattern was observed because of the noncompetitive profile of MVC inhibition with gp120 binding to CCR5, and the resistant virus acquired the ability to bind CCR5-drug complex (drug-bound CCR5) [78,79]. MVC-resistant recombinants retained sensitivity to both aplaviroc and enfuvirtide [78]. On the contrary, aplaviroc-resistant virus showed a rightward shift in the IC_{50} and the maximal inhibition level did not change. Mutations related to the aplaviroc sensitivity were shown not only in the V3 region but in the V1 and conserved regions in gp120 [80]. In multiple *in vitro* studies, CCR5-inhibitor-resistant viruses retained the same CCR5 tropism and no occurrence of X4 virus was observed, possibly because of its experimental circumstance (for example, cell lines suitable for R5-HIV-1 replication was used) [81,82]. Westby *et al.* reported that selection of an MVC-resistant R5-HIV-1 (SF162-derived) produced a tropism shift to X4-phenotype; however, this was observed in both MVC and control

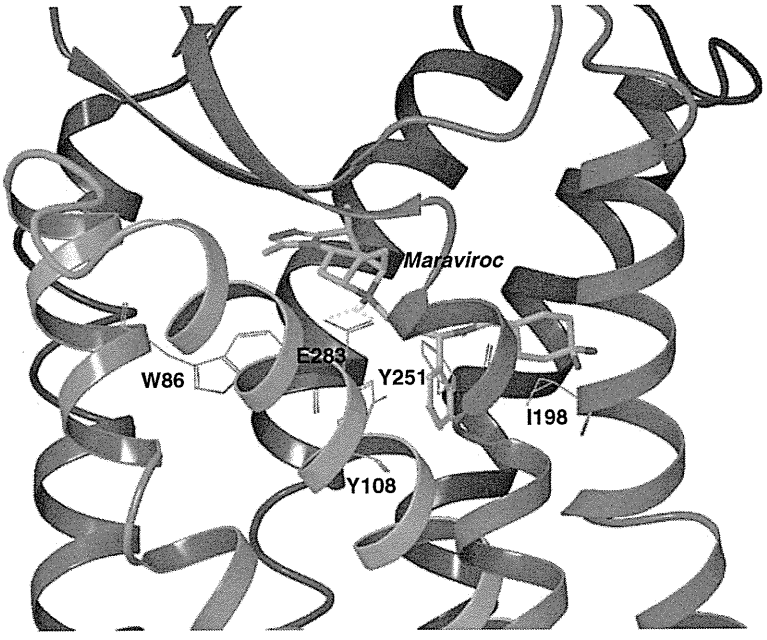

Figure 3. A docked structure of MVC within CCR5. A model of the binding mode of MVC (stick representation) illustrates the relative location of MVC within CCR5. Polar hydrogens are only shown. Note that MVC binds to the hydrophobic cavity shown by a red arrowhead in **Figure 2**, as does aplaviroc [90]. Site-directed mutagenesis experiments conducted by Garcia-Perez *et al.* [94] indicate that MVC has polar interactions with E283 residue, critical for the binding of MVC to CCR5. Other residues important for the binding of MVC are shown in wires.

(no drug) passage cultures, indicating that its appearance was not a result of selective pressure by MVC [78].

A clinical trial (MERIT study) showed that the level of MVC resistance is low if ever, and the virological failure observed was mainly caused by the emergence of X4-HIV-1 that preexisted in patients but was not detected by the tropism assay [83,84]. Other studies (MOTIVATE 1 and 2) showed that 60% patients, who failed treatment with MVC, had X4-HIV-1, while only 6% of patients in the control had X4-HIV-1 [60], indicating that most cases who experienced virological failure with MVC were related to the R5 to X4 tropism change.

6.2 HIV-1 tropism

Prior to initiation of therapy, all patients must take a blood test to determine the tropism (coreceptor usage) of HIV-1 the patient harbors, because CCR5 inhibitors are only active against R5-HIV-1 but not against X4-HIV-1. If some patients have small amounts of X4-HIV-1, even below the detection level, the exposure to a CCR5 inhibitor may allow outgrowth of the X4-HIV-1. The Trofile assay is used to determine the tropism of HIV-1 in patients. In the assay, the virus genome from the patient's HIV-1 is isolated and cloned, the infectious virus is produced, subsequently virus susceptibility to the CCR5 inhibitor is determined using CCR5 and CXCR4 expressing cell lines. In this assay, viral loads >1,000 copies/ml are needed to obtain reliable results, and using the Trofile® enhanced sensitivity assay, 0.3% CXCR4-using HIV-1 variants can be detected with 100% sensitivity [42,58,85]. The requirement of HIV-1 tropism testing before initiation of MVC treatment has been an obstacle for the widened use of MVC. Another way to determine HIV-1 tropism is by genotypic assay [45], which has been favored in European countries over the Trofile® enhanced sensitivity assay. This issue has been described in the "Market review" section of this article.

6.3 Immunological reconstitution

The administration of MVC results in an increase of $CD4^+$ T-cell counts in the blood, a possible advantage that may contribute to an increased immunological function. Of note, this effect was observed in both drug experienced and drug-naïve patients in clinical trials (MOTIVATE 1 & 2, and MERIT study), and even in the group who do not respond virologically to MVC (mean increases of 59 and 43 cells/mm^3 with MVC administration compared with 10 cells/mm^3 in patients treated with placebo; MOTIVATE 1 & 2 studies) [56,63,86]. Recently, Cuzin *et al.* reported a study to address the ability of a 24-week maraviroc intensification of stable and efficient HAART to increase the CD4 cell count slope. In this study, the median slope prior to intensification was +14 cells/µl/year and the slope increased to +23 cells/µl/year in patients who received 24-week maraviroc intensification [87].

6.4 Modeling analysis

While a crystal structure of CCR5 is currently not available, CCR5 structure models have been constructed by homology

modeling technique based on bovine rhodopsin crystal structure [88-90]. **Figure 2** illustrates the three-dimensional model of CCR5 that has a seven transmembrane helical structure. Six hydrophobic cavities were identified in the extracellular, transmembrane, and intracellular domains of CCR5. Among them, a hydrophobic cavity, to which CCR5 inhibitors highly likely bind, was identified (**Figure 2**, red arrow) [90]. In certain studies including ours [90,91], molecular interactions between CCR5 and small-molecule inhibitors were characterized, in which the computational modeling technique was combined with biological data including ligand binding assay using CCR5 mutants [90,91]. Several groups have reported binding modes for CCR5 inhibitors including MVC and aplaviroc. In those studies, all the CCR5 inhibitors have been shown to bind to the same hydrophobic cavity (**Figure 3**) [88-95]. Such approaches of combining site-directed mutagenesis-based data and molecular modeling should be useful for gaining structural insights for developing novel drug design.

7. Conclusion

As of this writing, MVC, a small-molecule CCR5 inhibitor, and enfuvirtide, an oligopeptide fusion inhibitor, are only drugs that have been approved for clinical use as entry inhibitors. Currently, there are only a few entry inhibitors in clinical/preclinical trials. Development of entry inhibitors will undoubtedly improve our ability to manage HIV-1 infection. In particular, development of potent and metabolically-stable novel CCR5 inhibitors with the possibility of once-daily (QD) dosing regimens is urgently needed. Development of CXCR4 inhibitors should complement the limitation of CCR5 inhibitors (i.e., the lack of activity to X4-HIV-1) and greatly improve the efficacy of CCR5 inhibitor-containing regimens.

8. Expert opinion

While the proof-of-principle has been established for the use of entry inhibitors including CCR5 inhibitors for treating HIV-1 infection and AIDS, further data of long-term efficacy of CCR5 inhibitors must be accumulated and examined. Although clinical trials have shown no clinically relevant differences in safety between individuals receiving MVC and those receiving the placebo, the long-term safety of blocking CCR5, a receptor whose function in healthy individuals is not fully understood, is to be determined. Considering the fact that CCR5 inhibitors are active only against R5-HIV-1 strains, there still exist several concerns upon the use of CCR5 inhibitors: i) virtually all HIV-1-infected individuals carry both R5-HIV-1 and X4-HIV-1 and/or dual-tropic HIV-1 species; ii) X4-HIV-1 is known to become predominant when HIV-1 diseases progress; and iii) long-term treatment with CCR5 inhibitors may accelerate the emergence/predominance of X4-HIV-1 and CCR5 inhibitor treatment becomes meaningless. Although the DHHS guideline [42] recommends MVC-containing regimens for treatment-naïve patients, the use of MVC has not been widened. MVC appears to have been used as one of drugs for salvage therapy in heavily ART-experienced patients rather than in drug-naïve patients. This is apparently due to its substantial disadvantages such as its twice-daily dosing schedule in addition to the concerns described above. Another significant disadvantage is that an HIV-1 tropism test such as the costly and time-consuming Trofile enhanced sensitivity assay must be performed prior to the initiation of MVC treatment, an inconvenience for both physicians and patients. The access to inexpensive, highly sensitive, and rapid HIV-1 tropism tests should be made available worldwide. As of this writing, only a few novel CCR5 inhibitors are on the pipeline. Development of more potent and more metabolically-stable novel CCR5 inhibitors with the possibility of once-daily (QD) dosing regimens is urgently needed.

Compared to CCR5 inhibitors, much fewer numbers of CXCR4 inhibitors have been reported as potential therapeutics for treating HIV-1 infection. In fact, no CXCR4 inhibitors have been approved for clinical use as of today. Hence, there is an urgent need for novel small-molecule inhibitors targeting CXCR4. Such CXCR4 inhibitors, if they become clinically available, should greatly improve the treatment options available to patients infected with X4- and/or dual-tropic HIV-1 strains in combination with a CCR5 inhibitor. In this regard, the crystal structures of CXCR4 in complex with CXCR4 inhibitors have recently been solved [96] and opened up the possibility of identifying and designing novel CXCR4 inhibitors.

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Declaration of interest

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