INTRODUCTION

Human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) is a key enzyme that converts single-stranded genomic RNA into double-stranded DNA, which in turn is transported to the nucleus and integrated into the host cell genome. The HIV-1 RT catalyzes both the RNA- and DNA-dependent DNA polymerase, and RNase H activities (11). The functional form of HIV-1 RT is a heterodimer consisting of p66 and p51 polypeptides (16). The p66 subunit has both enzymatic activities and includes the polymerase and the RNase H domains. The polymerase domain consists of the fingers, palm and thumb subdomains which are analogous to a right hand connected to the RNase H domain through the connection subdomain (42) (Fig. 1).

The p51 subunit lacks the RNase H domain and has been proposed to play a structural role (42), although recent work from our laboratory has also shown that mutations in the p51 subunit affect the polymerase and RNase H enzymatic functions of RT (38).

Highly active antiretroviral therapies (HAART) have been very effective in suppressing viral loads and having a significant impact on the life expectancy of HIV patients. Key components of HAART are drugs that target HIV RT. The two classes of RT inhibitors currently used in the clinic are nucleoside/nucleotide RT inhibitors (NRTI) and non-nucleoside RT inhibitors (NNRTI). NRTIs inhibit RT by acting as chain-terminators after they are incorporated into the nascent DNA chain. NNRTIs act non-competitively by binding to a hydrophobic pocket adjacent to, but distinct from the polymerase active site of RT and by imposing rigidity to the movements of thumb subdomain required for efficient polymerase function (20, 22, 35, 37, 39, 41).

Despite the phenomenal success of HAART regimens, continuous use of antivirals leads to the emergence of viruses that are resistant to all known anti-AIDS drugs. The mutations associated with NNRTI resistance are generally located at the NNRTI binding pocket (NNIBP). However, the mutations that cause resistance to NRTIs have been noted to be scattered in the polymerase domain (22, 39). While most NNRTI and NRTI resistance mutations are at the palm and fingers subdomains of HIV-1 RT, it has recently been shown that some mutations associated with NNRTI and NRTI resistance are at the connection and RNase H regions of RT (6, 8, 13, 15, 17, 29, 43). The most significant of these mutations is N348I, which confers moderate resistance to both NRTIs and NNRTIs, and is present in a significant number of clinical isolates, especially in the presence of other NRTI mutations.

In light of the new emerging drug resistance mutations, it is essential to identify inhibitors that are very potent and effective against viral strains that are resistant to all approved therapeutics. One such inhibitor is 4′-ethynyl-2-fluoro-2′-deoxyadenosine triphosphate (EFdA-TP) (18, 19). We have recently reported the mechanism of HIV inhibition by EFdA (26). In contrast to other approved NRTIs, which have a modification at 3′OH, EFdA contains a 3′OH moiety and blocks DNA synthesis by locking the primer terminus at the pre-translocation site of HIV-1 RT. In addition to EFdA, we have recently shown that ENdA also inhibits HIV RT potently acting as a TDRTI (data not shown).

Recently, using transient-state kinetic experiments we established the mechanism of NNRTI resistance of HIV-1 RT containing the N348I mutation at the connection subdomain of the enzyme (38). We showed that the resistance to the NNRTI nevirapine (NEV) is primarily the result of changes distant from the NNRTI binding pocket, which decrease inhibitor binding (increase $K_{d\text{-}NVP}$) by primarily decreasing the association rate of the inhibitor ($k_{on\text{-}NVP}$). Moreover, the N348I mutation increased nucleic acid binding affinity, enhanced processivity and lowered the catalytic turnover rate of the natural substrate. In this study we

determine the ability of TDRTIs to block reverse transcription by the multi-drug resistant N348I HIV-1 RT as well as other NRTI resistant RTs, D67N/K70R/L210Q/T215F (resistant to AZT by the excision mechanism) D67N/K70R/L210Q/T215F/N348I, and A62V/V75I/F77L/F116Y/Q151M (multidrug resistant to AZT and dideoxynucleotide RT inhibitors).

MATERIALS AND METHODS

Enzymes and Nucleic acids

The RT genes coding for p66 and p51 subunits of BH10 HIV-1 were cloned in the pETDuet-1 vector (Novagen) using restriction sites *NcoI* and *SacI* for the p51 subunit, and *SacII* and *AvrII* for the p66 subunit (2, 38). The sequences coding for a hexa-histidine tag and the 3C protease recognition sequence were added at the N-terminus of the p51 subunit. RT was expressed in BL21 (Invitrogen) and purified by nickel affinity chromatography and monoQ anion exchange chromatography (33). Oligonucleotides used in this study were chemically synthesized and purchased from Integrated DNA Technologies (Coralville, IA). Sequences of the DNA substrates are shown in Table 1. Deoxynucleotide triphosphates and dideoxynucleotide triphosphates were purchased from Fermentas (Glen Burnie, MD). EFdA and ENdA were synthesized by Yamasa Corporation (Chiba, Japan) as described before (30). Using EFdA and ENdA as starting material the triphosphate forms EFdA-TP and ENdA-TP were synthesized by TriLink BioTechnologies (San Diego, CA). Concentrations of nucleotides, EFdA-TP and ENdA-TP were calculated spectrophotometrically on the basis of absorption at 260 nm and their extinction coefficients. All nucleotides were treated with inorganic pyrophosphatase (Roche Diagnostics) as described previously (24) to remove traces of PPi contamination that might interfere with the rescue assay.

Primer extension assays

Inhibition of HIV-1 RT by TDRTIs—DNA template (T_{d31}) was annealed to 5′-Cy3 labeled DNA primer (P_{d18}). To monitor primer extension, the T_{d31}/5′Cy3-P_{d18} hybrid (20 nM) was incubated at 37°C with WT or drug-resistant HIV-1 RTs (20 nM) in a buffer containing 50 mM Tris (pH 7.8) and 50 mM NaCl (RT buffer). Varying amounts of EFdA-TP or ENdA-TP were added and the reactions were initiated by the addition of 6 mM $MgCl_2$ to a final volume of 20 μl. All dNTPs were present at a final concentration of 1 μM. The reactions were terminated after 15 minutes by adding equal volume of 100% formamide containing traces of bromophenol blue. The products were resolved on a 15% polyacrylamide 7 M urea gel. In this and in subsequent assays, the gels were scanned with a PhosphorImager (FujiFilm FLA 5000), the bands for fully extended product were quantified using Multi Gauge (FujiFilm) and results were plotted using one site competition equation on GraphPad Prism 4 to determine the IC_{50} for EFdA-TP and ENdA-TP.

Site-specific Fe^{2+} Footprinting Assay—Site-specific Fe^{2+} footprints were monitored on 5′-Cy3-labeled DNA templates. 100 nM of 5′Cy3-T_{d43}/P_{d20} was incubated with 600 nM WT or N348I RT in a buffer containing 120 mM sodium cacodylate (pH 7), 20 mM NaCl, 6 mM $MgCl_2$, and either of 5 μM ddATP or 1 μM EFdA-TP, to allow quantitative chain-termination. Prior to the treatment with Fe^{2+}, complexes were pre-incubated for 7 min with increasing concentrations of the next incoming nucleotide (dTTP). The complexes were treated with ammonium iron sulfate (1 mM) as previously described (21). This reaction relies on autoxidation of Fe^{2+} (3) to create a local concentration of hydroxyl radical which cleaves the DNA at the nucleotide closest to the Fe^{2+} specifically bound to the RNase H active site.

ATP-dependent Excision and Rescue assay—20 nM of purified T_{d31}/$P_{d18\text{-}EFdA\text{-}MP}$ or T_{r31}/$P_{d18\text{-}EFdA\text{-}MP}$ were incubated with 60 nM WT, N348I, D67N/K70R/L210Q/T215F

or D67N/K70R/L210Q/T215F/N348I RT in the presence of 3.5 mM ATP, 100 μM dATP, 0.5 μM dTTP, and 10 μM ddGTP in RT buffer and 10 mM $MgCl_2$. Aliquots of the reaction were stopped at different time points (0–90 min) and analyzed as described above.

RNase H Assays—RNase H assays were performed by incubating the RNA/DNA duplex5′Cy3-T_{r35}/P_{d25} or 5′Cy3-$T_{r35}/P_{d25\text{-}ddAMP}$ or 5′Cy3-$T_{r35}/P_{d25\text{-}EFdA\text{-}MP}$ (50 nM) with WT or N348I RT (50 nM) in RT buffer at 37 °C with $MgCl_2$ (6 mM). Reactions were quenched after incubation (1–5 min) with equal volumes of formamide containing trace amounts of bromophenol blue. Reaction products were analyzed as before. The primary RNase H cleavage product is mainly 18 nucleotides from the 3′-end of the DNA primer (18 nucleotides), and the secondary cleavage product is mainly 12 nucleotides from the 3′-end of the primer (12 nucleotides) as reported previously (10, 12, 38).

RESULTS

The inhibitors used here to characterize the susceptibility of N348I to various drugs are adenosine analogs. The structures of these analogs are shown in Fig. 2. The normal deoxynucleotide dA is shown in Fig. 2A. EFdA and ENdA are shown in Figs. 2B and 2C, respectively. It can be seen in these figures that unlike other anti-HIV NRTIs both EFdA and ENdA have a 3′OH. These compounds also contain an ethynyl group at the 4′ position. EFdA and ENdA differ in their substitutions at the 2 position of the purine ring. EFdA at this position has fluorine whereas ENdA has an amino group.

Inhibition of WT and N348I mutant of HIV-1 RT

The inhibition of WT, N348I, D67N/K70R/L210Q/T215F, D67N/K70R/L210Q/T215F/N348I mutants of HIV-1 RT by EFdA-TP and ENdA-TP was assessed by a primer extension assay. As shown in Fig. 3A–3D, EFdA-TP and ENdA-TP suppressed RT-catalyzed DNA synthesis in a dose-dependent manner. The IC_{50} values for both analogs are shown in Table 2. N348I, D67N/K70R/L210Q/T215F and D67N/K70R/L210Q/T215F/N348I RTs were inhibited by EFdA-TP and ENdA-TP with similar efficiency compared to the WT enzyme. In addition, another mutant HIV-1 RT (A62V/V75I/F77L/F116Y/Q151M) was included in drug susceptibility assays (Fig. 3E).

We have previously shown that EFdA inhibits DNA synthesis at the point of incorporation. Thus, we examined here the stopping patterns after incorporation products of the primer extension assay for the stopping patterns (Fig. 3). The primer synthesis shown in Fig. 3 clearly demonstrates that the stopping pattern follows the incorporation of adenosine analogs. Three distinct bands at positions 1, 6 and 10 indicate that both analogs inhibit RT mainly at the point of incorporation. Therefore, these compounds act primarily as obligate chain terminators. There is also an additional band at position 7, suggesting that in some instances EFdA may allow addition of one nucleotide after its incorporation, thus acting as a delayed chain terminator (Fig. 3). This type of inhibition is far less common and is sequence-dependent. These finding agrees with our previous studies on WT RT (26).

Effect of EFdA-MP on Translocation of WT and N348I mutant of HIV-1 RT

The connection subdomain mutation N348I has been related to the altered DNA binding affinity and processivity of the mutant enzyme compared to the WT RT (4, 38). Since EFdA is a TDRTI and its incorporation is assumed to affect the translocation and thereby DNA binding and processivity, we investigated the translocation of EFdA-containing template-primers using the hydroxyl radical site-specific footprinting assay (21). The results of the footprinting assay shown in Fig. 4 demonstrate that the presence of EFdA-MP at the 3′ end of the DNA primer blocks translocation and prevents incorporation of the next incoming

dNTP. Therefore, similar to WT RT, the mutant N348I RT is also inhibited by EFdA-TP *via* the same mechanism.

Effect of EFdA-MP on RNase H activity of WT and N348I RTs

The template/primers containing EFdA-MP, ddAMP, or without inhibitor incorporated at the 3′ end of the primer were used in RNase H assays with WT and N348I RTs in a time dependent manner. As previously noted, Fig. 5 shows that N348I mutant RT has decreased RNase H activity for all substrates used in this assay. The RNase H assays carried out in presence of T/P trap showed the disappearance of the secondary cuts for both enzymes used here. This is likely due to a defect in translocation that EFdA imposes on the enzyme. Interestingly, the primary cut of EFdA-terminated primers is a single band when the T/P has EFdA, but not ddA at the 3′ primer terminus. Moreover, the RNA cleavage of T_{r35}/$P_{d25-EFdA-MP}$ was less than that of T_{r35}/$P_{d25\text{-}ddAMP}$ or T_{r35}/P_{d25} possibly because of less favorable positioning at the RNase H of T/P with EFdA at the 3′ terminus.

ATP-dependent unblocking of EFdA-MP terminated primers by WT and N348I RTs

Since EFdA-MP-terminated primers bind predominantly in a pre-translocation mode we expected that EFdA-MP will be efficiently unblocked by both WT and N348I RTs. The ATP-dependent excision and subsequent rescue of EFdA-MP primers is shown in Fig. 6. The bands marked as 'Rescued Primer" have comparable product for the WT and N348I mutant enzyme for both DNA (Fig. 6A) and RNA (Fig. 6B) templates suggesting that resistance mutant N348I does not have any significant effect on the unblocking of EFdA-MP containing primers (RNA *vs.* DNA) (Fig. 6). However, the N348I mutation in the background of AZT resistance mutations D67N, K70R, L210Q and T215F showed a 2-fold increase in unblocking EFdA-MP containing primers both with DNA and RNA templates (Fig. 6).

DISCUSSION

There are currently more than 20 antiretrovirals that have been approved by the US Food and Drug Administration for the treatment of HIV infection. They fall into four categories, targeting HIV RT, protease, integrase, the entry step, and the fusion of the viral and cell membranes. RT inhibitors are either NRTIs or NNRTIs. The NRTIs such zidovudine (AZT) and lamivudine compete with the natural substrates and get incorporated into the nascent DNA chain, blocking further polymerization because they lack a 3′OH group required for DNA synthesis. NNRTIs such as nevirapine and efavirenz inhibit the polymerase activity of RT by binding at a hydrophobic pocket nearly 10 Å away from the polymerase active site (Fig. 1). This pocket is created after the binding of NNRTIs. The highly active antiretroviral therapy (HAART) introduced in the mid-90s contains the combination of antivirals (generally a protease inhibitor and two NRTIs or an NNRTI and two NRTIs) targets the replication of the resistant virus.

Extended or incomplete treatments with antiretrovirals result in the emergence of drug resistance mutations. In the case of drugs that target RT, most of the resistance mutations were found to be present in the polymerase domain of RT. These resistance mutations against NRTIs function primarily with two mechanisms: (i) they reduce the binding affinity/ incorporation of NRTI (34, 40) or (ii) enhance the selective excision of incorporated NRTI from a chain-terminated primer terminus (9, 23–25, 36). The resistance against NNRTIs is primarily through the mutations that reduce the binding affinity of NNRTIs (7, 31, 32, 35).

Recent studies showed that connection subdomain mutations can confer resistance to NRTIs. Nikolenko et al. suggested that some of these mutations increase AZT resistance by

reducing template RNA degradation, thereby preserving the RNA template and providing additional time for RT to excise AZT monophosphate (27, 28). Hachiya et al., (13) as well as another research group (43) identified a clinical isolate with phenotypic resistance to nevirapine (NVP) in the absence of known NNRTI mutations. This resistance was shown to be caused by N348I, a mutation at the connection subdomain of HIV-1 RT. This mutation is not a polymorphism, as it exists in more than 10% of drug-treated, but not drug-naïve HIV patients. The connection subdomain mutation N348I has been related to the altered DNA binding affinity and processivity of the mutant enzyme compared to the WT RT (4, 38). Ehteshami et al. showed that N348I enhances resistance to AZT through both RNase H-dependent and -independent mechanisms (10). Since EFdA is a TDRTI and its incorporation is assumed to affect the translocation and thereby DNA binding and processivity, we investigated the susceptibility of two highly potent antiretrovirals EFdA and ENdA.

We report that both EFdA-TP and ENdA-TP are very potent inhibitors of N348I, D67N/K70R/L210Q/T215F, D67N/K70R/L210Q/T215F/N348I, and A62V/V75I/F77L/F116Y/Q151M RTs. They inhibit RT primarily at the point of incorporation and since they prevent enzyme translocation they both belong to the TDRTI class of NRTIs. The D67N/K70R/L210Q/T215F set of mutations are the classical thymidine-associated mutations (TAMs), which are known to cause resistance to AZT by enhancing excision of AZT-terminated primers (1, 5, 23). The A62V/V75I/F77L/F116Y/Q151M set of mutations is known as the "Q151M" complex RT, and has been known as a multidrug-resistance mutation, since the latter mutations are known to be involved in resistant variants with reduced susceptibility to dideoxynucleotides and to AZT. Unlike D67N/K70R/L210Q/T215F RT, the Q151M complex decreases susceptibility to NRTIs by decreasing incorporation efficiency of the inhibitors rather than increasing excision and unblocking of chain-terminated primers (14). Finally, N348I is known to cause resistance to both NRTIs and NNRTIs. Hence, collectively, these mutants represent all mechanisms by which RT becomes resistant to available antivirals. Importantly, we find that they are all susceptible to the EFdA and ENdA TDRTIs. Hence, this new class of RT inhibitors should be able to efficiently block viruses that carry clinically relevant mutations, including the new connection domain mutation N348I.

Acknowledgments

This work was supported by the Ministry of Knowledge and Economy, Bilateral International Collaborative Research and Development Program, Republic of Korea, National Institutes of Health Grants AI076119, AI074389, and AI100890 (to S. G. S.) and AI079801 (to M.A.P.).

Abbreviations

EFdA	4′-ethynyl-2-fluoro-2′-deoxyadenosine
ENdA	4′-ethynyl-2-amino-2′-deoxyadenosine
NRTI	Nucleoside reverse transcriptase inhibitor
RT	Reverse transcriptase
T/P_{EFdA}	Template/Primer terminated with 4′-ethynyl-2-fluoro-2′-deoxyadenosine
TDRTI	Translocation defective reverse transcriptase inhibitor
WT	wild-type

References

1. Arion D, Kaushik N, McCormick S, Borkow G, Parniak MA. Phenotypic mechanism of HIV-1 resistance to 3′-azido-3′-deoxythymidine AZT: increased polymerization processivity and enhanced sensitivity to pyrophosphate of the mutant viral reverse transcriptase. Biochemistry. 1998; 37:15908–15917. [PubMed: 9843396]
2. Bauman JD, Das K, Ho WC, Baweja M, Himmel DM, Clark AD Jr, Oren DA, Boyer PL, Hughes SH, Shatkin AJ, Arnold E. Crystal engineering of HIV-1 reverse transcriptase for structure-based drug design. Nucleic Acids Res. 2008; 36:5083–5092. [PubMed: 18676450]
3. Biaglow JE, Kachur AV. The generation of hydroxyl radicals in the reaction of molecular oxygen with polyphosphate complexes of ferrous ion. Radiat Res. 1997; 148:181–187. [PubMed: 9254738]
4. Biondi MJ, Beilhartz GL, McCormick S, Gotte M. N348I in HIV-1 reverse transcriptase can counteract the nevirapine-mediated bias toward RNase H cleavage during plus-strand initiation. J Biol Chem. 285:26966–26975. [PubMed: 20530477]
5. Boyer PL, Sarafianos SG, Arnold E, Hughes SH. Selective excision of AZTMP by drug-resistant human immunodeficiency virus reverse transcriptase. J Virol. 2001; 75:4832–4842. [PubMed: 11312355]
6. Brehm JH, Koontz D, Meteer JD, Pathak V, Sluis-Cremer N, Mellors JW. Selection of mutations in the connection and RNase H domains of human immunodeficiency virus type 1 reverse transcriptase that increase resistance to 3′-azido-3′-dideoxythymidine. J Virol. 2007; 81:7852–7859. [PubMed: 17507476]
7. Das K, Ding J, Hsiou Y, Clark AD Jr, Moereels H, Koymans L, Andries K, Pauwels R, Janssen PA, Boyer PL, Clark P, Smith RH Jr, Kroeger Smith MB, Michejda CJ, Hughes SH, Arnold E. Crystal structures of 8-Cl and 9-Cl TIBO complexed with wild-type HIV-1 RT and 8-Cl TIBO complexed with the Tyr181Cys HIV-1 RT drug-resistant mutant. J Mol Biol. 1996; 264:1085–1100. [PubMed: 9000632]
8. Delviks-Frankenberry KA, Nikolenko GN, Barr R, Pathak VK. Mutations in human immunodeficiency virus type 1 RNase H primer grip enhance 3′-azido-3′-deoxythymidine resistance. J Virol. 2007; 81:6837–6845. [PubMed: 17428874]
9. Dharmasena S, Pongracz Z, Arnold E, Sarafianos SG, Parniak MA. 3′-Azido-3′-deoxythymidine-(5′)-tetraphospho-(5′)-adenosine, the product of ATP-mediated excision of chain-terminating AZTMP, is a potent chain-terminating substrate for HIV-1 reverse transcriptase. Biochemistry. 2007; 46:828–836. [PubMed: 17223704]
10. Ehteshami M, Beilhartz GL, Scarth BJ, Tchesnokov EP, McCormick S, Wynhoven B, Harrigan PR, Gotte M. Connection domain mutations N348I and A360V in HIV-1 reverse transcriptase enhance resistance to 3′-azido-3′-deoxythymidine through both RNase H-dependent and -independent mechanisms. J Biol Chem. 2008; 283:22222–22232. [PubMed: 18547911]
11. Goff SP. Retroviral reverse transcriptase: synthesis, structure, and function. J Acquir Immune Defic Syndr. 1990; 3:817–831. [PubMed: 1694894]
12. Gotte M, Rausch JW, Marchand B, Sarafianos S, Le Grice SF. Reverse transcriptase in motion: conformational dynamics of enzyme-substrate interactions. Biochim Biophys Acta. 1804:1202–1212. [PubMed: 19665597]
13. Hachiya A, Kodama EN, Sarafianos SG, Schuckmann MM, Sakagami Y, Matsuoka M, Takiguchi M, Gatanaga H, Oka S. Amino acid mutation N348I in the connection subdomain of human immunodeficiency virus type 1 reverse transcriptase confers multiclass resistance to nucleoside and nonnucleoside reverse transcriptase inhibitors. J Virol. 2008; 82:3261–3270. [PubMed: 18216099]
14. Hachiya A, Kodama EN, Schuckmann MM, Kirby KA, Michailidis E, Sakagami Y, Oka S, Singh K, Sarafianos SG. K70Q adds high-level tenofovir resistance to "Q151M complex" HIV reverse transcriptase through the enhanced discrimination mechanism. PloS one. 2011; 6:e16242. [PubMed: 21249155]
15. Hachiya A, Shimane K, Sarafianos SG, Kodama EN, Sakagami Y, Negishi F, Koizumi H, Gatanaga H, Matsuoka M, Takiguchi M, Oka S. Clinical relevance of substitutions in the connection subdomain and RNase H domain of HIV-1 reverse transcriptase from a cohort of antiretroviral treatment-naive patients. Antiviral Res. 2009; 82:115–121. [PubMed: 19428602]

16. Jacobo-Molina A, Ding J, Nanni RG, Clark AD Jr, Lu X, Tantillo C, Williams RL, Kamer G, Ferris AL, Clark P, et al. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 A resolution shows bent DNA. Proceedings of the National Academy of Sciences of the United States of America. 1993; 90:6320–6324. [PubMed: 7687065]
17. Julias JG, McWilliams MJ, Sarafianos SG, Alvord WG, Arnold E, Hughes SH. Mutation of amino acids in the connection domain of human immunodeficiency virus type 1 reverse transcriptase that contact the template-primer affects RNase H activity. J Virol. 2003; 77:8548–8554. [PubMed: 12857924]
18. Kawamoto A, Kodama E, Sarafianos SG, Sakagami Y, Kohgo S, Kitano K, Ashida N, Iwai Y, Hayakawa H, Nakata H, Mitsuya H, Arnold E, Matsuoka M. 2′-deoxy-4′-C-ethynyl-2-halo-adenosines active against drug-resistant human immunodeficiency virus type 1 variants. Int J Biochem Cell Biol. 2008; 40:2410–2420. [PubMed: 18487070]
19. Kodama EI, Kohgo S, Kitano K, Machida H, Gatanaga H, Shigeta S, Matsuoka M, Ohrui H, Mitsuya H. 4′-Ethynyl nucleoside analogs: potent inhibitors of multidrug-resistant human immunodeficiency virus variants in vitro. Antimicrob Agents Chemother. 2001; 45:1539–1546. [PubMed: 11302824]
20. Kohlstaedt LA, Wang J, Friedman JM, Rice PA, Steitz TA. Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science. 1992; 256:1783–1790. [PubMed: 1377403]
21. Marchand B, Gotte M. Site-specific footprinting reveals differences in the translocation status of HIV-1 reverse transcriptase. Implications for polymerase translocation and drug resistance. J Biol Chem. 2003; 278:35362–35372. [PubMed: 12819205]
22. Menendez-Arias L, Berkhout B. Retroviral reverse transcription. Virus Res. 2008; 134:1–3. [PubMed: 18299161]
23. Meyer PR, Matsuura SE, Mian AM, So AG, Scott WA. A mechanism of AZT resistance: an increase in nucleotide-dependent primer unblocking by mutant HIV-1 reverse transcriptase. Mol Cell. 1999; 4:35–43. [PubMed: 10445025]
24. Meyer PR, Matsuura SE, So AG, Scott WA. Unblocking of chain-terminated primer by HIV-1 reverse transcriptase through a nucleotide-dependent mechanism. Proceedings of the National Academy of Sciences of the United States of America. 1998; 95:13471–13476. [PubMed: 9811824]
25. Meyer PR, Matsuura SE, Tolun AA, Pfeifer I, So AG, Mellors JW, Scott WA. Effects of specific zidovudine resistance mutations and substrate structure on nucleotide-dependent primer unblocking by human immunodeficiency virus type 1 reverse transcriptase. Antimicrob Agents Chemother. 2002; 46:1540–1545. [PubMed: 11959594]
26. Michailidis E, Marchand B, Kodama EN, Singh K, Matsuoka M, Kirby KA, Ryan EM, Sawani AM, Nagy E, Ashida N, Mitsuya H, Parniak MA, Sarafianos SG. Mechanism of inhibition of HIV-1 reverse transcriptase by 4′-Ethynyl-2-fluoro-2′-deoxyadenosine triphosphate, a translocation-defective reverse transcriptase inhibitor. J Biol Chem. 2009; 284:35681–35691. [PubMed: 19837673]
27. Nikolenko GN, Delviks-Frankenberry KA, Palmer S, Maldarelli F, Fivash MJ Jr, Coffin JM, Pathak VK. Mutations in the connection domain of HIV-1 reverse transcriptase increase 3′-azido-3′-deoxythymidine resistance. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104:317–322. [PubMed: 17179211]
28. Nikolenko GN, Delviks-Frankenberry KA, Pathak VK. A novel molecular mechanism of dual resistance to nucleoside and nonnucleoside reverse transcriptase inhibitors. J Virol. 2010; 84:5238–5249. [PubMed: 20219933]
29. Nikolenko GN, Palmer S, Maldarelli F, Mellors JW, Coffin JM, Pathak VK. Mechanism for nucleoside analog-mediated abrogation of HIV-1 replication: balance between RNase H activity and nucleotide excision. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102:2093–2098. [PubMed: 15684061]
30. Ohrui H, Kohgo S, Hayakawa H, Kodama E, Matsuoka M, Nakata T, Mitsuya H. 2′-Deoxy-4′-C-ethynyl-2-fluoroadenosine: a nucleoside reverse transcriptase inhibitor with highly potent activity

against all HIV-1 strains, favorable toxic profiles and stability in plasma. Nucleic Acids Symp Ser (Oxf). 2006:1–2.

31. Ren J, Esnouf R, Hopkins A, Ross C, Jones Y, Stammers D, Stuart D. The structure of HIV-1 reverse transcriptase complexed with 9-chloro-TIBO: lessons for inhibitor design. Structure. 1995; 3:915–926. [PubMed: 8535785]
32. Ren J, Nichols C, Bird L, Chamberlain P, Weaver K, Short S, Stuart DI, Stammers DK. Structural mechanisms of drug resistance for mutations at codons 181 and 188 in HIV-1 reverse transcriptase and the improved resilience of second generation non-nucleoside inhibitors. J Mol Biol. 2001; 312:795–805. [PubMed: 11575933]
33. Sarafianos SG, Clark AD Jr, Tuske S, Squire CJ, Das K, Sheng D, Ilankumaran P, Ramesha AR, Kroth H, Sayer JM, Jerina DM, Boyer PL, Hughes SH, Arnold E. Trapping HIV-1 reverse transcriptase before and after translocation on DNA. J Biol Chem. 2003; 278:16280–16288. [PubMed: 12554739]
34. Sarafianos SG, Das K, Clark AD Jr, Ding J, Boyer PL, Hughes SH, Arnold E. Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance with beta-branched amino acids. Proceedings of the National Academy of Sciences of the United States of America. 1999; 96:10027–10032. [PubMed: 10468556]
35. Sarafianos SG, Das K, Hughes SH, Arnold E. Taking aim at a moving target: designing drugs to inhibit drug-resistant HIV-1 reverse transcriptases. Curr Opin Struct Biol. 2004; 14:716–730. [PubMed: 15582396]
36. Sarafianos SG, Hughes SH, Arnold E. Designing anti-AIDS drugs targeting the major mechanism of HIV-1 RT resistance to nucleoside analog drugs. Int J Biochem Cell Biol. 2004; 36:1706–1715. [PubMed: 15183339]
37. Sarafianos SG, Marchand B, Das K, Himmel DM, Parniak MA, Hughes SH, Arnold E. Structure and Function of HIV-1 Reverse Transcriptase: Molecular Mechanisms of Polymerization and Inhibition. J Mol Biol. 2009; 385:693–713. [PubMed: 19022262]
38. Schuckmann MM, Marchand B, Hachiya A, Kodama EN, Kirby KA, Singh K, Sarafianos SG. The N348I mutation at the connection subdomain of HIV-1 reverse transcriptase decreases binding to nevirapine. J Biol Chem. 285:38700–38709. [PubMed: 20876531]
39. Singh K, Marchand B, Kirby KA, Michailidis E, Sarafianos SG. Structural Aspects of Drug Resistance and Inhibition of HIV-1 Reverse Transcriptase. Viruses. 2010; 2:606–638. [PubMed: 20376302]
40. Sluis-Cremer N, Arion D, Parniak MA. Molecular mechanisms of HIV-1 resistance to nucleoside reverse transcriptase inhibitors (NRTIs). Cell Mol Life Sci. 2000; 57:1408–1422. [PubMed: 11078020]
41. Smerdon SJ, Jager J, Wang J, Kohlstaedt LA, Chirino AJ, Friedman JM, Rice PA, Steitz TA. Structure of the binding site for nonnucleoside inhibitors of the reverse transcriptase of human immunodeficiency virus type 1. Proceedings of the National Academy of Sciences of the United States of America. 1994; 91:3911–3915. [PubMed: 7513427]
42. Wang J, Smerdon SJ, Jager J, Kohlstaedt LA, Rice PA, Friedman JM, Steitz TA. Structural basis of asymmetry in the human immunodeficiency virus type 1 reverse transcriptase heterodimer. Proceedings of the National Academy of Sciences of the United States of America. 1994; 91:7242–7246. [PubMed: 7518928]
43. Yap SH, Sheen CW, Fahey J, Zanin M, Tyssen D, Lima VD, Wynhoven B, Kuiper M, Sluis-Cremer N, Harrigan PR, Tachedjian G. N348I in the connection domain of HIV-1 reverse transcriptase confers zidovudine and nevirapine resistance. PLoS Med. 2007; 4:e335. [PubMed: 18052601]

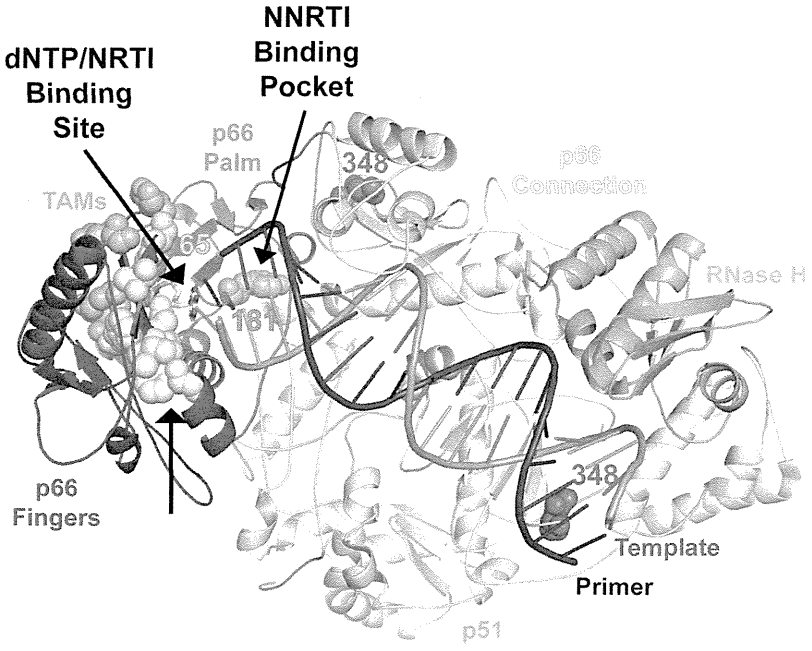


Figure 1. HIV-1 RT structure with highlighted residues of drug-resistance

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.

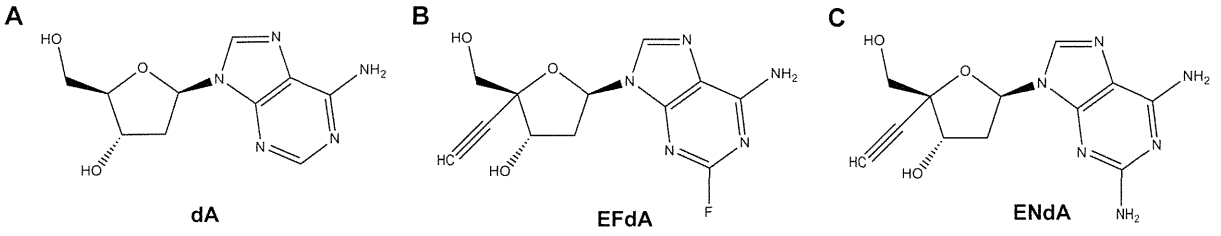


Figure 2.
Chemical structures of dA, EFdA and ENdA

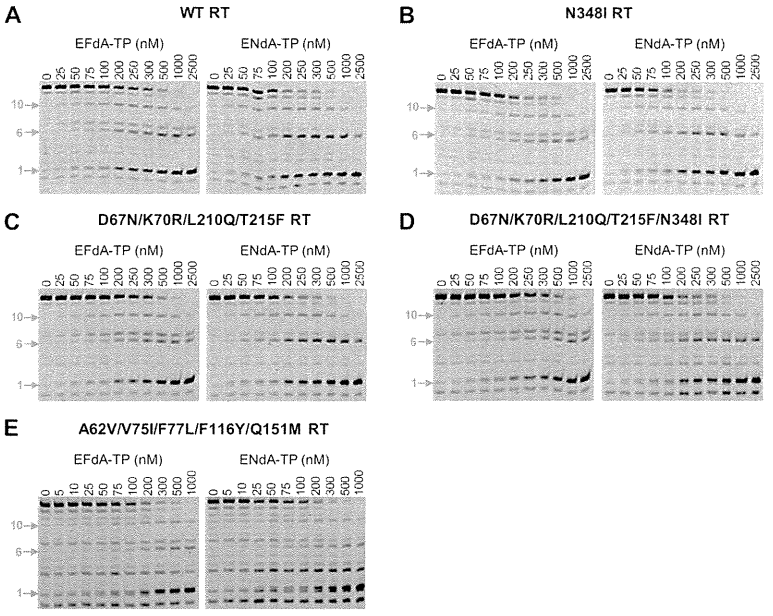


Figure 3. EFdA-TP and ENdA-TP inhibit WT and drug-resistant HIV-1 RTs

(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.

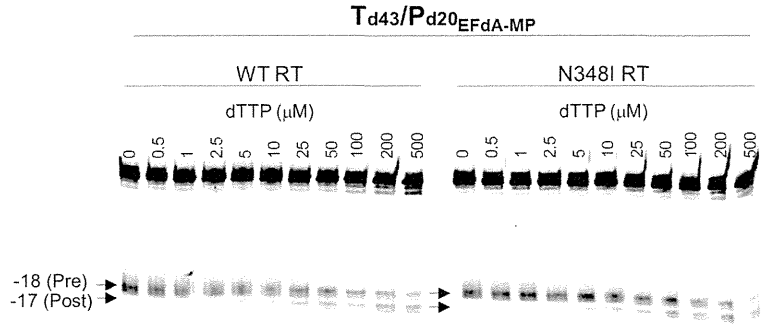


Figure 4. Determination of the translocation state of WT or N348I HIV-1 RT bound to T_{d43}/$P_{20\text{-}EFdA\text{-}MP}$

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.

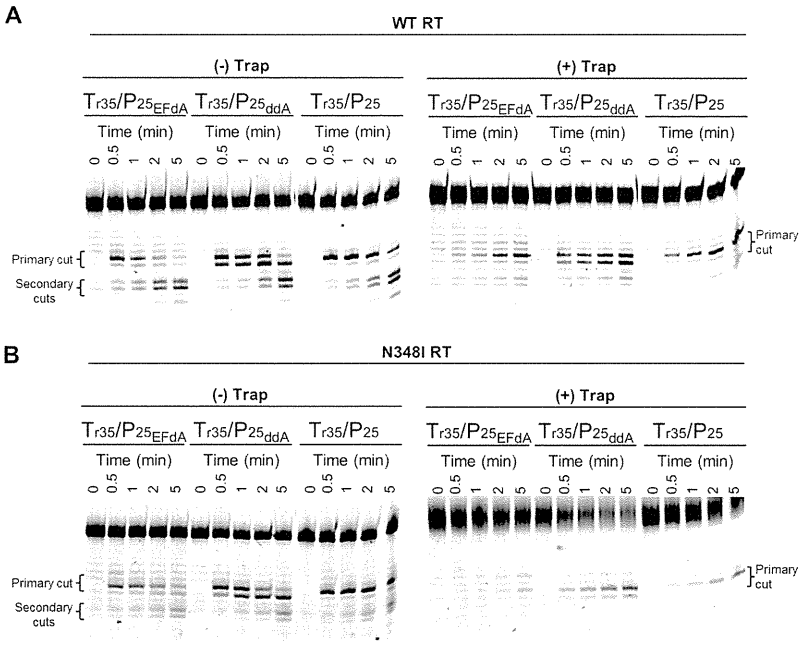
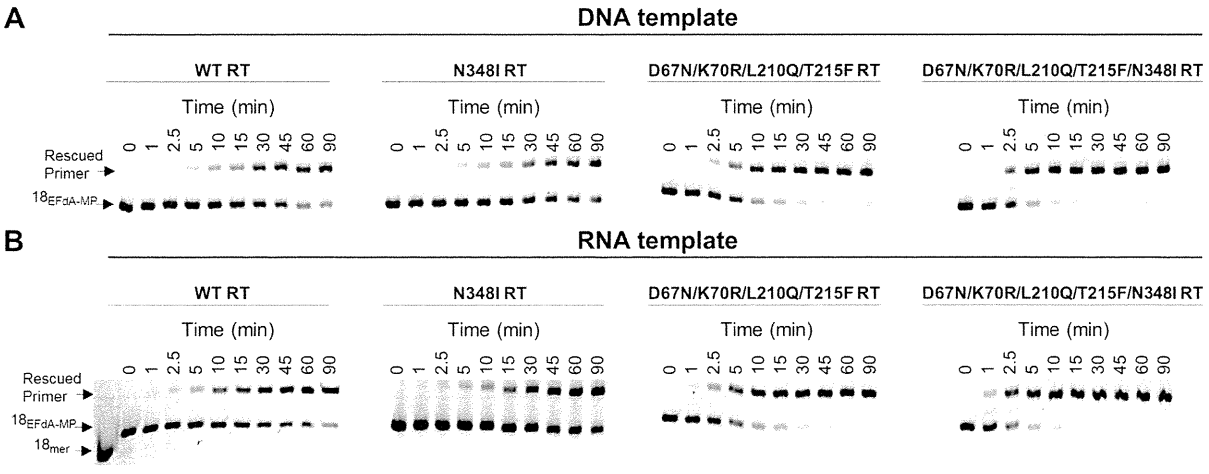


Figure 5. Effect of EFdA on RNase H activity of WT and N348I HIV-1 RTs

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

Cell Mol Biol (Noisy-le-grand). Author manuscript; available in PMC 2013 January 23.

EXPERT OPINION

CCR5 inhibitors: emergence, success, and challenges

Kenji Maeda[†], Debananda Das, Hirotomo Nakata & Hiroaki Mitsuya
[†]*National Cancer Institute, Experimental Retrovirology Section, HIV and AIDS Malignancy Branch, Bethesda, MD, USA*

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

Expert Opin. Emerging Drugs (2012) 17(2):135-145

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

informa
healthcare

10.1517/14728214.2012.673584 © 2012 Informa UK, Ltd. ISSN 1472-8214

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

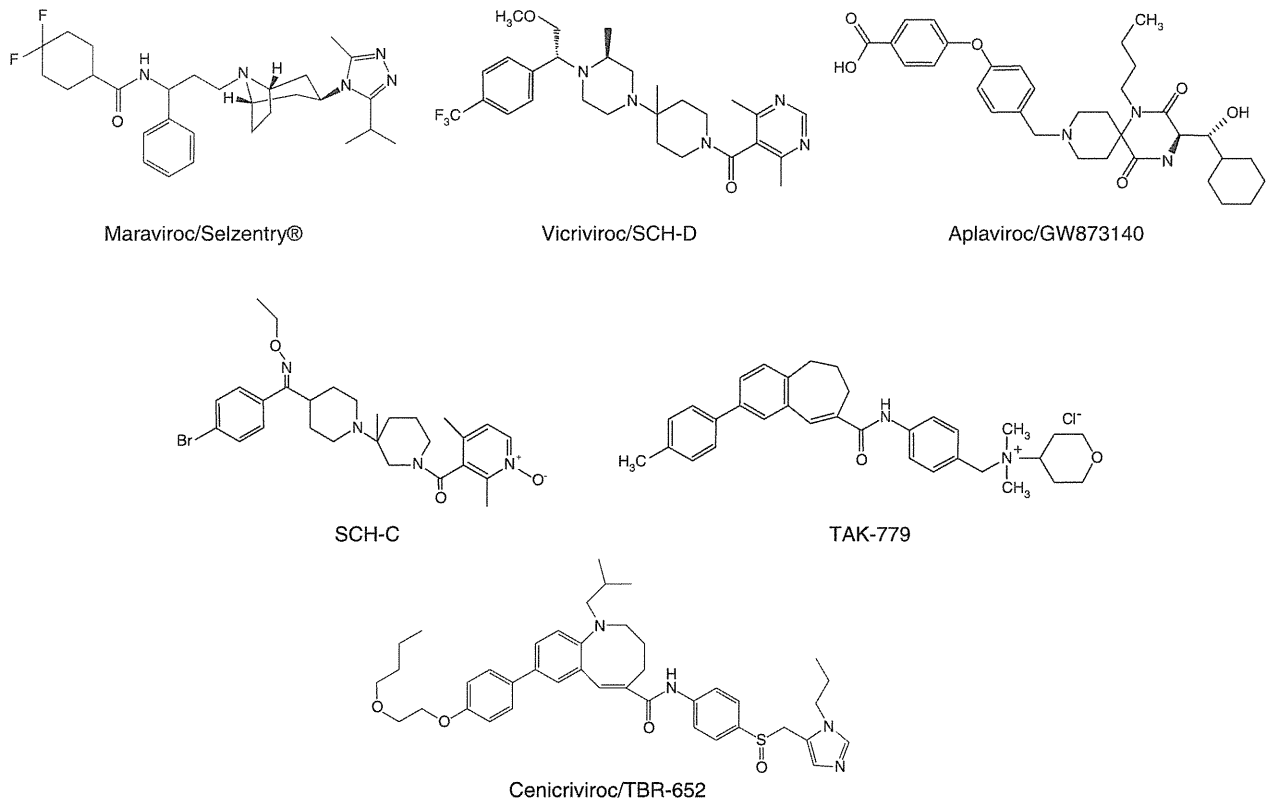


Figure 1. Structures of selected CCR5 inhibitors. MVC is the only clinically approved CCR5 inhibitor.

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,