TABLE 4. Sensitivities of infectious molecular HIV clones carrying various amino acid substitutions to GRL-02031[a]

Recombinant HIV-1 clone	EC_{50} (μM) of GRL-02031	Fold change in EC_{50}	Fold change in EC_{50} compared with that of other PIs	Fold change in EC_{50} of other PIs reported previously (reference)
pNL4-3 (wild-type)	0.023 ± 0.008	1.0		
L10F	0.037 ± 0.001	1.6	1.4 (APV)	1.5 (IDV) (42)
L33F	0.028 ± 0.005	1.2	1.0 (RTV)	1.4 (APV) (31)
M46I	0.028 ± 0.009	1.2	1.2 (RTV)	1.0 (APV) (40)
I47V	0.037 ± 0.006	1.6	1.2 (APV)	2.2 (APV) (31)
Q58E	0.033 ± 0.007	1.4	1.0 (APV)	Not previously reported
V82I	0.035 ± 0.001	1.5	1.5 (RTV)	1.9 (APV) (31)
I84V	0.030 ± 0.0001	1.3	2.2 (IDV)	10.6 (IDV) (41)
I85V	0.024 ± 0.011	1.0	2.1 (RTV)	Not previously reported
M46I/I47V	0.073 ± 0.009	3.2	1.3 (APV)	1.0 (APV) (40)
V82I/I85V	0.035 ± 0.002	1.5	1.6 (RTV)	Not previously reported
I84V/I85V	0.097 ± 0.010	4.2	14.8 (RTV)	Not previously reported
L10F/I47V/V82I/I85V	0.43 ± 0.06	18.7	1.9 (RTV)	Not previously reported
L10F/M46I/I47V/V82I/I85V	>1	>43	10.0 (APV)	Not previously reported
D30N	0.020 ± 0.009	0.9	5.6 (NFV)	6.0 (NFV) (41)
G48V	0.040 ± 0.0008	1.7	5.1 (SQV)	7.0 (SQV) (21)
I50V	0.015 ± 0.008	0.7	1.2 (APV)	3.5 (APV) (31)
L90M	0.032 ± 0.001	1.4	1.0 (SQV)	3.0 (SQV) (21)

[a] MT-4 cells (1×10^4/ml) were exposed to 100 $TCID_{50}$s of each infectious molecular HIV clone, and the inhibition of p24 Gag protein production by the drug was used as the endpoint on day 7 in culture. The fold change represents the ratio of the EC_{50} for each mutant clone to the EC_{50} for wild-type $HIV\text{-}1_{NL4\text{-}3}$. All assays were performed in triplicate, and the values shown are mean values (±1 standard deviation) derived from the results of three independent experiments.

tightly in the binding cavity and has favorable polar and nonpolar interactions with the active-site residues of the HIV-1 protease. The van der Waals surfaces of Ile47 and Ile47′ and of Ile84′ demonstrate that they form tight nonpolar interactions with GRL-02031. Our antiviral data showing that the I47V substitution is associated with HIV-1 resistance to GRL-02031 (Tables 3 and 4) are in agreement with this structural finding, in that the substitution should reduce GRL-02031's interaction with protease and helps develop HIV-1 resistance to the inhibitor.

DISCUSSION

In the present work, we demonstrated that GRL-02031 suppresses the replication of a wide spectrum of HIV-1 isolates and is potent against a variety of HIV-1 variants highly resistant to multiple PIs, with the differences in the EC_{50}s being less than twofold in comparison with the EC_{50} for wild-type strain $HIV\text{-}1_{ERS104pre}$ (Table 2). Additionally, when $HIV\text{-}1_{NL4\text{-}3}$ was propagated in the presence of increasing concentrations of IDV, APV, or GRL-02031, the time of emergence of HIV-1 variants highly resistant to GRL-02031 was substantially delayed compared to that of IDV- or APV-resistant HIV-1 variants (Fig. 2). Indeed, 21, 27, and 37 passages were required for HIV-1 to acquire the ability to propagate in the presence of APV, IDV, and GRL-02031 at 5 μM, respectively. In this regard, when we generated a variety of PI-resistant HIV-1 variants by propagating laboratory strain $HIV\text{-}1_{NL4\text{-}3}$ in the presence of increasing concentrations of a PI in MT-4 cells using the same procedure as that used in the present study, it required 27, 23, 22, 21, and 14 passages for the virus to propagate in the presence of 5 μM of SQV, APV, IDV, NFV, and RTV, respectively (26). However, it should be noted that the population size of HIV-1 in a culture is relatively small and that the viral acquisition of mutations can be affected by stochastic phenomena. For example, mutations take place at random and the rates of mutations in the HIV-1 genome may not be reproducible, although certain mutations that severely compromise viral replication would not remain in culture.

During the selection of $HIV\text{-}1_{NL4\text{-}3}$ with GRL-02031, the L10F substitution, one of the secondary substitutions, first appeared. The L10F mutation occurs distal to the active site of the enzyme and is thought to act in concert with active-site mutations and compensate for a possible functional deficit caused by the latter (6, 32). Mutations at Leu-10 reportedly occur in 5 to 10% of HIV-1 isolates recovered from untreated HIV-1-infected individuals but increase in prevalence by 60 to 80% in heavily treated patients (19, 22). However, the virological and structural significance of the L10F substitution in HIV-1 resistance to GRL-02031 is presently unknown.

By passage 37, two active-site mutations (V82I and I84V) emerged. These V82 and I84 residues represent active-site residues whose side chains are involved in the formation of the protease substrate cleft and that make direct contact with certain PIs (48), and the V82I substitution has been shown to be effective in conferring resistance when it is combined with a second active-site mutation, such as V32I (23). Another active-site mutation (I85V) and two flap mutations (M46I and I47V) also emerged by passage 30. Both Met46 and Ile47 are located in the flap region of the enzyme; the I47V substitution is reported to be associated with viral resistance to APV and JE-2147 (40, 48). The lipophilic potential of the computationally defined cavity for the binding of GRL-02031 within the HIV protease seems to be related to a finding that the van der Waals surfaces of Ile47 and Ile47′ and of Ile84′ form tight nonpolar interactions with GRL-02031 (Fig. 4C). Our antiviral data showing that the I47V substitution is associated with HIV-1 resistance to GRL-02031 (Table 3) are in agreement with this structural finding. However, it is also of note that HIV-1 acquires substantial resistance to GRL-02031 when the virus gains multiple mutations in the protease (Table 4), as

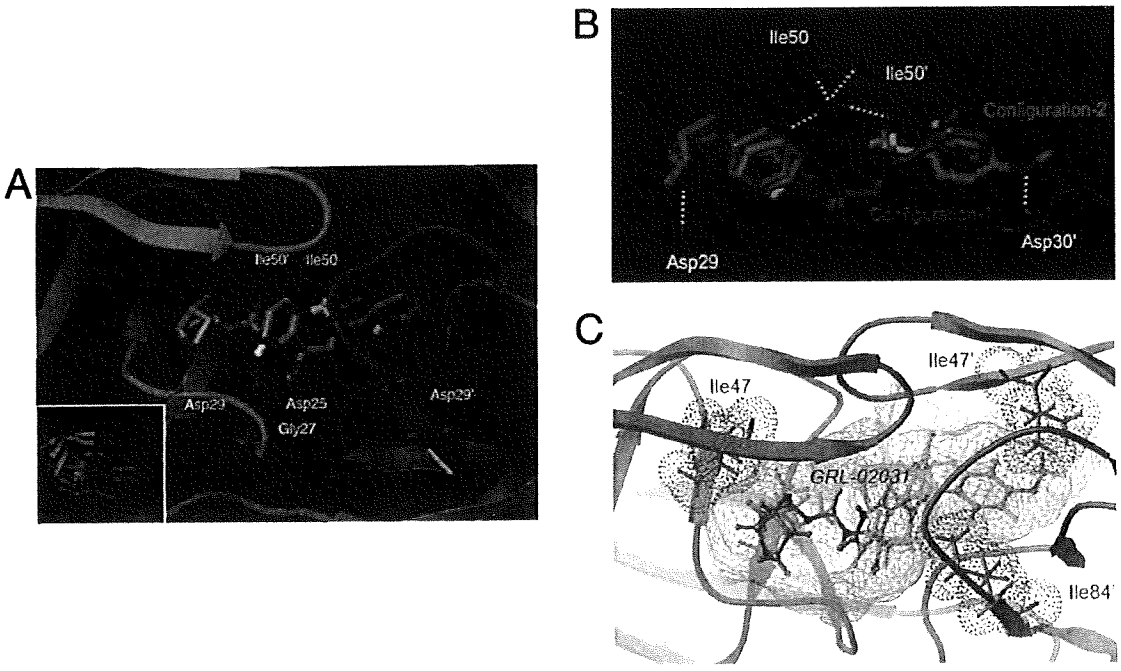

FIG. 4. Molecular interactions of GRL-02031 with HIV-1 protease. (A) A model of the interaction of GRL-02031 with HIV protease. The bird's-eye view of the docked pose (inset) is presented along with a blown-up figure highlighting the important hydrogen bond interactions. The inhibitor is predicted to have hydrogen bond interactions with Asp25, Gly27, Asp29, Ile50, Asp29′, and Ile50′. Note that the pyrrolidone oxygen (red stick) interacts with the S-2′ subpocket and forms a hydrogen bond interaction with Asp29′. (B) Superimposed binding configurations of GRL-02031 with the HIV-1 protease. The carbons are shown in gray in configuration 1 and in green in configuration 2. Selected hydrogen bond interactions of configuration 2 are shown. In configuration 2, the methoxybenzene interacts with the S-2′ site and forms a hydrogen bond interaction with Asp30′. The interaction of the P-2 ligand Cp-THF is the same in both configurations. (C) The binding cavity of HIV protease with lipophilic potential is shown. GRL-02031 fits tightly in the binding cavity and has favorable polar and nonpolar interactions with the active-site residues of the HIV-1 protease. The van der Waals surfaces of Ile47 and Ile47′ (both in magenta) and of Ile84′ (in purple) demonstrate that they form tight nonpolar interactions with GRL-02031. The protease residues are shown in stick representation. The following atoms are indicated by designated colors: C, gray; O, red; N, blue; S, yellow; H, cyan. Both protease chains are shown in green. The figure was generated with the MOLCAD program (Sybyl, version 8.0; Tripos, L.P, St. Louis, MO).

seen in the case of DRV (9). This resistance profile (i.e., the requirement of multiple mutations) of GRL-02031 may also confer certain advantage in the resistance profile of GRL-02031.

Two mutations at conserved residues, L33F and Q58E, also emerged by passage 37 and were present in 10 and 9 of 10 clones, respectively. L33F has primarily been reported in patients treated with RTV or APV (37). The L33F substitution alone did not change the susceptibility of HIV-1 to GRL-02031 (Table 4), although it has recently gained attention because of its association with resistance to the FDA-approved PI, tipranavir (33).

In the HIV-1 variants selected with GRL-02031, four amino acid substitutions in the Gag proteins (G62R, R409K, L363M, and I437T) were seen by passage 37. R409K within the p7 Gag seems to be associated with viral resistance to APV (14), although the significance of G62R within p17 is as yet unknown. The p7-p1 cleavage-site mutation I437T has been reported to be associated with ATV resistance (17). It is of note that by passage 15, an unusual amino acid substitution, L363M, emerged; this substitution has not previously been reported in relation to PI resistance. This L363M is located at the p24-p2 cleavage site, which represents the C terminus of the capsid (CA) p24 protein that is highly conserved and that is involved in virion assembly. The deletion of this cluster or the introduction of mutations such as L363A is known to cause significant impairment of particle formation and infectivity (34). It is noteworthy that L363M appears in HIV-1 variants resistant to a maturation inhibitor, PA-457 [3-*O*-(3′,3′-dimethylsuccinyl) betulinic acid], which binds to the CA-p2 cleavage site or its proximity, blocks the cleavage by protease during virion maturation, and exerts activity against HIV-1 (27, 44, 49).

It was noted that GRL-02031 and SQV remained active against most of the PI-selected HIV-1 variants and that SQV, IDV, and NFV remained potent against HIV-$1_{GRL\text{-}02031\text{-}5\ \mu M}$ (Table 3), suggesting that the combination of GRL-02031, SQV, IDV, and NFV can exert complementarily augmented activity against multi-PI-resistant HIV-1 variants. Such a difference in the resistance profile of GRL-02031 when it is used with SQV and NFV may be due to the differences in binding and antiviral potency associated with the D30N and G48V mutations (Table 4).

In an attempt to explain why GRL-02031 can exert potent activity against a wide spectrum of HIV-1 variants resistant to multiple PIs, we performed structural modeling and molecular docking of the interactions of GRL-02031 with protease (Fig. 4). Interestingly, our structural modeling analysis demonstrated that there are two distinct binding modes of GRL-

02031 in the S-2′ pocket of the protease. Either the 2-pyrrolidone group or the methoxybenzene moiety can orient toward Asp29′ and Asp30′ (configuration 1 and configuration 2, respectively) (Fig. 4B). It is presumed that such alternate binding modes provide distinct advantages to GRL-02031 in maintaining its antiviral activity against a wide spectrum of HIV-1 variants resistant to other currently available PIs. The alternate binding modes could explain the reason why the development of resistance to GRL-02031 is substantially delayed compared to the time to the development of resistance to APV or IDV (Fig. 2). In addition, the models of GRL-02031 indicated that it is capable of forming hydrogen bond interactions with the backbone atoms of Asp29, Asp29′, and/or Asp30′. Such backbone interactions have been shown to be important in maintaining potency not only against wild-type protease but also against drug-resistant mutant proteases (1, 15, 16, 36). This may also explain why GRL-02031 maintains its potency against a wide variety of drug-resistant mutant proteases.

It is of note that the difference seen with GRL-02031 (one- to twofold) seems substantially less than that seen with DRV (one- to sevenfold) (Table 2). Although this difference may not be translated into an actual difference in the clinical setting, it is worth noting that GRL-02031 may have certain advantages in its activity against highly drug-resistant HIV-1 variants. Considering that the acquisition of multiple amino acid substitutions is required for the emergence of HIV-1 resistance to GRL-02031, the profile of HIV-1 resistance to GRL-02031, which is apparently different from the profiles for the other PIs, might result in an advantage for GRL-02031, although further evaluations, including testing of the compound in the clinical setting, are required.

Taken together, GRL-02031 exerts potent activity against a wide spectrum of laboratory and clinical wild-type and multidrug-resistant HIV-1 strains without significant cytotoxicity in vitro and substantially delays the emergence of HIV-1 variants resistant to GRL-02031. These data warrant further consideration of GRL-02031 as a candidate as a novel PI for the treatment of AIDS.

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Chapter 2

Entry Inhibitors of Human Immunodeficiency Virus

MASANORI BABA

Highly active antiretroviral therapy (HAART) based on the combination of different classes of inhibitors has dramatically improved the prognosis of human immunodeficiency virus type 1 (HIV-1) infection after its establishment (52, 109). In fact, more than 20 drugs, targeting reverse transcriptase and protease, are available in clinic for the treatment of HIV-1 infection. Since reverse transcriptase and protease are virus-specific enzymes, the emergence of drug-resistant viruses caused by amino acid mutations of the enzymes often results in treatment failure of HAART (36). Furthermore, there are few drug options in the case of treatment failure because of cross-resistance to the same class of compounds (40). Therefore, a lot of attempts have been made to identify novel anti-HIV-1 agents targeting a molecule different from reverse transcriptase and protease. Among the potential targets, viral entry and proviral DNA integration processes are considered to be the most promising, and some inhibitors of these processes have already been licensed or are currently under phase II or III clinical studies (Table 1). In this review, I focus on the viral entry processes that play crucial roles in HIV-1 replication and describe inhibitors thereof.

TARGET IDENTIFICATION

The HIV-1 envelope glycoprotein complex consists of the surface subunit gp120 and the transmembrane subunit gp41. The two subunits remain noncovalently associated and oligomerize as trimers on the surface of virions (13). The entry of HIV-1 into the target cells is initiated by the binding of the envelope glycoprotein gp120 to the cellular receptor protein CD4 (Fig. 1a). Thus, it is easily predicted that blocking viral attachment to the target cells (gp120-CD4 interaction) results in complete protection of the cells from HIV-1 infection. In fact, a number of substances have been shown to interfere with this step and strongly inhibit HIV-1 replication in vitro (18). However, each of them appears to have its own problems for clinical development, which is discussed later. The binding of gp120 to CD4 results in a complex series of conformational changes in both gp120 and gp41, which includes exposure and increased affinity of the binding site of gp120 to a chemokine receptor, either CCR5 or CXCR4 (67, 107). Binding of gp120 to one of the chemokine receptors is indispensable for subsequent steps of viral entry to proceed (Fig. 1b). Although several chemokine receptors have been shown to interact with gp120, CCR5 and CXCR4 play a major role as coreceptors of HIV-1 (10).

HIV-1 using CCR5 as a coreceptor (R5 HIV-1) is isolated predominantly during the asymptomatic stage and is also responsible for viral transmission between individuals (14). In addition, R5 HIV-1 seems to play an important role even in the advanced stage of the disease (53, 95). Thus, it has been considered that CCR5 is an attractive target for inhibition of HIV-1 replication. This idea has also been encouraged by the observation that individuals having homozygous CCR5-Δ32, a truncated and nonfunctional form of CCR5, display profound resistance to HIV-1 infection without apparent immune dysfunction (16, 55, 77). These results suggest that CCR5 antagonists are effective as anti-HIV-1 agents without serious side effects despite targeting the host cellular molecule CCR5. On the other hand, the rationale of using CXCR4 antagonists for treatment of HIV-1 infection is still a matter of controversy. The emergence of CXCR4-using (X4) HIV-1 is clearly associated with accelerated $CD4^+$ T-cell depletion and rapid progress to the end stage of the disease (14, 73). However, animal studies demonstrated that the natural CXCR4 ligand SDF-1 was essential during fetal development, especially for B-cell lymphopoiesis, bone marrow myelopoiesis, and cardiac ventricular septum formation (68). Furthermore, CXCR4 is expressed in developing vascular endothelial

Masanori Baba • Division of Antiviral Chemotherapy, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima 890–8544, Japan.

Table 1. Entry inhibitors approved for clinical use or in clinical studies[a]

Stage	Name	Mechanism
Phase I	TAK-652	CCR5 antagonist
Phase I/IIa	PRO 140	Anti-CCR5 monoclonal antibody
	INCB9471	CCR5 antagonist
	HGS004	Anti-CCR5 monoclonal antibody
Phase IIb	TNX-355	Anti-CD4 monoclonal antibody
Phase III	Vicriviroc (SCH-D)	CCR5 antagonist
Approved	Maraviroc (UK-427, 857, Selzentry)	CCR5 antagonist
	Enfuvirtide (T-20, Fuzeon)	Fusion inhibitor targeting gp41

[a]The inhibitor list is based on the currently available information through the Internet. Therefore, it may not cover all of the entry inhibitors in clinical studies, or their development may have already proceeded to the next stage at the time of publication.

cells, and mice lacking CXCR4 or SDF-1 had defective formation of the large vessels supplying the gastrointestinal tract (86). Although the small-molecule CXCR4 antagonist AMD3100 was found to be well tolerated in humans (35), the safety and usefulness of CXCR4 antagonists should be carefully monitored in further clinical studies.

The transmembrane glycoprotein gp41 is composed of three domains: the ectodomain, the membrane-spanning domain, and the cytoplasmic tail (13, 102). The ectodomain contains an N-terminal fusion peptide that is essential for membrane fusion. It also contains two regions with a hydrophobic heptad repeat (HR-1 and HR-2), whose sequence motif is characterized as coiled coils. Binding of gp120 to either CCR5 or CXCR4 leads to conformational changes of gp41, which allows the fusion peptide to interact with the target cell membrane (Fig. 1c). The heptad repeat regions of gp41 fold into a six-helix bundle (Fig. 1d). Consequently, the viral envelope and cellular membrane come closer together and complete membrane fusion (Fig. 1e). Since synthetic peptides corresponding to either HR-1 or HR-2 amino acid sequences are capable of inhibiting membrane fusion, gp41 is also considered to be an attractive target for inhibition of HIV-1 replication.

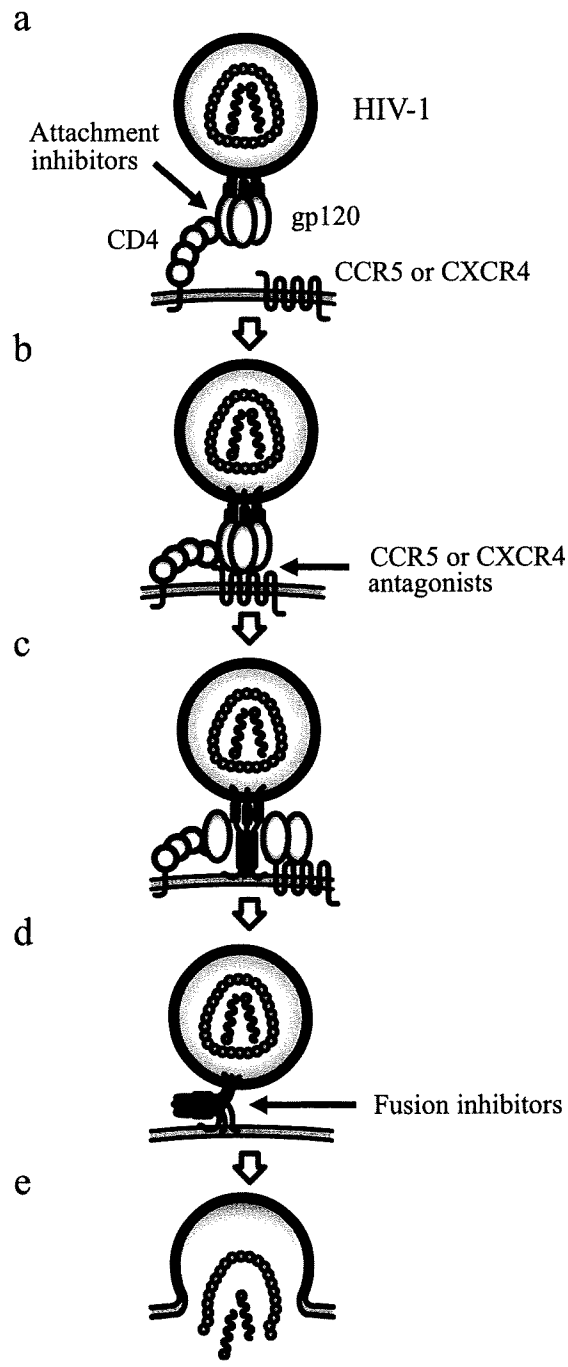

Figure 1. A schematic presentation of HIV-1 entry. (a) The first step of HIV-1 entry to the host cells is gp120 binding to the primary receptor CD4. (b) After binding, the conformation of gp120 changes, which induces exposure of the coreceptor binding domain. Consequently, gp120 binds to a coreceptor, either CCR5 or CXCR4. (c) The binding of gp120 to a coreceptor leads to conformational changes of gp41 that allow insertion of its fusion peptide into the target cell membrane. (d) Then, the heptad repeat (HR-1 and HR-2) regions of gp41 fold into a six-helix bundle (hairpin structure). (e) The bundle formation brings viral envelope and cell membrane closer together and facilitates their fusion. The closed arrows indicate the molecules or steps that entry inhibitors interact with.

INHIBITORS AND MECHANISMS OF ACTION

Viral Attachment Inhibitors

A variety of polyanionic compounds have been reported to inhibit HIV-1 replication in vitro through blocking virus attachment to the target cells (3, 17). Representative molecules are dextran sulfate and heparin. Early studies demonstrated that dextran sulfate exerted its anti-HIV-1 activity by inhibiting the adsorption of viral particles to the target cells (7, 63). Later, their mechanism of action was found to be electrostatic

neutralization to the positively charged residues in the V3 loop of gp120 (65, 105). Although sulfated polyanions are highly potent inhibitors of X4 HIV-1 replication in cell cultures, their activity proved to be much weaker against R5 HIV-1 (62). Furthermore, the results of clinical trials with dextran sulfate were quite disappointing due to its poor oral bioavailability and high toxicity upon intravenous administration (28).

Cyanovirin-N (CV-N) is a small (11-kDa) carbohydrate-binding protein (lectin) isolated from the cyanobacterium *Nostoc ellipsosporum* with highly potent anti-HIV-1 activity (12). The antiviral activity of CV-N is mediated via high-affinity interactions with the mannose-rich oligosaccharides of gp120 (11, 25). In addition to CV-N, several carbohydrate-binding agents have been identified as effective inhibitors of HIV-1 replication in vitro (8). However, their clinical development does not seem to be feasible because of poor oral bioavailability, high toxicity, and potential immunogenicity in vivo; nevertheless, the use of CN-V as a topical microbicide to prevent HIV-1 transmission is of considerable interest (93).

BMS-378806 (Fig. 2a) is a small-molecule HIV-1 inhibitor that blocks viral attachment to the target cells. The compound exhibited potent antiviral activity against a panel of R5, X4, and R5X4 (dualtropic) HIV-1 strains in vitro (31, 54). However, HIV-2 and simian immunodeficiency virus were not susceptible to this compound. Initially, it was thought that BMS-378806 bound to gp120 and inhibited the interaction between gp120 and CD4. However, a recent study suggests that the compound binds to gp120 and blocks the induction of HR-1 exposure without significantly affecting the binding of CD4 (81). In animal studies, BMS-378806 displayed favorable pharmacokinetic profiles, including low protein binding, minimal effect of human serum on anti-HIV-1 activity, and good oral bioavailability (54, 108). Although clinical studies have been conducted with BMS-488043 (Fig. 2b), a derivative of BMS-378806, development of this drug as an anti-HIV-1 agent was halted. Nevertheless, like other viral attachment inhibitors, this compound may have potential as a topical microbicide for prevention of HIV-1 transmission (41).

Coreceptor Inhibitors

The first small-molecule and nonpeptide chemokine receptor antagonists having potent and selective anti-HIV-1 activity in vitro were a series of bicyclams (19). The prototype compound AMD3100 (Fig. 3a) proved inhibitory to the replication of several laboratory and clinical HIV-1 strains at nanomolar concentrations or lower (20). Although bicyclams were initially reported to interfere with a viral uncoating event, these compounds were found to be highly specific inhibitors of CXCR4 (21, 79). AMD3100 potently inhibited the intracellular calcium signaling induced by the CXCR4 ligand SDF-1 but did not induce signaling itself, indicating that the compound behaves as a CXCR4 antagonist. Furthermore, the inhibition was strictly restricted to CXCR4 and was not observed for any other chemokine receptors (33). Since this compound is not orally bioavailable, an intravenous single-dose phase I clinical study was performed with healthy and seronegative volunteers. As mentioned above, intravenous administration of AMD3100 was well tolerated, and its plasma concentrations were sustained above the 90% inhibitory concentrations for HIV-1 replication in vitro for 12 h (35). To evaluate the in vivo efficacy, an open-label dose-escalation study was conducted for 40 HIV-1-infected subjects. AMD3100 was administered for 10 days by continuous intravenous infusion at various doses. Only one patient, whose virus was confirmed to use solely CXCR4 and who also received the highest

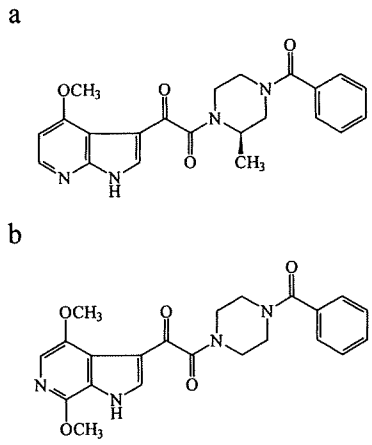

Figure 2. Chemical structures of viral attachment inhibitors. (a) BMS-378806; (b) BMS-488043.

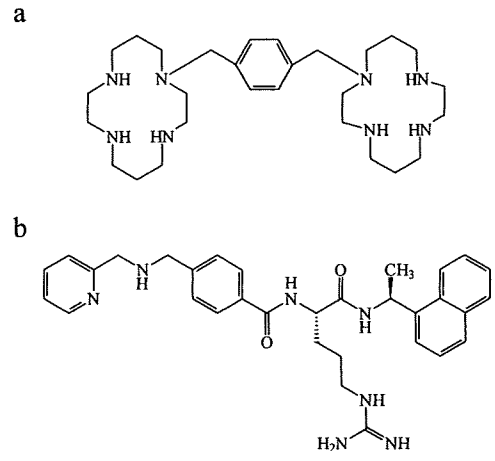

Figure 3. Chemical structures of CXCR4 inhibitors. (a) AMD3100; (b) KRH-1636.

dose, had a significant (0.9-$\log_{10}$) reduction of plasma viral RNA from baseline (34). However, the average change in viral load across all patients was the minimum. Given such results, AMD3100 is not being further developed as an anti-HIV-1 agent.

In addition to AMD3100 and its derivatives, several molecules have been shown to inhibit HIV-1 replication by interacting with CXCR4. These include T22, ALX40-4C, CGP64222, and KRH-1636. T22 is a synthetic peptide that consists of 18 amino acids and an analog of polyphemusin II, which is isolated from hemocyte debris of American horseshoe crabs (66). This peptide specifically inhibits X4 HIV-1 replication in vitro. ALX40-4C (*N*-α-acetyl-nona-D-arginine amide acetate) was a peptide initially developed as a competitive inhibitor of the binding of the HIV-1 Tat protein to the transactivation response (TAR) RNA structure (85). Later, it was found that ALX40-4C inhibited HIV-1 envelope-mediated membrane fusion and viral entry at the level of CXCR4 use (22). CGP64222 is a hybrid peptoid/peptide oligomer of 9 residues, which was also selected as a potent inhibitor of Tat-TAR interaction (32). In fact, it was able to block the formation of the Tat/TAR RNA complex in vitro at nanomolar concentrations. However, CGP64222 proved inactive against HIV-1 strains resistant to the bicyclams, suggesting that CGP64222 inhibits HIV-1 replication through selective interaction with CXCR4 (15). Clinical development of these molecules does not seem to be feasible due to various disadvantages derived from their structures (peptides), such as high cost, possible immunogenicity, and no oral bioavailability.

KRH-1636 (Fig. 3b) is a small-molecule and nonpeptide CXCR4 antagonist with potent and selective anti-HIV-1 activity in vitro and in vivo (38). The compound efficiently inhibited the replication of various X4 HIV-1 in cell cultures by blocking viral entry and membrane fusion via CXCR4 but not via CCR5. It also inhibited binding of SDF-1 to CXCR4 and subsequent intracellular signal transduction. The anti-HIV-1 activity of KRH-1636 could be reproduced in human peripheral blood lymphocyte severe combined immunodeficiency (PBL-SCID) mice. Furthermore, KRH-1636 was absorbed into the blood after intraduodenal administration. However, the clinical efficacy of KRH-1636 and its derivatives is still unclear and remains to be further investigated.

The first small-molecule CCR5 antagonist described in the literature is TAK-779 (Fig. 4a), which is a potent and selective inhibitor of HIV-1 replication (5). This compound inhibited R5 HIV-1 replication at nanomolar concentrations in vitro. TAK-779 antagonized the binding of RANTES and other natural CCR5 ligands to CCR5 and blocked CCR5-mediated signaling at nanomolar concentrations. TAK-779 inhibits HIV-1 replication at the membrane fusion stage by blocking the interaction of gp120 with CCR5. Furthermore, it was found that the binding site for TAK-779 on CCR5 is located near the extracellular surface of the receptor and within a cavity formed between transmembrane helices 1, 2, 3, and 7 (24). Since TAK-779 is an anilide derivative with a quaternary ammonium moiety, it is not orally bioavailable. Therefore, it could not be further developed as an anti-HIV-1 agent. In the meantime, several groups have identified different classes of small-molecule and orally bioavailable CCR5 antagonists. Among the compounds, a small-molecule CCR5 antagonist to be described is SCH-C (Fig. 4b). This compound is an oxime-piperidine derivative and was shown to selectively bind to CCR5 and potently inhibit R5 HIV-1 replication at nanomolar concentrations (84). Although SCH-C displayed excellent oral bioavailability and was well tolerated in early-phase clinical studies, this compound was found to affect cardiac functions, such as dose-dependent prolongation of the QT interval in electrocardiogram. Thus, further development of SCH-C was terminated.

Maraviroc (Fig. 4c) is the first CCR5 antagonist that has recently been licensed by the U.S. Food and Drug Administration for the treatment of HIV-1-infected patients. It was approved for use in combination with other antiretroviral medications for the treatment of R5 HIV-1 in adults whose viral loads remain detectable despite existing antiretroviral treatment or who have multidrug-resistant HIV-1. The currently approved dosage of maraviroc is 300 mg twice daily. Maraviroc has potent anti-HIV-1 activity and favorable pharmacological properties (23). This compound displayed potent antiviral activity against a wide range of R5 HIV-1 clinical isolates at nanomolar concentrations. Maraviroc had potent cross-subtype anti-HIV-1 activity; nevertheless, a three- to sixfold reduction of antiviral activity was observed against subtype G clinical isolates. Its inhibitory effect was highly specific to CCR5, and it did not affect the functions of a wide range of receptors and enzymes, such as CCR2 and the cardiac potassium channel hERG. Pharmacokinetic and metabolic properties of maraviroc were reported to be different from one animal species to another (99). For instance, the compound was incompletely absorbed in rats (20 to 30%) but was well absorbed in dogs (more than 70%). In vitro studies suggested the involvement of P-glycoprotein in restricting oral absorption. In initial clinical studies, maraviroc had good oral bioavailability, and its terminal half-life on multiple dosing was approximately 17 h and did not alter significantly with dose. When treatment-naïve R5 HIV-1-infected patients received maraviroc monotherapy at multiple doses (up to 300 mg) for 10 days, the compound was well tolerated at all doses. Maximum reduction in viral load was observed at a median of 10 to 15 days, with a mean reduction of more than

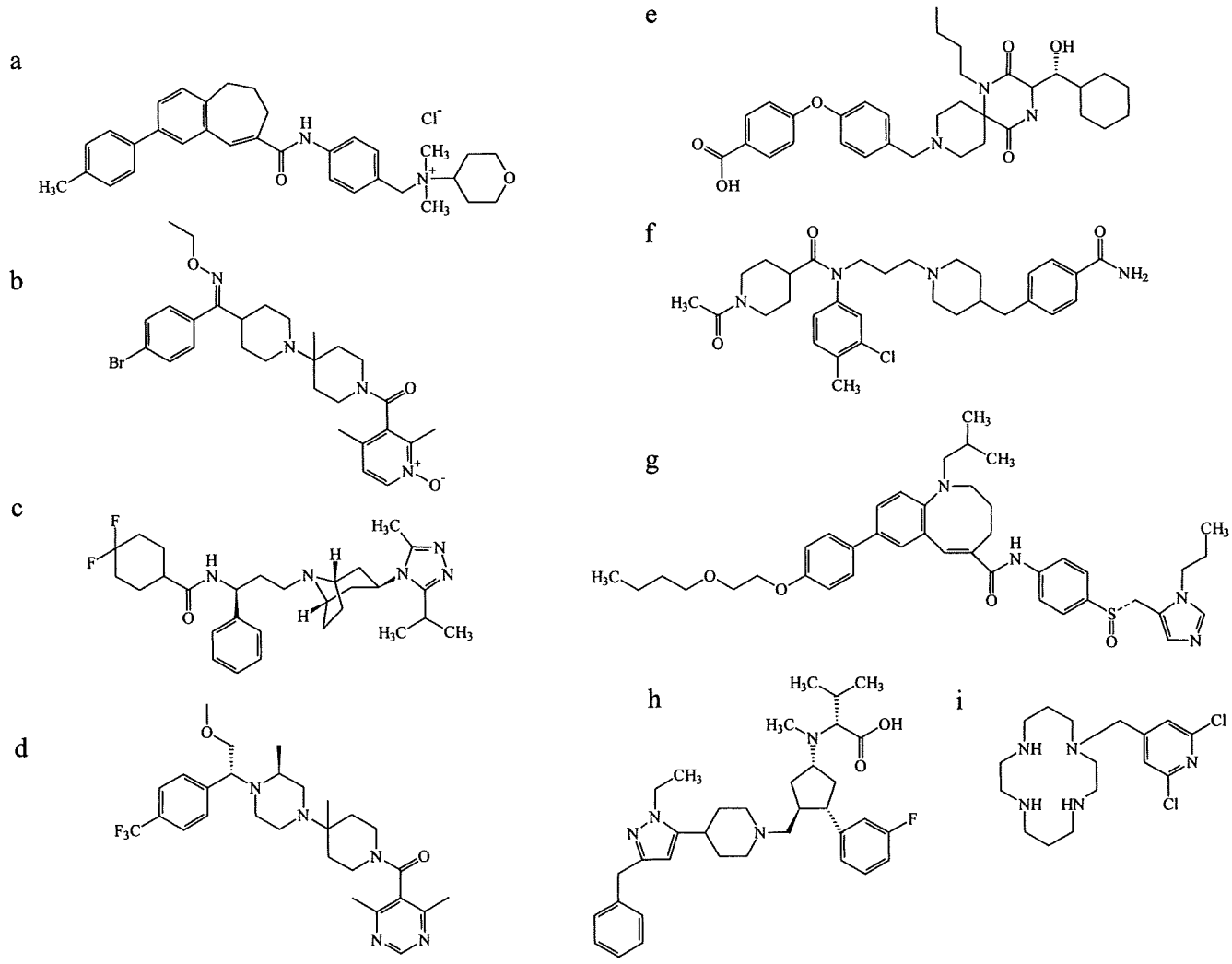

Figure 4. Chemical structures of CCR5 inhibitors. (a) TAK-779; (b) SCH-C (SCH 351125); (c) maraviroc (UK-427,857); (d) vicriviroc (SCH-D); (e) aplaviroc (GW873140/ONO4128); (f) TAK-220; (g) TAK-652; (h) CMPD167; (i) AMD3451.

1.6 $\log_{10}$ at all twice-daily doses of more than 100 mg (27). Recently, the 24-week results of two identical, randomized, and controlled phase IIb/III clinical studies in heavily treatment-experienced patients with triple-class antiretroviral resistance have been reported (48, 71). The patients were randomly assigned to receive oral maraviroc at doses of 150 mg once daily or 150 mg twice daily or to receive a placebo in combination with an optimized background therapy. Both once-daily and twice-daily maraviroc demonstrated significantly greater virologic suppression than the placebo, when used in combination with optimized background therapy.

Vicriviroc (Fig. 4d) is an SCH-C derivative and a more potent inhibitor of HIV-1 replication than SCH-C, which also acts as a CCR5-specific antagonist. This compound exhibited broad-spectrum antiviral activity against a genotypically diverse panel of R5 HIV-1 isolates at subnanomolar concentrations (83). This compound showed synergistic anti-HIV-1 activity in combination with all other classes of approved antiretrovirals. Vicriviroc appeared to have less potential for cardiac effects than did SCH-C. Furthermore, vicriviroc was found to be fully active against the subtype G isolate RU570, which had reduced susceptibility to SCH-C. A phase I clinical study of vicriviroc monotherapy for 14 days demonstrated that the compound was well tolerated and produced significant (1.0-$\log_{10}$ or greater) declines in plasma HIV-1 RNA at total oral daily doses of 50 or 100 mg (80). In a double-blind, randomized phase II study in antiretroviral-treatment-experienced individuals experiencing virologic failure, vicriviroc at 5, 10, or 15 mg or placebo was added to the failing regimen for 14 days, after which the antiretroviral regimen was optimized. Mean changes in plasma HIV-1 RNA level were greater in the vicriviroc groups than in the placebo group (30). Recently, the 48-week results of this study have

been presented, in which sustained viral suppression was achieved with a vicriviroc-containing regimen in treatment-experienced patients with advanced disease (29). Based on results from phase II clinical trials, the manufacturer will select a dose of vicriviroc to move forward into phase III clinical development in treatment-experienced patients.

Aplaviroc (Fig. 4e) is a spirodiketopiperazine derivative, which specifically blocked the binding of macrophage inflammatory protein 1α (MIP-1α) to CCR5 with high affinity (59). The compound had potent antiviral activity against a wide range of laboratory and primary R5 HIV-1 isolates, including multidrug-resistant mutants at subnanomolar concentrations in vitro. Different from TAK-779 or SCH-C, the compound was found to preserve RANTES and MIP-1β binding to CCR5-expressing cells and their functions (59, 100). The antiviral activity was also confirmed in human peripheral blood mononuclear cell (PBMC)-transplanted R5 HIV-1-infected nonobese diabetic-SCID interleukin-2 receptor γ-chain-knocked-out mice, in which massive and systemic HIV-1 replication was observed (69). An approach of combining the site-directed mutagenesis and molecular modeling of CCR5 revealed that the binding site of aplaviroc as well as other CCR5 antagonists (TAK-779 and SCH-C) was located predominantly in a lipophilic pocket in the interface of extracellular loops and the upper transmembrane domain of CCR5 (58). A double-blind, randomized, placebo-controlled oral-dose escalation study was conducted with healthy subjects (1). Single and repeated (every 12 h for 8 days) doses of 50 to 800 mg were well tolerated without serious adverse events, including significant changes in electrocardiogram. In treatment-naïve and -experienced R5 HIV-1-infected subjects, aplaviroc achieved a mean maximum viral load reduction of 1.66 $\log_{10}$ from baseline (49). Unfortunately, the manufacturer announced that further development of aplaviroc was terminated because of its severe hepatotoxicity to some infected patients during phase IIb/III clinical studies.

The orally bioavailable CCR5 antagonists TAK-220 and TAK-652 (current names, TBR-220 and TBR-652, respectively) are successors of TAK-779. TAK-220 (Fig. 4f) is a novel series of compounds whose chemical structures totally differ from that of TAK-779. TAK-220 strongly inhibited the binding of RANTES and MIP-1α to CCR5 but had no effect on MIP-1β binding (87). The compound inhibited the replication of R5 HIV-1 clinical isolates in PBMCs at nanomolar concentrations. The activity of TAK-220 was found to be HIV-1 subtype independent. Pharmacokinetic studies of TAK-220 in animals demonstrated that its oral bioavailability was dependent on animal species (9.5% in rats and 28.9% in monkeys). TAK-220 displayed synergistic antiviral activity in vitro when combined with several licensed antiretrovirals (89). Analysis for the interacting molecules of CCR5 revealed that TAK-220 shared some amino acid residues of CCR5 with TAK-779 (72). However, the involvement of additional amino acid residues was identified, which appeared to further enhance the affinity of TAK-220 to the binding pocket in CCR5. The other compound, TAK-652 (Fig. 4g), is a derivative of TAK-779. Different from TAK-220, TAK-652 was equally inhibitory to the binding of RANTES, MIP-1α, and MIP-1β to CCR5 (6). Furthermore, like TAK-779, TAK-652 also strongly inhibited ligand binding to CCR2b. The compound was highly active against R5 HIV-1, including NRTI-, NNRTI-, and PI-resistant clinical isolates at subnanomolar concentrations. The activity of TAK-652 was HIV-1 subtype independent, and it was fully active against the subtype G isolate RU570, which was reported to be resistant to some CCR5 antagonists. Furthermore, this compound was found to have favorable drug interactions with other antiretrovirals in vitro (88). A single oral administration of TAK-652 of up to 100 mg was safe and well tolerated in humans. The compound also displayed favorable pharmacokinetics (a long half-life) in vivo, suggesting that once-daily administration may be feasible. Further clinical studies of TAK-220 and TAK-652 are currently under consideration.

Other CCR5 antagonists to be described are CMPD167 and AMD3451. CMPD167 (Fig. 4h) is a small-molecule CCR5 antagonist with potent antiviral activity against R5 HIV-1 and simian immunodeficiency virus in vitro (96). Based on a concept of vaginal microbicides for prevention of HIV-1 transmission, this compound was administered intravaginally to macaques and examined for its inhibitory effect on simian-human immunodeficiency virus infection. Significant protection was achieved with CMPD167 alone and in combination with other entry inhibitors having different mechanisms of action (97). Furthermore, oral CMPD167 was able to protect a substantial proportion of macaques from vaginal infection with simian-human immunodeficiency virus (98). The macaques that became infected despite receiving CMPD167 had reduced plasma viremia levels during the earliest stages of infection. However, taking account of the fact that a microbicide must be active against multiple HIV-1 variants, double and triple combinations with different classes of entry inhibitors, such as viral attachment and fusion inhibitors, would be recommended (41). AMD3451 (Fig. 4i) is not a CCR5-specific antagonist but a CCR5/CXCR4 antagonist. It inhibited a wide range of R5, R5X4 (dualtropic), and X4 HIV-1 and HIV-2 at micromolar concentrations in vitro (74). Although such antiviral profiles of this compound seem to be attractive, further optimization, including structural modification for increasing activity, and pharmacokinetic and toxicity tests in vivo are required for its clinical development.

Fusion Inhibitors

As described above, gp41 plays a key role in fusion between viral envelope and cell membrane. A series of peptides were synthesized based on various regions of gp41 and examined for their anti-HIV-1 activity. Among the peptides, enfuvirtide (T-20 or DP-178), a 36-amino-acid peptide corresponding to amino acid residues 643 to 678 (Fig. 5), was found to be a highly potent and selective inhibitor of HIV-1 replication in vitro (104). It blocked cell fusion and viral entry at concentrations of <5 ng/ml. Enfuvirtide proved inhibitory to primary clinical isolates as well as laboratory strains of HIV-1. The active peptides, including enfuvirtide, likely work by binding to the trimeric coiled coils of gp41, thereby acting through a dominant-negative mechanism (13, 57, 102). A clinical study of intravenous enfuvirtide monotherapy for 14 days in 16 HIV-1-infected adults at four doses (3, 10, 30, and 100 mg twice daily) revealed significant, dose-related declines in plasma viral RNA in all subjects who received higher doses (42). Thus, this study has provided proof-of-concept that viral entry can be successfully blocked not only in vitro but also in vivo. In a 28-day randomized study involving 78 HIV-1-infected adults with extensive treatment experiences, dose-related decreases in viral load were observed for patients who received enfuvirtide in addition to a failing regimen (43). Two phase III clinical studies, T-20 versus Optimized Regimen Only study 1 (TORO 1) and TORO 2, were conducted with a large number of treatment-experienced patients in North and South America and in Europe and Australia, respectively (50, 51). These studies clearly demonstrated that the addition of enfuvirtide to an optimized antiretroviral regimen provided significant antiretroviral (decreasing HIV-1 RNA copy number) and immunological (increasing $CD4^+$ T-cell count) benefits over a 24-week period in patients who had previously received multiple antiretrovirals and had multidrug-resistant HIV-1. Furthermore, 48-week follow-up studies of TORO 1 and TORO 2 also demonstrated the durable efficacy of enfuvirtide plus optimized antiretroviral regimens and no exacerbation of adverse events commonly associated with existing antiretrovirals (70, 92). Based on the favorable results in clinical studies, enfuvirtide became the first entry inhibitor licensed for treatment of HIV-1 infection, although there were certain disadvantages of this compound, such as the necessity for twice-daily subcutaneous administration, injection-site skin reactions, and high production cost.

RESISTANCE TO ENTRY INHIBITORS

Viral Attachment Inhibitors

There may be no compound that will prove to be an exception to the fact that drug-resistant HIV-1 will emerge under selective pressure of any single antiretroviral agent. The selection of drug-resistant strains in vitro and their characterization are extremely important, since they may be able to predict the emergence of drug resistance in vivo. In addition, the analysis of resistant viruses for their amino acid mutations often provides useful information on the inhibitor's mechanism of action. An HIV-1 strain resistant to dextran sulfate was selected by cultivation of infected cells in the presence of the compound (26). This study demonstrated that several mutations were found in gp120 of the dextran sulfate-resistant strain but not in a wild-type strain. Cross-resistance was observed for polyanionic compounds structurally related to dextran sulfate. These results suggest that the molecular determinants of polyanion resistance seem to be located in the HIV-1 envelope proteins, especially in the V3 loop domain. A CV-N-resistant strain was also selected by serial passages of X4 HIV-1 with increasing concentrations of CV-N (106). The selected virus exhibited a variety of amino acid mutations that eliminated *N*-linked glycans from gp120

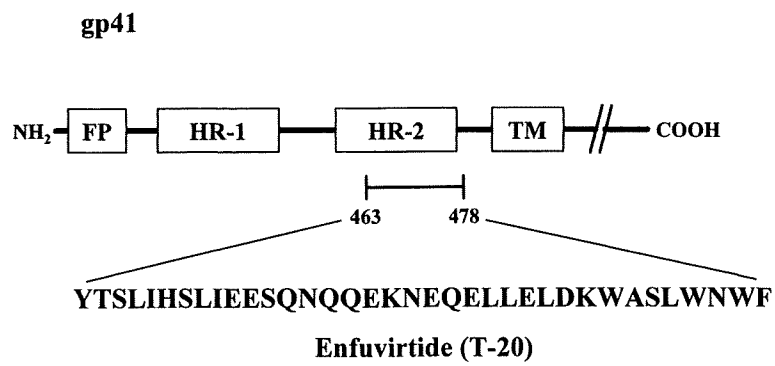

Figure 5. A schematic presentation of gp41 and enfuvirtide structure. gp41 consists of the fusion peptide (FP), two regions of the hydrophobic heptad repeat (HR-1 and HR-2), the transmembrane segment (TM), and the cytoplasmic region (not drawn in this schema). Enfuvirtide is a synthetic 36-amino-acid peptide corresponding to residues 643 to 678 in HR-2.

and had cross-resistance to concanavalin-A, another mannose-binding lectin. However, the virus did not have cross-resistance to the CXCR4 antagonist AMD3100. A recent study of resistance to CV-N identified eight different mutations exclusively located at *N*-glycosylation sites in gp120 (9). Six of the eight mutations resulted in the deletion of high-mannose type *N*-glycans, and two mutations deleted a complex type *N*-glycan from the variable V1/V2 domain of gp120. HIV-1 variants resistant to CV-N or cross-resistant to additional carbohydrate-binding agents were generated and examined for their biological properties (37). This study demonstrated that the resistant variants had increased susceptibility to immunoglobulins and sera obtained from HIV-1-infected patients and particularly to V3-loop-directed monoclonal antibodies. With regard to resistance to BMS-378808, initial studies reported that the resistant variants had several amino acid mutations within the CD4-binding pocket of gp120 (31, 54).

Coreceptor Inhibitors

HIV-1 variants with reduced susceptibility to the CXCR4 ligand SDF-1 or the CXCR4 antagonist AMD3100 could be isolated in vitro, and they have multiple amino acid mutations in gp120. However, these variants still utilized CXCR4 for entering the cells (78). Relative resistance to AMD3100 was conferred by different single amino acid substitutions in the second extracellular loop (ECL2) or in the adjacent membrane-spanning domain (TM4) of CXCR4 (47). Only substitutions of a neutral residue for aspartic acid and of a nonaromatic residue for phenylalanine were associated with drug resistance, suggesting a direct interaction of AMD3100 with these amino acids rather than indirect effects of their substitutions on the structure of CXCR4. Interestingly, a T22 analog, which represents another class of CXCR4 antagonists, retained full activity against AMD3100-resistant strains (2).

HIV-1 resistant to CCR5 antagonists was obtained when an R5 clinical isolate was passaged in PBMCs with increasing concentrations of AD101, a CCR5 antagonist structurally related to SCH-C (91). The strain was highly resistant to AD101 and cross-resistant to SCH-C, yet it was unable to use CXCR4. Genetic and phenotypic analyses revealed that four amino acid changes in the V3 region of gp120 were necessary and sufficient to confer resistance (45). Furthermore, there was a correlation between the susceptibility of R5 clinical isolates to RANTES and AD101 (44). HIV-1 strains highly resistant to vicriviroc were also isolated through cultivation of R5 HIV-1 isolates in mitogen-stimulated primary $CD4^+$ T cells (60). These strains were found to be completely dependent on CCR5 for entry. Interestingly, they had cross-resistance to not only SCH-C but also RANTES derivatives, which were inhibitory to R5 HIV-1 replication. Unlike AD101-resistant strains, the vicriviroc-resistant mutants had no amino acid changes in the V3 region compared to their parental viruses. Instead, several sequence changes were present elsewhere in gp120 (V2, C3, and V4). In phase IIb clinical studies, protocol-defined virologic failure in six of seven treatment-experienced subjects receiving a vicriviroc-containing regimen was not associated with phenotypic evidence of vicriviroc resistance (94). V3 loop sequence changes occurred in these subjects, but neither consistent nor stereotypic amino acid substitutions were observed, suggesting that some other mechanisms explain vicriviroc failure.

Serial passages of infected cells in vitro with increasing maraviroc concentrations failed to select drug-resistant variants from some laboratory-adapted and clinical isolates of HIV-1. However, high-level resistance to maraviroc was selected from three of six primary isolates passaged in PBLs (103). The SF162 strain acquired resistance to maraviroc in both treated and control cultures; all resistant variants were able to use CXCR4 as a coreceptor. In contrast, maraviroc-resistant viruses derived from isolates CC1/85 and RU570 remained CCR5-tropic. The maraviroc-resistant R5 HIV-1 retained full susceptibility to SCH-C and aplaviroc, suggesting that although the CCR5 binding sites for these agents are similar, their impacts on the surface conformation of the receptor are different. The results of this study also suggest that the envelope proteins of maraviroc-resistant viruses are able to recognize and utilize inhibitor-bound CCR5, which involves the ordered accumulation of mutations in the viral envelope, both in the V3 loop and elsewhere within gp120. This mechanism of resistance is characterized phenotypically by dose-response curves with a reduced maximal percentage inhibition. These observations were confirmed by the analysis of the samples obtained from treatment failure patients carrying R5 HIV-1 in phase III clinical studies (64).

A long-term culture experiment with R5 HIV-1-infected PBMCs was conducted with escalating concentrations of TAK-652. After serial passages of the infected cells for more than 1 year, an escape virus was obtained (4). This virus displayed complete resistance to TAK-652, in contrast to the wild type. The escape virus appeared to have cross-resistance to the structurally related compound TAK-779 but retained full susceptibility to TAK-220, which is a totally different class of CCR5 antagonists. Furthermore, the escape virus was unable to use CXCR4 as a coreceptor. Analysis for Env amino acid sequences of the escape viruses at certain points of passage revealed that amino acid changes accumulated with an increasing number of passages. Several amino acid changes not only in the V3 region but also in other Env regions seemed to be required for R5 HIV-1 to acquire

complete resistance to TAK-652. A similar experiment was also conducted with TAK-220. However, no viruses with reduced susceptibility to TAK-220 could be obtained even after 2 years (my unpublished observations).

Fusion Inhibitors

It was reported that enfuvirtide-resistant HIV-1 emerged after in vitro passage for 6 weeks in the presence of increasing concentrations of the compound (75). Sequence analysis of the resistant isolates suggested that a contiguous 3-amino-acid sequence at positions 36 to 38 within the HR-1 of gp41 was associated with enfuvirtide resistance. Site-directed mutagenesis studies confirmed this observation and indicated that changes in two of these three residues were necessary for development of the resistant phenotype. A similar result was obtained in a phase I clinical study (101). All four patients who received an intermediate dose of enfuvirtide (30 mg twice daily) showed an initial decline in plasma viral load over the first 10 days but a rising trend by day 14. HIV-1 obtained from one patient developed a mutation in the 3-amino-acid sequence after 14 days of therapy, specifically an aspartic acid substitution for glycine at position 36. Viruses obtained from phase II clinical study patients treated with enfuvirtide as functional monotherapy for 28 days or in combination with oral antiretrovirals for more than 48 weeks were analyzed for their amino acid substitutions at positions 36 to 45 (82). The viruses from patients experiencing the rebound of viral load exhibited reduced susceptibility to enfuvirtide and substitutions in amino acids 36 to 45. Furthermore, on-treatment changes in the phenotypic susceptibility of virus isolates to the compound were generally associated with genotypic changes in amino acids 36 to 45. A study using recombinant viruses carrying enfuvirtide resistance mutations revealed that their replicative fitness and drug susceptibility were inversely correlated (56). In phase III clinical studies, the amino acid sequence at positions 36 to 45 was highly conserved at baseline except for polymorphism at position 42. In contrast, amino acid substitutions within positions 36 to 45 on treatment were observed for the viruses obtained from most of the patients who met protocol-defined virological failure criteria (61).

FUTURE DIRECTIONS

In addition to the inhibitors described in this chapter, several antibodies have been shown to interact with the molecules essential for HIV-1 entry to the host cells. These include the anti-CD4 monoclonal antibody TNX-355 (46) and the anti-CCR5 monoclonal antibody PRO 140 (90). They may exert synergistic antiviral activity in combination with other entry inhibitors (110). A phase I clinical study in a small number of HIV-1-infected subjects demonstrated that single doses of TNX-355 reduced plasma viral RNA levels and increased $CD4^+$ T-cell counts (46). In a phase II clinical study, TNX-355 was administered intravenously once a week for the first 9 weeks and then once every other week. On the other hand, a randomized double-blind placebo-controlled study of PRO 140 was conducted with subjects infected with exclusively R5 HIV-1 (76). In this study, the participants received single intravenous infusions of PRO 140, and viral load, pharmacokinetics, and safety assessments were performed through day 59. Single-dose PRO 140 was well tolerated, and potent and dose-dependent antiviral activity was demonstrated in HIV-1-infected subjects, suggesting that the antibody is able to act as a potent and long-acting antiretroviral agent.

Another class of entry inhibitors that should be extensively explored is small-molecule compounds that bind to gp41 and block the fusion between viral envelope and cell membrane. Although enfuvirtide has been used in clinical settings, a small-molecule fusion inhibitor will have several advantages over the peptide in terms of oral bioavailability and production cost. Furthermore, HIV-1 mutants resistant to either CCR5 or CXCR4 antagonists may not have cross-resistance to a small-molecule fusion inhibitor. However, only a few compounds have been reported as fusion inhibitors until now. Two N-substituted pyrroles, designated NB-2 and NB-64, inhibited HIV-1 replication in vitro (39). They blocked fusion and entry of HIV-1 by interfering with the gp41 six-helix bundle formation and disrupting the α-helical conformation. Although the anti-HIV-1 potency of NB-2 and NB-64 are not high enough for them to be considered as candidates for clinical development, they have broad anti-HIV-1 activity against a variety of primary HIV-1 isolates and high specificity to gp41. Thus, NB-2 and NB-64 may be used as lead compounds for designing more-potent small-molecule fusion inhibitors.

Since the machinery of HIV-1 entry to the host cells is complex, the viral and cellular factors involved in this process have not been fully elucidated yet. Therefore, it would not be surprising that more-potent and specific inhibition of viral entry could be achieved by small-molecule compounds with a novel mechanism of action. Hopefully, such compounds can be identified in the near future and added as new members of HIV-1 entry inhibitors.

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Short communication

Inhibition of porcine endogenous retrovirus (PERV) replication by HIV-1 gene expression inhibitors

Minyi Shi[a,b,1], Xin Wang[b,2], Mika Okamoto[b], Sonshin Takao[a], Masanori Baba[b,*]

[a] Frontier Science Research Center, Kagoshima University, Kagoshima 890-8544, Japan
[b] Division of Antiviral Chemotherapy, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima 890-8544, Japan

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ABSTRACT

Porcine endogenous retrovirus (PERV) is persistently integrated into the host genomic DNA as a provirus and released from a variety of porcine cells. PERV infects a certain range of human cells, which is a major concern in xenotransplantation. Therefore, the use of viral gene expression inhibitors could be envisaged, if they reduce PERV production from porcine organs and minimize viral transmission to human recipients. In the present study, four HIV-1 gene expression inhibitors were examined for their inhibitory effect on PERV replication in porcine cells constitutively producing the virus. Among the compounds, the fluoroquinolone derivative K-37 and the bacterial product EM2487 displayed potent and selective inhibition of PERV replication in the cells mediated by the suppression of viral mRNA synthesis. Thus, retroviral gene expression inhibitors may be able to reduce the risk of PERV transmission.

Xenotransplantation, the grafting of cells, tissues, or organs into different species, is a possible solution to overcome the extreme shortage of human allografts for transplantation (Cooper and Keogh, 2001). Among the animals, non-human primates and pigs are considered to be suitable donors for xenotransplantation. The use of non-human primates as organ donors is associated with a high risk of transmitting various infectious pathogens to humans (Allan, 2003). Apart from immunological rejection, pigs may be more suitable donors than non-human primates because of the resemblance of their organ sizes and a lower risk of transmitting various infectious pathogens. However, porcine endogenous retrovirus (PERV) is still a major obstacle to successful xenotransplantation with sufficient safety. PERV is a type C retrovirus persistently integrated into the host genomic DNA as a provirus. Multiple copies of PERV proviral DNA exist in all of the breeds examined to date (Louz et al., 2008). PERV is classified into three subtypes, such as PERV-A, -B, and -C, based on the divergence of its envelope genes.

It has been demonstrated that PERV particles are released from a variety of porcine cells and infect a certain range of human cells (Martin et al., 1998; Patience et al., 1997; Wilson et al., 1998). There are a number of patients who received porcine tissues, such as pancreatic islet cells, skin, liver, and kidney; nevertheless PERV infection has not been observed in these individuals (Heneine et al., 1998; Paradis et al., 1999; Patience et al., 1998). However, long-lived microchimerism was found in some patients treated by extracorporeal splenic perfusion, which might increase a potential risk of PERV infection through the activation of viral replication (Paradis et al., 1999). An immunosuppressive treatment upon organ transplantation may also increase a risk of PERV transmission. The use of antiretrovirals would be the first option to minimize the possibility of PERV transmission to recipients, if they could have an inhibitory effect on PERV replication without serious side effects. Among the antiretrovirals, zidovudine (AZT) and didanosine (ddI) proved to be active against PERV replication in cell cultures (Powell et al., 2000; Qari et al., 2001). We have previously demonstrated that the acyclic nucleoside phosphonate tenofovir (PMPA), an HIV-1 reverse transcriptase (RT) inhibitor, selectively inhibits PERV replication in human cells (Shi et al., 2007). However, such RT inhibitors cannot suppress the production of PERV from the porcine cells in which its proviral DNA is integrated. Therefore, it would be very useful if an inhibitor of PERV gene expression could be identified. In the present study, we have examined four inhibitors of HIV-1 gene expression for their antiviral activity against PERV replication in porcine cells persistently infected with the virus and found that the fluoroquinolone derivative K-37 (Baba et al., 1998) and the bacterial product EM2487 (Baba et al., 1999) are potent and selective inhibitors of PERV replication.

K-37 and the nuclear factor κB (NF-κB) inhibitor cepharanthine (Okamoto et al., 1998) were provided by Daiichi Pharmaceutical

* Corresponding author. Tel.: +81 99 275 5930; fax: +81 99 275 5932.
E-mail address: m-baba@vanilla.ocn.ne.jp (M. Baba).
[1] Present address: Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT 06520, USA.
[2] Present address: Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06520, USA.

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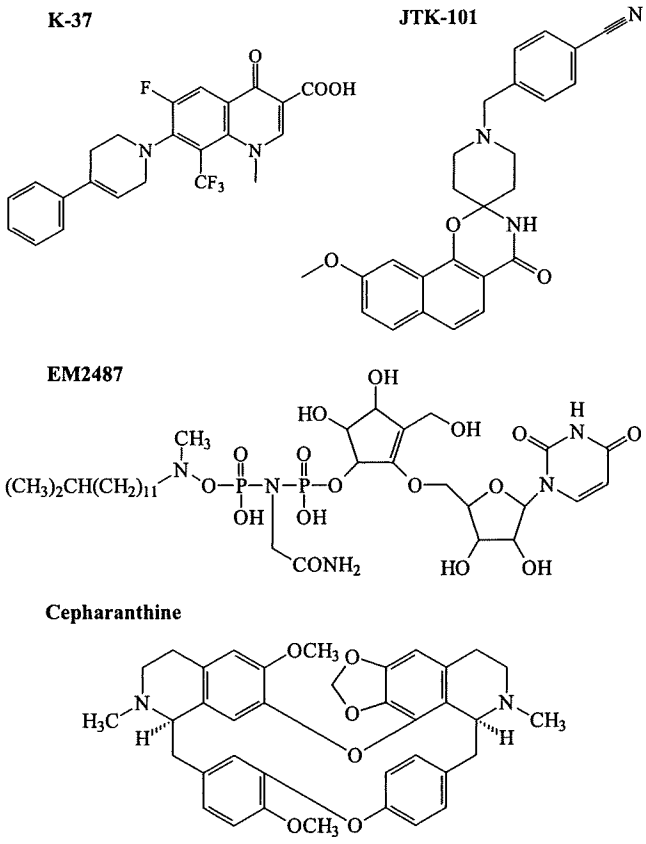

Fig. 1. Chemical structures of test compounds.

Co. (Tokyo, Japan) and Kaken Shoyaku (Mitaka, Japan), respectively. JTK-101 (Wang et al., 2007) was synthesized by Japan Tobacco Co. (Takatsuki, Japan). EM2487 was provided by Esai Co. (Tsukuba, Japan). These compounds (Fig. 1) were chosen for this study, because their antiviral activity against HIV-1 replication in chronically infected cells had been demonstrated (Baba et al., 1998, 1999; Okamoto et al., 1998; Wang et al., 2007). All compounds were dissolved in dimethyl sulfoxide (DMSO) at 10 mM or higher concentrations to exclude any antiviral or cytotoxic effect of DMSO and stored −20 °C until use. The porcine embryonic kidney cell line PK15, which produces PERV particles, was obtained from the American Type Culture Collection. The cells were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 1.5 g/l sodium bicarbonate, and antibiotics.

The activity of the compounds against persistent PERV infection was based on the inhibition of PERV particle production from PK15 cells. PK15 cells were seeded in a 24-well plate (2.5×10^4 cells/well). After incubation for 16 h at 37 °C, the culture supernatants were removed and the cell monolayer was washed by phosphate-buffered saline (PBS), and then 2 ml of fresh medium containing various concentrations of the test compounds was added to each well. After a 48-h incubation period, the culture supernatants were collected and filtered (0.45 μm pore size). Then the filtrates were mixed with 22% (w/v) polyethylene glycol 6000 solution. After incubation for 5 h at 4 °C with continuous stirring, the mixture was centrifuged at 15,000 rpm for 15 min at 4 °C. The pellets contained PERV particles released from PK15 cells. The inhibition of PERV particle production was determined by the decrease of PERV reverse transcriptase activity using a commercial RT assay kit (Roche, Mannheim, Germany). The pellets obtained above were resuspended in lysis buffer supplied by the assay kit and subjected to reverse transcription reaction for 2 h, according to the Manufacturer's instructions, except that $MgCl_2$ in the reaction mixture was replaced by $MnCl_2$ (Phan-Thanh et al., 1992). All experiments were carried out in duplicate.

The antiviral activity of test compounds was also determined by the inhibition of PERV mRNA expression in PK15 cells. PK15 cells were seeded and cultured in the medium containing test compounds in the same manner as described for the antiviral assay. After a 48-h incubation, the culture supernatants were removed, and the cells were extensively washed with PBS, trypsinized, and washed again with PBS. Total RNA was extracted from the cells with RNeasy Mini Kit (Qiagen) and subjected to real-time RT-PCR analysis. The PERV mRNA level was determined using the sense primer (5′-AGCTCCGGGAGGCCTACTC-3′), the antisense primer (5′-ACAGCCGTTGGTGTGGTCA-3′), and the Taqman® probe (5′-FAM-CCACCGTGCAGGAAACCTCGAGACT-TAMRA-3′). The primer pair amplifies a region of the *pol* gene of PERV (Paradis et al., 1999). The nucleotide sequences used for the construction of the primers and probe were based on the reports by B. Bartosch, R.A. Weiss and Y. Takeuchi (GeneBank accession numbers: AY099323 and AY099324). The final concentrations of the primer pairs and probe were 200 and 100 nM, respectively. The Taqman® PCR reagent kit and Taqman® Multiscribe™ reverse transcription reagent kit (Applied Biosystems, Roche, Branchburg, NJ) were used according to the Manufacturer's instructions. Each sample was run in triplicate. Nonspecific inhibition of host cellular mRNA synthesis by the test compounds was determined with the Taqman 18S rRNA reagent kit (Applied Biosystems).

Cytotoxicity of the test compounds was determined by a tetrazolium dye method (Tetracolor One®, Seikagaku Corporation, Tokyo, Japan) (Yamamoto et al., 2001). PK15 cells were seeded and cultured in the medium containing test compounds in the same manner, as described in the antiviral assay. After a 48-h incubation, 1.5 ml of the culture supernatants were removed and 25 μl of the dye was added to each well. After a 4-h incubation at 37 °C, the specific (450 nm) and reference (630 nm) absorbances were monitored for each well by a microplate reader.

When four HIV-1 gene expression inhibitors were examined for their inhibitory effect on PERV replication in PK15 cells, K-37 and EM2487 displayed dose-dependent reduction of PERV RT activity in culture supernatants (Fig. 2A and C). K-37 and EM2487 did not show a direct inhibitory effect on PERV RT activity (data not shown). These compounds did not display apparent cytotoxicity to PK15 cells at concentrations up to 1 and 10 μM, respectively, indicating that K-37 and EM2487 are selective inhibitors of PERV replication in porcine cells. In contrast, JTK-101 and cepharanthine did not show any activity against PERV replication at the highest concentration tested (1 μM) (Fig. 2B and D). Since PERV proviral DNA is integrated in the genome of the host cells, the compounds were also examined for their inhibitory effect on viral mRNA synthesis in PK15 cells. As shown in Fig. 3, dose-dependent suppression of PERV mRNA synthesis was observed for K-37 and EM2487 but not for JTK-101 or cepharanthine. These results are in accordance with those obtained in the RT assay (Fig. 2). The 50% effective concentration (EC_{50}) of K-37 for PERV replication and its 50% inhibitory concentration (IC_{50}) for viral mRNA synthesis were 0.35 ± 0.04 and 0.34 ± 0.05 μM, respectively (Table 1). On the other hand, its 50% cytotoxic concentration (CC_{50}) was 4.63 ± 1.62 μM, suggesting that K-37 is a selective inhibitor of PERV gene expression. Similarly, the EC_{50}, IC_{50}, and CC_{50} of EM2487 were 5.44 ± 1.40, 4.36 ± 0.30, and >10 μM, respectively.

K-37 is a potent and selective inhibitor of HIV-1 replication in both acutely and chronically infected cells at submicromolar concentrations (Baba et al., 1998). K-37 could inhibit Tat-dependent transactivation, yet it was not an inhibitor of Tat itself or its cofactor CDK9/cyclin T1. Since PERV does not generate Tat protein, it is apparent that the anti-PERV activity of K-37 is not due to the inhi-