Isolation of TAK-779-Resistant HIV-1 from an R5 HIV-1 gp120 V3 Loop Library

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The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein gp120 interacts with CD4 and the CCR5 coreceptor for viral entry. The V3 loop in gp120 is a crucial region for determining coreceptor usage during viral entry, and a variety of amino acid substitutions have been observed in clinical isolates. To construct an HIV-1 V3 loop library, we chose 10 amino acid positions in the V3 loop and incorporated random combinations (27,648 possibilities) of the amino acid substitutions derived from 31 R5 viruses into the V3 loop of HIV-1JR-FL proviral DNA. The constructed HIV-1 library contained 6.6 x 10^6 independent clones containing a set of 0-10 amino acid substitutions in the V3 loop. To address whether restricted steric alteration in the V3 loop could confer resistance to an entry inhibitor, TAK-779, we selected entry inhibitor-resistant HIV-1 by increasing the concentration of TAK-779 from 0.10 μM to 0.30 μM in PM1-CCR5 cells with high expression of CCR5. The selected viruses at passage 8 contained 5 amino acid substitutions in the V3 loop without any other mutations in gp120 and showed 15-fold resistance compared to the parental virus. These results indicate that a certain structure of the V3 loop containing amino acid substitutions derived from 31 R5 viruses can contribute to the acquisition of resistance to entry inhibitors binding to CCR5. Taken together, this type of HIV-1 V3 loop library is useful for isolating and analyzing the specific biological features of HIV-1 with respect to alterations of the V3 loop structure.

Entry of R5 human immunodeficiency virus type 1 (HIV-1) into target cells requires sequential interaction of the envelope glycoprotein gp120 with CD4 and the coreceptor CCR5 (1). The binding of gp120 to CD4 leads to exposure of the coreceptor binding site, designated the third hypervariable loop (V3 loop), which is composed of approximately 35 amino acid residues (2,3). Subsequently, the interaction of gp120 with the coreceptor induces the exposure of a fusion peptide of transmembrane glycoprotein gp41, and allows membrane fusion and entry of the viral core into the target cell (3-6). In the series of viral entry events, the gp120 V3 loop has been regarded as the major determinant of coreceptor usage (7-15). Single amino acid changes in the V3 loop can switch viral coreceptor usage (16-19), although changes in this region alone are not always necessary or sufficient to confer a particular phenotype on the viruses (20-24). In fact, an increase in the positive charge of the V3 loop is often associated with CXCR4 usage (7,25). The presence of at least one basic substitution at V3 position 11 or 25 can change coreceptor usage (26,27), and sequence changes in the GPG motif in the V3 loop can modulate coreceptor usage (17,18). Variation in the residues flanking the GPG motif can alter the stability of the beta sheet and/or alter the surface accessibility of this element, thereby influencing coreceptor usage (28-30). If we can manipulate a mixed population of HIV-1 with diversity in the V3 loop in vitro, it would be a useful system for screening and analyzing the biological features of HIV-1 with regard to V3 variation. There has been a report regarding the construction of a heterogeneous retrovirus population in vitro (31) and we recently constructed an in vitro system that created an HIV-1 library containing mutations associated with protease inhibitor resistance (32). In the current report, we constructed an R5 HIV-1 V3 loop library containing diverse structures of the V3 loop. We chose 10 amino acid positions in the V3 loop (residues 302, 303, 304, 305, 306, 312, 314, 317, 318 and 321; V3 loop starting from Cys93 to Cys327 within V3 of HIV-1JR-FL Env) (Fig 1A), and incorporated the amino acid substitutions in 31 R5 HIV-1 strains obtained from the Los Alamos HIV Database (33). Theoretically, the number of substitution combinations in a library carrying a set of random combinations of 0-10 substitutions is 27,648 possibilities.

A new class of antiretroviral drugs that prevent virus entry are being developed. TAK-779 inhibits HIV-1 replication by blocking the interaction of gp120 with CCR5 (34). This compound has previously been shown to inhibit cell-to-cell fusion.
and cell-free virus infectivity in vitro (34). The binding site for TAK-779 is located near the CCR5 extracellular surface, within a cavity between transmembrane helices (35). Recently, the viruses resistant to an entry inhibitor enfuvirtide (T20) were sensitive to TAK-779 (36). However development of TAK-779 resistance remained to be elucidated. To address the contribution of the V3 loop to susceptibility to an entry inhibitor, we selected TAK-779-resistant variants from the HIV-1 library, and isolated R5 entry inhibitor-resistant HIV-1 with mutations restricted to the V3 loop.

EXPERIMENTAL PROCEDURES

Cell culture and molecular clones—PM1 (37) and Hela-CD4-LTR-β-gal (MAGI) cells (38) were provided by the NIH AIDS Research and Preference Reagent Program. PM1-CCR5 cells were generated by standard retrovirus-mediated transduction of PM1 cells with pBABE-CCR5 provided by the NIH AIDS Research and Preference Reagent Program. PM1 and PM1-CCR5 cells were grown in RPMI 1640-based complete medium supplemented with 10% fetal calf serum (FCS; Vitromex, Bayern, Germany), 200 U/ml penicillin and 200 U/ml streptomycin. The transduced cells were selected with complete medium plus 1 µg/ml of puromycin (Sigma-Aldrich, St. Louis, MO), and a single clone was selected based upon the expression level of CCR5 assessed by flow cytometry analysis. Similarly, MAGI-CCR5 was isolated by standard retrovirus-mediated transduction of MAGI cells with pBABE-CCR5 (39). MAGI and MAGI-CCR5 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 200 U/ml penicillin and 200 U/ml streptomycin.

The HIV-1 proviral expression vector pJR-FL was kindly provided by Dr. Y. Koyanagi (Kyoto University). pJR-FL_m was created from pJR-FL by incorporation of AflII and Nhel sites into env at nt 6395 and 6564, respectively. There were no amino acid substitutions caused by incorporation of the AflII site (CTG AAA (Leu Lys) -> CTT AAG (Leu Lys)). Incorporation of the Nhel site led to two amino acid substitutions (GTT ATA (Val Ile) -> GCT AGC (Ala Ser)). HIV-1_RJR-FL_m was used as the parental virus. Construction of an R5 HIV-1 V3 loop library—The strategy used to construct the HIV-1 library carrying a set of random amino acid substitutions is shown in Fig. 2A. The 2077 bp Stul-Xhol fragment from pJR-FL_m was subcloned into pCRAZ digested with Stul and Xhol, resulting in pCR-SANX. pCRAZ was created from pCR-blunt (Invitrogen, Carlsbad, CA) by deleting a PmlI fragment containing a Zeocin cassette. Six oligonucleotides, V31, V32, V33, V34, V35 and V36, were commercially synthesized to generate 3 short DNA fragments with sticky ends. The respective pairs of complementary oligonucleotides at 0.35 µg/µl were denatured for 2 min at 90°C in a heat block, and then left in the switched-off heat block for 30 min in 10 mM MgCl₂ and 10 mM Tris-HCl (pH 8.0) at room temperature for annealing. The resultant short DNA fragments were designated V312 (48 bp), V334 (65 bp) and V356 (64 bp), respectively. V334 contained 10 degenerated codons (ARA for Lys or Arg; RGT for Ser or Gly; RTA for Ile or Val; HMY for His, Pro, Asn, Ser, Thr or Tyr; MTR for Ile, Met or Leu; TKK for Phe, Leu, Trp or Cys; RCW for Thr or Ala; SMM for Glu, Asp, Gln, Ala, His or Pro; RTA for Ile or Val; and RAT for Asp or Asn) (Fig. 2A). Here, the single letter code is follows: R represents A or G; H represents A, C or T; M represents A or C; Y represents C or T; K represents G or T; W represents A or T; and S represents C or G. The 3 DNA fragments (0.25 µg each) were ligated by T4 DNA ligase (New England Biolabs Inc., Beverly, MA) and the resultant 185-bp DNA fragment was purified by 1.5% agarose electrophoresis. One hundred nanograms of the purified DNA fragment was used for polymerase chain reaction (PCR) as the DNA template with PfX DNA polymerase (Invitrogen). The upstream primer was VV-Af (5'-ACAGCTTAAGGAATC TGTAGAAATATTG-3') and the downstream primer was VV-Nh (5'-ATTTCATAGCTATC TGTTTAAAGGTGTCAT-3'). The amplification conditions for the first-round PCR were 94°C for 1 min, 16 cycles of 94°C for 30 s, 58°C for 30 s and 68°C for 60 s, and a final extension at 72°C for 10 min. PCR was performed using a Gene Amp PCR System 9700 (Applied Biosystems Inc., Foster City, CA). The PCR product (5.0 µg) was purified using the Concert Rapid PCR Clean-up System (Invitrogen), digested with AflII and Nhel and then ligated into the AflII and Nhel sites of pCR-SXZAN (10.0 µg), pCR-SXZAN was created from pCR-SANX by replacing the AflII-Nhel fragment with a linker. The ligation mixture was purified by Microcon-30 (Millipore Corp., Bedford, MA) and used to transform E. coli strain DH5alpha by electroporation with an ECM 600 (BTX, San Diego, CA) at 2.5 kV, generating pCR-SX-V3Lib containing 7.1 x 10⁶ independent clones with an AflII-Nhel fragment. After purification of the pCR-SX-V3Lib DNA, the Stul-Xhol fragment from 15 µg of the plasmid was cloned into the Stul and Xhol sites of pJR-FLASX. pJR-FLASX was created from pJR-FL by replacing the Stul-Xhol fragment of pJR-FL with a linker. The ligation products (0.2 µg) were transformed into E. coli strain JM109 by electroporation as described above. Finally, the HIV-1 library, designated pJR-FL-V3Lib, contained 6.6 x 10⁶ independent clones containing a 2077 bp...
(Stu-I-XhoI) env DNA fragment (ligation efficiency, 77%) (Table I).

**Analysis of replication**— All viral stocks, including the virus library, were prepared by transfecting 293T cells as described previously (32). Viral stocks for analysis of the replication of the virus library HIV-1V3Lib were generated by transient transfection of 293T cells with pJR-FL-V3Lib. Then 5 x 10^5 of PM1-CCR5 or MT-2 cells were infected with HIV-1JR-FLan or HIV-1V3Lib stocks containing 600 ng of p24 Gag antigen/ml. Viral replication was monitored by measuring the p24 Gag antigen concentration in the supernatants of the challenged cells. p24 Gag antigen was determined by p24 Gag antigen ELISA system using RETRO-TEK (ZeptoMetrix, Co., Buffalo, NY).

**Selection of TAK-779-resistant HIV-1V3Lib**— PM1-CCR5 cells (1 x 10^6) infected with HIV-1V3Lib (600 ng of p24 Gag antigen) at passage 1 and incubated for 3 d in the presence of 0.1 mM TAK-779. Virus passages were then performed at 3-4 d intervals using PM1-CCR5 cells (2 x 10^5) in the absence or presence of 0.10 mM TAK-779. The concentration of TAK-779 was increased from 0.10 to 0.30 mM at passage 3. The infectivity of the supernatant at each passage was evaluated using MAGI-CCR5 cells in the presence or absence of 0.1 mM TAK-779.

**Determination of susceptibilities**— The susceptibilities of the viruses to entry inhibitors were determined by the MAGI assay using previously titrated virus preparations (80 blue foci/well). MAGI-CCR5 cells (1 x 10^4 cells/well) were plated into 48-well tissue culture plates at 1 d prior to infection. After absorption of the virus for 2 h at 37°C in the presence or absence of 0.001-1.0 mM TAK-779 or AMD3100, the cells were washed twice with PBS (phosphate-buffered saline), and then further incubated for 48 h in the presence or absence of 0.0010 nM - 3.0 μM TAK-779 or AMD3100 in fresh medium. The cells were stained, and the number of blue foci in each well was counted (37). All experiments were performed in triplicate.

**Sequencing**— The nucleotide sequences of the V3 loops in the virus library were determined as follows. The virus mixture was precipitated and subjected to RT-PCR using the ImProm-II transcription system (Promega Co., Madison, WT). A 380-bp fragment containing a V3 loop sequence was amplified by PCR in a 50 μl reaction volume comprising 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM MgCl₂, 0.01% gelatin and 2 U ampliTaq (Applied Biosystems Inc.) with primers VV1 (5'- AATGGCAGTCTAGCAAGAAG-3') and VV2 (5'-TTTCTGGTGCCCCTCCTGAGGA-3'). The PCR products were purified by 1% agarose electrophoresis and cloned into the pCR-TOPO vector (Invitrogen). The cloned sequences were sequenced using an ABI Prism 310 (Applied Biosystems Inc.).

**RESULTS**

**Construction of the proviral DNA for the HIV-1 V3 loop library**

A variety of amino acid substitutions were observed in the V3 loop of 31 R5 HIV-1 derived from the Los Alamos HIV Database (33) (Fig. 1). To construct a proviral DNA mixture containing a set of random substitution combinations derived from the envelope V3 loops of the R5 isolates, we chose 10 amino acid positions for substitutions (residues 302, 303, 304, 305, 306, 312, 314, 317, 318 and 321; V3 loop starting from CyS293 to CyS327 in HIV-1JR-FL) (Fig. 2A). AflII-NheI DNA fragments containing a set of random combinations of these mutations were prepared with overlapping oligodeoxynucleotides comprising both DNA strands, and subsequently annealed and ligated to form the complete gene. In the AflII-NheI DNA fragments, the residues at positions 302, 303, 304, 314, 318 and 321 contained 2 possible amino acid residues, while the residues at positions 305, 306, 312 and 317 contained 6, 3, 4 and 6 possibilities, respectively (Fig. 2A). Amino acid substitutions (Phe to Cys at 312, Gln to His or Pro at 3317; Fig. 2A, underlined) that were not detected in the R5 isolates (Fig. 1) were inevitably incorporated into the library due to unintended combinations of nucleotide substitutions. Theoretically, the number of possible combinations of 0-10 amino acid substitutions was calculated to be 27,648 (Table 1) and the lowest frequency combination of the substitutions was 0.00204%. We finally obtained a proviral DNA, designated pJR-FL-V3Lib, for the HIV-1 library that contained 6.6 x 10^6 independent clones. This size of the library contained all possible combinations of the recombinants. Incorporation of random combinations of the amino acid substitutions into the V3 loops was confirmed by sequencing of randomly selected clones from the library DNA (Fig. 3A). There was no marked difference in the composition of the amino acid residues at the 10 positions in V3 between the library DNA and the virus library prepared for the following experiments (Fig. 3B).

**Coreceptor usage of the virus library**

We tested the coreceptor usage of the virus library using a single-round assay (Fig. 4A). HIV-1JR-FLan, which was used as a background for the construction of the V3 loop library, showed similar infectivity to HIV-1JR-FL. The infectivity of the virus library HIV-1V3Lib into MAGI-CCR5 cells was 26% lower than that of parental HIV-1JR-FLan. This result suggested that most of the viral clones
in the library were replication-competent. In fact, 16 of 21 (76%) viral clones randomly selected from the library were competent in PM1-CCR5 cells (data not shown). No blue-stained foci were detected in MAGI cells infected with HIV-1JR-FLan and HIV-1JR-FL, whereas X4 virus HIV-1X4.L4.3 did generate blue foci. To examine whether the library contained viral clones that acquired the ability to use CXCR4 as a coreceptor by combination of mutations restricted to the V3 loop, a large amount of p24 was used for infection of MAGI cells. No blue stained cells were obtained with HIV-1V3Lib (Fig. 4A). To further confirm this result, MT-2 cells were infected with HIV-1V3Lib (Fig. 4B). p24 production from PM1-CCR5 cells infected with HIV-1JR-FLan was saturated on d 3 due to the intense cytopathic effect caused by the high efficiency of infection at time 0. p24 generation from HIV-1V3Lib-infected PM1-CCR5 cells reached a higher level than that of HIV-1JR-FLan-infected cells due to a slower cell death rate. Whereas no increase in p24 was observed in the supernatant of MT-2 cells infected with the same supernatant of p24 of HIV-1V3Lib, even at d 7 post-infection. Similarly, there was no increase in the amount of p24 released from MT-2 cells infected with HIV-1JR-FLan. We repeated the same experiments, but did not obtain any viral clones from the library that could use CXCR4 for entry. These results indicated that none of the combinations of the 0-10 substitutions (27,648 possibilities) in the V3 loop derived from R5 viruses could use CXCR4 as a coreceptor in an HIV-1JR-FLan background. The V3 loop of clinical isolates has a global positive charge that can vary from +2 to +10, and an increase in the positive charge of the V3 loop enables the use of CXCR4 (7,25). The virus library had V3 loops with a positive charge from +3 to +7 and did not contain the X4 type.

Selection of TAK-779-resistant HIV-1 from the library

In the presence of 0.1 μM TAK-779, infectivity of HIV-1JR-FLan or unselected HIV-1V3Lib was suppressed by 86-92%, as evaluated by a single-round assay using MAGI-CCR5 cells (Fig. 5, passage 0). After three passages of HIV-1V3Lib in the presence of 0.1 μM TAK-779, we increased the concentration of TAK-779 to 0.30 μM at passage 3. HIV-1JR-FLan could not be passaged in PM1-CCR5 cells in the presence of 0.1 μM TAK-779 due to poor replication, whereas HIV-1V3Lib started to replicate after a delay of 3 or 4 d compared with HIV-1V3Lib in the absence of 0.30 μM TAK-779 at passage 3 (data not shown). The inhibitor-sensitive population of the virus library seemed to be replaced with the inhibitor-resistant variants from passages 3-8 (Fig. 5). The infectivities of the variants at passages 8 and 10 were not even inhibited by the presence of 0.10 μM TAK-779. In contrast, HIV-1V3Lib at passage 8 in the same passage schedule without the inhibitor did not acquire resistance to TAK-779. We determined the IC50 of the selected HIV-1V3Lib using a single-round assay (Table 2). The selected variants showed 15-fold resistance to TAK-779 compared with HIV-1JR-FLan, whereas unselected HIV-1V3Lib at passage 8 did not. To elucidate the alteration of the V3 loop responsible for the resistance in the selected HIV-1V3Lib, we subcloned and sequenced the V3 loops of the variants. The V3 loops contained 5 incorporated substitutions, namely I304V, H305N, I306M, F312L and E317D (Fig. 6A). In addition to these substitutions, 25% of the clones (3/12) contained A332D in the gp120 C3 region. No other mutations were detected in other regions of gp120 in the selected viruses by direct sequencing (data not shown). In the absence of the entry inhibitor, the viruses at passage 8 only contained 3 types of V3 loop in 13 clones (Fig. 6A), although the virus at passage 0 contained complexity in all 10 amino acid positions (Fig. 3A). These V3 loops, especially V3 loop #01, containing I304V, H305Y, I306L, E317H, I318V and D324N (85%; 11/13), may confer a substantial replicative advantage among the 27,648 possible substitution combinations in PM1-CCR5 cells without selective pressure.

To confirm that resistance was conferred by changes in the V3 loop, we cloned the AflIII-NheI DNA fragment into env genes containing the V3 loop of #31 and #32. These were then used to replace the V3 loop of the parental virus HIV-1JR-FLan, and their drug sensitivities were determined (Fig. 6A). The recombinants showed 16-fold resistance to TAK-779 compared with the parental virus and recombinants carrying the #01, #02 and #03 V3 loops, indicating that the set of 5 substitutions in V3 loop #31 was sufficient for resistance and A332D in the C3 region was not required (Table 3). To confirm a key amino acid substitution to resistance, we constructed HIV-1JR-FLan with one amino acid substitution detected in HIV-1V3Lib-P8-. However, one amino acid substitution could not confer TAK-779 resistance to the parental virus (Fig. 6B). Gly307-Pro308-Gly309-Arg310-Ala311 forms the tip of the V3 loop. The recombinant virus containing two substitutions at the both sides of proximal residues of the tip could confer low resistance (3.1-fold), compared to HIV-1V3Lib-P8+/#31.

DISCUSSION

We developed an alternative in vitro system for screening and analyzing an R5 HIV-1 library containing diverse structures of the V3 loop using HIV-1JR-FLan as the background. Using the R5
HIV-1 library, we could isolate the virus resistance to TAK-779 containing five amino acid substitutions in the V3 loop by 8 passages in PM1-CCR5 cells. For construction of the virus library, we reduced the number of substitution combinations by considering the following observations for the limited size of the library (< 10^3) (32). The residues in the GPG motif (Gly320-Pro321-Gly322 in HIV-1 JR-FLan Env) (Fig. 1) are important for soluble gp120-CD4 complex binding to cell surface CCR5 (39). Loss of an N-linked glycosylation site in the V3 loop (Asn298-Asn299-Thr300) has an influence on switching from R5 to the X4 phenotype (41). We did not alter these amino acid residues. Instead, we randomly substituted the amino acids Lys302, Ser303, Ile304, His305, Ile306, Phe312, Thr314, Glu317, Ile318 and Asp321 with amino acid residues derived from 31 R5 isolates. An increase in the positive charge of the V3 loop is often associated with CXC4R usage (7,25). In particular, previous studies have determined that the presence of at least one basic substitution at V3 position 11 or 25 (at position 303 or 317 in HIV-1 JR-FLan gp120) is associated with the X4 and R5X4 phenotypes (26,27). There were no basic substitutions at positions 303 or 317 in the library (Fig. 1). For incorporation of the mutations Leu or Trp at 325 and Glu, Gin or Ala at 330, the library inevitably contained the unintended mutations of Cys at 325 and His or Pro at 330. In addition to these mutations, 6.5% of the viral clones in the library contained at least one random mutation, which was considered to be incorporated through the PCR procedure due to low fidelity (Fig. 2A). As demonstrated, replacement of the HIV-1 JR-FL V3 region containing amino acid substitutions (27,648 combinations) derived from 31 R5 HIV isolates did not generate the R5X4 or X4 phenotype (Fig. 4), although replacement of the HIV-1 JR-FL V3 region with that of HIV-1NL4-3 was sufficient to use CXC4R as a coreceptor (data not shown). Various combinations of 0 - 10 amino acid substitutions derived from R5 viruses and random mutations incorporated by PCR were not enough to generate X4 virus. Alternative mutations were required for acquisition of the ability to use CXC4R from the parental virus.

To manipulate the library for the selection of escape viruses, recipient cells with a high susceptibility to infection were required to retain the diversity of the virus library. The diversity of the virus library could not be maintained in activated peripheral blood mononuclear cells (PBMC) from healthy donors due to their low susceptibility (data not shown). PM1 cells (42) endogenously express CCR5, suggesting that an increase in CCR5 expression may be possible. We used PM1-CCR5 cells generated from PM1 cells that were highly susceptible to the virus library and formed large frequent syncitia when infected with R5 HIV-1. After 8 passages, the virus population contained a restricted number of V3 loop types, even in the absence of the compound (Fig. 6A), suggesting that there was a V3 loop structure that conferred distinctive superior fitness to HIV-1. It is considered that there are two possible ways to acquire resistance to CCR5 entry inhibitors: one is coreceptor switching from CCR5 to CXCR4/CCR5 or CXCR4 usage, and the other is to develop resistance by altering the molecular environment of CCR5 to avoid steric hindrance by the inhibitor (43). Several escape HIV-1 variants have been isolated by CCR5 entry inhibitors (43), a CC chemokine (44) or an anti-CCR5 mAb (45). The escape mutants isolated with the CCR5-specific small molecule inhibitor AD101 (SCH 350581) in vitro did not switch to CXCR4 use and continued to use CCR5 in an inhibitor-insensitive manner (43,46). The escape mutants with significant (>100-fold) resistance to the anti-CCR5 mAb 2D7 did not lead to coreceptor switching to CXCR4 (45). A MIP-1alpha-resistant virus was selected from HIV-1 JR-FL molecular clones cultured in a cell line expressing both CCR5 and CXCR4, but did not acquire CXCR4 usage (44). Similarly, in this report, the escape mutant selected from the V3 loop library continued to use CCR5 for entry, but was at least 15-fold more resistant to TAK-779 than the parental virus. We cloned the partial env genes containing the V3 loop from the escape mutant isolates and made recombinant infectious molecular clones that fully recapitulated the phenotypes of the corresponding escape viruses without the mutation in C3 detected in clone #03. Introduction of amino acid change(s) in V3 loop of NL4-3/CC85Env chimeric virus could alter susceptibility to AD101 (46). One amino acid change in V3 loop (H305P in HIV-1 JR-FL V3 loop) conferred 500-fold resistance to AD101, and the chimeric virus with four amino acid changes (K302R, H305P, A311V, G316E in HIV-1 JR-FL V3 loop) showed extremely high resistance (> 5 x 10^6-fold). In the present study, TAK-779 selected variants did not contain K302R or H305P, though the library contained these two amino acid changes. Furthermore 8 passages were required to reach > 10 fold resistance, suggesting that the amino acid substitutions derived from polymorphisms in the V3 loop of R5 viruses may not confer high resistance to TAK-779 with HIV-1 JR-FLan background. We continued the selection of the virus library with TAK-779 up to 22 passages, but could not obtain variants replicating in the presence of > 0.30 µM TAK-779 (data not shown). According to the mutagenesis and modeling data, TAK-779 binding site is located near the CCR5 extracellular surface, within a cavity surrounded by transmembrane helices of 1, 2, 3, and 7 (35). This
binding site may be less influenced by interaction of V3 loop in gp120 than that of AD101. To acquire relatively higher resistance to TAK-779, other mutations that are not classified as polymorphisms may be needed for authentic interaction with CCR5 without the inhibition caused by entry inhibitors.

This type of HIV-1 library based on molecular clones containing combinations of mutations localized in a certain domain of the genes in HIV-1 may be a useful tool for analyzing the acquisition of resistance to entry inhibitors.

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**FOOTNOTES**

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FIGURE LEGENDS

**Fig. 1.** gp120 V3 loop sequences from 31 R5 HIV-1 described in the HIV Database (33). The top line shows the V3 loop amino acid residues in HIV-1JR-FL. The numbers shown beside the residues indicate the frequency of the substitutions in 31 R5 viruses. The 10 residues in bold are the positions incorporated with amino acid substitutions.

**Fig. 2.** Construction of the HIV-1 V3 loop library. A, Oligonucleotides used to construct the library. The nucleotides in bold indicate the positions incorporated with substitutions. The single letters in bold indicate the degenerated nucleotides: R represents A or G; H represents A, C or T; M represents A or C; Y represents C or T; K represents G or T; W represents A or T; and S represents C or G. The underlined nucleotides indicate the V3 loop-encoding sequence. The residues in bold indicate the substitutions that are randomly incorporated in the V3 loop of pJR-FL-V3Lib. The underlined residues indicate the substitutions that are not detected in the 31 R5 viruses but are inevitably incorporated into the library due to combinations of nucleotide substitutions. B, Schematic procedures for the construction of pJR-FL-V3Lib.

**Fig. 3.** A, Amino acid sequences of the V3 loops of viral clones in the virus library HIV-1V3Lib used for the selection of escape viruses. B, Compositions of the amino acid residues at positions 305, 306, 308, 309, 315, 320, 321 and 324 in the V3 loop in the library DNA and the generated virus library used for selection.

**Fig. 4.** Coreceptor usage of the virus library HIV-1V3Lib. A, Infectivities of HIV-1V3Lib and HIV-1JR-FL to MAGI and MAGI-CCR5 cells. The cells (1 x 10⁶) were infected with HIV-1V3Lib or HIV-1JR-FLan (0.6 μg of p24 Gag) and stained at 48 h post-infection. B, PM1-CCR5 and MT-2 cells (1 x 10⁵) were infected with HIV-1V3Lib or HIV-1JR-FLan (0.6 μg of p24 Gag) and virus replication was monitored by measuring the p24 antigen production. PM1 cells (1 x 10⁵) were also infected with HIV-1V3Lib, HIV-1JR-FLan, or HIV-1NL4-3 (0.6 μg of p24 Gag) and virus replication was monitored.

**Fig. 5.** Selection of TAK-779 escape viruses from the HIV-1 V3 loop library. The virus library (Fig. 3) was infected into PM1-CCR5 cells. At 3-4 d post-infection in the presence or absence of TAK-779, the released viruses at passages 0, 3, 6, 8 and 10 were used for single-round assays with MAGI-CCR5 cells in the presence of 0.1 μM TAK-779. The number of blue foci obtained in the presence of TAK-779 was divided by that in the absence of TAK-779.

**Fig. 6.** A, Sequences of the V3 loop in TAK-779 escape viruses (passage 8) in the absence (HIV-1V3Lib-P8-) or presence (HIV-1V3Lib-P8+) of TAK-779. The number above the sequences indicate the positions of amino acid residues in JR-FLan gp120. HIV-1JR-FLan has been also passaged for the same duration without drug pressure, however, no mutations were detected in the V3 loop (data not shown). B, Susceptibilities of recombinant viruses containing TAK-779 resistance-associated mutation(s). IC₅₀: concentration required to inhibit 50% of the blue foci formation in MAGI-CCR5 cells at two days post-infection. Degree of resistance (x-fold) as compared with HIV-1JR-FLan.
Table 1. HIV-1 library (pJR-FL-V3Lib) containing combinations of 0-10 amino acid substitutions in the V3 loop

| No. of combinations of amino acid substitutions | aNo. of independent clones | No. of clones containing a mutated V3 loop | bLigation efficiency |
|-----------------------------------------------|-----------------------------|----------------------------------------|---------------------|
| 27,648                                        |                             |                                        |                     |
| pCR-SX-V3Lib                                  | 8.5 x 10^6                  | 7.1 x 10^6                             | 84%                 |
| pJR-FL-V3Lib                                  | 9.3 x 10^6                  | 6.6 x 10^6                             | 77%                 |

aAfter transformation, 1/1000 volume of the cell suspension was inoculated on an LB agar plate containing 100 µg/ml ampicillin and incubated overnight. The number of independent clones was then counted.

bThe ligation efficiency was obtained from > 70 independent clones.
| Virus                | aIC<sub>50</sub> (µM) | TAK-779 | AMD3100 |
|---------------------|-----------------------|---------|---------|
| HIV-1<sub>JR-FL</sub>an | 0.032 ± 0.07 (1.0)    | > 3     | > 3     |
| HIV-1<sub>JR-FL</sub> | 0.034 ± 0.05 (1.1)    | > 3     | > 3     |
| HIV-1<sub>NL4-3</sub> | > 1                   | 0.0023 ± 0.0003 |          |
| bHIV-1<sub>V3Lib-P8</sub>- TAK-779 | 0.041 ± 0.12 (1.4)    | > 3     | > 3     |
| dHIV-1<sub>V3Lib-P8</sub>+ TAK-779 | 0.48 ± 0.15 (15)      | > 3     | > 3     |

aIC<sub>50</sub>: concentration required to inhibit 50% of the blue foci formation in MAGI-CCR5 cells at 2 days post-infection.
bVirus library passaged 8 times without TAK-779.
cDegree of resistance (x-fold) as compared with HIV-1<sub>JR-FL</sub>an.
dVirus library selected with 0.1 µM TAK-779 from passages 1-3 and 0.3 µM TAK-779 from passages 4-8.
Table 3. Susceptibilities of recombinant HIV-1\textsubscript{V3Lib} to entry inhibitors

| bVirus                  | IC\textsubscript{50} (μM) |   |
|-------------------------|---------------------------|---|
|                         | TAK-779                   | AMD3100 |
| HIV-1\textsubscript{JR-FLan} | 0.031 ± 0.080 (1.0)       | > 3    |
| HIV-1\textsubscript{V3Lib\#01} | 0.008 ± 0.010 (0.3)       | > 3    |
| HIV-1\textsubscript{V3Lib\#02} | 0.009 ± 0.004 (0.3)       | > 3    |
| HIV-1\textsubscript{V3Lib\#03} | 0.035 ± 0.012 (1.2)       | > 3    |
| HIV-1\textsubscript{V3Lib\#31} | 0.49 ± 0.09 (16)          | > 3    |
| HIV-1\textsubscript{V3Lib\#32} | 0.43 ± 0.15 (14)          | > 3    |

\( ^{a} \text{IC}_{50} \): concentration required to inhibit 50% of the blue foci formation in MAGI-CCR5 cells at 2 days post-infection.

\( ^{b} \)The recombinant viruses HIV-1\textsubscript{V3Lib\#01}, HIV-1\textsubscript{V3Lib\#02}, HIV-1\textsubscript{V3Lib\#03}, HIV-1\textsubscript{V3Lib\#31} and HIV-1\textsubscript{V3Lib\#32} contain V3 with amino acid substitutions detected in the viral clones, as shown in Fig. 6A.

\( ^{c} \)Numbers in parentheses represent degree of resistance (x-fold) as compared with HIV-1\textsubscript{JR-FLan}.
Annealing and ligation of degenerated oligonucleotides, PCR and digestion with *Afl*II and *Nhe*I

**Fig. 2A**
Fig. 2B
Fig. 3A
JR-FL an V3 loop

CTRPN

Library DNA

\[ X_{302} : K \ 32.7\%, \ R \ 67.3\% \]
\[ X_{303} : S \ 75.0\%, \ G \ 25.0\% \]
\[ X_{304} : I \ 44.2\%, \ V \ 55.8\% \]
\[ X_{305} : H \ 23.1\%, \ P \ 19.2\%, \ N \ 15.3\%, \ S \ 7.7\%, \ T \ 23.1\%, \ Y \ 11.5\% \]
\[ X_{306} : I \ 15.3\%, \ M \ 51.9\%, \ L \ 32.7\% \]
\[ X_{312} : F \ 32.7\%, \ L \ 25.0\%, \ W \ 17.3\%, \ C \ 25.0\% \]
\[ X_{314} : T \ 48.1\%, \ A \ 51.9\% \]
\[ X_{317} : E \ 19.2\%, \ D \ 15.4\%, \ Q \ 7.7\%, \ A \ 17.3\%, \ H \ 11.5\%, \ P \ 28.8\% \]
\[ X_{318} : I \ 59.6\%, \ V \ 40.4\% \]
\[ X_{321} : D \ 42.3\%, \ N \ 57.7\% \]

Virus library

\[ X_{302} : K \ 34.8\%, \ R \ 65.2\% \]
\[ X_{303} : S \ 47.8\%, \ G \ 52.2\% \]
\[ X_{304} : I \ 50.0\%, \ V \ 47.8\%, \ G \ 2.2\% \]
\[ X_{305} : H \ 19.6\%, \ P \ 21.7\%, \ N \ 28.2\%, \ S \ 6.5\%, \ T \ 6.5\%, \ Y \ 15.2\%, \ V \ 2.2\% \]
\[ X_{306} : I \ 19.6\%, \ M \ 34.8\%, \ L \ 45.6\% \]
\[ X_{312} : F \ 32.6\%, \ L \ 30.4\%, \ W \ 21.7\%, \ C \ 15.2\% \]
\[ X_{314} : T \ 45.6\%, \ A \ 54.4\% \]
\[ X_{317} : E \ 12.8\%, \ D \ 6.5\%, \ Q \ 6.5\%, \ A \ 32.6\%, \ H \ 10.9\%, \ P \ 30.4\% \]
\[ X_{318} : I \ 50.0\%, \ V \ 50.0\% \]
\[ X_{321} : D \ 52.2\%, \ N \ 47.8\% \]

Fig. 3B
Fig. 4A
Figure 4B

MT2 cells
HIV-1 JR-FLan
HIV-1 V3Lib
0
10
20
30
40
50
0 2 4 6 8 10
Time (days)
p24 (ng/ml)

PM1-CCR5 cells
HIV-1 JR-FLan
HIV-1 V3Lib
PM1 cells
HIV-1 JR-FLan
HIV-1 V3Lib
HIV-1 NL4-3
MT2 cells
HIV-1 JR-FLan
HIV-1 V3Lib

Fig. 4B
% replication in the presence of 0.1 µM TAK-779

Fig. 5
|       | C2    | V3   | C3    |
|-------|-------|------|-------|
| HIV-1 |       |      |       |
| JR-FLan | -LKESVEINCTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHCNISRRAKWNDTLKQIAS- |      |       |
| HIV-1 |       |      |       |
| V3Lib-P8- | #01  | VYL. | HV..N. | 11/13 |
| -TAK-779 | #02  | M...A..AV..N.. | 1/13  |
| #03  | GVYM...W...H...N.. | 1/13  |
| HIV-1 |       |      |       |
| V3Lib-P8+ | #31  | VN...L...D... | 9/12  |
| +TAK-779 | #32  | VN...L...D... | 3/12  |

Fig. 6A
| HIV-1 JR-FLan | V3 sequence | IC<sub>50</sub> (μM) | (-fold resistance) |
|---------------|-------------|----------------------|--------------------|
| HIV-1 v3Lib-P8+P31 | CTRPNNNTRKSIHIGPGRAFYTTEIIGDIRQAHC | 0.031 ± 0.007 | 1.0 |
| HIV-1 JR-FLan+I304V | VNM.L.D........ | 0.43 ± 0.12 | 14 |
| HIV-1 JR-FLan+H305N | V................ | 0.036 ± 0.013 | 1.2 |
| HIV-1 JR-FLan+I306M | N................ | 0.042 ± 0.010 | 1.4 |
| HIV-1 JR-FLan+F312L | M................ | 0.033 ± 0.018 | 1.1 |
| HIV-1 JR-FLan+E317D | L................ | 0.011 ± 0.005 | 0.4 |
| HIV-1 JR-FLan+F306M/E317D | M............................D........ | 0.047 ± 0.003 | 1.5 |
| HIV-1 JR-FLan+F306M/E317D | M............................D........ | 0.098 ± 0.011 | 3.1 |