form of CXCR4. The first mechanism comprises a shift in coreceptor usage from CXCR4 to CCR5, which is induced by selective pressure from CXCR4 antagonists. However, this is unlikely to occur frequently because coreceptor switching from CCR5 to CXCR4, and *vice versa*, requires multiple mutations throughout gp160 via transitional intermediates with poor replication fitness [77].

There is an evolutionary gap in viral fitness between viruses using CXCR4 and those using CCR5. However, an R5X4 dual-tropic virus can shift from X4-dominated tropism to R5-dominated tropism [83]. The R5X4 dual-tropic 89.6 mainly uses CXCR4 as a coreceptor, but after selection with the CXCR4 antagonist T140, coreceptor usage shifted from a phenotype that mainly used CXCR4 to one mainly using CCR5 due to a single amino acid substitution (R308S) in the V3 loop in vitro. These results indicated that the R5X4 virus could shift its main coreceptor usage due to a low genetic barrier to the development of resistance. In contrast, an outgrowth of the pre-existing minority of the R5 virus caused by CXCR4 antagonists, is expected to lead to virologic failure. AMD3100 is a small molecule compound called a bicyclam that has potent antiviral activity against a variety of X4-tropic strains [94-99]. However, it is not clinically available because of low oral bioavailability [100]. After treatment of clinical isolates *in vitro* with AM3100 for 28 days, the major population of viruses using CXCR4 was promptly replaced by the pre-existing minor population using CCR5 with multiple mutations in the V3 loop in vitro [101].

The third possible pathway results from accumulation of mutations in the viral envelope that allow interaction between gp120 and the coreceptor in the presence of the inhibitor. AMD3100-resistant viruses selected *in vitro* from NL4-3 strain still used CXCR4 as a coreceptor and contained several mutations in the V3 loop and showed poor fitness [102]. In contrast, other viruses resistant to POL3026, a specific β-hairpin mimetic CXCR4 antagonist, did not show any fitness cost and contained four mutations (Q310H, I320T, N325D, and A329T) in the gp120 V3 loop [70]. These four mutations were shared by viral strains resistant to SDF-1α [103] and T134 [104], indicating that the V3 loop is a crucial region for the acquisition of CXCR4 antagonist resistance.

The fourth possible mechanism involves acquisition of the ability to utilize the inhibitor-bound form as well as the drug-free form of CXCR4 for viral entry. Several clinical isolates demonstrate infection through the AMD3100-bound form of CXCR4, indicating a non-competitive mode of drug resistance [99]. The V1/V2 region of one of the isolates is responsible for this property, suggesting that baseline resistance to this kind of CXCR4 antagonist should be considered while developing CXCR4 antagonists. Recent advances have led to the development of orally-active CXCR4 antagonists, including AMD11070 [105], KRH-3955 [106], and GSK81297 [107]. Therefore, to prevent the possible emergence of pre-existing forms of the CCR5 virus, it is likely that CXCR4 antagonists will be effective only in combination with a CCR5 antagonist or other antiviral drugs.

Fusion inhibitory peptides and their mechanisms of action

Fusion inhibitors: Enfuvirtide (T-20) was approved by the FDA in 2003 as the first fusion inhibitor that efficiently suppresses the replication of HIV-1 resistant to available classes of anti-HIV-1 drugs (Figure 1), such as reverse transcriptase inhibitors (RTIs) and protease inhibitors (PIs). Hence, it has been widely used for treatment of HIV-1 infected patients where treatment with other antiretroviral drugs has failed [108]. T-20 comprises a 36 amino acid peptide derived from the gp41 HIV-1 C-terminal heptad repeat (C-HR), as shown in Figure 7.

During HIV-1 entry, binding of gp120 to CD4 and either CCR5 or CXCR4 initiates penetration of the hydrophobic fusion peptide domain at the N-terminal heptad repeat (N-HR) of gp41 into the target

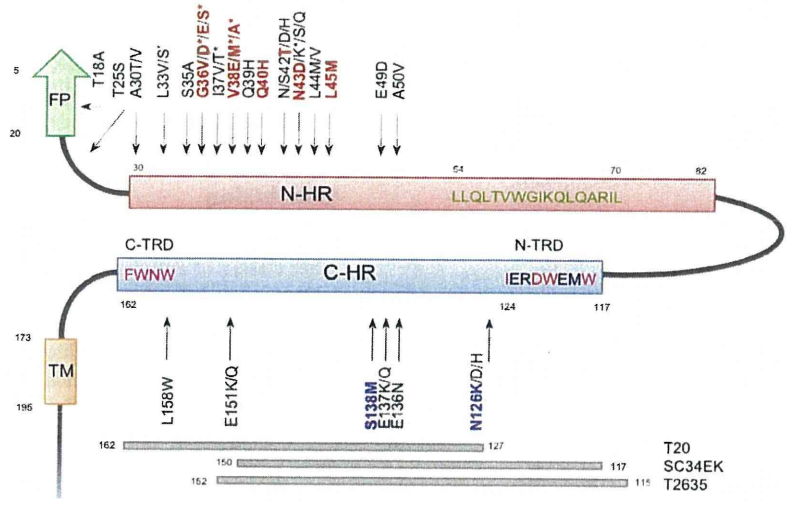

Figure 7: Schematic view of HIV-1 gp41 functional domains and mutation map for T-20. Putative hydrophobic pocket region of the N-HR is shown (green) and may form a leucine-zipper-like domain. In the C-HR, two tryptophan-rich domains (TRD; pink) are located at the N- and C-terminal regions (N-TRD and C-TRD, respectively). The N-TRD binds to the hydrophobic pocket in the N-HR, whereas the C-TRD plays a key role in membrane association. FP; fusion peptide domain, which penetrates into the target cell membrane. TM; transmembrane region. The amino acid sequence of the HXB2 clone is shown as a representative HIV-1 sequence. Only mutations located in the extracellular domain of gp41 are shown. Mutations observed in in vitro and in vivo selections are indicated by an asterisk (*). I37T was only selected in vitro. Primary and secondary mutations were most frequently associated with T-20 resistance (red and blue, respectively). In addition, T25S/A, S35A/T, R46K, L55F, Q56R/K, V72L, A101I/T/V/G, L108Q, N109D, D113G/N, E119Q, L130V, I135L, N140I, and L158W were selected in patients under T-20 containing regimens, but observed in some drug-naïve HIV-1 strains (Los Alamos HIV Sequence Data Bank, http://www.hiv.lanl.gov/content/index (natural polymorphisms). Corresponding regions of T-20, SC34EK, and T2635 are shown. T-20 is comprised of the original sequence but others are extensively modified.

cell membrane [6]. In the gp41 extra-cellular domain, the α-helical region at the C-HR begins to fold and interact with a trimeric form of the N-HR in an anti-parallel manner. This intramolecular folding forms a stable six-helix bundle and facilitates the fusion of the virus envelope and cellular membranes. During the fusion step of HIV-1 replication, T-20 can interfere with the formation of the six-helix bundle consisting of a trimeric N-HR/C-HR complex.

In the C-HR, two tryptophan-rich domains (TRDs) are located in close proximity to the connection loop (N-TRD) and the membrane-spanning or transmembrane region (C-TRD). Both TRDs resemble a leucine zipper structure and are believed to be important for interactions of the N-HR and the C-HR. T-20 contains the amino acid sequence of the C-TRD, whereas C34-based peptides, such as SC34EK and T2635, contain the N-TRD. T-20 is believed to bind to the N-HR as a decoy and prevents the formation of the six-helix bundle [109], resulting in the inhibition of HIV-1 entry. This mode of action has been well documented with another fusion inhibitory peptide, C34, and remains controversial whether the mechanisms of action of T-20 and C34 are in fact the same.

Primary and secondary mutations for fusion inhibitors: Although some fusion peptides, such as N36 [110] and IQN17 [111], are designed using the N-HR sequence, most have been designed using the C-HR sequence. Primary mutations for a representative C-HR derived peptide, T-20, are generally introduced within the N-HR, a putative binding site of T-20 [112,113]. Mutations frequently reported *in vivo* are located at amino acid positions 36–45 of the gp41, including G36D/S/E/V, V38A/M/E, Q40H, N42T, and N43D/K (Figure 7) [114]. Using circular dichroism analysis, others and we clearly demonstrated that these primary mutations reduce the binding affinity of C-peptides with the N-HR [112,115]. This mutation also impairs physiological intra-molecular binding of the C-HR with the N-HR, providing a replication cost [116]. Therefore, HIV-1 develops secondary or compensatory mutations in the C-HR to restore the reduced stabilities of the six-helix bundle by the introduction of primary mutations. N126K, E137K/Q, and S138A [115,117] have been reported *in vivo*, usually in combination with N-HR mutations. Mutations in the C-HR restore the intra-molecular folding/interaction of the C-HR with the N-HR. The enhanced binding affinity by the secondary mutations can be applied to peptide design, such as C34 with N126K and T-20 with S138A, which maintain anti-HIV-1 activity, even to drug-resistant HIV-1 [115].

Secondary mutations of the N-HR are not only non-synonymous, but also synonymous. A part of the RNA coding region for the env gene, including gp41, also encodes the Rev-responsible element (RRE), which is an RNA secondary structure important for unspliced RNA export from the nucleus that is required for efficient viral protein synthesis and packaging of genomic RNA [118,119]. Primary mutations at positions 36 and 38 for stem II and at 43 for stem III affect the RRE structure. Synonymous and non-synonymous mutations introduced into the gp41 compensate for RRE structure stability, such as T18A for V38A [120] and A30V for G36D [116], and Q41 (CAG to CAA) and L44 (UUG to CUG) for N43D [121]. This association between the gp41 and RRE results in some genetic restrictions.

Impact of mutations on clinical potency: Only one or two amino acid substitutions in gp41 appear to be sufficient for clinical treatment failure, where after the emergence of mutations, viral load gradually increases [122]. For example, G36E, V38A, Q40H, and N43D were shown to confer 39.3-, 16-, 21-, and 18-fold reductions in susceptibility to T-20, respectively [123]. Double or triple substitutions have also been identified in clinical isolates from patients undergoing therapy with T-20. Mutations such as N42T+N43S, V38A+N42D, and Q40H+L45M confer 61-, 140-, and 67-fold reductions in susceptibility to T-20, respectively [123]. Mutations at codons 36 (G36E/D/S) and 38 (V38A/G/M) seem to emerge relatively rapidly *in vivo*, whereas Q40H and N43D emerge more slowly [122]. After prolonged therapy, HIV-1 has been shown to develop secondary mutations and may confer more apparent resistance with improved replication kinetics. Therefore, combination regimens with other inhibitors, such as RTIs and PIs, are indispensable for sufficient positive viral responses.

T-20 appears to inhibit replication of HIV-1 subtype independently [124-126], since T-20 has mainly been used for subtype B HIV-1 infected patients. Based on the mechanism of action of T-20, interference of N-and C-HR interactions may be expected, where amino acid sequences are highly conserved across all subtypes. However, in non-B subtype HIV-1, N42S predominantly emerged as a resistance-related mutation [124,125].

Resistance to the next generation inhibitors: Next generation inhibitors have been designed using several strategies, such as the introduction of specific amino acid motifs and secondary mutations into the sequence of the original peptide inhibitors [115] to enhance the stability of the α-helical structure between inhibitors and fusion domain at the N-HR. In contrast to T-20, primary mutations to third generation inhibitors were not selected *in vitro* [127,128]; therefore, the accumulation of multiple mutations is likely necessary for the development of resistance. In the case of SC34EK, 13 amino acid substitutions (D36G, Q41R, N43K, A96D, N126K, E151K, H132Y, V182I, P203S, L204I, S241F, H258Q, and A312T) were introduced and single amino acid substitutions only conferred weak resistance (<6-fold) [127]. For another peptide, T-2635, 12 amino acids in 10 positions (A6V, L33S, Q66R/L, K77E/N, T94N, N100D, N126K, H132Q, E136G, and E151G) were selected, and single mutations did not confer resistance to T-2635 [128]. Interestingly, some of these mutations were located outside the N-HR and C-HR. Cross-resistance between SC34EK and T-2635 was only examined for the SC34EK-resistant virus and revealed little cross-resistance [127]. Further studies of resistance profiles might be helpful in defining new strategies for the design of fusion inhibitors that can suppress the replication of resistant variants of HIV-1.

Conclusion

The emergence of viruses resistant to entry inhibitors, as well as other classes of antiviral agents (reverse transcriptase or protease inhibitors), has been reported *in vitro* and *in vivo*. Resistance to entry inhibitors, including attachment inhibitors and coreceptor antagonists, is mainly conferred as a result of missense mutations within the gp120 subunit of the *env* gene, which differ from one inhibitor to another. Alternatively, treatment failure can occur through the expansion of pre-existing CXCR4-using virus for CCR5 antagonists, and vice versa. Agents that target gp41-dependent fusion select for HIV-1 variants with mutationswithin the gp41 envelope gene. These results indicate the incredible flexibility of the HIV-1 genome to escape from a variety of entry inhibitors. Therefore, the development of novel entry inhibitors for clinical use is needed to limit escape mutants by effective combination therapy.

References

1. Potter SJ, Chew CB, Steain M, Dwyer DE, Saksena NK (2004) Obstacles to successful antiretroviral treatment of HIV-1 infection: problems & perspectives. Indian J Med Res 119: 217-237.

2. Shafer RW, Schapiro JM (2008) HIV-1 drug resistance mutations: an updated framework for the second decade of HAART. AIDS Rev 10: 67-84.
3. Gupta RK, Gibb DM, Pillay D (2009) Management of paediatric HIV-1 resistance. Curr Opin Infect Dis 22: 256-263.
4. Wild C, Greenwell T, Matthews T (1993) A synthetic peptide from HIV-1 gp41 is a potent inhibitor of virus-mediated cell-cell fusion. AIDS Res Hum Retroviruses 9: 1051-1053.
5. Chan DC, Fass D, Berger JM, Kim PS (1997) Core structure of gp41 from the HIV envelope glycoprotein. Cell 89: 263-273.
6. Chan DC, Kim PS (1998) HIV entry and its inhibition. Cell 93: 681-684.
7. Westby M, van der Ryst E (2010) CCR5 antagonists: host-targeted antiviral agents for the treatment of HIV infection, 4 years on. Antivir Chem Chemother 20: 179-192.
8. Dorr P, Westby M, Dobbs S, Griffin P, Irvine B, et al. (2005) Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. Antimicrob Agents Chemother 49: 4721-4732.
9. Fatkenheuer G, Pozniak AL, Johnson MA, Plettenberg A, Staszewski S, et al. (2005) Efficacy of short-term monotherapy with maraviroc, a new CCR5 antagonist, in patients infected with HIV-1. Nat Med 11: 1170-1172.
10. Moore JP, Kuritzkes DR (2009) A piece de resistance: how HIV-1 escapes small molecule CCR5 inhibitors. Curr Opin HIV AIDS 4: 118-124.
11. Wyatt R, Sodroski J (1998) The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science 280: 1884-1888.
12. Sattentau QJ, Moore JP (1993) The role of CD4 in HIV binding and entry. Philos Trans R Soc Lond B Biol Sci 342: 59-66.
13. Ugolini S, Mondor I, Sattentau QJ (1999) HIV-1 attachment: another look. Trends Microbiol 7: 144-149.
14. ES, Li XL, Moudgil T, Ho DD (1990) High concentrations of recombinant soluble CD4 are required to neutralize primary human immunodeficiency virus type 1 isolates. Proc Natl Acad Sci U S A 87: 6574-6578.
15. Orloff SL, Kennedy MS, Belperron AA, Maddon PJ, McDougal JS (1993) Two mechanisms of soluble CD4 (sCD4)-mediated inhibition of human immunodeficiency virus type 1 (HIV-1) infectivity and their relation to primary HIV-1 isolates with reduced sensitivity to sCD4. J Virol 67: 1461-1471.
16. McKeating J, Balfe P, Clapham P, Weiss RA (1991) Recombinant CD4-selected human immunodeficiency virus type 1 variants with reduced gp120 affinity for CD4 and increased cell fusion capacity. J Virol 65: 4777-4785.
17. Yoshimura K, Harada S, Shibata J, Hatada M, Yamada Y, et al. (2010) Enhanced exposure of human immunodeficiency virus type 1 primary isolate neutralization epitopes through binding of CD4 mimetic compounds. J Virol 84: 7558-7568.
18. Jacobson JM, Israel RJ, Lowy I, Ostrow NA, Vassilatos LS, et al. (2004) Treatment of advanced human immunodeficiency virus type 1 disease with the viral entry inhibitor PRO 542. Antimicrob Agents Chemother 48: 423-429.
19. Bodart V, Anastassov V, Darkes MC, Idzan SR, Labrecque J, et al. (2009) Pharmacology of AMD3465: a small molecule antagonist of the chemokine receptor CXCR4. Biochem Pharmacol 78: 993-1000.
20. Kuritzkes DR, Jacobson J, Powderly WG, Godofsky E, DeJesus E, et al. (2004) Antiretroviral activity of the anti-CD4 monoclonal antibody TNX-355 in patients infected with HIV type 1. J Infect Dis 189: 286-291.
21. Toma J, Weinheimer SP, Stawiski E, Whitcomb JM, Lewis ST, et al. (2011) Loss of asparagine-linked glycosylation sites in variable region 5 of human immunodeficiency virus type 1 envelope is associated with resistance to CD4 antibody ibalizumab. J Virol 85: 3872-3880.
22. Zwick MB, Jensen R, Church S, Wang M, Stiegler G, et al. (2005) Anti-human immunodeficiency virus type 1 (HIV-1) antibodies 2F5 and 4E10 require surprisingly few crucial residues in the membrane-proximal external region of glycoprotein gp41 to neutralize HIV-1. J Virol 79: 1252-1261.
23. Calarese DA, Lee HK, Huang CY, Best MD, Astronomo RD, et al. (2005) Dissection of the carbohydrate specificity of the broadly neutralizing anti-HIV-1 antibody 2G12. Proc Natl Acad Sci U S A 102: 13372-13377.
24. Walker LM, Phogat SK, Chan-Hui PY, Wagner D, Phung P, et al. (2009) Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. Science 326: 285-289.
25. DR, Pyati J, Koduri R, Sharp SJ, Thornton GB, et al. (1994) Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science 266: 1024-1027.
26. T, Xu L, Dey B, Hessell AJ, Van Ryk D, et al. (2007) Structural definition of a conserved neutralization epitope on HIV-1 gp120. Nature 445: 732-737.
27. H, Stamatatos L, Ip JE, Barbas CF, Parren PW, et al. (1997) Human immunodeficiency virus type 1 mutants that escape neutralization by human monoclonal antibody IgG1b12. off. J Virol 71: 6869-6874.
28. Wu X, Zhou T, O'Dell S, Wyatt RT, Kwong PD, et al. (2009) Mechanism of human immunodeficiency virus type 1 resistance to monoclonal antibody B12 that effectively targets the site of CD4 attachment. J Virol 83: 10892-10907.
29. Wu X, Yang ZY, Li Y, Hogerkorp CM, Schief WR, et al. (2010) Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. Science 329: 856-861.
30. Zhou T, Georgiev I, Wu X, Yang ZY, Dai K, et al. (2010) Structural basis for broad and potent neutralization of HIV-1 by antibody VRC01. Science 329: 811-817.
31. Li Y, O'Dell S, Walker LM, Wu X, Guenaga J, et al. (2011) Mechanism of neutralization by the broadly neutralizing HIV-1 monoclonal antibody VRC01. J Virol 85: 8954-8967.
32. Guo Q, Ho HT, Dicker I, Fan L, Zhou N, et al. (2003) Biochemical and genetic characterizations of a novel human immunodeficiency virus type 1 inhibitor that blocks gp120-CD4 interactions. J Virol 77: 10528-10536.
33. Lin PF, Blair W, Wang T, Spicer T, Guo Q, et al. (2003) A small molecule HIV-1 inhibitor that targets the HIV-1 envelope and inhibits CD4 receptor binding. Proc Natl Acad Sci U S A 100: 11013-11018.
34. Fransen S, Bridger G, Whitcomb JM, Toma J, Stawiski E, et al. (2008) Suppression of dualtropic human immunodeficiency virus type 1 by the CXCR4 antagonist AMD3100 is associated with efficiency of CXCR4 use and baseline virus composition. Antimicrob Agents Chemother 52: 2608-2615.
35. Zhou N, Nowicka-Sans B, Zhang S, Fan L, Fang J, et al. (2011) *In vivo* patterns of resistance to the HIV attachment inhibitor BMS-488043. Antimicrob Agents Chemother 55: 729-737.
36. A, Madani N, Klein JC, Hubicki A, Ng D, et al. (2006) Thermodynamics of binding of a low-molecular-weight CD4 mimetic to HIV-1 gp120. Biochemistry 45: 10973-10980.
37. Q, Ma L, Jiang S, Lu H, Liu S, et al. (2005) Identification of N-phenyl-N'-(2,2,6,6-tetramethyl-piperidin-4-yl)-oxalamides as a new class of HIV-1 entry inhibitors that prevent gp120 binding to CD4. Virology 339: 213-225.
38. Lalonde JM, Elban MA, Courter JR, Sugawara A, Soeta T, et al. (2011) Design, synthesis and biological evaluation of small molecule inhibitors of CD4-gp120 binding based on virtual screening. Bioorg Med Chem 19: 91-101.
39. Madani N, Schon A, Princiotto AM, Lalonde JM, Courter JR, et al. (2008) Small-molecule CD4 mimics interact with a highly conserved pocket on HIV-1 gp120. Structure 16: 1689-1701.
40. Narumi T, Ochiai C, Yoshimura K, Harada S, Tanaka T, et al. (2010) CD4 mimics targeting the HIV entry mechanism and their hybrid molecules with a CXCR4 antagonist. Bioorg Med Chem Lett 20: 5853-5858.
41. Yamada Y, Ochiai C, Yoshimura K, Tanaka T, Ohashi N, et al. (2010) CD4 mimics targeting the mechanism of HIV entry. Bioorg Med Chem Lett 20: 354-358.
42. EE, Lin X, Li W, Cotter R, Klein MT, et al. (2006) Inhibition of highly productive HIV-1 infection in T cells, primary human macrophages, microglia, and astrocytes by Sargassum fusiforme. AIDS Res Ther 3: 15.
43. Lee DY, Lin X, Paskaleva EE, Liu Y, Puttamadappa SS, et al. (2009) Palmitic Acid Is a Novel CD4 Fusion Inhibitor That Blocks HIV Entry and Infection. AIDS Res Hum Retroviruses 25: 1231-1241.
44. Paskaleva EE, Xue J, Lee DY, Shekhtman A, Canki M (2010) Palmitic acid analogs exhibit nanomolar binding affinity for the HIV-1 CD4 receptor and nanomolar inhibition of gp120-to-CD4 fusion. PLoS One 5: e12168.
45. Lin X, Paskaleva EE, Chang W, Shekhtman A, Canki M (2011) Inhibition of HIV-

1 infection in ex vivo cervical tissue model of human vagina by palmitic acid; implications for a microbicide development. PLoS One 6: e24803.

46. Connor RI, Sheridan KE, Ceradini D, Choe S, Landau NR (1997) Change in coreceptor use correlates with disease progression in HIV-1-infected individuals. J Exp Med 185: 621-628.

47. Liu R, Paxton Wa, Choe S, Ceradini D, Martin SR, et al. (1996) Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell 86: 367-377.

48. Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, et al. (1996) Resistance to HIV-1 infection in Caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature 382: 722-725.

49. Baba M, Nishimura O, Kanzaki N, Okamoto M, Sawada H, et al. (1999) A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. Proc Natl Acad Sci U S A 96: 5698-5703.

50. Imamura S, Ichikawa T, Nishikawa Y, Kanzaki N, Takashima K, et al. (2006) Discovery of a piperidine-4-carboxamide CCR5 antagonist (TAK-220) with highly potent Anti-HIV-1 activity. J Med Chem 49: 2784-2793.

51. Maeda K, Yoshimura K, Shibayama S, Habashita H, Tada H, et al. (2001) Novel low molecular weight spirodiketopiperazine derivatives potently inhibit R5 HIV-1 infection through their antagonistic effects on CCR5. J Biol Chem 276: 35194-35200.

52. Strizki JM, Xu S, Wagner NE, Wojcik L, Liu J, et al. (2001) SCH-C (SCH 351125), an orally bioavailable, smallmolecule antagonist of the chemokine receptor CCR5, is a potent inhibitor of HIV-1 infection *in vitro* and *in vivo*. Proc Natl Acad Sci U S A 98: 1-6.

53. Tagat JR, McCombie SW, Nazareno D, Labroli MA, Xiao Y, et al. (2004) Piperazine-Based CCR5 Antagonists as HIV-1 Inhibitors. IV. Discovery of 1-[(4,6-Dimethyl-5-pyrimidinyl)carbonyl]- 4-[4-{2-methoxy-1(R)-4-(trifluoromethyl)phenyl}ethyl-3(S)-methyl-1-piperazinyl]- 4-methylpiperidine (Sch-417690/Sch-D), a Potent, Highly Sele. J Med Chem 47: 2405-2408.

54. Dragic T, Trkola a, Thompson Da, Cormier EG, Kajumo Fa, et al. (2000) A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. Proc Natl Acad Sci USA 97: 5639-5644.

55. Maeda K, Nakata H, Koh Y, Miyakawa T, Ogata H, et al. (2004) Spirodiketopiperazine-based CCR5 inhibitor which preserves CC-chemokine/CCR5 interactions and exerts potent activity against R5 human immunodeficiency virus type 1 in vitro. J Virol 78: 8654-8662.

56. Imamura S, Nishikawa Y, Ichikawa T, Hattori T, Matsushita Y, et al. (2005) CCR5 antagonists as anti-HIV-1 agents. Part 3: Synthesis and biological evaluation of piperidine-4-carboxamide derivatives. Bioorg Med Chem 13: 397-416.

57. Seibert C, Ying W, Gavrilov S, Tsamis F, Kuhmann SE, et al. (2006) Interaction of small molecule inhibitors of HIV-1 entry with CCR5. Virology 349: 41-54.

58. Tsamis F, Gavrilov S, Kajumo F, Seibert C, Kuhmann S, et al. (2003) Analysis of the mechanism by which the small-molecule CCR5 antagonists SCH-351125 and SCH-350581 inhibit human immunodeficiency virus type 1 entry. J Virol 77: 5201-5208.

59. Baba M, Miyake H, Wang X, Okamoto M, Takashima K (2007) Isolation and characterization of human immunodeficiency virus type 1 resistant to the small-molecule CCR5 antagonist TAK-652. Antimicrobial agents and chemotherapy 51: 707-715.

60. Trkola A, Kuhmann SE, Strizki JM, Maxwell E, Ketas T, et al. (2002) HIV-1 escape from a small molecule, CCR5-specific entry inhibitor does not involve CXCR4 use. Proc Natl Acad Sci U S A 99: 395-400.

61. Westby M, Smith-Burchnell C, Mori J, Lewis M, Mosley M, et al. (2007) Reduced maximal inhibition in phenotypic susceptibility assays indicates that viral strains resistant to the CCR5 antagonist maraviroc utilize inhibitor-bound receptor for entry. J Virol 81: 2359-2371.

62. Yuan Y, Maeda Y, Terasawa H, Monde K, Harada S, et al. (2011) A combination of polymorphic mutations in V3 loop of HIV-1 gp120 can confer noncompetitive resistance to maraviroc. Virology 413: 293-299.

63. Yusa K, Maeda Y, Fujioka A, Monde K, Harada S (2005) Isolation of TAK-779-resistant HIV-1 from an R5 HIV-1 GP120 V3 loop library. J Biol Chem 280: 30083-30090.

64. Ogert RA, Wojcik L, Buontempo C, Ba L, Buontempo P, et al. (2008) Mapping resistance to the CCR5 co-receptor antagonist vicriviroc using heterologous chimeric HIV-1 envelope genes reveals key determinants in the C2-V5 domain of gp120. Virology 373: 387-399.

65. Ogert RA, Hou Y, Ba L, Wojcik L, Qiu P, et al. (2010) Clinical resistance to vicriviroc through adaptive V3 loop mutations in HIV-1 subtype D gp120 that alter interactions with the N-terminus and ECL2 of CCR5. Virology 400: 145-155.

66. Tilton JC, Wilen CB, Didigu Ca, Sinha R, Harrison JE, et al. (2010) A maraviroc-resistant HIV-1 with narrow cross-resistance to other CCR5 antagonists depends on both N-terminal and extracellular loop domains of drug-bound CCR5. J Virol 84: 10863-10876.

67. Tsibris AMN, Sagar M, Gulick RM, Su Z, Hughes M, et al. (2008) *In vivo* emergence of vicriviroc resistance in a human immunodeficiency virus type 1 subtype C-infected subject. J Virol 82: 8210-8214.

68. Gulick RM, Su Z, Flexner C, Hughes MD, Skolnik PR, et al. (2007) Phase 2 study of the safety and efficacy of vicriviroc, a CCR5 inhibitor, in HIV-1-Infected, treatment-experienced patients: AIDS clinical trials group 5211. J Infect Dis 196: 304-312.

69. Marozsan AJ, Kuhmann SE, Morgan T, Herrera C, Rivera-Troche E, et al. (2005) Generation and properties of a human immunodeficiency virus type 1 isolate resistant to the small molecule CCR5 inhibitor, SCH-417690 (SCH-D). Virology 338: 182-199.

70. Moncunill G, Armand-Ugón M, Pauls E, Clotet B, Esté Ja (2008) HIV-1 escape to CCR5 coreceptor antagonism through selection of CXCR4-using variants in vitro. Aids 22: 23-31.

71. Briggs DR, Tuttle DL, Sleasman JW, Goodenow MM (2000) Envelope V3 amino acid sequence predicts HIV-1 phenotype (co-receptor usage and tropism for macrophages). Aids 14: 2937-2939.

72. Hu Q, Trent JO, Tomaras GD, Wang Z, Murray JL, et al. (2000) Identification of ENV determinants in V3 that influence the molecular anatomy of CCR5 utilization. J Mol Biol 302: 359-375.

73. N, Haraguchi Y, Takeuchi Y, Soda Y, Kanbe K, et al. (1999) Changes in and discrepancies between cell tropisms and coreceptor uses of human immunodeficiency virus type 1 induced by single point mutations at the V3 tip of the env protein. Virology 259: 324-333.

74. Verrier F, Borman AM, Brand D, Girard M (1999) Role of the HIV type 1 glycoprotein 120 V3 loop in determining coreceptor usage. AIDS Res Hum Retroviruses 15: 731-743.

75. Resch W, Hoffman N, Swanstrom R (2001) Improved success of phenotype prediction of the human immunodeficiency virus type 1 from envelope variable loop 3 sequence using neural networks. Virology 288: 51-62.

76. Maeda Y, Foda M, Matsushita S, Harada S (2000) Involvement of both the V2 and V3 regions of the CCR5-tropic human immunodeficiency virus type 1 envelope in reduced sensitivity to macrophage inflammatory protein 1alpha. J Virol 74: 1787-1793.

77. Pastore C, Ramos A, Mosier DE (2004) Intrinsic obstacles to human immunodeficiency virus type 1 coreceptor switching. J Virol 78: 7565-7574.

78. Kuhmann S, Pugach P, Kunstman K (2004) Genetic and phenotypic analyses of human immunodeficiency virus type 1 escape from a small-molecule CCR5 inhibitor. J Virol 78: 2790-2807.

79. Pugach P, Marozsan AJ, Ketas TJ, Landes EL, Moore JP, et al. (2007) HIV-1 clones resistant to a small molecule CCR5 inhibitor use the inhibitor-bound form of CCR5 for entry. Virology 361: 212-228.

80. Anastassopoulou CG, Marozsan AJ, Matet A, Snyder AD, Arts EJ, et al. (2007) Escape of HIV-1 from a small molecule CCR5 inhibitor is not associated with a fitness loss. PLoS pathogens 3: e79.

81. Ogert RA, Ba L, Hou Y, Buontempo C, Qiu P, et al. (2009) Structure-function analysis of human immunodeficiency virus type 1 gp120 amino acid mutations associated with resistance to the CCR5 coreceptor antagonist vicriviroc. J Virol 83: 12151-12163.

82. Huang C-c, Tang M, Zhang M-Y, Majeed S, Montabana E, et al. (2005) Structure of a V3-Containing HIV-1 gp120 Core. Science 310: 1025-1028.

83. Maeda Y, Yusa K, Harada S (2008) Altered sensitivity of an R5X4 HIV-1 strain 89.6 to coreceptor inhibitors by a single amino acid substitution in the V3 region of gp120. Antiviral Res 77: 128-135.

84. Berro R, Sanders RW, Lu M, Klasse PJ, Moore JP (2009) Two HIV-1 variants

resistant to small molecule CCR5 inhibitors differ in how they use CCR5 for entry. PLoS pathogens 5: e1000548.

85. Anastassopoulou CG, Ketas TJ, Klasse PJ, Moore JP (2009) Resistance to CCR5 inhibitors caused by sequence changes in the fusion peptide of HIV-1 gp41. Proc Natl Acad Sci USA 106: 5318-5323.

86. Lee B, Sharron M, Blanpain C, Doranz BJ, Vakili J, et al. (1999) Epitope mapping of CCR5 reveals multiple conformational states and distinct but overlapping structures involved in chemokine and coreceptor function. J Biol Chem 274: 9617-9626.

87. Berro R, Klasse PJ, Lascano D, Flegler A, Nagashima KA, et al. (2011) Multiple CCR5 conformations on the cell surface are used differentially by human immunodeficiency viruses resistant or sensitive to CCR5 inhibitors. J Virol 85: 8227-8240.

88. Feng Y, Broder CC, Kennedy PE, Berger EA (1996) HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science 272: 872-877.

89. Salazar-Gonzalez JF, Salazar MG, Keele BF, Learn GH, Giorgi EE, et al. (2009) Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. J Exp Med 206: 1273-1289.

90. Moore JP, Kitchen SG, Pugach P, Zack JA (2004) The CCR5 and CXCR4 coreceptors--central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. AIDS Res Hum Retroviruses 20: 111-126.

91. Schuitemaker H, Koot M, Kootstra NA, Dercksen MW, de Goede RE, et al. (1992) Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population. J Virol 66: 1354-1360.

92. Gorry PR, Sterjovski J, Churchill M, Witlox K, Gray L, et al. (2004) The role of viral coreceptors and enhanced macrophage tropism in human immunodeficiency virus type 1 disease progression. Sex Health 1: 23-34.

93. Lapidot T (2001) Mechanism of human stem cell migration and repopulation of NOD/SCID and B2mnull NOD/SCID mice. The role of SDF-1/CXCR4 interactions. Ann N Y Acad Sci 938: 83-95.

94. De Clercq E, Yamamoto N, Pauwels R, Balzarini J, Witvrouw M, et al. (1994) Highly potent and selective inhibition of human immunodeficiency virus by the bicyclam derivative JM3100. Antimicrob Agents Chemother 38: 668-674.

95. Donzella GA, Schols D, Lin SW, Este JA, Nagashima KA, et al. (1998) AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. Nat Med 4: 72-77.

96. Labrosse B, Labernardiere JL, Dam E, Trouplin V, Skrabal K, et al. (2003) Baseline susceptibility of primary human immunodeficiency virus type 1 to entry inhibitors. J Virol 77: 1610-1613.

97. Schols D, Este JA, Henson G, De Clercq E (1997) Bicyclams, a class of potent anti-HIV agents, are targeted at the HIV coreceptor fusin/CXCR-4. Antiviral Res 35: 147-156.

98. Schols D, Struyf S, Van Damme J, Este JA, Henson G, et al. (1997) Inhibition of T-tropic HIV strains by selective antagonization of the chemokine receptor CXCR4. J Exp Med 186: 1383-1388.

99. Harrison JE, Lynch JB, Sierra LJ, Blackburn LA, Ray N, et al. (2008) Baseline resistance of primary human immunodeficiency virus type 1 strains to the CXCR4 inhibitor AMD3100. J Virol 82: 11695-11704.

100. Hendrix CW, Collier AC, Lederman MM, Schols D, Pollard RB, et al. (2004) Safety, pharmacokinetics, and antiviral activity of AMD3100, a selective CXCR4 receptor inhibitor, in HIV-1 infection. J Acquir Immune Defic Syndr 37: 1253-1262.

101. Este JA, Cabrera C, Blanco J, Gutierrez A, Bridger G, et al. (1999) Shift of clinical human immunodeficiency virus type 1 isolates from X4 to R5 and prevention of emergence of the syncytium-inducing phenotype by blockade of CXCR4. J Virol 73: 5577-5585.

102. Armand-Ugon M, Quinones-Mateu ME, Gutierez A, Barretina J, Blanco J, et al. (2003) Reduced fitness of HIV-1 resistant to CXCR4 antagonists. Antivir Ther 8: 1-8.

103. Schols D, Este JA, Cabrera C, De Clercq E (1998) T-cell-line-tropic human immunodeficiency virus type 1 that is made resistant to stromal cell-derived factor 1alpha contains mutations in the envelope gp120 but does not show a switch in coreceptor use. J Virol 72: 4032-4037.

104. Kanbara K, Sato S, Tanuma J, Tamamura H, Gotoh K, et al. (2001) Biological and genetic characterization of a human immunodeficiency virus strain resistant to CXCR4 antagonist T134. AIDS Res Hum Retroviruses 17: 615-622.

105. Moyle G, DeJesus E, Boffito M, Wong RS, Gibney C, et al. (2009) Proof of activity with AMD11070, an orally bioavailable inhibitor of CXCR4-tropic HIV type 1. Clin Infect Dis 48: 798-805.

106. Murakami T, Kumakura S, Yamazaki T, Tanaka R, Hamatake M, et al. (2009) The novel CXCR4 antagonist KRH-3955 is an orally bioavailable and extremely potent inhibitor of human immunodeficiency virus type 1 infection: comparative studies with AMD3100. Antimicrob Agents Chemother 53: 2940-2948.

107. Jenkinson S, Thomson M, McCoy D, Edelstein M, Danehower S, et al. (2010) Blockade of X4-tropic HIV-1 cellular entry by GSK812397, a potent noncompetitive CXCR4 receptor antagonist. Antimicrob Agents Chemother 54: 817-824.

108. Lazzarin A, Clotet B, Cooper D, Reynes J, Arasteh K, et al. (2003) Efficacy of enfuvirtide in patients infected with drug-resistant HIV-1 in Europe and Australia. N Engl J Med 348: 2186-2195.

109. Wild C, Oas T, McDanal C, Bolognesi D, Matthews T (1992) A synthetic peptide inhibitor of human immunodeficiency virus replication: correlation between solution structure and viral inhibition. Proc Natl Acad Sci U S A 89: 10537-10541.

110. Chen CH, Matthews TJ, McDanal CB, Bolognesi DP, Greenberg ML (1995) A molecular clasp in the human immunodeficiency virus (HIV) type 1 TM protein determines the anti-HIV activity of gp41 derivatives: implication for viral fusion. J Virol 69: 3771-3777.

111. Eckert DM, Malashkevich VN, Hong LH, Carr PA, Kim PS (1999) Inhibiting HIV-1 entry: discovery of D-peptide inhibitors that target the gp41 coiled-coil pocket. Cell 99: 103-115.

112. Rimsky LT, Shugars DC, Matthews TJ (1998) Determinants of human immunodeficiency virus type 1 resistance to gp41-derived inhibitory peptides. J Virol 72: 986-993.

113. Sista PR, Melby T, Davison D, Jin L, Mosier S, et al. (2004) Characterization of determinants of genotypic and phenotypic resistance to enfuvirtide in baseline and on-treatment HIV-1 isolates. AIDS 18: 1787-1794.

114. Marcial M, Lu J, Deeks SG, Ziermann R, Kuritzkes DR (2006) Performance of human immunodeficiency virus type 1 gp41 assays for detecting enfuvirtide (T-20) resistance mutations. J Clin Microbiol 44: 3384-3387.

115. Izumi K, Kodama E, Shimura K, Sakagami Y, Watanabe K, et al. (2009) Design of peptide-based inhibitors for human immunodeficiency virus type 1 strains resistant to T-20. J Biol Chem 284: 4914-4920.

116. Nameki D, Kodama E, Ikeuchi M, Mabuchi N, Otaka A, et al. (2005) Mutations conferring resistance to human immunodeficiency virus type 1 fusion inhibitors are restricted by gp41 and Rev-responsive element functions. J Virol 79: 764-770.

117. Xu L, Pozniak A, Wildfire A, Stanfield-Oakley SA, Mosier SM, et al. (2005) Emergence and evolution of enfuvirtide resistance following long-term therapy involves heptad repeat 2 mutations within gp41. Antimicrob Agents Chemother 49: 1113-1119.

118. Fischer U, Meyer S, Teufel M, Heckel C, Luhrmann R, et al. (1994) Evidence that HIV-1 Rev directly promotes the nuclear export of unspliced RNA. EMBO J 13: 4105-4112.

119. Daugherty MD, Liu B, Frankel AD (2010) Structural basis for cooperative RNA binding and export complex assembly by HIV Rev. Nat Struct Mol Biol 17: 1337-1342.

120. Svicher V, Aquaro S, D'Arrigo R, Artese A, Dimonte S, et al. (2008) Specific enfuvirtide-associated mutational pathways in HIV-1 Gp41 are significantly correlated with an increase in CD4(+) cell count, despite virological failure. J Infect Dis 197: 1408-1418.

121. Ueno M, Kodama EN, Shimura K, Sakurai Y, Kajiwara K, et al. (2009) Synonymous mutations in stem-loop III of Rev responsive elements enhance HIV-1 replication impaired by primary mutations for resistance to enfuvirtide. Antiviral Res 82: 67-72.

122. Lu J, Deeks SG, Hoh R, Beatty G, Kuritzkes BA, et al. (2006) Rapid emergence of enfuvirtide resistance in HIV-1-infected patients: results of a clonal analysis. J Acquir Immune Defic Syndr 43: 60-64.

123. Mink M, Mosier SM, Janumpalli S, Davison D, Jin L, et al. (2005) Impact of human immunodeficiency virus type 1 gp41 amino acid substitutions selected during enfuvirtide treatment on gp41 binding and antiviral potency of enfuvirtide in vitro. J Virol 79: 12447-12454.

124. Hanna SL, Yang C, Owen SM, Lal RB (2002) Variability of critical epitopes within HIV-1 heptad repeat domains for selected entry inhibitors in HIV-infected populations worldwide [corrected]. AIDS 16: 1603-1608.

125. Pessoa LS, Valadao AL, Abreu CM, Calazans AR, Martins AN, et al. (2011) Genotypic analysis of the gp41 HR1 region from HIV-1 isolates from enfuvirtide-treated and untreated patients. J Acquir Immune Defic Syndr 57 Suppl 3: S197-201.

126. Roman F, Gonzalez D, Lambert C, Deroo S, Fischer A, et al. (2003) Uncommon mutations at residue positions critical for enfuvirtide (T-20) resistance in enfuvirtide-naive patients infected with subtype B and non-B HIV-1 strains. J Acquir Immune Defic Syndr 33: 134-139.

127. Shimura K, Nameki D, Kajiwara K, Watanabe K, Sakagami Y, et al. (2010) Resistance profiles of novel electrostatically constrained HIV-1 fusion inhibitors. J Biol Chem 285: 39471-39480.

128. Eggink D, Bontjer I, Langedijk JP, Berkhout B, Sanders RW (2011) Resistance of Human Immunodeficiency Virus Type 1 to a Third-Generation Fusion Inhibitor Requires Multiple Mutations in gp41 and Is Accompanied by a Dramatic Loss of gp41 Function. J Virol 85: 10785-19797.

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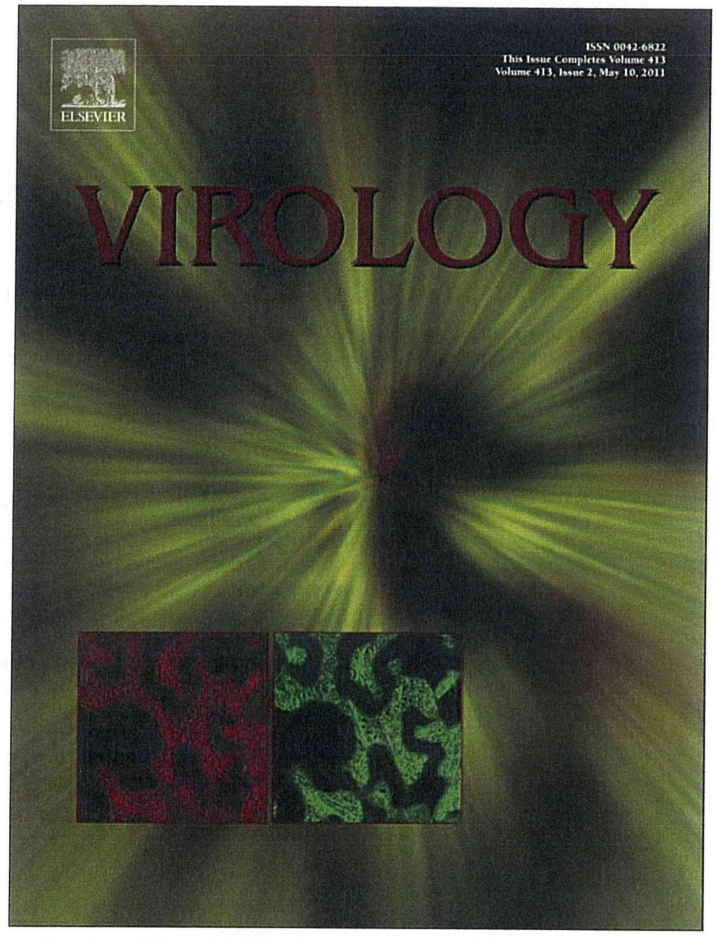

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A combination of polymorphic mutations in V3 loop of HIV-1 gp120 can confer noncompetitive resistance to maraviroc

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ABSTRACT

Maraviroc binds to the pocket of extracellular loops of the cell surface CCR5 and prevents R5 HIV-1 from using CCR5 as a coreceptor for entry into CD4-positive cells. To evaluate the contribution of the V3 loop structure in gp120 to maraviroc resistance, we isolated maraviroc-resistant variants from the V3 loop library virus (HIV-1_{V3Lib}) containing a set of random combinations of 0–10 polymorphic mutations *in vitro*. HIV-1_{V3Lib} at passage 17 could not be suppressed even at 10 μM (>1400-fold resistance), while HIV-1_{JR-FL} at passage 17 revealed an 8-fold resistance to maraviroc. HIV-$1_{V3Lib-P17}$ contained T199K and T275M plus 5 mutations in the V3 loop, I304V/F312W/T314A/E317D/I318V. The profile of pseudotyped virus containing I304V/F312W/T314A/E317D/I318V in V3 loop alone revealed a typical noncompetitive resistance, although T199K and/or T275M could not confer noncompetitive resistance. This type of library virus is useful for isolation of escape viruses from effective entry inhibitors.

Introduction

The entry of human immunodeficiency virus type 1 (HIV-1) in target cells is a feasible step where small compounds could be used to block viral replication (Donzella et al., 1998; Dragic et al., 2000; Strizki et al., 2001; Trkola et al., 2002). To completely suppress viral entry *in vivo*, various antiviral agents have been developed that target unique viral proteins and receptors (Kuhmann and Hartley, 2008; Tsibris and Kuritzkes, 2007; Westby and van der Ryst, 2010). Enfuvirtide (Fuzeon) is an antiviral peptide that prevents HIV entry by blocking gp41-mediated fusion through interaction with the gp41 N-heptad repeat domain to form a heterologous trimer of heterodimer complex (Chan et al., 1997; Chan and Kim, 1998; Wild et al., 1993). Another target to block viral entry is CCR5. Small compounds that can bind to the pockets of the extracellular loops of CCR5 are expected to be potent antiviral agents. Several small-molecule CCR5 inhibitors have progressed through clinical development (Westby and van der Ryst, 2010). Maraviroc (Dorr et al., 2005; Fatkenheuer et al., 2005) is the first and only CCR5 antagonist approved by the U.S. Food and Drug Administration in 2007 for treatment-experienced patients with an R5-tropic virus.

The emergence of viruses resistant to entry inhibitors as well as other classes of antiviral agents has been reported *in vitro* and *in vivo* (Moore and Kuritzkes, 2009; Westby and van der Ryst, 2010). The intuitive manner of resistance to small-molecule CCR5 inhibitors depends on coreceptor switching from a CCR5-using virus to a dual-tropic virus or a CXCR4-using virus, but these are rare cases *in vitro* and *in vivo* (Maeda et al., 2008; Westby and van der Ryst, 2010). Virologic failure in clinical aspects is an outgrowth of the pre-existing minority population of the CXCR4-using virus (Gulick et al., 2007; Moore and Kuritzkes, 2009; Westby and van der Ryst, 2010). These results indicate that the acquisition of the other type of resistance occurs preferentially in R5 viruses because coreceptor switching requires multiple mutations throughout gp160 through transitional intermediates with poor replication fitness (Pastore et al., 2004). Two types of genetic pathways for virus escape have been reported *in vitro* (Marozsan et al., 2005; Pugach et al., 2007; Trkola et al., 2002). The first is the accumulation of multiple amino acid substitutions in Env including 2–4 substitutions in the gp120 V3 domain. Unique changes have been detected in different isolates (Baba et al., 2007; Kuhmann et al., 2004; Marozsan et al., 2005; Ogert et al., 2008; Pugach et al., 2007; Trkola et al., 2002; Westby et al., 2007). Some of these resistant viruses revealed noncompetitive resistance (Kuhmann et al., 2004; Trkola et al., 2002; Westby et al., 2007). In noncompetitive resistance, the escape variants could use the inhibitor-bound form of CCR5 as well as free CCR5 for entry. The second is a genetic pathway independent of V3 mutations. Resistance to vicriviroc has developed through multiple

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amino acid substitutions throughout gp160 without any changes in the V3 loop (Marozsan et al., 2005). The determinants of resistance induced by vicriviroc have been mapped on a 200-residue stretch of gp120 spanning the C2–V5 region (Ogert et al., 2008). These reports indicate that resistance to small-molecule CCR5 inhibitors is complicated and there appears to be no common key mutations.

In this study, we used the V3 loop library virus (HIV-1_{V3Lib}), which carries a set of random combinations from 0 to 10 substitutions (27,648 possibilities) in the V3 loop (residues 302, 303, 304, 305, 306, 312, 314, 317, 318, and 321; V3 loop from Cys^{293} to Cys^{327}) (Yusa et al., 2005) (Fig. 1A). They were polymorphic mutations derived from 31 R5 clinical isolates. To further elucidate the contribution of the V3 loop to resistance to small-molecule CCR5 inhibitors, we selected maraviroc-resistant variants from HIV-1_{V3Lib}. We describe the isolation of maraviroc-resistant variants after 17 passages with a gradual increase in maraviroc concentration *in vitro*, and discuss the finding that the resistant variants from HIV-1_{V3Lib} revealed noncompetitive resistance to maraviroc.

Results

Selection of maraviroc-resistant variants from HIV-$1_{JR\text{-}FL}$ and HIV-1_{V3Lib}

We used the replication-competent HIV-1_{V3Lib} for selection of maraviroc-resistant viruses. Each virus clone in the library contains a set of 0–10 amino acid substitutions in the gp120 V3 loop from Cys^{293} to Cys^{327} (Fig. 1A). We used PM1/CCR5 cells for virus passages because they have two advantages. First, PM1/CCR5 cells are highly sensitive to the R5 virus compared to the parental PM1 cells; second, prominent cell fusion caused by viral infection is a straightforward sign of virus proliferation. EC_{50}s of HIV-$1_{JR\text{-}FL}$ and HIV-1_{V3Lib} to maraviroc were 0.0069 ± 0.0019 μM and 0.0055 ± 0.0007 μM, respectively (Table 1). The susceptibility of HIV-1_{V3Lib} to maraviroc was similar to that of the wild type. To select maraviroc-resistant variants, PM1/CCR5 cells were infected with HIV-$1_{JR\text{-}FL}$ or HIV-1_{V3Lib} in the presence of 0.003 μM maraviroc in passage 1 (Fig. 1B). After infection, 4 to 7 days were required for the viruses to sufficiently replicate for the next passage. During the passages, the concentration of maraviroc was gradually increased up to 0.1 μM until passage 14 for HIV-$1_{JR\text{-}FL}$ and HIV-1_{V3Lib} in the same manner. At passage 15, the library virus could replicate in 4 days in the presence of 0.2 μM maraviroc, but the wild type could not. The concentration of maraviroc was increased up to 0.7 μM for HIV-1_{V3Lib} and up to 0.1 μM for HIV-$1_{JR\text{-}FL\text{-}P17}$ at passage 17.

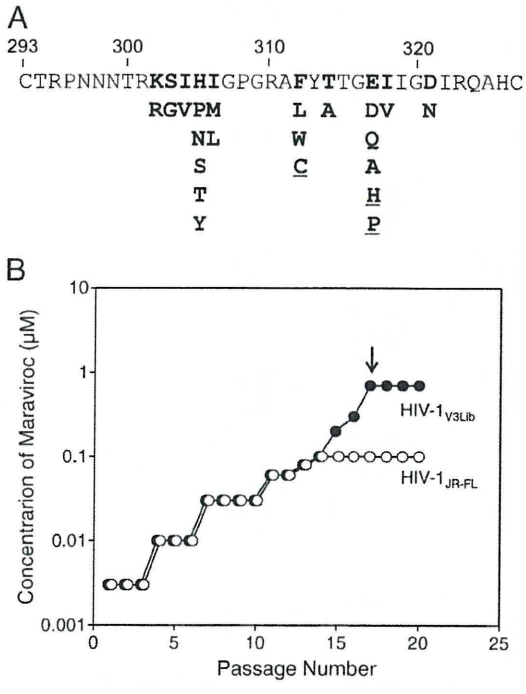

Fig. 1. (A) Amino acid substitutions in HIV-1_{V3Lib}. Residues in boldface indicate the substitutions that were randomly incorporated in HIV-1_{V3Lib}. Underlined residues indicate the substitutions that were not detected in 31 R5 viruses (Yusa et al., 2005). F312C, E317H, and E317P were inevitably incorporated in HIV-1_{V3Lib} due to combinations of nucleotide substitutions. (B) Induction of maraviroc-resistant variants from HIV-1_{V3Lib}. HIV-$1_{JR\text{-}FL}$ and HIV-1_{V3Lib} were passaged in PM1/CCR5 cells in the presence of maraviroc increasing from 0.003 μM to 0.1 μM for HIV-$1_{JR\text{-}FL}$ and from 0.003 μM to 0.7 μM for HIV-1_{V3Lib}.

HIV-1_{V3Lib} did not inherently contain V3 mutants that could use CXCR4 as a coreceptor (Yusa et al., 2005). To address whether coreceptor switching occurred in HIV-$1_{V3Lib\text{-}P17}$, MT-2 cells, which could support X4 virus HIV-$1_{NL4\text{-}3}$ but not R5 virus HIV-$1_{JR\text{-}FL}$ (Fig. 2B), were infected with the virus. It was clearly shown that HIV-$1_{V3Lib\text{-}P17}$ could not replicate in MT-2 cells using CXCR4, indicating that the high resistance to maraviroc in HIV-$1_{V3Lib\text{-}P17}$ was not due to coreceptor switching.

Mutations in HIV-$1_{JR\text{-}FL\text{-}P17}$ and HIV-$1_{V3Lib\text{-}P17}$ at passage 17

To identify the mutations responsible for complete resistance to maraviroc, we sequenced *env* genes at passage 10 and 17 (Table 2). At passage 10, S303G was partially detected in HIV-$1_{JR\text{-}FL\text{-}P10}$ gp120 by direct sequencing. Actually, 2 of 4 clones of HIV-$1_{JR\text{-}FL\text{-}P10}$ contained S303G alone in the V3 loop, and no other common mutations were detected in the other regions of gp120 and gp41 (data not shown). Virus clones containing S303G did not become a major population after further selection at passage 17. T314P (4 of 8 clones), S303G (2 of 8 clones), N299S (2 of 8 clones), K302E (1 of 8 clones), and A311L (1 of 8 clones) were detected in the V3 loop, indicating that the V3

Table 1
Susceptibility of the viruses selected with maraviroc.

	EC_{50}[a] (μM)	
	Maraviroc[b]	TAK-779[b]
HIV-$1_{JR\text{-}FL}$	0.0069 ± 0.0019[c] (1.0)	0.043 ± 0.009 (1.0)
HIV-$1_{JR\text{-}FL\text{-}P17}$	0.055 ± 0.0055 (8.0)	0.15 ± 0.033 (3.5)
HIV-1_{V3Lib}	0.0055 ± 0.0007 (0.80)	0.025 ± 0.007 (0.58)
HIV-$1_{V3Lib\text{-}P17}$	>10 (>1400)	>10 (>230)

[a] PM1/CCR5 cells were infected at 100 TCID50 of viruses in the presence of the CCR5 inhibitor on day 0. Cytopathic effect was determined on day 6 by MTT method.
[b] Drug concentration of 50% growth inhibition of the cells (CC50) was >10 μM.
[c] Mean ± SD (n = 3).

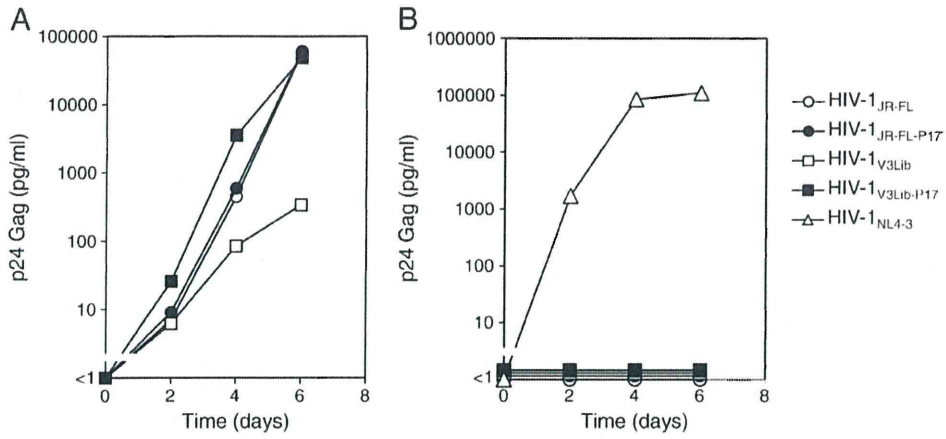

Fig. 2. Replication of HIV-$1_{JR\text{-}FL\text{-}P17}$ and HIV-$1_{V3Lib\text{-}P17}$ in PM1/CCR5 cells (A) or MT2 cells (B). Cells (5×10^4) were infected with 10 ng of p24 Gag. Viral replication was monitored by measuring p24 Gag in the supernatant. Experiments were performed in triplicate.

structure was not strictly focused on the selection pressure. Instead, T199K in the C2 region was the only mutation detected by direct sequencing, and the mutation was confirmed in 7 of 8 clones. The mixture of these clones (HIV-$1_{JR\text{-}FL\text{-}P17}$) revealed 8-fold resistance to maraviroc (Table 1), suggesting that T199K may be a responsible mutation for the low resistance in HIV-$1_{JR\text{-}FL\text{-}P17}$.

The mutation profile of HIV-1_{V3Lib} at passage 10 was different from that at passage 17. In passage 10, S303G (4 of 4), I306M (3 of 4), F312W (3 of 4), T314A (3 of 4), and I322N (4 of 4) were detected in a major population, and 1 of 4 clones contained G149R/T199A in the non-V3 region. Thus, suggesting that the low concentrations of maraviroc from 0.003 to 0.01 μM compelled the condensation of the V3-mutant mixture to a small number of V3 structures. After further selection, the V3 structures detected in passage 10 were lost at passage 17, and 5 mutations in the V3 loop, I304V/F312W/T314A/E317D/I318V (designated as V3-M5) and T199K/T275M (all 8) were detected by direct sequencing. The amino acid substitutions of V3-M5 were polymorphic mutations inherently incorporated into the library virus. All of the clones from HIV-$1_{V3Lib\text{-}P17}$ contained these 7 common mutations, although some of them contained minor mutations such as T262L (3 of 8). There were no other mutations detected in the other regions of gp120 and gp41 (data not shown). HIV-$1_{V3Lib\text{-}P17}$ revealed a >1400-fold

Table 2
Mutations in gp120 of V3 loop library virus selected with maraviroc.

maraviroc (μM)	non-V3 mutations	V3 mutations[a]
		293 300 310 320
		CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC
HIV-$1_{JR\text{-}FL}$		
P10[b] 0.03	**-**	.....**G**.....
CL#01	D227V	.....G.....
CL#02	-	.....
CL#03	V267I	.....G.....
CL#04	Y174H/T199K	.....
P17[b] 0.1	**T199K**	.....**P**.....
CL#01	L124F/V197A/T199K/E220L/S240G	.....P.....
CL#02	V83L/N87Y/T199K/G442E	.....P.....
CL#03	V83I/T199K/C436R/N452D	.....P.....
CL#04	V83L/T199K/F378Y	.....P.....
CL#05	V166A/T199K/P209L/L256R/N351D	.....G.....L.....
CL#06	-	.....S.....
CL#07	N140D/T199K	.....S.....
CL#08	N134I/T199K/K233E	.....EG.....
HIV-$1_{JR\text{-}FL\text{-}V3Lib}$		
P10 0.03	**-**	.....**G..M.....W.A.....N**.....
CL#01	-	.....G..M.....W.A.....N.....
CL#02	G149R/T199A	.....G..M.....L.A..A...N.....
CL#03	-	.....G..M.....W.A.....N.....
CL#04	-	.....GV.L.....W....Q...N.....
P17 0.7	**T199K, T275M**	.....**V.....W.A..DV**.....
CL#01	T199K/T275M	.....V.....W.A..DV.....
CL#02	T199K/T275M	.....V.....W.A..DV.....
CL#03	T199K/T275M	.....V.....W.A..DV.....
CL#04	T199K/E265K/T275M	.....V.....W.A..DV.....
CL#05	T199K/T262L/T275M	.....V.....GW.A..DV.....
CL#06	T199K/E208K/G219S/T262L/T275M	.....V.....W.A..DV.....
CL#07	T199K/T262L/T275M	.....V.....W.A..DV.....
CL#08	T199K/T275M	.....V.....W.A..DV.....

[a] Amino acid residues underlined are the mutation positions in HIV-$1_{JR\text{-}FL\text{-}V3Lib}$.
[b] P10, P17 direct sequencing was performed to detect mutations (in bold) in Env.

resistance to maraviroc compared with HIV-1_{JR-FL} (Table 1). These results strongly suggested that T199K/T275M plus V3-M5 conferred complete resistance to maraviroc.

Susceptibilities of recombinant viruses to maraviroc

To confirm which mutations were responsible for complete resistance, we constructed molecular clones containing combinations of T199K, T275M, and/or V3-M5, and measured their susceptibilities to maraviroc (Fig. 3). EC_{50} of HIV-1_{JR-FL} was 0.018 ± 0.004 μM, while those of HIV-1_{T199K} and HIV-1_{T275M} were 0.042 ± 0.007 μM and 0.074 ± 0.011 μM. Thus HIV-1_{T199K} and HIV-1_{T275M} were 2.3- and 4.1-fold more resistant than HIV-1_{JR-FL}. HIV-$1_{T199K/T275M}$ was 3.3-fold more resistant, indicating that without V3 mutations, T199K, T275M, or T199K/T275M could confer low resistance, but not lead to complete resistance. On the other hand, the V3-M5 alone could confer complete resistance to maraviroc, although its viral fitness was lower than that of HIV-1_{JR-FL} (Fig. 4). p24 Gag produced in the absence of maraviroc in HIV-1_{V3-M5} was 1040 pg/ml and that in HIV-1_{JR-FL} was 8600 pg/ml. T199K combined with V3-M5 can confer complete resistance, and increase its viral fitness. p24 Gag production in HIV-$1_{T199K/V3-M5}$ in the absence of maraviroc was 8.5-fold higher than that in HIV-1_{V3-M5}. T275M was detected in all 8 clones at passage 17, however, the combination of T275M with V3-M5 resulted in marked decrease of viral fitness (Fig. 4), although the viral replication could not be suppressed by 3 or 10 μM maraviroc. These results indicated that T275M with V3-M5 could confer complete resistance. T275M/V3-M5 plus T199K restored the decreased viral fitness with complete resistance. The replication of HIV-$1_{T199K/T275M/V3-M5}$ in the presence of 3 or 10 μM maraviroc was comparable to that of HIV-1_{JR-FL}. Taken together, V3-M5 is responsible for the acquisition of complete resistance, and T199K and/or T275M have a strong effect on viral replication under drug selection pressure.

Susceptibilities of pseudotyped viruses: single-round entry assay

To confirm the noncompetitive resistance mechanism, we determined the susceptibilities of the recombinant viruses with a single-round entry assay using MAGIC-5 cells (Hachiya et al., 2001). EC_{50} of pseudotyped HIV-$1_{Env-JR-FL}$ was 0.00035 ± 0.00007 μM. The pseudotyped viruses HIV-$1_{Env-T199K/T275M/V3-M5}$, HIV-$1_{Env-T199K}$, HIV-$1_{Env-T275M}$, HIV-$1_{Env-T199K/T275M}$, HIV-$1_{Env-V3-M5}$, HIV-$1_{Env-T199K/V3-M5}$, and HIV-$1_{Env-T275M/V3-M5}$ revealed a ≤3.4-fold resistance compared with HIV-$1_{Env-JR-FL}$. The competent molecular clones containing T199K/T275M/V3-M5, V3-M5, T199K/V3-M5, and T275M/V3-M5 could not be blocked by 3 or 10 μM maraviroc (Fig. 4), while single-round entry of these pseudotyped viruses could be inhibited by 50% with ≤0.0012 μM of maraviroc (Table 3). However, in the presence of 0.1 or 1 μM maraviroc, inhibition of viral entry could not be completely blocked (Fig. 5), indicating that the viruses could utilize the maraviroc-bound form of CCR5. HIV-1_{V3-M5}, HIV-$1_{T199K/V3-M5}$, and HIV-$1_{T199K/T275M/V3-M5}$ retained 19, 26, and 36%, respectively, of their entry ability at 1 μM maraviroc than those of the pseudotyped virus in drug-free conditions. These results indicated that these viruses acquired noncompetitive resistance by interacting with the maraviroc-binding CCR5 complex as a second receptor.

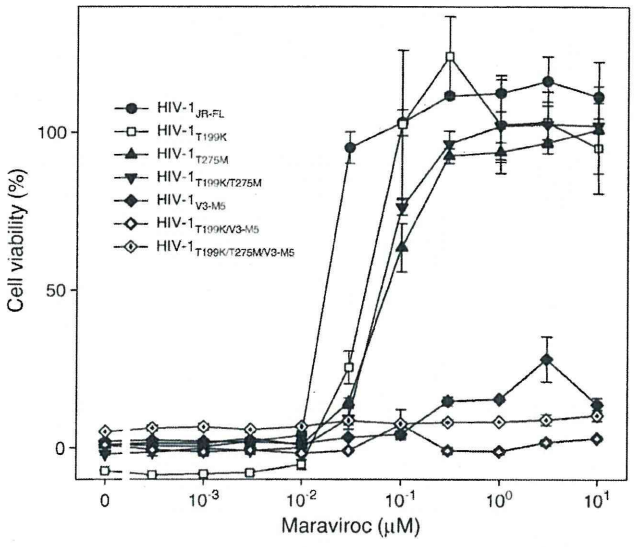

Fig. 3. Susceptibilities of replication-competent recombinant viruses. PM1/CCR5 cells were infected with recombinant virus at 100 $TCID_{50}$ in the presence of maraviroc and cultured for 6 days, and the cytopathic effect was determined by the MTT assay. Susceptibility of HIV-$1_{T275M/V3-M5}$ could not be examined because of its low replication. Mean ± SD (n = 3).

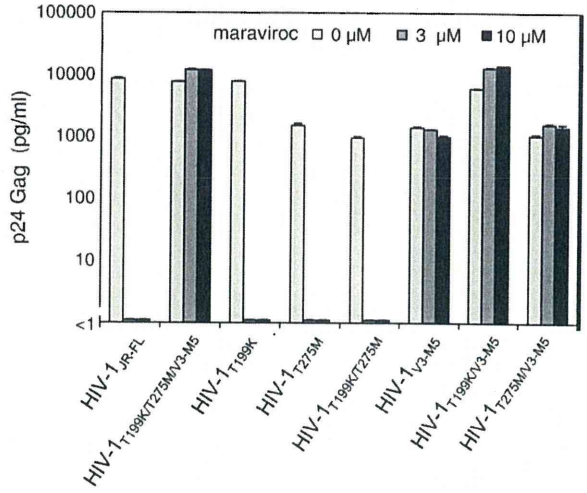

Fig. 4. The effect of 3 or 10 μM of maraviroc on the production of p24 Gag in the recombinant viruses. Cells (5×10^4) were infected with 10 ng of p24 Gag in the presence of 3 or 10 μM of maraviroc. After 6 days, the amount of p24 Gag in the supernatant was measured with HIV-1 p24 Gag ELISA. Mean ± SD (n = 3).

Discussion

Maraviroc is a highly potent antiviral agent targeting CCR5 to block the viral entry step (Kuhmann and Hartley, 2008; MacArthur and Novak, 2008). Primary R5 isolates cultured in stimulated PBMC are usually used to induce CCR5 inhibitor-resistant variants (Baba et al., 2007; Kuhmann et al., 2004; Marozsan et al., 2005; Ogert et al., 2008; Pugach et al., 2007; Trkola et al., 2002; Westby et al., 2007). Here we used PM1/CCR5 cells with the HIV-1_{V3Lib} constructed from a laboratory strain to further focus on the contribution of the V3 loop in gp120 in acquisition of maraviroc resistance. If HIV-1_{V3Lib} originally contained maraviroc-resistant viruses without additional mutations,

Table 3
Susceptibility of recombinant viruses to maraviroc determined by single-round entry assay.

	Maraviroc	
	EC_{50}[a]	(μM)
HIV-$1_{Env-JR-FL}$	0.00035 ± 0.00007[b]	(1.0)
HIV-$1_{Env-T199K/T275M/V3-M5}$	0.00090 ± 0.00014	(2.6)
HIV-$1_{Env-T199K}$	0.00050 ± 0.00007	(1.4)
HIV-$1_{Env-T275M}$	0.00085 ± 0.00015	(2.4)
HIV-$1_{Env-T199K/T275M}$	0.00064 ± 0.00018	(2.6)
HIV-$1_{Env-V3-M5}$	0.00071 ± 0.00022	(2.0)
HIV-$1_{Env-T199K/V3-M5}$	0.0012 ± 0.0005	(3.4)
HIV-$1_{Env-T275M/V3-M5}$	0.00064 ± 0.00021	(1.8)

[a] MAGIC-5 cells (2 x 10^4) were infected with pseudotyped virus on day 0, and 48 h postinfection luciferase activity was measured to determine effective concentration of 50% entry inhibition (EC_{50}).
[b] Mean ± SD (n = 3).

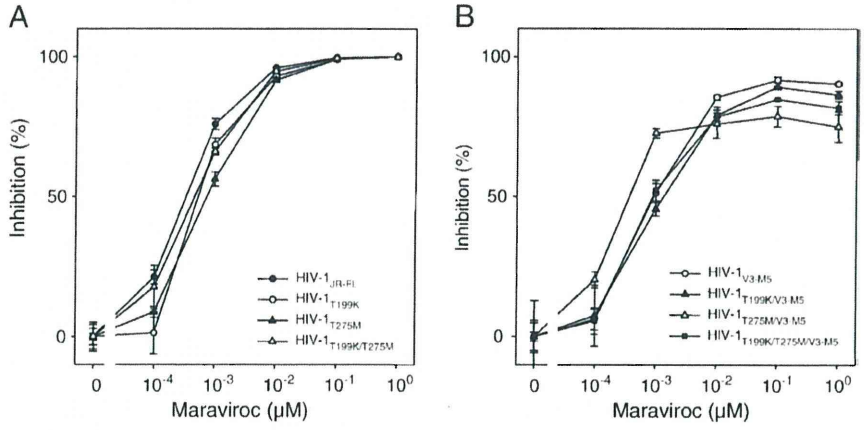

Fig. 5. Inhibition of viral entry. Pseudotyped viruses were prepared with 293T cells by transfection with pNL-luc and pCNX-FLenv. MAGIC-5 cells were infected with pseudotyped viruses in the presence of 0.0001–1 μM of maraviroc. Mean ± SD (n = 3).

the selection periods could be shortened compared to the use of the wild type for selection of the virus *in vitro*. In reality, it took more than 15 passages until we obtained the resistant variants that could replicate in the presence of ≥0.10 μM, while resistant variants could not be isolated using HIV-1_{JR-FL} in the same manner. The library virus inherently confers lower viral fitness in various virus clones replicating in PM1/CCR5 cells compared to the wild type; 36% of replication-deficient virus clones (<0.5% p24 Gag generated of that of wild type on day 6 after infection), 17% of 0.5–10% replication-competent virus clones, 38% of 10–50% replication-competent virus clones, and 9% of >50% replication-competent virus clones (Monde et al., 2007). From selection with 0.003 to 0.1 μM for HIV-1_{JR-FL}, mutations including T199K that conferred low resistance were condensed in the viral population, and a similar condensation of variants carrying such mutations occurred in HIV-1_{V3Lib} (1 of 4 clones contained T199K at passage 10). Maraviroc from 0.1 to 0.7 μM (passage 11 to 17) could suppress the proliferation of relatively low-resistant variants and enabled the chance for a variant containing V3-M5 combined with T199K/T275M to command a majority of the viral population. These sequential events needed more than 15 passages to obtain highly resistant variants.

HIV-1 $_{V3Lib-P17}$ contained 5 amino acid substitutions in the V3 loop. We have reported the resistant virus from the same V3 library virus with TAK-779, which contained five mutations I304V/H305V/I306M/F312L/E317D in V3 loop (Yusa et al., 2005). The TAK-779 isolated virus revealed relatively low resistance (15-fold). Two of the five mutations, I304V and E317D were common mutations of V3-M5, and additional F312L, T314A and I318V in V3 loop could confer noncompetitive resistance to maraviroc and TAK-779. A preclinical precursor of vicriviroc AD101-resistant variants from the CC1/85 clinical isolate revealed noncompetitive resistance, which contained 4 amino acid substitutions – K305R (K302R numbering from HV-1_{JR-FL} gp120), H308P (H305P), A316V (A311V), and G321E (G316E) – in the V3 region (Berro et al., 2009; Kuhmann et al., 2004). These substitutions were not included in the V3-M5 mutations. They introduced the 4 mutations in the V3 region of HV-1_{JR-FL}, but the mutant V3 did not affect AD101 susceptibility in the different context (Moore and Kuritzkes, 2009). Another study reported that A316T (A311T numbering from HV-1_{JR-FL} gp120) and I323V (I318V) were particularly influential on resistance to vicriviroc (Westby et al., 2007). I323V (I318V) was also included in the V3-M5 mutations in HIV-$1_{V3Lib-P17}$. It has been proposed that the multiple mutations at both sides of the V3 loop in vicriviroc-resistant HIV-1 CC101.19 decreased interactions between the V3 tip and the second extracellular loop (ECL2) of CCR5 and interactions with the CCR5 N-terminus were enhanced (Berro et al., 2009). Similarly vicriviroc-resistant HIV-1 subtype C carried K305R (K302R numbering from HV-1_{JR-FL} gp120), S306P (S303P), T307I (T304I), F318I (F313I), T320R (T315R), G321E (G316E) and H330Y (H326Y) accumulated sequentially on both sides of the V3 stem; particularly incorporation of S306P and/or K305R is crucial for efficient usage of the compound-CCR5 complex (Henrich et al., 2010; Tsibris et al., 2008). In HIV-1 subtype D, Q315E (Arg_{308} in HV-1_{JR-FL} gp120) and R321G (Glu_{315}) are essential for resistance to vicriviroc, which is supposed to influence interaction of gp120 with both the N-terminus and the ECL-2 region of CCR5 (Ogert et al., 2010). Our results also revealed that 5 amino acid substitutions at both sides of the V3 stem could confer noncompetitive resistance, conceivably through modified interactions of the V3 loop with the ECL2 and the N-terminus of CCR5. Further experiments are necessary to elucidate the contribution of each amino acid substitutions of V3-M5 for noncompetitive resistance.

HIV-1_{V3-M5}, HIV-$1_{T199K/V3-M5}$, and HIV-$1_{T199K/T275M/V3-M5}$ displayed full resistance with maximum concentration of maraviroc (10 μM), suggesting noncompetitive resistance (Pugach et al., 2007; Westby et al., 2007). In the case of noncompetitive resistance, the inhibitor concentration no longer has any further inhibitory effect on viral replication. The escape variant uses the inhibitor-bound form of CCR5 for entry, as well as a free receptor usually with lower efficiency. Single-entry assays with the three pseudotyped viruses showed that 19–36% viral entry activity was retained at 1 μM of maraviroc. HIV-$1_{T199K/V3-M5}$ could use the maraviroc-bound form of CCR5 with 26% of efficiency, whereas HIV-$1_{T199K/T275M/V3-M5}$ could use it with 36% efficiency, indicating that T199K/T275M with V3-M5 finally prevailed for selection at passage 17. These results indicate that V3-M5 mutations alone can confer complete resistance, and non-V3 mutations like T199K and/or T275M in the C2 domain intensively modify viral fitness.

In these experiments, we obtained a combination of multiple mutations in the V3 loop containing V3-M5, I304V/F312W/T314A/E317D/I318V from HIV-1_{V3Lib}. Other types of V3 mutations in combination with non-V3 mutations may be selected to support their viral fitness. To test this possibility, we may be able to select various combinations of V3 mutants from a V3 library constructed with HIV-1_{T199K} or HIV-$1_{T199K/T275M}$ as a vector. We could not fully explain the condition of the V3 structure that confers noncompetitive resistance. To address this question, further studies involving the analysis of mutants containing various combinations of mutations in the V3 loop are necessary.

Materials and methods

Cells and viruses

PM1/CCR5 cells were generated from the human $CD4^+$ T-cell line PM1 (Lusso et al., 2005) by standard retrovirus-mediated transduction

with pG1TKneo-CCR5 (Maeda et al., 2000). The cells were maintained in RPMI1640 (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; Vitromex). MAGIC-5 cells (HeLa-CD4$^+$-CCR5$^+$-LTR-ß-galactosidase) (Hachiya et al., 2001), used as reporter cells for HIV-1 infection, and 293T cells were maintained in Dulbecco's modified Eagle's medium (ICN Biomedicals) supplemented with 10% heat-inactivated FCS.

For construction of the viral competent library of pJR-FL$_{V3Lib}$, 176-bp V3-loop DNA fragments containing 0–10 random combinations of amino acid substitutions were introduced in pJR-FL, as previously described (Yusa et al., 2005). For virus preparation, 293T cells (2×10^6) were transfected with 10 μg of pJR-FL or pJR-FL$_{V3Lib}$ using the calcium phosphate ProFection Mammalian Transfection System (Promega). The supernatant was collected 28 h after transfection, filtered through a 0.22-μm filter (Millipore), and stored at −80 °C until further use. p24 Gag in the supernatant was measured using a p24 Gag ELISA (Zeptometrix).

Selection of maraviroc-resistant variants

Maraviroc was provided by the NIH AIDS Research and Reference Reagent Program, Division of AIDS National Institute of Allergy and Infectious Diseases. For selection of maraviroc-resistant viruses, 5×10^5 of PM1/CCR5 cells were infected with 300 ng of p24 Gag in passage 1. After washing twice with phosphate-buffered saline (PBS), the infected cells were incubated with 0.003 μM of maraviroc at 37 °C in 5% CO_2. Virus passages were performed at 4- to 7-d intervals using 1×10^5 PM1/CCR5 cells from passage 2 to 17 in the presence of maraviroc gradually increasing up to 0.7 μM for HIV-1$_{V3Lib}$ and 0.1 μM for HIV-1$_{JR\text{-}FL}$ at passage 17.

Sequencing

The nucleotide sequences of *env* genes in the virus selected with maraviroc at passage 10 and 17 were determined as follows. The virus mixture was precipitated and subjected to reverse transcription-PCR using the ImProm-II Reverse Transcription System (Promega). A 2.5-kb fragment of the *env* gene including a viral envelope-encoding sequence in 50 μl reaction volume consisting of 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 0.01% gelatin, and 2 U AmpliTaq (Applied Biosystems Inc.) was amplified by PCR with primers JREnvF1 (5′-GAGAGAGAGCAGAAGACAGTGGCAATGA-3′) and JREnvR2 (5′-CACTACGTTTTGACCACTTGCCACCCA-3′). For direct sequencing, a 1/100 volume of the first PCR mixture was amplified with primers tagged with M13 tails, and the products were purified using a PCR purification kit (Marlingen). Then, the second batch of PCR products was used as the sequencing template. To sequence the virus clones, the first PCR products were purified by 1% agarose electrophoresis and subcloned in the pCR-TOPO vector (Invitrogen). The cloned DNA was sequenced using an ABI Prism 310 (Applied Biosystems Inc.).

Determination of drug susceptibilities

Susceptibilities of the viruses to the entry inhibitor was determined by the MTT assay using PM1/CCR5 cells for replication-competent viruses as previously described (Pauwels et al., 1988). Susceptibilities in the single-round viral entry assay were determined using previously titrated pseudotyped virus preparations using MAGIC-5 cells. Briefly, MAGIC-5 cells were plated in 48-well tissue culture plates 1 day prior to infection. After absorption of the pseudotyped virus for 2 h at 37 °C in the presence or absence of 0.0001–10 μM maraviroc, the cells were washed twice with PBS, and then further incubated for 48 h in the presence or absence of the inhibitor in fresh medium. EC_{50} was determined by measuring luciferase activity.

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References

Baba, M., Miyake, H., Wang, X., Okamoto, M., Takashima, K., 2007. Isolation and characterization of human immunodeficiency virus type 1 resistant to the small-molecule CCR5 antagonist TAK-652. Antimicrob. Agents Chemother. 51 (2), 707–715.

Berro, R., Sanders, R.W., Lu, M., Klasse, P.J., Moore, J.P., 2009. Two HIV-1 variants resistant to small molecule CCR5 inhibitors differ in how they use CCR5 for entry. PLoS Pathog. 5 (8), e1000548.

Chan, D.C., Kim, P.S., 1998. HIV entry and its inhibition. Cell 93 (5), 681–684.

Chan, D.C., Fass, D., Berger, J.M., Kim, P.S., 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell 89 (2), 263–273.

Donzella, G.A., Schols, D., Lin, S.W., Este, J.A., Nagashima, K.A., Maddon, P.J., Allaway, G.P., Sakmar, T.P., Henson, G., De Clercq, E., Moore, J.P., 1998. AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. Nat. Med. 4 (1), 72–77.

Dorr, P., Westby, M., Dobbs, S., Griffin, P., Irvine, B., Macartney, M., Mori, J., Rickett, G., Smith-Burchnell, C., Napier, C., Webster, R., Armour, D., Price, D., Stammen, B., Wood, A., Perros, M., 2005. Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. Antimicrob. Agents Chemother. 49 (11), 4721–4732.

Dragic, T., Trkola, A., Thompson, D.A., Cormier, E.G., Kajumo, F.A., Maxwell, E., Lin, S.W., Ying, W., Smith, S.O., Sakmar, T.P., Moore, J.P., 2000. A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. Proc. Natl Acad. Sci. USA 97 (10), 5639–5644.

Fatkenheuer, G., Pozniak, A.L., Johnson, M.A., Plettenberg, A., Staszewski, S., Hoepelman, A.I., Saag, M.S., Goebel, F.D., Rockstroh, J.K., Dezube, B.J., Jenkins, T.M., Medhurst, C., Sullivan, J.F., Ridgway, C., Abel, S., James, I.T., Youle, M., van der Ryst, E., 2005. Efficacy of short-term monotherapy with maraviroc, a new CCR5 antagonist, in patients infected with HIV-1. Nat. Med. 11 (11), 1170–1172.

Gulick, R.M., Su, Z., Flexner, C., Hughes, M.D., Skolnik, P.R., Wilkin, T.J., Gross, R., Krambrink, A., Coakley, E., Greaves, W.L., Zolopa, A., Reichman, R., Godfrey, C., Hirsch, M., Kuritzkes, D.R., 2007. Phase 2 study of the safety and efficacy of vicriviroc, a CCR5 inhibitor, in HIV-1-Infected, treatment-experienced patients: AIDS clinical trials group 5211. J. Infect. Dis. 196 (2), 304–312.

Hachiya, A., Aizawa-Matsuoka, S., Tanaka, M., Takahashi, Y., Ida, S., Gatanaga, H., Hirabayashi, Y., Kojima, A., Tatsumi, M., Oka, S., 2001. Rapid and simple phenotypic assay for drug susceptibility of human immunodeficiency virus type 1 using CCR5-expressing HeLa/CD4 (+) cell clone 1-10 (MAGIC-5). Antimicrob. Agents Chemother. 45 (2), 495–501.

Henrich, T.J., Tsibris, A.M., Lewine, N.R., Konstantinidis, I., Leopold, K.E., Sagar, M., Kuritzkes, D.R., 2010. Evolution of CCR5 antagonist resistance in an HIV-1 subtype C clinical isolate. J. Acquir. Immune Defic. Syndr. 55 (4), 420–427.

Kuhmann, S.E., Hartley, O., 2008. Targeting chemokine receptors in HIV: a status report. Annu. Rev. Pharmacol. Toxicol. 48, 425–461.

Kuhmann, S.E., Pugach, P., Kunstman, K.J., Taylor, J., Stanfield, R.L., Snyder, A., Strizki, J.M., Riley, J., Baroudy, B.M., Wilson, I.A., Korber, B.T., Wolinsky, S.M., Moore, J.P., 2004. Genetic and phenotypic analyses of human immunodeficiency virus type 1 escape from a small-molecule CCR5 inhibitor. J. Virol. 78 (6), 2790–2807.

Lusso, P., Earl, P.L., Sironi, F., Santoro, F., Ripamonti, C., Scarlatti, G., Longhi, R., Berger, E.A., Burastero, S.E., 2005. Cryptic nature of a conserved, CD4-inducible V3 loop neutralization epitope in the native envelope glycoprotein oligomer of CCR5-restricted, but not CXCR4-using, primary human immunodeficiency virus type 1 strains. J. Virol. 79 (11), 6957–6968.

MacArthur, R.D., Novak, R.M., 2008. Reviews of anti-infective agents: maraviroc: the first of a new class of antiretroviral agents. Clin. Infect. Dis. 47 (2), 236–241.

Maeda, Y., Foda, M., Matsushita, S., Harada, S., 2000. Involvement of both the V2 and V3 regions of the CCR5-tropic human immunodeficiency virus type 1 envelope in reduced sensitivity to macrophage inflammatory protein 1alpha. J. Virol. 74 (4), 1787–1793.

Maeda, Y., Yusa, K., Harada, S., 2008. Altered sensitivity of an R5X4 HIV-1 strain 89.6 to coreceptor inhibitors by a single amino acid substitution in the V3 region of gp120. Antiviral. Res. 77 (2), 128–135.

Marozsan, A.J., Kuhmann, S.E., Morgan, T., Herrera, C., Rivera-Troche, E., Xu, S., Baroudy, B.M., Strizki, J., Moore, J.P., 2005. Generation and properties of a human immunodeficiency virus type 1 isolate resistant to the small molecule CCR5 inhibitor, SCH-417690 (SCH-D). Virology 338 (1), 182–199.

Monde, K., Maeda, Y., Tanaka, Y., Harada, S., Yusa, K., 2007. Gp120 V3-dependent impairment of R5 HIV-1 infectivity due to virion-incorporated CCR5. J. Biol. Chem. 282 (51), 36923–36932.

Moore, J.P., Kuritzkes, D.R., 2009. A piece de resistance: how HIV-1 escapes small molecule CCR5 inhibitors. Curr. Opin. HIV AIDS 4 (2), 118–124.

Ogert, R.A., Wojcik, L., Buontempo, C., Ba, L., Buontempo, P., Ralston, R., Strizki, J., Howe, J.A., 2008. Mapping resistance to the CCR5 co-receptor antagonist vicriviroc using heterologous chimeric HIV-1 envelope genes reveals key determinants in the C2-V5 domain of gp120. Virology 373 (2), 387–399.

Ogert, R.A., Hou, Y., Ba, L., Wojcik, L., Qiu, P., Murgolo, N., Duca, J., Dunkle, L.M., Ralston, R., Howe, J.A., 2010. Clinical resistance to vicriviroc through adaptive V3 loop mutations in HIV-1 subtype D gp120 that alter interactions with the N-terminus and ECL2 of CCR5. Virology 400 (1), 145–155.

Pastore, C., Ramos, A., Mosier, D.E., 2004. Intrinsic obstacles to human immunodeficiency virus type 1 coreceptor switching. J. Virol. 78 (14), 7565–7574.
Pauwels, R., Balzarini, J., Baba, M., Snoeck, R., Schols, D., Herdewijn, P., Desmyter, J., De Clercq, E., 1988. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J. Virol. Methods 20 (4), 309–321.
Pugach, P., Marozsan, A.J., Ketas, T.J., Landes, E.L., Moore, J.P., Kuhmann, S.E., 2007. HIV-1 clones resistant to a small molecule CCR5 inhibitor use the inhibitor-bound form of CCR5 for entry. Virology 361 (1), 212–228.
Strizki, J.M., Xu, S., Wagner, N.E., Wojcik, L., Liu, J., Hou, Y., Endres, M., Palani, A., Shapiro, S., Clader, J.W., Greenlee, W.J., Tagat, J.R., McCombie, S., Cox, K., Fawzi, A.B., Chou, C.C., Pugliese-Sivo, C., Davies, L., Moreno, M.E., Ho, D.D., Trkola, A., Stoddart, C.A., Moore, J.P., Reyes, G.R., Baroudy, B.M., 2001. SCH-C (SCH 351125), an orally bioavailable, small molecule antagonist of the chemokine receptor CCR5, is a potent inhibitor of HIV-1 infection in vitro and in vivo. Proc. Natl Acad. Sci. USA 98 (22), 12718–12723.
Trkola, A., Kuhmann, S.E., Strizki, J.M., Maxwell, E., Ketas, T., Morgan, T., Pugach, P., Xu, S., Wojcik, L., Tagat, J., Palani, A., Shapiro, S., Clader, J.W., McCombie, S., Reyes, G.R., Baroudy, B.M., Moore, J.P., 2002. HIV-1 escape from a small molecule, CCR5-specific entry inhibitor does not involve CXCR4 use. Proc. Natl Acad. Sci. USA 99 (1), 395–400.
Tsibris, A.M., Kuritzkes, D.R., 2007. Chemokine antagonists as therapeutics: focus on HIV-1. Annu. Rev. Med. 58, 445–459.
Tsibris, A.M., Sagar, M., Gulick, R.M., Su, Z., Hughes, M., Greaves, W., Subramanian, M., Flexner, C., Giguel, F., Leopold, K.E., Coakley, E., Kuritzkes, D.R., 2008. In vivo emergence of vicriviroc resistance in a human immunodeficiency virus type 1 subtype C-infected subject. J. Virol. 82 (16), 8210–8214.
Westby, M., van der Ryst, E., 2010. CCR5 antagonists: host-targeted antiviral agents for the treatment of HIV infection, 4 years on. Antivir. Chem. Chemother. 20 (5), 179–192.
Westby, M., Smith-Burchnell, C., Mori, J., Lewis, M., Mosley, M., Stockdale, M., Dorr, P., Ciaramella, G., Perros, M., 2007. Reduced maximal inhibition in phenotypic susceptibility assays indicates that viral strains resistant to the CCR5 antagonist maraviroc utilize inhibitor-bound receptor for entry. J. Virol. 81 (5), 2359–2371.
Wild, C., Greenwell, T., Matthews, T., 1993. A synthetic peptide from HIV-1 gp41 is a potent inhibitor of virus-mediated cell-cell fusion. AIDS Res. Hum. Retroviruses 9 (11), 1051–1053.
Yusa, K., Maeda, Y., Fujioka, A., Monde, K., Harada, S., 2005. Isolation of TAK-779-resistant HIV-1 from an R5 HIV-1 GP120 V3 loop library. J. Biol. Chem. 280 (34), 30083–30090.

ARTICLES

バイオ医薬品の品質・安全性評価シリーズ（第5回）

バイオ医薬品の外来性感染性物質について

Adventitious agents in Biopharmaceuticals

国立医薬品食品衛生研究所 生物薬品部

遊佐敬介，新見伸吾，橋井則貴

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はじめに

バイオ医薬品の品質・安全性確保において，外来性感染性物質の管理は重要な要件の１つである。外来性感染性物質として細菌，真菌，ウイルス，異常プリオンがある。ここでは組換えDNA技術や細胞培養技術を用いて生産される医薬品のウイルス安全性がどのようにして確保されているのかを中心に述べる。

１．外来性感染性物質

外来性感染性物質には，細菌，真菌，マイコプラズマ，異常型プリオン，ウイルスがある。そのなかでも除去，否定試験が困難なものは，異常型プリオンとウイルスである（図１）。プリオンは，分子量33～35kDaのタンパク質である。正常型プリオンがβ構造リッチな立体構造である異常型プリオンに変換され，凝集体となったものが，

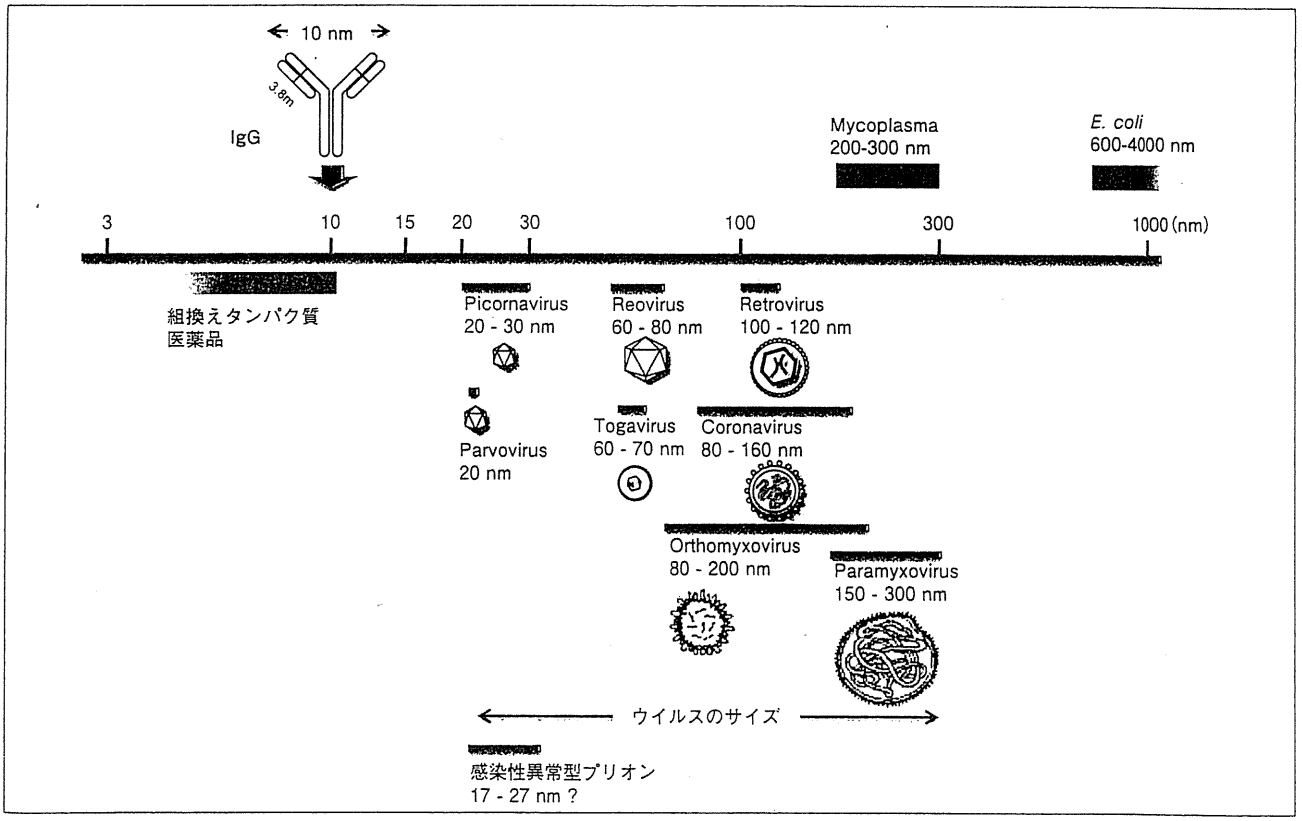

図１　外来性感染性物質

致死性神経変性疾患であるプリオン病（伝達性海綿状脳症）を引き起こすと考えられている。また感染性をもつ異常型プリオンは，凝集体のサイズによって感染性が異なるとの報告[1]もあるが，詳しいことはよくわかっていない。異常型プリオンが，ひとたび製造工程に混入するとその検出や不活化／除去が困難なため，迷入させないための方策が取られ，無血清培地への切り替えや培地に含まれる反芻動物由来原材料に関しても最大限の注意が払われている[2~4]。

ウイルスの基本構造は，粒子の中心にあるウイルス核酸と，それを取り囲むカプシドと呼ばれるタンパク質の殻から構成された粒子である。その大きさは最も小さいものはパルボウイルス（直径20nm）やピコルナウイルス（20～30nm）で，大きいものは最近見つかったミミウイルス（400nm）までその分布範囲は広い（図１）。これに対して比較的大きな分子量をもつイムノグロブリンは，２本のH鎖と２本のL鎖からなり，そのサイズは約10nmである。したがって，イムノグロブリンと最も小さなウイルスとの差はそれほどないことがわかる。またウイルスはサイズが異なるばかりでなく，その核酸の種類も形態（エンベロープの有無など）も多様である。2005年には1,950種のウイルスが記載されているが[5]，ヒトに感染し，病原性をもつウイルスは必ずしも多い訳ではない。ところが，その一方で新しい人獣共通感染症を引き起こすウイルスが次々報告されており，未知のウイルスによる医薬品の汚染が起きる可能性を常に孕んでいる。ウイルスフィルター，培養技術や精製工程の改良に伴いウイルス安全性は向上しているものの，細胞基材のウイルス試験や医薬品の製造工程における不活化工程や除去工程を組み合わせることによって，さらに安全性を高める工夫がなされている[6]。

2．バイオ医薬品におけるウイルス安全性

日本では1985年に承認されたインスリンやインターフェロンβ以来，約80品目もの組換え・細胞培養医薬品が国内で承認され，臨床現場で使われている[8]。これらの医薬品のうち動物細胞を用いて製造される医薬品は，生体成分を利用して製造されるために生産基材や原材料が感染性物質を含んでいる可能性があり，またその製造工程で感染性物質が混入すると，重大な感染事故を引き起こす懸念がある。そのため，バイオ医薬品の安全性は極めて厳格，かつ合理的なルールに沿って守られてきた。バイオ医薬品のウイルスの汚染を防ぐための基準作りが日米欧で行われ，平成12年にガイドライン「ヒト又は動物細胞株を用いて製造されるバイオテクノロジー応用医薬品のウイルス安全性評価」（Q5A）が通知され[6]，原材料は「生物由来原料基準」[2]および細胞基材については「生物薬品（バイオテクノロジー応用医薬品／生物起源由来医薬品）製造用細胞基材の由来，調製及び特性解析」（Q5D）[7]などを考慮してウイルス安全性に努めることになっている。ガイドラインに示されている特徴的な考えは，低濃度のウイルス検出感度に限界があるため，ウイルスクリアランス試験というプロセス評価を加えることにより，最終製品のウイルスに対する安全性を担保するという点にある。そのために，適切な工程でのウイルス試験や精製工程でのウイルス不活化／除去能を定量的に

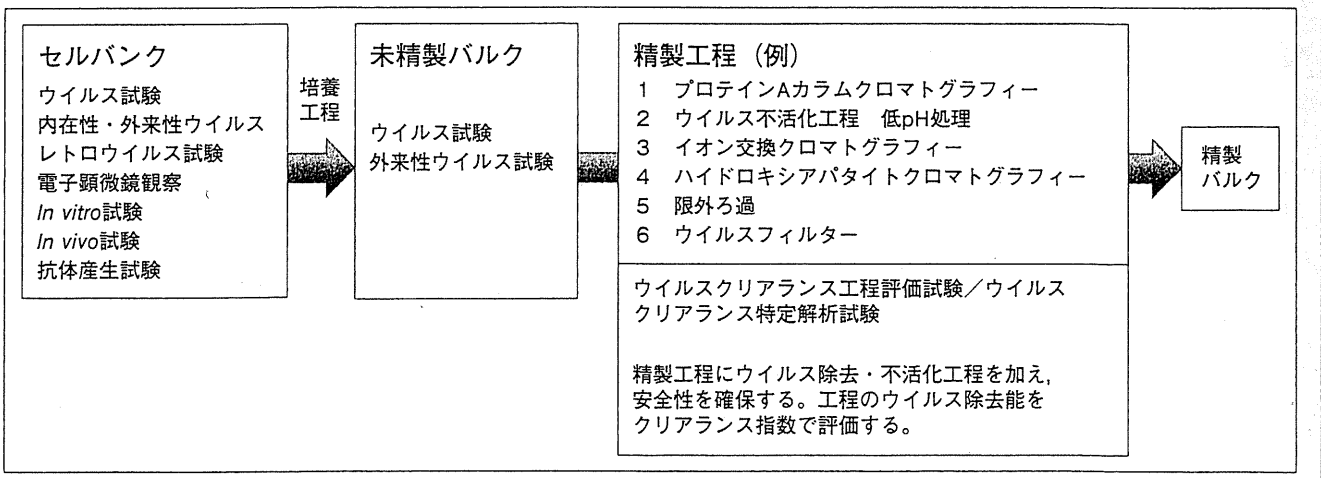

図２　バイオ医薬品の製造工程

示すことによって，工程全体を通じてその妥当性を明らかにする必要がある(図2)。

ウイルスによる汚染の可能性として，①医薬品生産に用いられる細胞が内在性，あるいは外来性ウイルスによって汚染されているケース(例としてレトロウイルスなどの潜伏感染やヘルペスウイルスの持続感染などによるもの)，②細胞培養に用いられる培地，トリプシン等の生物由来原料がウイルスで汚染されているケース，③細胞培養や精製工程における外界からのウイルスが迷入するケース(培養操作時のウイルス迷入や精製カラムの汚染や製剤化での汚染など)がある。したがって，こうした可能性を否定できるような合理的な根拠が提示されなくてはならない。そのためには，製造に用いられる細胞のウイルス安全性を十分調べること，原材料の生物由来原料の基準に基づいた使用，製造工程でのウイルス不活化除去能の評価，製造工程での適切な段階でのウイルス試験が必要になる。

3．医薬品製造用細胞のウイルス安全性

医薬品製造に用いられる細胞は，管理の基本となるMCB(マスターセルバンク)とこのMCBを増幅・分注・ストックし，実際の製造の度に用いられるWCB(ワーキングセルバンク)の2種類の細胞バンクとして管理される。MCBの純度試験では，無菌性やマイコプラズマの否定試験に加えて，多様なウイルス検出のためのウイルス試験が必要である。MCBは，バイオ医薬品製造の起点となる細胞なので，厳しい管理が必要になる。MCBのウイルス試験は，電子顕微鏡による直接観察やウイルスに対する感受性の高い細胞による感染性試験，実験小動物を用いたウイルス試験など多岐にわたる。MCBの純度試験に要求されるものには，①レトロウイルスおよび内在性ウイルス試験，②*In vitro*試験，③*In vivo*試験，④抗体産生試験，④その他細胞種特異ウイルス試験(適宜実施)がある(表1)。これに加えてCAL(医薬品製造のために*In vitro*細胞例の上限までに培養された細胞)についても①～③のウイルス試験が必要となる。

レトロウイルスは，そのライフサイクルに，感染宿主のゲノムに組み込まれる過程がある。そのため，いったん感染が成立して，ゲノムへの組み込みが起きると，ゲノムから取り除かれることなく，宿主細胞に潜み続けることができる。実際，ほ乳類のゲノムでは実にその8～10%は，レトロウイルスに由来する構造からなるといわれている。そのほとんどが，すでに進化の途上に複製能を失ったかつてのウイルスゲノムの残骸である。ところ

表1　各細胞レベルで1度は実施するべきウイルス試験

	MCB	WCB	CAL[a]
レトロウイルスおよび内在性ウイルス試験感染性試験			
感染性試験	+	−	+
電子顕微鏡観察	+	−	+
逆転写酵素活性[b]	+	−	+
その他細胞種特異ウイルス試験[c]	適宜実施	−	適宜実施
非内在性ウイルスまたは外来性ウイルス試験			
*In vitro*試験	+	−[d]	+
*In vivo*試験	+	−[d]	+
抗体産生試験[e]	+	−	−
その他細胞種特異ウイルス試験[f]	+	−	−

a. CAL：医薬品製造のために*In vitro*細胞齢の上限にまで培養された細胞。
b. レトロウイルス感染性試験が陽性のときは不要。
c. 細胞株個々の起源・由来から存在が予測されるウイルスを検出するために適した試験。
d. 第1回目のWCBについては，CALの段階で実施すること。それ以降のWCBについては，それ自体またはCALの段階で*In vitro*試験および*In vivo*試験をそれぞれ1種類ずつ実施する。
e. げっ歯類由来細胞株に対する試験の例として，マウス抗体産生(MAP)試験，ラット抗体産生(RAP)試験，ハムスター抗体産生(HAP)試験がある。
f. ヒト由来細胞株，ヒト以外の霊長類由来細胞株あるいはげっ歯類以外の動物由来細胞株である場合は，それぞれの細胞株に適切な試験を適宜実施すること。

表2　レトロウイルスおよび内在性ウイルス試験

感染性試験	S^+L^-フォーカスアッセイ(異種指向性，両指向性のマウスのレトロウイルスを調べる試験法) XCプラークアッセイ(ラット横紋筋腫由来細胞を使う同種指向性マウスレトロウイルス試験法)
電子顕微鏡観察	透過型電子顕微鏡で，細胞の超薄切片を観察する。げっ歯類の細胞株の多くは，内在性レトロウイルス粒子，レトロウイルス様粒子(A type，R type)が観察されるが，感染性がなく，医薬品製造に使用される。
逆転写酵素活性	レトロウイルスは，RNAを鋳型にしてDNA合成するために逆転写酵素をもっている。この活性は宿主にはないためレトロウイルスの検出に用いられる。
その他の細胞種特異ウイルス試験	その他の内在性ウイルスに関しては，各ウイルスに特異的な検出系がある。NATなどは，高感度であるが，感染性のないウイルスの検出や混入したDNAの非特異的増幅などの可能性を考慮するべきである。

表3　非内在性ウイルスまたは外来性ウイルス試験

*In vitro*試験	*In vitro*試験は広範囲のウイルスに感受性の高い培養細胞（MRC-5細胞，Vero細胞等）を使用する。 cell lysateを添加するか，混合培養してCPEの有無や上清での血球凝集反応を観察する。 ①MRC-5細胞（ヒト胎児正常肺組織由来） アデノウイルス，コクサッキーウイルスA，B，エコーウイルス，インフルエンザ，パラインフルエンザ，単純ヘルペスウイルス，CMV，エンテロウイルス，ポリオウイルス，麻疹ウイルス，ワクシニアウイルス，風疹ウイルス，RSウイルス，アルボウイルスなどが検出できる。 ②Vero細胞（アフリカミドリザル腎臓上皮由来） アデノウイルス，コクサッキーウイルスA，B，エコーウイルス，インフルエンザ，パラインフルエンザ，単純ヘルペスウイルス，CMV，エンテロウイルス，ポリオウイルス，麻疹ウイルス，ワクシニアウイルス，風疹ウイルス，RSウイルス，日本脳炎ウイルス，ポックスウイルス，フラビウイルス，ポリオーマウイルス，ロタウイルス，フィロウイルス，レオウイルス，アレナウイルス，ブンヤウイルス，コロナウイルス，ジステンパーウイルス，アルボウイルスなどが検出できる。
*In vivo*試験	①発育鶏卵接種試験：インフルエンザ，麻疹ウイルス，風疹ウイルス，ヘルペスウイルス，ラブドウイルス，ワクシニアウイルスなどを検出できる。 ②成熟マウス接種試験：ヘルペスウイルス，ラブドウイルス，ワクシニアウイルス，リンパ性脈絡髄膜炎ウイルスなどを検出できる。 ③乳のみマウス接種試験：コクサッキーウイルスA，B，単純ヘルペスウイルス，ラブドウイルス，ワクシニアウイルス，リンパ性脈絡髄膜炎ウイルスなどを検出できる。 ④モルモット接種試験：パラミクソウイルス，レオウイルス，ワクシニアウイルス，リンパ性脈絡髄膜炎ウイルス等を検出できる。
抗体産生試験	マウス，ハムスター，ラットなどに経口，経鼻，腹腔などから接種して4週間後の血清中の抗体産生の有無を調べ，ウイルスの有無を確認する。
その他細胞種特異ウイルス試験	特定のウイルスを標的にしたNAT等

が中には，感染性は失われたものの，ウイルス様の粒子を細胞内に産生しているものや細胞外に放出している細胞がある。例えばバイオ医薬品製造でよく使われるCHO細胞は，このようなウイルス様粒子を絶えず産生しており，電子顕微鏡観察によって細胞内にウイルス様粒子が認められる。この粒子は，感染性をもたず，安全性がすでに明らかになっており，CHO細胞は医薬品製造に最も多く使われている細胞である。ウイルス試験は，内在性のレトロウイルスのほか，外来性のレトロウイルスの試験も必要である（表2）。この他に*In vitro*試験によって，多様なウイルスに対してそれぞれ高感受性の細胞を用いてMCBやCALの感染の有無を調べる必要がある（表3）。また*In vivo*試験では，発育鶏卵や小動物を用いてウイルスの感染の有無を同様に調べる。さらにMCBに関しては，抗体産生試験によっても感染の有無を調べる。

4．医薬品製造工程における ウイルスクリアランス試験

ウイルスクリアランス試験は，未精製バルクから，精製バルクまでの精製工程でのウイルスの不活化，除去能を評価することである。出発点となる未精製バルクは培養後の上清であり，ウイルス汚染の有無を検出するのに適した段階であると考えられる。Q5Aでは精製バルクにおける適切なウイルス試験を求めている。ここでは未精製バルクのウイルス試験では外来性ウイルスが存在しないことを高い感度をもつ検出法によって確認しなくてはならない。細胞や未精製バルクでの外来性ウイルス汚染がわかったものについては，そのウイルスのヒトへの感染の有無にかかわらず，その利用は例外的なケースを除いて通常認められない。ICH Q5Aガイドラインには，細胞や未精製バルクでウイルスが検出された場合を5段階に分けてウイルスクリアランス工程評価の実施要領が示されている。例えばCHO細胞を用いて得られた未精製バルクには，レトロウイルス様粒子が認められるが，感染性はなく非病原性であると認められている。ウイルスクリアランス試験では非特異的モデルウイルスを用いたウイルスクリアランス工程特性解析試験とともに特異的モデルウイルスを用いたウイルスクリアランス工程評価試験（例えばマウス白血病ウイルス）が必要になる。つまりウイルスクリアランス工程評価試験ではMCBに存在することが知られている，ないし存在が予想されるウイルスのクリアランスを証明するために行われ，ウイルスクリアランス工程特性解析試験では製造工程中に迷入する可能性がある外来性ウイルスのクリアランスに関して保証を与えるためのものである。

ウイルスクリアランス工程特性解析試験で用いられるのは非特異的モデルウイルスと呼ばれる既知のウイルスである。この試験では，その性質や形態（エンベロープの有無や核酸），サイズが異なるウイルスを選定して実施する。使われるウイルスの例を表4にあげてある。実

表4　ウイルスクリアランス試験に用いられるウイルス

ウイルス	宿主	ゲノム	env	サイズ(nm)	形状	抵抗性
マウス微小ウイルス(MVM) パルボウイルス科	マウス	DNA	有	22-25	球形	高
水疱性口内炎ウイルス(VSV) ラブドウイルス科	ウマ ウシ	RNA	有	70×150	弾丸	低
マウス白血病ウイルス(MuLV) レトロウイルス科	マウス	RNA	有	80-120	多様／球形	低
シンドビスウイルス(Sindbis Virus) トガウイルス科	ヒト	RNA	有	60-70	球形	低
ウシ下痢症ウイルス(BVDV) フラビウイルス科	ウシ	RNA	有	50-70	多様／球形	低
ポリオウイルスSabin 1型(Poliovirus) ピコルナウイルス科	ヒト	RNA	無	25-30	正20面体	中
ネコカリシウイルス(FCV) カリシウイルス科	ネコ	RNA	無	30-38	正20面体	中
仮性狂犬病ウイルス(PRV) ヘルペスウイルス科	ブタ	DNA	有	120-200	球形	中

際にはこれらのウイルスをスパイクし，医薬品の精製工程の不活化，除去能を評価する。クリアランス試験は，２つ以上の製造工程について，どのようなウイルス不活化，除去能力を有するかを評価することが望ましいとされる。

5．外来性感染性物質による汚染

実際今日まで，医薬品製造細胞が培養時にウイルスに感染し，出荷が停止した例を始めとするいくつかの事例が海外で報告されている(表５)。最近では2008，2009年にライソゾーム酵素欠損疾患の治療薬を製造していたGenzyme社が，製造に使われていたバイオリアクターのウイルス汚染によって，一時操業停止に追い込まれ，CerezymeとFabrazymeの供給不足を招いた[9]。これはウイルス感染によって，CHO細胞の増殖性が低下したためにウイルス汚染が見つかった例である。原因となったウイルスは分離され，Vesivirus2117と名づけられた。このウイルスはエンベロープをもたない，正20面体構造(40nm)をもつカリシウイルス科のウイルスで，その塩基配列の解析から同じベジウイルス属に分類されるミンクカリシウイルスやネコカリシウイルスによく似ていることがわかった。幸いこのウイルスはヒト細胞には感染せず，出荷が停止されたため，製品の汚染はなかったが，動物細胞を使ったバイオ医薬品製造のウイルス安全性にとって大きな教訓となった。

おわりに

細胞株由来のバイオテクノロジー応用医薬品製造業者

表５　報告のあるバイオ医薬品製造におけるウイルス汚染事例

ウイルス	細胞	汚染源	会社	文献
EHDV	CHO cells	contaminated FBS	Recombinant protein for phase I clinical trials (Biofen GmbH& Co.)	Burstyn, 1996 (ref 10)
MVM	CHO cells	contaminated raw materials	Pulmozyme® (Genentech)	Garnick, 1996 (ref 11)
Vesivirus 2117	CHO cells	contaminated FBS	(BoehringerIngelheim Pharmaceuticals)	Oehming, et al., 2003 (ref. 12)
Reovirus	Unprocessed bulk harvest (CHO cells)	contaminated FBS	undiclosed	Nims, 2006 (ref. 13)
CVV	CHO cells	contaminated FBS	undisclosed	Nims, 2006 (ref. 13)
Vesivirus 2017	CHO cells	contaminated raw materials	Cerezyme® and Fabrazyme® (Genzyme)	Genzyme Corp, 2008, 2009 (ref. 9)

EHDV, Epizootic hemorrhagic disease virus(伝染性出血熱ウイルス)，レオウイルス科；MVM, Minute virus of mice(マウス微小ウイルス)，パルボウイルス科；CVV, Cache Valley virus(カシェ渓谷ウイルス)，ブニヤウイルス科；Vesivirus 2017(ベジウイルス2017)，ベジウイルス科.

は，それぞれの製品や製造工程について，ウイルスに対する安全性を保証するため採用したウイルス安全性のための方策を説明し，その妥当性を示す必要がある。そして承認審査に必要な書類には，詳細なデータに加えて，ウイルス安全性評価に関する総括を記載する必要がある。しかしウイルス試験法やウイルスクリアランス試験を行うには高度の専門性が必要とされ，その上使用するウイルスを扱うための生物学的封じ込め基準を満たした施設が必要となる。そのためウイルス安全試験はその多くが委託機関によって行われている。

■参考文献

1) Silveira, J.R., Raymond, G.J., Hughson, A.G., Race, R.E., Sim, V.L., Hayes, S.F. and Caughey, B.：The most imfections prion protein particles., *Nature*, **437**, 257-261, 2005.
2)「生物由来原料基準」平成15年厚生労働省告示第210号，平成15年5月20日
3)「ウシ等由来原材料を使用した医薬品，医療用具等の一部変更承 認申請におけるリスク評価等の取扱いについて」薬食審査発第801001号 薬食安発第0801001号 平成15年8月1日
4)「生物由来原料基準の規定を満たさないマスターセルバンク又はマスターシードを使用した医薬品等の取扱いについて」事務連絡 平成21年3月27日 厚生労働省医薬食品局審査管理課
5) Fauquet, C.M., Fargette, D. International committee on taxonomy of viruses and the 3, 142 unassigned species., *Virol. J.*, **2**, 64, 2005.
6)「ヒト又は動物細胞株を用いて製造されるバイオテクノロジー応用医薬品のウイルス 安全性評価」医薬審第329号通知 平成12年2月22日（ICH Q5Aガイドライン）
7)「生物薬品（バイオテクノロジー応用医薬品／生物起源由来医薬品）製造用細胞基材の由来，調製及び特性解析」医薬審第873号，平成12年7月14日（ICH Q5Dガイドライン）
8) http://www.nihs.go.jp/dbcb/approved_biologicals.html
9) Oehmig, A., Büttner, M., Weiland, F., Werz, W., Bergemann, K., Pfaff E.：Identification of a calicivirus isolate of unknown origin., *J.Gen.Virol.*, **84**, 2837-2845, 2003.
10) Burstyn, D.G.：Contamination of genetically engineered Chinese hamster ovary cells., *Dev. Biol. Stand.*, **88**, 199-203, 1996.
11) Garnick, R.L.：Experience with viral contamination in cell culture., *Dev. Biol. Stand.*, **88**, 49-56, 1996.
12) Oehmig, A., Büttner, M ., Weiland, F ., Werz, W ., Bergemann, K ., Pfaff, E.：Identification of a calicivirus isolate of unknown origin., *J. Gen., Virol.*, **84**, 2837-2845, 2003.
13) Nims, R.W.：Detection of adventitious viruses in biologicals - a rare occurrence., *Dev. Biol.*, **123**, 153-64, 2006.