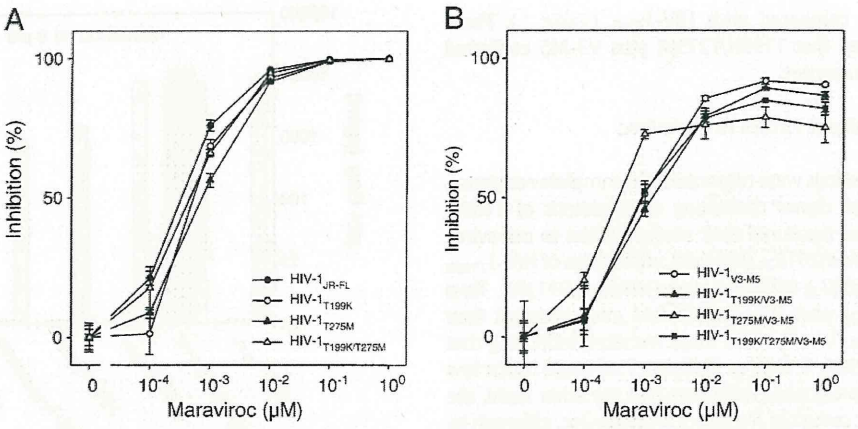

Fig. 5. Inhibition of viral entry. Pseudotyped viruses were prepared with 293T cells by transfection with pNL-luc and pCNX-FLenv. MAGIC-5 cells were infected with pseudotyped viruses in the presence of 0.0001–1 μM of maraviroc. Mean ± SD (n = 3).

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$_{JR-FL}$ 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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Review Article Open Access

In vitro and *In vivo* Resistance to Human Immunodeficiency Virus Type 1 Entry Inhibitors

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Abstract

Viral entry is one of the most important targets for the efficient treatment of Human immunodeficiency virus type 1 (HIV-1)-infected patients. The entry process consists of multiple molecular steps: attachment of viral gp120 to CD4, interaction of gp120 with CCR5 or CXCR4 co-receptors, and gp41-mediated fusion of the viral and cellular membranes. Understanding the sequential steps of the entry process has enabled the production of various antiviral drugs to block each of these steps. Currently, the CCR5 inhibitor, maraviroc, and the fusion inhibitor, enfuvirtide, are clinically available. However, the emergence of HIV-1 strains resistant to entry inhibitors, as commonly observed for other classes of antiviral agents, is a serious problem. In this review, we describe a variety of entry inhibitors targeting different steps of viral entry and escape variants that are generated *in vitro* and *in vivo*.

Keywords: CD4-gp120 binding inhibitor; CCR5 antagonist; CXCR4 antagonist; Fusion inhibitor; Resistance; HIV-1

Introduction

The development of chemotherapy with antiretroviral agents has reduced the morbidity and mortality of Human immunodeficiency virus type 1 (HIV-1)-infected individuals. Successful treatment of HIV-1-infected patients using chemotherapy is partly due to a combination of different classes of antiviral agents against the viral protease or reverse transcriptase. However, successful eradication of the virus from infected individuals has not been achieved by antiviral treatment, and is often limited by the emergence of drug-resistant HIV-1 strains [1-3]. These problems highlight the need to develop novel anti-HIV-1 drugs that target different steps of the viral replication process. Viral entry is currently one of the most attractive targets for the development of new drugs to control HIV-1 infection. Viral entry proceeds through Env (gp120, gp41)-mediated membrane fusion, and consists of sequential steps: (i) attachment of viral gp120 to the CD4 receptor; (ii) binding of gp120 to CCR5 or CXCR4 co-receptors; and (iii) fusion of the viral and cellular membranes (Figure 1). A large number of inhibitors targeting different steps of the viral entry process have been developed, including peptides/peptide mimics, small molecules, and monoclonal antibodies (MAb).

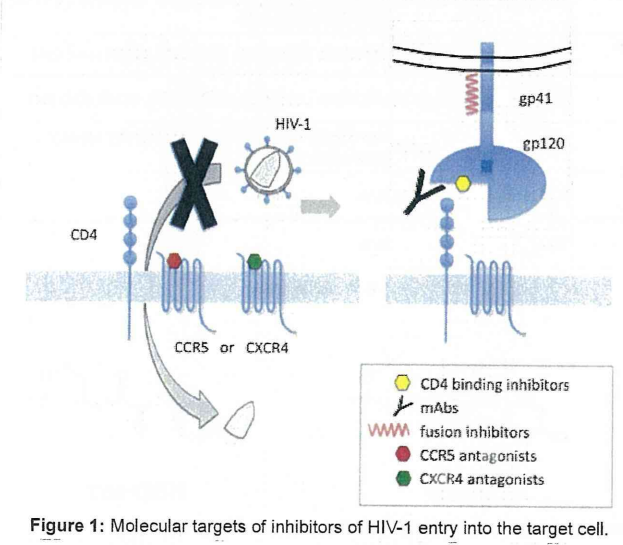

Figure 1: Molecular targets of inhibitors of HIV-1 entry into the target cell.

Enfuvirtide (also known as T-20) was the first of a new class of drugs known as fusion inhibitors, which was approved by the U.S. Food and Drug Administration (FDA) in 2003. Approval was given for the use of this drug in combination with other anti-HIV-1 medications to treat advanced HIV-1 infection in adults and children aged six years and older. The drug is an antiviral peptide that prevents HIV-1 entry by blocking gp41-mediated fusion [4-6]. Small compounds that can bind to the pockets of the extracellular loops of a coreceptor are expected to be potent antiviral agents. Several small-molecule CCR5 inhibitors have progressed through clinical development [7]. Maraviroc [8,9], a CCR5 antagonist, is the second entry inhibitor approved by the FDA in 2007 for treatment-experienced patients infected with a CCR5-tropic (R5-tropic) virus. Extensive research is currently underway to develop the next generation of entry inhibitors, however, the emergence of viral strains resistant to entry inhibitors, as well as other classes of antiviral

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agents, has been reported *in vitro* and *in vivo* [7,10]. In this review, we describe the current status of *in vitro* and *in vivo* resistance to HIV-1 entry inhibitors.

Resistance to CD4-gp120 binding inhibitors

Inhibition of CD4-gp120 binding: Entry of HIV-1 into target cells is mediated by the trimeric envelope glycoprotein complex, each monomer consisting of a gp120 exterior envelope glycoprotein and a gp41 transmembrane envelope glycoprotein [11]. Attachment of HIV-1 to the cell is initiated by the binding of gp120 to its primary CD4 receptor, which is expressed on the surface of the target cell. The gp120-CD4 interaction induces conformational changes in gp120 that facilitate binding to additional coreceptors (for example, CCR5 or CXCR4). Attachment inhibitors are a novel class of compounds that bind to gp120 and interfere with its interaction with CD4 [12]. Thus, these agents can prevent HIV-1 from attaching to the CD4+ T cell and block infection at the initial stage of the viral replication cycle (Figure 1). There are two primary types of HIV-1 attachment inhibitors: nonspecific attachment inhibitors and CD4-gp120 binding inhibitor [13].

In this section, we focus on the CD4-gp120 binding inhibitors, the soluble form of CD4 (sCD4), a fusion protein of CD4 with Ig (PRO542), a monoclonal anti-CD4 antibody (Ibalizumab, formerly TNX-355), CD4 binding site (CD4bs) monoclonal antibodies (b12 and VRC01), small-molecule HIV-1 attachment inhibitors (BMS-378806 and BMS-488043), and a new class of small-molecule CD4 mimics (NBD-556 and NBD-557) and a natural small bioactive molecule (Palmitic acid) (Figure 2). We also describe the resistance profiles against these CD4-gp120 binding inhibitors *in vivo* and/or in vitro.

Soluble CD4 (sCD4) and PRO542: In the late 1980s, various recombinant, soluble proteins derived from the N-terminal domains of CD4 were shown to be potent inhibitors of laboratory strains of HIV-1 [14]. Based on the potential of sCD4 to inhibit HIV-1 infection in vitro, this protein was tested for clinical efficacy in HIV-1-infected individuals; however, no effect on plasma viral load was observed [14]. Further examination revealed that doses of sCD4 significantly higher than those achieved in the clinical trial were required to neutralize primary clinical isolates of HIV-1, in contrast to the relatively sensitive, laboratory-adapted strains [15].

The first report of sCD4-resistant variants induced by *in vitro* selection showed that the resistant variant had a single mutation (M434T) in the C4 region [16]. During selection with sCD4, it was also reported that, seven mutations (E211G, P212L, V255E, N280K, S375N, G380R, and G431E) appeared during *in vitro* passage [17]. Further, a recombinant clone containing a V255E mutation was found to be highly resistant to sCD4 compared with the wild-type virus (114-fold higher 50% inhibitory concentration [IC_{50}] value). To determine the mutation profiles obtained during *in vitro* selection with sCD4, the atomic coordinates of the crystal structure of gp120 bound to sCD4 was retrieved from public protein structure database (PDB entry: 1RZJ). From these analyses, it was determined that almost all the described resistance mutations were located the inside the CD4-binding cavity of gp120 [17].

Recently, a novel recombinant antibody-like fusion protein (CD4-1gG2; PRO542) was developed in which the Fv portions of both the heavy and light chains of human IgG2 were replaced with the D1D2 domains of human CD4 [18]. PRO542 was shown to broadly and po-

Profile of CD4-gp120 binding inhibitors

	Structure	Feature	Target	Resistant related mutations (region of gp160) [ref]
sCD4	Solbule form of CD4 domain1-4	First CD4-gp120 binding inhibitor	CD4 binding site of gp120	M434T (C4) [16], V255E(C2) [17]
PRO542	Tetravalent CD4 (domain1-2)-IgG	Developing for microbicide	CD4 binding site of gp120	* N/A
Ibalizumab	Anti-CD4 monoclonal antibody (MAb)	First-in-class, MAb inhibitor of CD4-mediated HIV entry	Domain 2 of CD4	N/A
b12	Anti-CD4 binding site Mab	Neutralizing around 40% of HIV-1 primary isolates	CD4 binding site of gp120	P369L (C3) [27]
VRC01	Anti-CD4 binding site Mab	Neutralizing over 90% of diverse HIV-1 primary isolates	CD4 binding site of gp120	K121A(C1), L179A(V2), T202A(C2), D279A(C2), R304A(V3), I420A(C4), I423A(C4), Y435A(C4), G471A (C5), D474A(C5) [31]
BMS-378806	see below Figure	First small molecule HIV-1 CD4 attachemnt inhibitor	CD4 binding site of gp120	V68A(C1), M426L(C4), M475I(V5), I595F(gp41) [33]
BMS-488043	see below Figure	Improved in vitro antiviral activity and PK properties compared to BMS-378806	CD4 binding site of gp120	V68A(C1), L116I(C1), S375I/N(C3), M426L(C4) [34]
NBD-556	see below Figure	Inhibition of HIV-1 entry and enhancing neutralizing potency of Abs	CD4 binding site of gp120	S377N(C3), A433T(C4) [17], S375W(C3), I424A(C4), W427A(C4), V475A(C5) [38]
NBD-557	see below Figure	Inhibition of HIV-1 entry and enhancing neutralizing potency of Abs	CD4 binding site of gp120	N/A
Palmitic acid	$CH_3(CH_2)_{14}COOH$	A natural small bioactive molecule from *Sargassum fusiforme*	Domain 1 of CD4	N/A

* N/A : not available

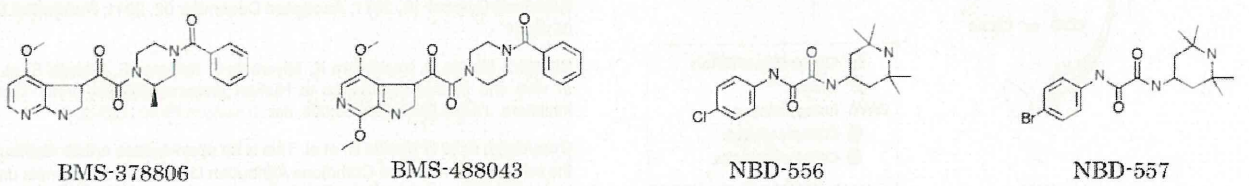

Figure 2: Profile of CD4-gp120 binding inhibitors including molecular structures of selected small molecular inhibitors.

tently neutralize HIV-1 subtype B isolates, and was also able to neutralize strains from non-B isolates with the same breadth and potency as for subtype B strains. PRO542 blocks attachment and entry of the virus into CD4+ target cells and were mainly developed for the prevention and transmission of HIV-1 through external application agents, such as microbicides.

Ibalizumab (TNX-355): Monoclonal anti-CD4 antibodies block the interaction between gp120 and CD4 and, therefore, inhibit viral entry [19]. Ibalizumab (formerly TNX-355) was a first-in-class, monoclonal antibody inhibitor of CD4-mediated HIV-1 entry [20]. By blocking CD4-dependent HIV-1 entry, ibalizumab was shown to be active against a broad spectrum of HIV-1 isolates, including recombinant subtypes, as well as both CCR5-tropic and CXCR4-tropic HIV-1 isolates. Many clinical trials with HIV-1-infected patients have demonstrated the antiviral activity, safety, and tolerability of ibalizumab. A nine-week phase Ib study investigating the addition of ibalizumab monotherapy to failing drug regimens showed transient reductions in HIV-1 viral loads and the evolution of HIV-1 variants with reduced susceptibility to ibalizumab. Further, clones with reduced susceptibility to ibalizumab contained fewer potential N-linked glycosylation sites (PNGSs) within the V5 region of gp120. Reduction in ibalizumab susceptibility due to the loss of V5 PNGSs was confirmed by site-directed mutagenesis [21].

Monoclonal antibodies, b12 and VRC01: Several broadly neutralizing MAbs isolated from HIV-1-infected individuals define conserved epitopes on the HIV-1 Env. These include the membrane proximal external region of gp41 targeted by MAbs 4E10 and 2F5 [22]; the carbohydrate-specific outer domain epitope targeted by 2G12 [23]; a V2-V3-associated epitope targeted by PG9/PG16 [24]; and the CD4bs [25] targeted by b12 and VRC01. The CD4bs overlaps with the conserved region on gp120 that is involved in the engagement of CD4. The prototypical CD4bs-directed MAb, b12, neutralizes around 40% of primary isolates, and its structure (in complex with the core of gp120) has been defined [26]. However, Mo et al. [27] reported the first resistant variant induced by *in vitro* selection with b12 that showed a P369L mutation in the C3 region of HIV-1_{JRCSF}. Further, several b12-resistant viruses commonly display an intact b12 epitope on the gp120 subunits [28], suggesting that quaternary packing of Env also confers resistance to b12.

A recently described CD4bs-directed MAb, VRC01, had been shown to be able to neutralize over 90% of diverse HIV-1 primary isolates [29]. The structure of VRC01 in complex with the gp120 core reveals that the VRC01 heavy chain binds to the gp120 CD4bs in a manner similar to that of CD4 [30]. The gp120 loop D and V5 regions contain substitutions uniquely affecting VRC01 binding, but not b12 or CD4-Ig binding. In contrast to the interaction of CD4 or b12 with the HIV-1 Env, occlusion of the VRC01 epitope by quaternary constraints was not a major factor limiting neutralization. Interestingly, many Ala substitutions at non-contact residues increased the potency of CD4- or b12-mediated neutralization; however, few of these substitutions enhanced VRC01-mediated neutralization [31]. This study suggests that VRC01 approaches its cognate epitope on the functional spike with less steric hindrance than b12 and, surprisingly, with less hindrance than the soluble form of CD4 itself. These differences might be related to the distinctly different angle of approach to the CD4bs employed by VRC01, in contrast to the more loop-proximal approach employed by CD4 and b12.

BMS-378806 and BMS-488043: BMS-378806 (Figure 2) is a recently identified small-molecule HIV-1 attachment inhibitor with good antiviral activity and pharmacokinetic properties [32]. BMS-378806 binds directly to gp120 with a stoichiometry of approximately 1:1 and with a binding affinity similar to that of soluble CD4. The potential BMS-378806 target site was localized to a specific region within the CD4 binding pocket of gp120 using HIV-1 gp120 variants carrying either compound-selected resistant substitutions or gp120-CD4 contact site mutations [32]. M426L (C4) and M475I (V5) substitutions located at or near gp120/CD4 contact sites were shown to confer high levels of resistance to the *in vitro* mutated HIV-1 variants, suggesting that the CD4 binding pocket of gp120 was the antiviral target. M434I and other secondary changes (V68A and I595F) also affect the drug susceptibility of recombinant viruses, presumably by influencing the gp120 conformation [33]. BMS-378806 (Figure 2) exhibited decreased, but still significant activity against subtype C viruses, low activity against viruses from subtypes A and D, and poor or no activity against subtypes E, F, G, and Group O viruses [33].

BMS-488043 (Figure 2) is a novel and unique small-molecule that inhibits the attachment of HIV-1 to $CD4^+$ lymphocytes. BMS-488043 exhibits potent antiviral activity against macrophage-, T-cell-, and dual-tropic HIV-1 laboratory strains (subtype B) and potent antiviral activity against a majority of subtype B and C clinical isolates [34]. Data from a limited number of clinical isolates showed that BMS-488043 exhibited a wide range of activity against the A, D, F, and G subtypes, with no activity observed against three subtype AE isolates [34]. The antiviral activity, pharmacokinetics, viral susceptibility, and safety of BMS-488043 were evaluated in an eight-day monotherapy trial that demonstrated significant reductions in viral load. To examine the effects of BMS-488043 monotherapy on HIV-1 sensitivity, phenotypic sensitivity assessment of baseline and post-dosing (day 8) samples were performed. The analyses revealed that four subjects showed emergent phenotypic resistance. Population sequencing and sequence determination of the cloned envelope genes revealed five gp120 mutations at four loci (V68A, L116I, S375I/N, and M426L) associated with BMS-488043 resistance; the most common (substitution at the 375 locus) located near the CD4 binding pocket [35].

NBD-556 and NBD-557: Targeting the functionally important and conserved CD4bs on HIV-1 gp120 represents an attractive potential approach to HIV-1 therapy or prophylaxis. Recently, a new class of small-molecule CD4 mimics was identified [36-38]. These compounds, which include the prototypic compound, NBD-556, and its derivatives, mimic the effects of CD4 by inducing the exposure of the co-receptor-binding site on gp120 [17,39]. NBD-556 and -557 (Figure 2) show potent cell fusion and virus-cell fusion inhibitory activity at low (micromolar) concentrations. A mechanistic study showed that both compounds target viral entry by inhibiting the binding of gp120 to its cellular receptor, CD4. A surface plasmon resonance study showed that these compounds bind to unliganded HIV-1 gp120, but not to CD4 [37]. Another recent study identified NBD-analogs as CD4 mimetics that were used for the prophylaxis and treatment of HIV-1 infection [39]. These compounds inhibited HIV-1 transmission by inhibiting the binding of the natural ligand, CD4, and prematurely triggering the envelope glycoprotein to undergo irreversible conformational changes. NBD-556 binds to the F43 cavity, which is formed by binding of gp120 to the CD4 receptor in a highly conserved manner [17,39].

Recently, our group reported that NBD-556 has potent neutralizing antibody-enhancing activity toward plasma antibodies that cannot access neutralizing epitopes hidden within the trimeric Env, such as gp120-CD4 induced epitope (CD4i) and anti-V3 antibodies [17]. Therefore, to investigate the binding site of NBD-556 on gp120, we in-

duced HIV-1 variants that were resistant to NBD-556 in vitro. Two amino acid substitutions (S375N in C3 and A433T in C4) were identified at passage 21 in the presence of 50 μM NBD-556. The profiles of the resistance mutations after selection with NBD-556 and sCD4 were very similar with regard to their three-dimensional positions.

Elucidation of the detailed molecular mechanisms governing the interaction between gp120 and NBD compounds will enable the optimization and evaluation of this strategy in more complex biological models of HIV-1 infection. Consequently, we will continue to synthesize NBD analogs and search for drugs with greater potency to change the tertiary structure of the envelope glycoproteins and reduce host cytotoxicity [40,41].

Palmitic acid : Previous studies with whole *Sargassum fusiforme* (*S. fusiforme*) extract and with the bioactive SP4-2 fraction demonstrated inhibition of HIV-1 infection in several primary and transformed cell lines [42]. Palmitic acid (PA), which was isolated from the SP4-2 bioactive fraction, specifically block productive X4 and R5-tropic HIV-1 infection [43]. PA occupies a novel hydrophobic cavity on the CD4 receptor that is constrained by amino acids F52-to-L70 [44], which encompass residues that have been previously identified as a region critical for gp120 binding. PA is mainly developed as microbicides [45].

Resistance to CCR5 antagonists

CCR5 antagonists: The binding of HIV-1 to CD4 molecules induces conformational change in gp120, resulting in the recognition of either CCR5 or CXCR4 as a coreceptor for HIV-1 (Figure 1). It has been shown that CCR5-utilizing HIV-1 (R5 virus) is associated with human-to-human transmission that predominate throughout the infection, while CXCR4-utilizing HIV-1 (X4 virus) emerges during the late stage of infection in approximately half of HIV-1-infected individuals and is associated with disease progression [46]. Most strikingly, it had been shown that homozygous individuals having a 32-bp deletion in the CCR5 coding region (CCR5Δ32) were found to be resistant to R5 HIV-1 and remained apparently healthy [47,48]. These findings suggested that CCR5 would be an attractive therapeutic target for treating HIV-1 infection, although it is a host factor. Several small molecule compounds have been developed and were found to bind CCR5 and inhibit R5 virus replication [49-53]. Molecular studies using CCR5 mutants indicated that these compounds bind to a cavity formed by transmembrane helices of CCR5, and thereby inducing the conformational change in an allosteric manner that is not recognized by gp120 of HIV-1 [54-58]. Among these, TAK-779 (Figure 3) was the first compound developed [49] that could inhibit not only HIV-1 infection, but also binding of RANTES (CCR5 ligand) to CCR5-expressing cells at nanomolar concentrations, but was terminated due to poor oral bioavailability. Maraviroc (MVC, UK427, 857) (Figure 3), however, has been approved and used in the clinic for the treatment of HIV-1 infection [8]. Another promising drug, vicriviroc (VCV, SCH-D, SCH-417690) (Figure 3), recently completed phase III trials but has not yet been approved [53].

Resistance to CCR5 antagonists: Although CCR5 antagonists target

Profile of CCR5 antagonist-resistant mutants

drug	virus used		resistant-related mutations		references
	virus name or *in vivo*	subtype	V3	Non-V3	
AD101	CC1/85	B	H305R, H308P, A316V, G321E	none	[60, 78]
TAK-779	JR-FL$_{V3lib}$	B	I304V, H305N, I306M, F312L, E317D	none	[63]
TAK-652	KK	unknown	ND[a]	ND	[59]
VVC	CC1/85	B	none	G516V, M518V, F519I (gp41)	[69, 84, 85]
VVC	RU570	G	K305R, R315Q, K319T	P437S (C4)	[64, 81]
VVC	S91	D	Q315E, R321G	E328K, G429R (C4)	[65]
VVC	*in vivo*	C	K305R, T307I, F316I, T318R, G319E	none	[67]
MVC	CC1/85	B	A316T, I323V	ND	[61]
MVC	JR-FL$_{V3lib}$	B	I304V, F312W, T314A, E317D, I318V	T199K, T275M (C2)	[62]
MVC	*in vivo*	B	P/T308H, T320H, I322V	D407G, Δ[b]N386 (V4)	[66]

[a]ND, not determined; [b]Δ, deletion

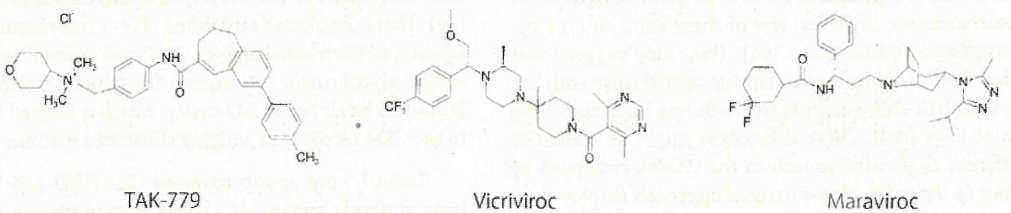

Figure 3: Profile of CCR5 antagonist-resistant mutants. The CCR5 antagonist-resistant mutants were isolated in vitro and in vivo across different subtypes of HIV-1. Resistance-related mutations were found in the V3 and non-V3 regions including the C2, V4, C4, and gp41. Chemical structures of representative CCR5 antagonists are shown.

a host cell receptor, the *in vitro* [59-64] and *in vivo* [65-67] emergence of viruses resistant to CCR5 antagonists in different subtypes has been reported, as shown in Figure 3. The most intuitive mechanism of resistance to CCR5 antagonists is likely to be the acquisition of CXCR4 use or selection of minority variants of CXCR4- or dual/mixed-tropic viruses [61,68-70]. Numerous studies showed that coreceptor selectivity of HIV-1 is primarily dependent on the third hypervariable region (V3 loop) of gp120 [71-74]. Furthermore, there is a simple rule to predict HIV-1 coreceptor usage called the 11/25 rule: if either the 11th or 25th amino acid position of V3 is positively charged, the virus will use CXCR4 as the coreceptor, otherwise it will use CCR5 [75]. Thus, a single amino acid substitution in the V3 loop is sufficient to acquire usage of CXCR4. However, these are rare cases when the viruses exclusively use CCR5.

Indeed, escape variants from selective pressure by natural ligand for CCR5, such as MIP-1α (CCL3) [76], or CCR5 antagonists [60], still use CCR5 and do not involve acquisition of CXCR4 usage. These studies indicate that acquisition of CXCR4 usage conferred by mutations in the V3 loop of gp120 results in the loss of replication fitness, as previously described [77]. However, the escape variants from CCR5 antagonists usually retain CCR5 usage [60,61,69,78], and recognize the antagonist-bound form of CCR5 as well as the free CCR5 form for entry by the accumulation of multiple amino acid mutations, called non-competitive resistance [61,79]. In non-competitive resistance, once saturating concentrations of antagonists were achieved, further inhibition was not observed, resulting in the plateau of inhibition, while competitive resistance can achieve inhibition of viral replication by a sufficient inhibitor concentration, resulting in a shift in the IC_{50} value (Figure 4). A principal determinant for the reduced sensitivity to CCR5 antagonists has been shown to be the V3 loop of gp120 although the mutations appear to be isolate-specific and antagonist-dependent [33].

In general, primary R5 viruses or laboratory-adapted R5 infectious clones cultured in stimulated peripheral mononuclear cells (PBMCs) have been used for the selection of CCR5 antagonist-resistant variants. However, the use of PBMCs for virus passage is donor-dependent and labor-intensive. Additionally, the use of a single clone for selection would need long-term passage to induce resistant viruses. To overcome these problems, we constructed R5-tropic infectious clones containing a V3 loop library, HIV-1_{V3Lib}. To construct replication competent HIV-1_{V3Lib}, we chose 10 amino acid positions in the V3 loop and incorporated random combinations of the amino acid substitutions derived from 31 subtype B R5 viruses into the V3 loop library (Figure 5). This novel *in vitro* system enabled the selection of escape variants from CCR5 antagonists over a relatively short time period.

In addition to the V3 library, we are currently using PM1/CCR5 cells for virus passages. The PM1/CCR5 cell line was generated by standard retrovirus-mediated transduction of parental PM cell line with the CCR5 gene, as previously described [63,76], and is highly sensitive to the R5 viruses compared to the parental PM1 cell line. Remarkably, the infection of PM1/CCR5 cells with R5 viruses induces prominent cell fusion, which is clear sign of virus proliferation. Thus, the use of PM1/CCR5 cells with the HIV-1_{V3Lib} allows us to focus on the contribution of the V3 loop in gp120 in CCR5 antagonist-resistance with a shortened selection period compared to the use of PBMCs with wild-type virus. As expected, we were able to isolate TAK-779- [63] and MVC-resistant [62] variants using replication competent HIV-1_{V3Lib}. Indeed, TAK-779- and MVC-resistant variants were determined to contain several amino acid substitutions within the V3 loop sequence. However, MVC-resistant variants also contained several amino acid substitutions in non-V3 regions (T199K and T275M), such as elsewhere in the gp120 to retain infectivity [80,81]. However, these mutations could not confer non-competitive resistance, indicating the importance of the V3 loop for non-competitive resistance.

Mechanisms of resistance: It is thought that docking of gp120 to CCR5 without CCR5 antagonists involves interactions of both the V3 tip with the second extracellular loop of CCR5 (ECL2) and the V3 stem-C4 region (bridging sheet) with the CCR5 N-terminus (NT) [82]. Since small molecule inhibitors interact with the pocket formed by transmembrane helices, thereby inducing allosteric conformational change in the ECL2, the wild-type virus can no longer interact with the ECL2. It is assumed that binding of small molecule inhibitors alters orientation between the ECL2 and NT regions, disrupting multipoint binding sites for gp120, thereby impeding gp120-CCR5 interaction [83]. Indeed, studies using CCR5 mutants showed that the escape variants were more dependent on tyrosine-sulfated CCR5 NT than wild-type viruses [65,66,84]. Furthermore, these escape variants were more sensitive to monoclonal antibodies recognizing the NT portion of CCR5 [65]. These studies indicated that the escape variants from CCR5 antagonists showed enhanced interactions with the NT that may be a consequence of a weakened interaction with the ECL2 (Figure 6).

Another genetic pathway is independent of V3 mutations. Vicriviroc-resistant mutants have been developed with multiple amino acid substitutions throughout the gp120 spanning the C2-V5 region without any changes in the V3 loop [69]. Recently, three amino acid changes in the fusion peptide domain of gp41 have been shown to be responsible for resistance although the effect of these mutations was

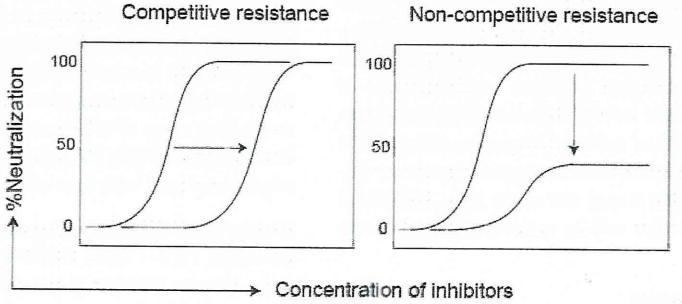

Figure 4: Typical competitive and non-competitive resistance profiles. Competitive resistance can achieve inhibition of viral replication by a sufficient inhibitor concentration, resulting in a shift in the IC50 value (left panel). In non-competitive inhibition, increasing concentrations of inhibitors have no effect, resulting in no increase in the inhibitory effect (right panel).

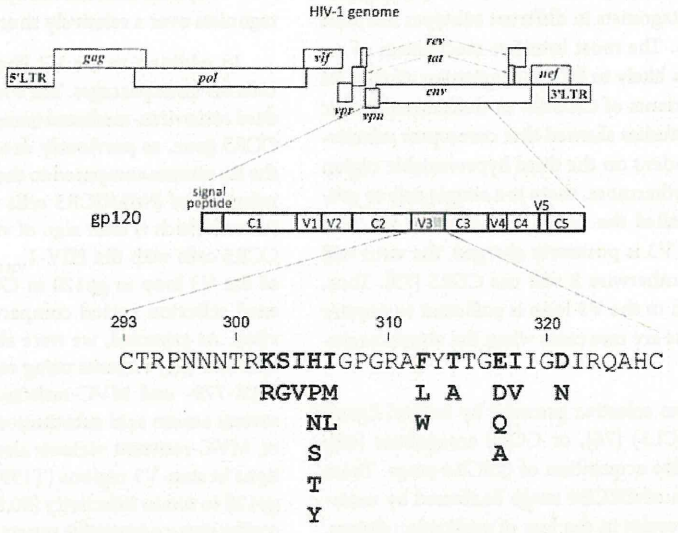

Figure 5: Schematic structure of HIV-1 V3 loop library showing introduced mutations in V3 for the analysis of escape mutants. Residues in boldface indicate the substitutions that were randomly incorporated in the V3 loop, possible >2 x 104 combinations. The amino acid substitutions were detected in 31 R5 clinical isolates.

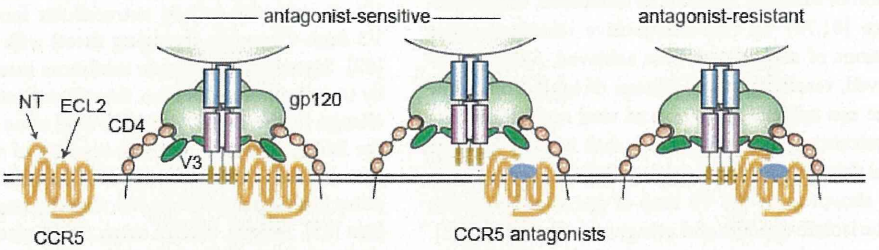

Figure 6: Resistant HIV-1 viruses can enter host cells in the presence of the CCR5 antagonist. The successful viral fusion requires the interaction of the V3 loop in gp120 with the ECL2 and NT of CCR5. CCR5 antagonists bind to the pocket formed by TM helices and induce allosteric conformational changes in the ECL2, thereby disrupting the interaction of gp120 with CCR5. The CCR5 antagonists-resistant viruses containing multiple amino acid substitutions in the V3 loop can recognize antagonist-bound forms of CCR5 by enhanced interaction with the NT.

context-dependent [84,85]. Thus, the mechanisms by which changes in the fusion peptide alter the gp120-CCR5 interaction still remain to be determined.

As previously mentioned, the patterns of mutations in escape variants against CCR5 antagonists were hypervariable and context-dependent, due in part to extensive sequence heterogeneity of HIV-1 *env*. Resistance to CCR5 antagonists was also found to be dependent upon cellular conditions such as cell tropism and the availability of CCR5. The differential staining of CCR5-expressing cells by various CCR5 monoclonal antibodies suggested that CCR5 exists in heterogeneous forms [86] and compositions of these multiple forms differed in cell type [87]. These findings suggested that different conformations of CCR5 with CCR5 antagonists might induce different substitutions in gp120. Moreover, the development of cross-resistance to other CCR5 antagonists is inconsistent, where some studies suggest that it may occur [69,78,79] and some suggest that it may not occur [61]. Additional data from *in vitro* and *in vivo* studies will be needed to elucidate the meaning of these studies.

Resistance to CXCR4 antagonists

CXCR4 as a target: CXCR4 is a coreceptor that is used for entry by X4-tropic viruses [88]; however, it is not always regarded as a suitable therapeutic target molecule for HIV-1 infection (Figure 1). R5 and X4 HIV-1 variants are both present in transmissible body fluids; however, R5-tropic HIV-1 transmits infection and dominates the early stages of HIV-1 pathogenesis [89], whereas X4-tropic HIV-1 evolves during the later stages and leads to acceleration of disease progression due to faster decline in $CD4^+$ T lymphocytes [90,91]. Coreceptor switching from CCR5 to CXCR4 occurs in approximately 40–50% of infected individuals [92]; in addition, the R5 virus is still present as a minor viral population even after emergence of the X4 virus. Furthermore, CXCR4 deletion in mice was shown to induce a variety of severe disorders and resulted in embryonic lethality [93], suggesting that CXCR4-targeting drugs may be less well tolerated than CCR5 inhibitors. These studies indicate that administration of CXCR4 inhibitors is relatively restricted to the later stage of infection after coreceptor switching. Therefore, the development of CXCR4 antagonists has proceeded at a deliberate pace when compared with that of other types of entry inhibitors.

Escape from CXCR4 antagonists: Based on the manner of escape of R5-tropic HIV-1 from CCR5 antagonists, four main resistance pathways may be intuitively possible for X4 HIV-1 escape from CXCR4 antagonists: (i) coreceptor switching from CXCR4 to CCR5; (ii) outgrowth of the pre-existing R5 virus; (iii) decrease in CXCR4 susceptibility by mutation(s) in Env; and (iv) utilization of the drug-bound

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

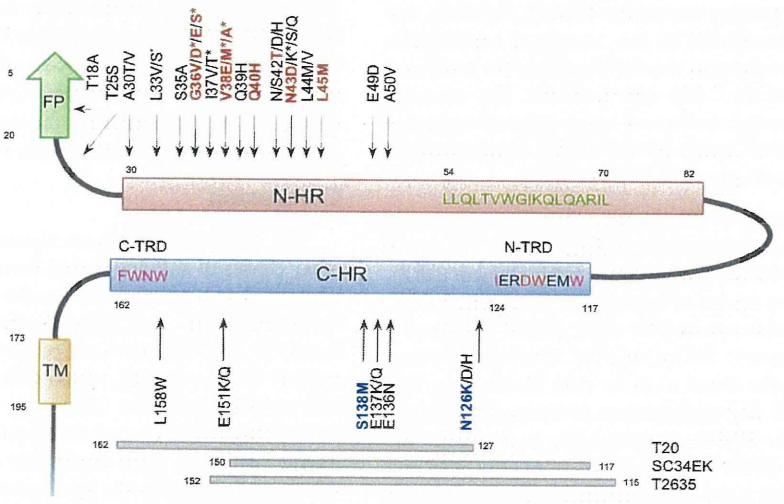

Figure 7: Schematic view of HIV-1 gp41 functional domains and mutation map for T-20. Putative hydrophobic pocket region of the N-HR is shown (green) and may form a leucine-zipper-like domain. In the C-HR, two tryptophan-rich domains (TRD; pink) are located at the N- and C-terminal regions (N-TRD and 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.

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ヒトに感染が疑われているレトロウイルスとウイルス安全性

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Xenotropic Murine Leukemia Virus-related Virus and Viral Safety

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1. はじめに

エイズ患者がはじめて報告されたのは1981年である．不幸な血液製剤汚染の例をあげるまでもなく，生物製剤におけるウイルス安全性の問題は常に問われ続けている大きな課題のひとつである．ここ30年を振り返ってもエイズウイルスの他に新規のウイルス感染症が相次いで報告された．SARSウイルス，ニパウイルス，ウェストナイルウイルスなどは大きな社会問題になった．SARS，ニパウイルスは，今後感染者を出す可能はあるものの，幸いなことにこれらはすばやい対応によって局所的な流行で封じ込められてきている．一方感染者の一部に脳炎を発症させるウェストナイルウイルスは，アフリカ北東部から既に北米に侵入し，2010年には全米で981人の感染者が確認されており，メキシコにも広がりつつある．このウイルスは野鳥に感染して運ばれ，蚊によって感染が広がる．こうした人獣新興感染症は感染が広がると，輸血，臓器移植，血液製剤を含めた生物製剤のウイルス安全性に脅威となるので常に監視をしていく必要がある．

最近ある種のレトロウイルスが前立腺癌[1]や慢性疲労症候群[2,3]の患者群で高率に感染しているという報告が米国であった．しかも健常人でもその3.7%にウイルス感染が見つかったという報告[2]があり，議論を呼んでいる．だが，その後ウイルス感染が確認できないとする否定的な報告が相次いだ[4~8]．ヒトの疾患とウイルスの因果関係を即断するには，注意深さも必要だ．過去にはウイルスとの関係を疑われるものの，その後確認することができず，結局否定された事例が過去にいくつもあるからである[9]．

2. レトロウイルス感染の報告

2.1 前立腺癌患者でのレトロウイルス感染報告

ことの始まりは，前立腺癌発症に関係する遺伝子を探索する過程で見つかったRNase L遺伝子である[1,10]．この遺伝子を調べてみると，興味深いことにRNase Lの462番目のアミノ酸は通常アルギニンだが，これがグルタミンに変わっている変異R462Qをもっている人がいることがわかった．RNase Lというのは，RNAの分解酵素のひとつである．ウイルス感染によってインターフェロンtype Iの分泌を介して，RNase Lが活性化され，外界からのRNAを分解し，感染を防ぐ．ちなみにこの抗ウイルスシステムを担うRNase Lの遺伝子をノックアウトしたマウスでは，ピコルナウイルス，コクサッキーウイルスB4などに感染しやすくなることが確かめられている．

常染色体上の遺伝子は通常母方と父方から一つずつ受け継いでいる．R462Qの変異RNase Lを持つ人の前立腺癌の発症率を見ていくと，この変異遺伝子を一つ持った人（RQ）は前立腺癌の発症率が，1.5倍にあがり，二つ持った人（QQ）では2倍にあがる．実際，この変異のためにRNase Lの活性は本来の1/3にまで低下して

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しまう．そのため変異RNase Lを二つもっている人は，病原体に対する防御機能が低下していると考えられる．そこでQQの人で，前立腺癌を発症した患者の癌細胞にウイルスが感染しているかどうかを調べたところ，患者の40%からマウス白血病ウイルスによく似た未知のレトロウイルスが検出された．そのウイルスは，異種指向性マウス白血病ウイルス関連レトロウイルス（XMRV）と名付けられた．その塩基配列は従来までよく知られているXMLV系統のものとは違うが，極めて近い．もちろんRNase活性の低下によって感染しやすくなった結果，ウイルスが見つかっただけかもしれず，癌発症との因果関係を議論するには十分ではない．不思議な点はXMRVが癌細胞そのものではなく，周辺のストローマ細胞に感染している点である[1]．その後別の研究グループが，ウイルスはストローマ細胞よりむしろ末期の前立腺癌細胞に感染しており，RNase Lの変異とウイルスの感染率は関係がないと報告している[10]．またヨーロッパの他のグループは，前立腺癌を同様に調べたがRNase Lの変異の有無に関わらず，ウイルスの感染を検出することはできなかった[4,11]．XMRV感染者は北米にのみ存在しているのか，ウイルスはストローマ細胞にのみ感染し，癌細胞には感染していないのか，R462Q変異は発癌過程と関係しているのかといった点も含め，まだ不明な点が多い．

2.2 慢性疲労症候群患者でのレトロウイルス感染の報告

慢性疲労症候群とは，強度の疲労が長期にわたって続く原因不明の疾患である．Lombardi *et al.*[2]は，慢性疲労症候群の患者で67%，健常人では3.7%の血液サンプルからXMRVを核酸増幅法によって容易に検出することができたと報告した．おどろいたことに感染細胞からは，ウイルス抗原が容易にみつかり，ウイルスが分離された．ウイルスは血中に比較的高いコピー数で存在し，末梢血中のリンパ球に感染が確認された．感染者のリンパ球を培養すると実際に調べた5人の患者すべての細胞でウイルスのエンベロープ抗原が確認されている．もしこれが本当に特異的なウイルス抗原をみているとすると，XMRVはほかのどんなレトロウイルスよりも末梢リンパ球に高い感染率を示していることになる．ところが，この報告に続く複数の独立した研究グループからは，慢性疲労症候群の患者でXMRV感染の証拠を見つけることはできないという報告が相次いだ[5~8]．

昨年の12月に慢性疲労症候群の患者でXMRVとは異なるが同じマウスレトロウイルスの一種であるMLV関連ウイルス（MLVに近縁のウイルス）が高率でみつかり，感染と発症の間に関係があるのではないかと報告された[3]．従来まで慢性疲労症候群の発症は，ウイルス感染のほか多様な原因で起きるのではないかと考えられていた．そのため患者でのウイルス感染の二つの報告は驚きとともに受け止められた．もし慢性疲労症候群とレトロウイルス感染とのあいだになんらかの関係があることが裏づけられれば，極めて重要な発見となるのはいうまでもない．慢性疲労症候群がレトロウイルス感染によって引き起こされるとするなら，抗ウイルス剤による有効な治療が可能になるからである．

まとめるとこうなる．現在ヒトに感染が疑われているウイルスはマウスのレトロウイルスによく似ており，2種類が報告されている．一つは，XMRV（異種指向性マウス白血病ウイルス関連ウイルス）もう一つはMLV関連ウイルスである．前者のXMRVは，前立腺癌と慢性疲労症候群の発症との関係が疑われている．このウイルスは，マウス白血病ウイルスに似てはいるがマウス細胞にもはや感染できない．おそらく進化の過程で，げっ歯類から異なる宿主に感染したものと考えられる．異種指向性とは，ヒト細胞などの異種の細胞に感染することができるのに，マウスの細胞にはもはや感染できなくなっているウイルスをいう[12,13]．テナガザルやコアラで見つかっているレトロウイルスは，南東アジアのげっ歯類のウイルスが，進化のある時点でテナガザルやコアラに感染したものと考えられている[12,14]．だからといって，今回報告されたXMRVがごく最近マウスからヒトに人間に感染したとは考えにくい[15]．また後者のMLV関連ウイルスは，いまのところ慢性疲労症候群患者で高率に感染していると報告されており，マウスゲノムに見いだされる内在性レトロウイルスとごく近縁のウイルスである．

3. ウイルス検出の真偽

3.1 近縁ウイルスによる実験室の汚染

今回問題になっているレトロウイルスは，生命科学系の実験室では非常に身近なマウスのレトロウイルスの近縁種である．あまりに身近なために，実験室で用いられる細胞や試薬は，レトロウイルスの核酸で汚染されているといってよいくらいなのである．そのため核酸増幅検査では，既知のマウスレトロウイルスの核酸の混入に十分注意する必要がある．多くのヒト癌細胞株は，ヌードマウスに移植片として移植されたのちに樹立されたという経緯がある．そのため，マウスに移植されたときにマウス個体内で移植細胞への感染がおきた可能性がある[15]．また実験室内で感染細胞株から感染していないヒトの細胞株に，ウイルスが水平感染を起こすことも考えられる．

通常ウイルスを扱わない実験室では厳密にウイルスを封じ込めることはないので，ウイルス感染が知らぬ間にひろがっている可能性は十分ある．

またレトロウイルス汚染 DNA は，実験試薬にも含まれている．モノクローナル抗体を産生するマウスハイブリドーマの 50%が異種指向性のマウスのレトロウイルスを分泌していたという報告もある[15]．しかも核酸増幅試薬のなかには，低温でのポリメラーゼ活性をブロックするためにマウスモノクローナル抗体が，核酸増幅試薬に含まれているものがある[16]．つまりウイルス核酸の有無を調べる際に用いられる試薬が，レトロウイルス DNA で汚染されている可能性があることになる．実際こうした核酸増幅試薬を使い，鋳型をいれずに，核酸増幅を行うと，バンドが検出できるという報告もある[16]．したがって，マウスレトロウイルス近縁のウイルス検出を実験室で行う場合は細心の注意を払わなくてはならない．

似たようなことが過去に何度も起きている．1972 年には，ある小児横紋筋肉腫に由来するヒト細胞株で新規のレトロウイルスが発見され，最初のヒト由来の RNA 腫瘍ウイルスとして脚光を浴びた．ところがこのウイルスは，のちにネコ由来の異種指向性のレトロウイルスであることがわかったのである．じつは問題の細胞株は樹立される前に一時的に猫の脳内で異種移植片として継代されており，ウイルスはこの細胞が移植片として継代されていたときに宿主であるネコから感染したものだった[13]．

3.2 マウスレトロウイルス核酸の混入の可能性

前立腺癌，慢性疲労症候群患者からのレトロウイルスが，マウスレトロウイルスの核酸の混入によるものではないかという疑いが出てきた．その一つは，XMRV の検出には，XMRV でのみ 24 塩基欠損している gag の leader 配列をターゲットにするプライマーが使われてきたが，この欠損は必ずしも XMRV に特異的ではないことがわかったことだ．Hue ら[17] によると，欠損領域を標的としたプライマーを使って一般的に実験室で使用される 12 種類の野生由来近交系マウス DNA から内在性の MLV を容易に増幅することができた．同様にヒトの 411 株のがん細胞のうち 5 株から MLV の配列を増幅できることも示された．つまり，もしマウス細胞株やヒト癌細胞株の DNA が検体に混入しているとその検体は擬陽性となる可能性が十分あることがわかったのである．

それでは患者から得られた XMRV の塩基配列は，実験室における近縁ウイルスとどんな関係にあるのだろう[17]．患者由来ウイルスの塩基配列をヒトの前立腺癌由

Table 1 前立腺癌，慢性疲労症候群患者での XMRV の検出

	患者	健常人	結論	文献
前立腺がん	86 人中 9 人が RT-PCR 陽性			Urisman *et al.*: *PLoS Pathog.*, 2, e25 (2006) Mar
	87 人中 1 人が RT-PCR 陽性	70 人中 1 人が陽性	否定	Fischer *et al.*: *J. Clin. Virol.*, 43, 277-283 (2008) Nov
	233 人中 14 人が PCR 陽性，54 人がウイルス抗原陽性	101 人中 2 人が PCR 陽性，4 人がウイルス抗原陽性		Schlaberg *et al.*: *Proc. Natl. Acad. Sci. USA*, 106, 16351-16356 (2009) Sep
	589 人が PCR, RT-PCR 陰性，146 人のウイルス抗原を検査し陰性	5 人全員が抗原陰性	否定	Hohn *et al.*: *Retrovirology*, 6, 92 (2009) Oct 16
	800 人弱中陽性 0		否定	Aloia *et al.*: *Cancer Res.*, 70, 10028-10033 (2010) Oct
	144 人中 32 人が PCR で陽性			Danielson *et al.*: *J. Infect. Dis.*, 202, 1470-1477 (2010) Oct
慢性疲労性症候群	101 人中 68 人が PCR 陽性	218 人中 8 人が PCR 陽性		Lombardi *et al.*: *Science*, 326, 585-589 (2009) Oct
	136 人検査し，PCR 陰性	95 人検査し，PCR 陽性	否定	Groom *et al.*: *Retrovirology*, 7, 10 (2010) Feb
	32 人検査し，RT-PCR で陰性	43 人検査し，RT-PCR 陰性	否定	van Kuppeveld *et al.*: *BMJ*, 6, c1018 (2010) Feb
	186 人検査し，PCR で陰性		否定	Erlwein *et al.*: *PLoS One*, 5, e8519 (2010) Mar
	51 人検査し，PCR 陰性	56 人検査し，PCR 陰性	否定	Switzer *et al.*: *Retrovirology*, 7, 57 (2010) Jul
	37 人中 32 人が PCR 陽性	44 人中 3 人が PCR 陽性		Lo *et al.*: *Proc. Natl. Acad. Sci. USA*, 107, 1470-1477 (2010) Sep
	151 人検査し，PCR で陰性，79 人を検査し，RT-PCR で陰性	43 人検査し，RT-PCR 陰性	否定	Barnes *et al.*: *J. Infect. Dis.*, 202, 1482-1485 (2010) Nov
	198 人検査，PCR 陰性	95 人検査し，PCR 陰性	否定	Henrich *et al.*: *J. Infect. Dis.*, 202, 1478-1481 (2010) Nov

来の 22Rv1 細胞株に感染しているウイルスの塩基配列を比較してみると，22Rv1 細胞株に感染しているウイルスに極めてよく似ていることが分かった．22Rv1 細胞株は，細胞株樹立の過程で XMRV 近縁ウイルスに感染したものと推定されている．以上から患者由来ウイルスは 22Rv1 細胞株のプロウイルスと起源を同じくする可能性でてきた．その上患者由来の XMRV は，22Rv1 細胞由来のウイルスに比べて，多様性に乏しく独立のコホートに属する複数の患者由来のウイルスと考えるには極めて不自然に思える．

国内では慢性疲労症候群の患者に関して XMRV 感染の有無を調べた報告があるが，いずれも感染に否定的な発表であった[18,19]．既に述べた状況を考慮すると，XMRV や MLV 関連ウイルス感染は，前立腺癌，慢性疲労症候群といった疾患とは関係がない可能性が高い．一部には感染ウイルスが分離されているので，すべてが近縁ウイルス核酸の混入が原因だと断言はできないが，これらウイルスの実在を疑うのに十分な証拠が蓄積されている段階であるといってよい（Table 1）．そのため米国では 150 人の慢性疲労症候群の患者検体と同数の健常人の検体を使って複数の研究機関で同じ検出法によって調べる大規模な計画が進行中である[20]．これによってある程度の最終的な結論がでるものと期待されている．

4. 血液製剤の安全性

HIV，HCV，HBV に関しては，採血された血液は核酸増幅検査によって調べられ，ウイルス汚染血液は除去される体制が既に整っている．もし新規ウイルスのヒトへの感染があれば，広範なスクリーニングを行う体制を整備することは技術上問題ないと考えられる．血漿分画製剤は原料を一部海外からの輸入に頼っているが，たとえ原料に新規レトロウイルスが含まれていたとしても，血漿分画製剤製造工程には，ウイルス不活化，除去工程があり，血漿分画製剤の安全性を脅かす可能性は低い．レトロウイルスはエンベロープをもつウイルスなので，製造工程における界面活性剤処理，熱処理などによって容易に感染性を失うことが予想されるからだ．

Table 2 新規ヒト疾患関連ウイルス検出の際の注意点

1. バックグラウンドの少ない特異的な核酸増幅用プライマーを準備する．
2. 検出する新規ウイルスが，MLV，SV40 などに近縁である場合はこれら近縁のウイルス核酸による汚染に注意する．
3. 標準となる陽性，陰性核酸増幅用サンプルとプライマーを準備し，研究機関配布してそれらを対照とする．

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Microarray analysis of responsible genes in increased growth rate in the subline of HL60 (HL60RG) cells

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ABSTRACT

HL60RG, a subline of human promyelocytic leukemia HL60 cells, has a increased growth rate than their parental cells. To gain information of the mechanisms involved in the increased growth rate of HL60RG, we performed a multiplex fluorescence in situ hybridization (M-FISH), standard cytogenetics analysis (G-banding) and genome scan using 10K SNP mapping array on both cell types. Characteristic genomic alterations in HL60RG cells were identified including uniparental disomy (UPD) of chromosome 1, and hemizygous deletion in 10p and 11p. However, no such defects were observed in HL60 cells. Changes in gene expression in HL60RG cells were determined using expression arrays (Affymetrix GeneChip, HU133A). Candidate genes associated with the rapid growth of HL60RG cells were identified. Two tumor necrosis factor receptors, *TNFRSF1B* (type II tumor necrosis factor-α receptor) and *TNFRSF8* (also known as a tumor marker CD30), which are adjacently located on chromosome 1 showed opposing changes in gene expression in HL60RG cells—over-expression of *TNFRSF8* and repression of *TNFRSF1B*. Differences in the DNA methylation status in the transcriptional regulatory regions of both genes between HL60 and HL60RG was detected by a methylation-specific PCR assay. In conclusion, alterations in chromosome and gene expression in HL60RG may be associated with increased growth rate.

1. Introduction

The HL60 cell line, derived from human acute promyelocytic leukemia, can be induced to differentiate into morphological and functionally mature granulocytes. The cell line was extensively characterized when it was established in 1977 and exhibits hallmarks typical of tumorigenic cells including aneuploidy, karyotypic heterogeneity, chromosomal aberrations and instability [1–3]. Genomic alterations include the defective *TP53* gene and the amplified *MYC* gene [2,4–7]. HL60RG, the sub-cell-line of the HL60 cells, was isolated after continuous culturing of HL60 for long periods. These cells exhibit a increased growth rate with doubling time around 20 h, which is approximately half that of HL60 cells. The differentiation potential characteristics of HL60 cells [8] decreased in this cell line. HL60RG can be viewed more as a progressive type tumor cell line compared with the HL60 parental cell line. The goal of this study was to investigate the mechanism underlying increased growth rate in HL60RG which may contribute to malignant progression of leukemia.

In an earlier study, we compared the two cell lines using various cytogenetic methods including G-banding, metaphase comparative genome hybridization (CGH) and array CGH. A comprehensive set of analyses revealed chromosomal alterations in these cell lines. In this study, we carried out G-banding, M-FISH and a genome-wide scan using Affymetrix 10K SNP mapping array. The SNP array methodology allowed simultaneous measurement of both DNA copy number and allelic ratios in samples being investigated [9,10]. Loss of heterozygosity (LOH) without changes in chromosomal dosage can also be detected by SNP array. Therefore, the SNP array methodology provides detailed cytogenetic information which was not possible by other methods. Furthermore, the application of 10K SNP array with an average resolution of 0.210 Mb was able to detect more detailed copy number changes compared with a conventional CGH. Changes in RNA levels with expression arrays (Affymetrix

Abbreviations: M-FISH, multiplex fluorescence in situ hybridization; CGH, comparative genome hybridization; DM, double minute; HSR, homogenously staining region; LOH, loss of heterozygosity; SNP, single nucleotide polymorphism; UPD, uniparental disomy.

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