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-IgG2; 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-
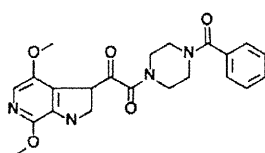
Profile of CD4-gp120 binding inhibitors

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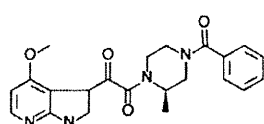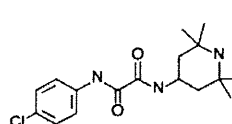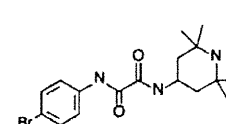
Figure 2: Profile of CD4-gp120 binding inhibitors including molecular structures of selected small molecular inhibitors.

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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 anti-

viral 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
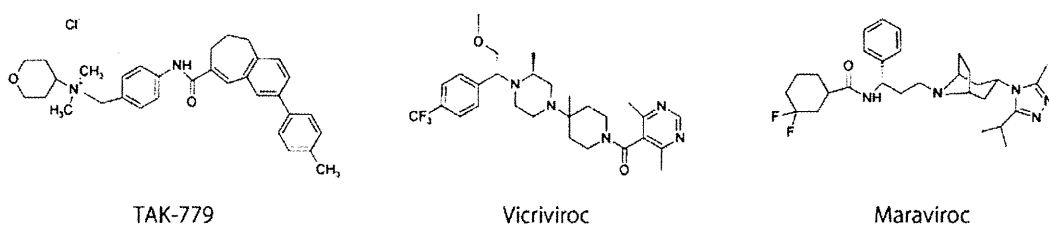

TAK-779   Vicriviroc   Maraviroc

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
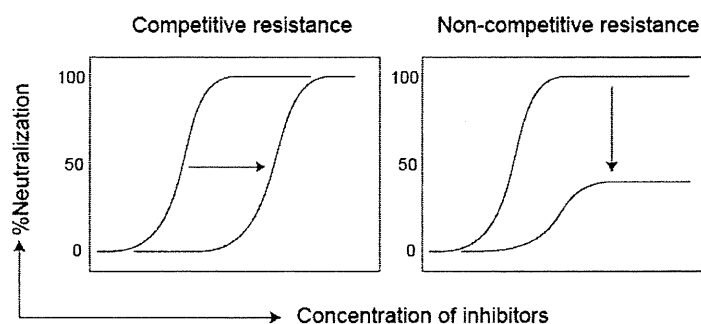
Competitive resistance   Non-competitive resistance

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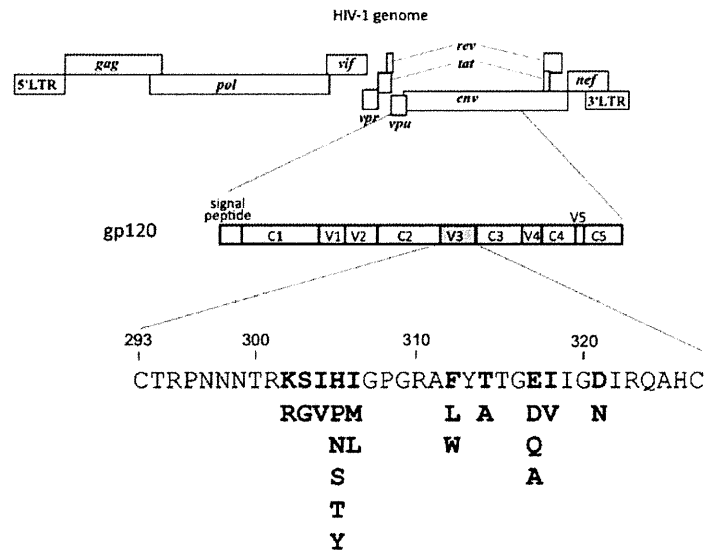
Figure 5: Schematic structure of HIV-1 V3 loop library showing introduced mutations in V3 for the analysis of escape mutants. Residues in boldface indicate the substitutions that were randomly incorporated in the V3 loop, possible >2 x 104 combinations. The amino acid substitutions were detected in 31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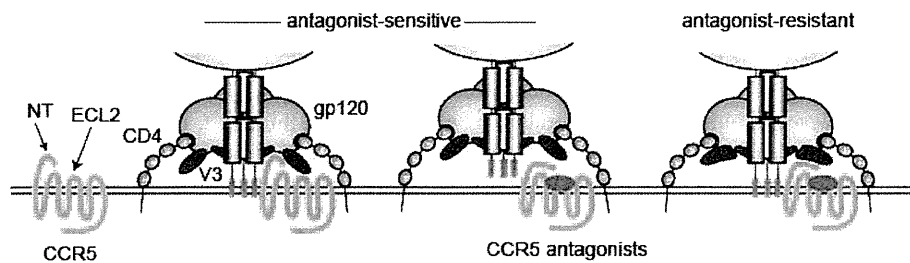

Figure 6: Resistant HIV-1 viruses can enter host cells in the presence of the CCR5 antagonist. The successful viral fusion requires the interaction of the V3 loop in gp120 with the ECL2 and NT of CCR5. CCR5 antagonists bind to the pocket formed by TM helices and induce allosteric conformational changes 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

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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 co-receptor 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
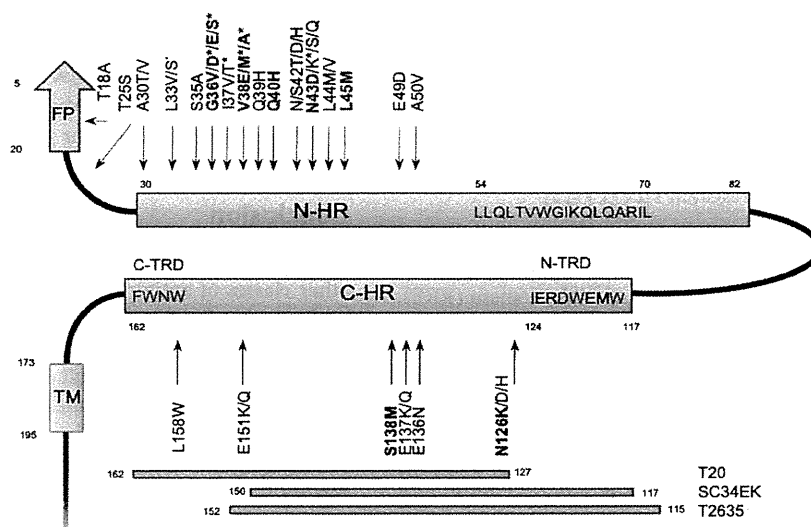

Figure 7: Schematic view of HIV-1 gp41 functional domains and mutation map for T-20. Putative hydrophobic pocket region of the N-HR is shown (green) and may form a leucine-zipper-like domain. In the C-HR, two tryptophan-rich domains (TRD; pink) are located at the N- and C-terminal regions (N-TRD and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

apy 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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INTERNAL MEDICINE

Open-Label Randomized Multicenter Selection Study of Once Daily Antiretroviral Treatment Regimen Comparing Ritonavir-Boosted Atazanavir to Efavirenz with Fixed-Dose Abacavir and Lamivudine

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Abstract

Background The side-effects of anti-retroviral drugs are different between Japanese and Caucasian patients. Severe central nerve system (CNS) side-effects to efavirenz and low rate of hypersensitivity against abacavir characterize the Japanese.

Objective The objective of this study was to select a once daily regimen for further non-inferior study comparing the virological efficacy and safety of the first line once daily antiretroviral treatment regimens in the current HIV/AIDS guideline.

Methods The study design was a randomized, open label, multicenter, selection study. One arm was treated with efavirenz and the other with ritonavir-boosted atazanavir. A fixed-dose lamivudine plus abacavir were used in both arms. The primary endpoint was virologic success (viral load less than 50 copies/mL) rate at 48 weeks. Patients were followed-up to 96 weeks with safety as the secondary endpoint. Clinicaltrials.Gov (NCT 00280969) and the University hospital Medical Information Network (UMIN000000243).

Results A total of 71 participants were enrolled. Virologic success rates in both arms were similar at week 48 [efavirenz arm 28/36 (77.8%); atazanavir arm 27/35 (77.1%)], but were decreased at week 96 to 55.6% in the efavirenz arm and 68.8% in the atazanavir arm (p=0.33). At the 96-week follow-up, 52.8% of the EFV arm and 34.3% of the ATV/r arm reached total cholesterol more than 220 mg/dL and required treatment. None of the patients developed cardiovascular complications in this study by week 96.

Conclusion There was no significant difference in the efficacy of efavirenz and ritonavir-boosted atazanavir combined with lamivudine plus abacavir at 48 weeks. The evaluation of safety was extended to 96 weeks, which also showed no significant difference in both arms.

Key words: HIV, antiretroviral treatment, efavirenz, atazanavir, abacavir, lamivudine

Introduction

The use of a non-nucleoside transcriptase inhibitor (NNRTI) or ritonavir-boosted protease inhibitor as the key drug, combined with two nucleoside reverse-transcriptase inhibitors (NRTI), as the backbone drugs, is recommended as an initial therapy in human immunodeficiency virus type 1 (HIV-1) infection. For the key drug, when efavirenz (EFV) or ritonavir-boosted atazanavir (ATV/r) is selected, once daily therapy is possible. EFV is a widely used NNRTI, however, in some clinical studies conducted in Asia, a higher rate of adverse events, especially central nervous system-related symptoms, has been noted (1-3).

In terms of backbone drugs, didanosine (ddI), stavudine (d4T) and zidovudine (ZDV) were widely used NRTIs.

However, their mitochondrial toxicity made long-term use difficult (4-7). Due to HLA-B*5701-related hypersensitivity, abacavir (ABC) is listed as the second line drug under the United States Department of Health and Human Services (DHHS) guidelines. However, HLA-B*5701 is quite rare among Japanese, and thus the incidence of hypersensitivity to ABC in Japanese patients is lower than that of Caucasians (8-10). Although tenofovir (TDF) is widely used as the first line drug, the dose-dependent nephrotoxicity is a major concern in Japanese because Japanese body weight is lighter than that of Caucasians (11, 12).

The present study was designed in 2006, when the combination of TDF, lamivudine (3TC) or entiricitabine (FTC), and EFV was the first line regimen of antiretroviral treatment (13). To explore the optimal antiretroviral combination for the best clinical outcome among Japanese HIV-1 patients (14), a selection study was designed to compare the efficacy and safety of once daily treatment with EFV or ATV/r combined with a fixed-dose ABC and 3TC (ABC/3TC).

Objective

The objective of this study was to select a once daily regimen for further non-inferior study comparing the virological efficacy and safety of the first line once daily antiretroviral treatment regimens in the current HIV/AIDS guideline.

Subjects and Methods

Study design

The study was designed as a randomized, open label, multicenter selection study, which means the superior regimen at the end point is to be selected as alternate arm to compare with the current first line regimen in the next step. Therefore, this study was not to compare superiority or non-inferiority of both arms. As the selection study, the main objective is to select a treatment regimen for further pivotal study and the secondary objective is safety. The primary endpoint was the proportion of patients in each arm who achieved virologic success (HIV-1 RNA less than 50 copies/mL in plasma) at week 48. The secondary endpoints were death, AIDS and serious non-AIDS events, non-AIDS defining cancer, treatment-related serious or grade 3 to 4 adverse events, and discontinuation of antiretroviral treatment before week 96.

The inclusion criteria of this study were those who were treatment-naïve, HIV-1 positive Japanese men with a CD4+ count ranging from 100 to 300 cell/mm^3. The exclusion criteria included current active AIDS, acute retroviral syndrome and persistent active hepatitis B infection (HBs-Ag positive). Patients with a history of 3TC treatment for hepatitis B infection were also excluded. After obtaining informed consent, eligible participants were randomized into once daily

600 mg EFV or 100 mg RTV and 300 mg ATV (EFV arm vs ATV/r arm). All participants received a fixed dose of 600 mg of ABC and 300 mg 3TC (ABC/3TC).

At baseline, the demographic characteristics and a complete medical history were recorded, physical examination was performed, and various laboratory tests were obtained (CD4+ count, HIV-1 RNA, complete blood count, biochemistry, liver and renal function tests, and total cholesterol). Participants were examined at baseline, then every 4 weeks until week 96. Careful clinical examination was provided at each visit, including history taking of any adverse event, adherence to treatment, and physical examination. Furthermore, blood tests were obtained including complete blood count, biochemistry, liver and renal function tests, CD4+ count and HIV-1 RNA. When HIV-1 RNA became less than 50 copies/mL, participants were rescheduled to be seen every 4 to 12 weeks. All participants underwent clinical examination at week 48 as the primary endpoint, then every 12 weeks until week 96 as the secondary follow-up period for evaluation of safety.

The study recruitment period was started on September 1st of 2005 for 2 years. The study protocol was originally designed to follow patients for 48 weeks, however, during the study period, cardiovascular adverse events of ABC-containing regimen were reported (15, 16). Considering the importance of adherence to safety, the follow-up period was extended to 96 weeks.

Independent data and safety monitoring board reviewed virology and safety data by treatment allocation were obtained when all participants had completed 24 weeks of the study. A total of 18 academic medical institutions in Japan participated in this study. The study protocol was approved by the ethics committee of each site and was registered at Clinicaltrials. Gov (NCT00280969) and the University Hospital Medical Information Network (UMIN000000243).

Statistical analyses

The estimated proportion of virologic failure, representing HIV-1 RNA of more than 50 copies/mL at 48 weeks of treatment, was 30% over one year. To choose one treatment group with a probability of 0.90, if it is superior to another treatment by >10%, if any, a sample size of 40 participants per group was necessary according to the selection design (17).

To assess differences in proportions, we used Fisher's exact test and calculated exact confidence intervals (CIs). We conducted intent-to-treat analysis and used the T test to compare the efavirenz arm and the ritonavir boosted atazanavir arm, unless the data showed skewed distribution, in which case the Wilcoxon's test was used. All analyses used a two-sided alfa of 0.05. No adjustment for each test was made for multiple comparisons due to the fact that we have several tests to compare the efficacies and safeties of two groups. All analyses, unless otherwise specified, were determined a priori and were hypothesis driven. Statistical analyses were performed using SAS version 9.1.

Table 1. Baseline Characteristics of Participants

Variable	efavirenz	atazanavir/r	p
Number of patients	36	35	NS
Age (yrs) median	35	36	NS
HIV-RNA (log_{10} copies/mL)			
median	4.6	4.4	NS
range	2.8–5.4	3.0–5.3	
CD4 count (cells/mm³)			
median	220	226	NS
range	121–323	103–324	
Total Cholesterol (mg/dL)			
median	155.5	159.5	NS
range	122–208	112–215	
Total bilirubin (mg/dL)			
median	0.6	0.5	NS
range	0.3–1.7	0.3–1.5	
ALT (IU/L)			
median	24	20	NS
range	8–71	8–78	
Creatinine (mg/dL)			
median	0.80	0.75	NS
range	0.6–1.03	0.6–1.02	

Results

Participants

In the study recruitment period, 71 participants were randomly assigned to two groups (36 in EFV arm and 35 in ATV/r arm). The baseline characteristics of the subjects are listed in Table 1. Among the 71 participants, 62 (87.3%) for the primary endpoint and 58 (80.6%) for the secondary endpoint completed the study protocol. By week 96, 9 participants had withdrawn due to clinical events, 2 declined to continue the study for personal reasons, one died by accident and 3 were transferred to other non-participating institutions.

Primary endpoint

At week 48, by intent-to-treat, missing-equals-failure analysis, 28 of 36 participants (77.8%, 95% CI: 60.9-89.9) in the EFV arm and 27 of 35 (77.1%, 95% CI: 59.9-89.9) in the ATV/r arm achieved the goal of HIV-1 RNA less than 50 copies/mL. There was no significant difference between the two arms (p=0.95).

Virologic success over time

Figure 1 shows the intent-to-treat analysis of participants who reached virologic success. At week 96, the rates of virologic success in the EFV arm were 55.6% (20 of 36) and 68.6% (24 of 35) in the ATV/r arm (p=0.33). The number of participants with a baseline HIV-1 RNA level of more than 100,000 copies/mL was 5 in the EFV arm and 2 in the ATV/r arm. One participant in each arm withdrew from the study at week 4 due to skin rash. The rest of the participants achieved virologic success in the EFV arm (4 out of 4) and in ATV/r arm (1 out of 1).

Secondary endpoints

In the EFV arm, 7 of 36 participants did not complete the study; 5 of the 7 developed psychiatric symptoms, including suicidal idealization, insomnia and irritation, 2 developed skin rashes and the remaining 2 were lost to follow-up because they were transferred to non-affiliated hospitals. In the ATV/r arm, 6 of 35 patients could not complete the study; one died by accident for unknown reason (the cause of death according to the coroner's report was not related to the cardiovascular system), 2 participants required treatment change (this was due to suicidal idealization in one and to skin rash in the other), one participant withdrew by own wish, one enrolled into another study, and one was transferred to another non-affiliated medical care facility.

Figure 2 shows the change of total cholesterol, liver function and total bilirubin from the baseline. At enrollment in the study, the median total cholesterol in the EFV arm was 155.5 mg/dL (range: 122-208) and in the ATV/r arm was 159.5 mg/dL (range: 112-215). The total cholesterol was not more than 220 mg/dL in any of the participants of both arms at baseline, and there was no significant difference between the two arms. During the study period, the total cholesterol increased to more than 220 mg/dL and required treatment with hypolipidemic agents in 52.8% of the EFV arm and 34.3% of the ATV/r arm. There was a significant increase in total cholesterol from the baseline in both arms (p < 0.05). There was no significant change in liver function tests during the study. New onset grade 3 hyperbilirubinemia was noted in 27 of 35 (77.1%) of the ATV/r arm but in none of the EFV arm. None of the hyperbilirubinemia in the ATV/r arm was associated with altered liver function, altered renal function, nephrolithiasis, or cholelithiasis.

Discussion

This study was designed as selection study, which means the superior regimen at the endpoint is to be selected as an alternate arm to compare with the current first line treatment in the next step. By definition of the selection study, the superior arm does not require statistical significance (17). At week 48, 77.8% of ATV/r arm and 77.1% of EFV arm reached HIV-VL of less than 50 copies/mL. Based on the definition of the selection study, the combination ABC/3TC/EFV was selected to compare the current first line treatment while the efficacy of each arm was almost even in this study.

In this clinical trial of 71 participants over a period of 96 weeks, no cardiovascular events or severe hypersensitivity reaction against ABC was observed. In this study, the efficacy of EFV combined with ABC/3TC and ATV/r combined with ABC/3TC was similar. Therefore, ABC based regimen can be selected as a safe combination to compare the efficacy of the first line combinations, such as EFV plus TDF/FTC or ATV/r plus TDF/FTC (18-20), in the next step for the best clinical benefits in Japanese patients.
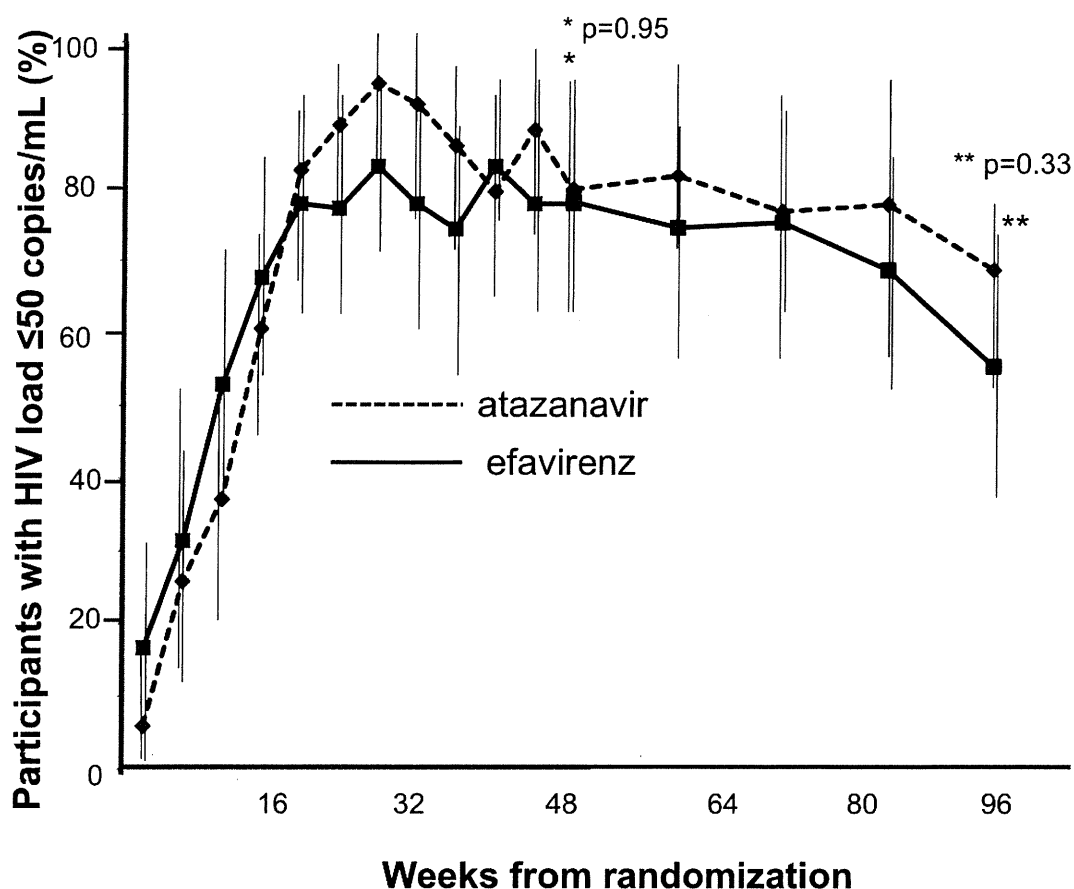
Figure 1.   Proportions of participants with HIV-RNA less than 50 copies/mL.
The efficacies of the efavirenz arm and ritonavir-boosted atazanavir arm were compared with in-
tent-to-treat analysis. There were no significant difference between arms at both week 48 (p=0.95)
and week 96 (p=0.33).

In February 2008, the United States National Institution of Allergy and Infectious Disease announced that the data and safety monitoring board of ACTG 5202 recommended a modification of the study design because they found that among participants with high viral loads (100,000 or more copies/mL) at the time of screening, treatment combinations that included ABC/3TC were not as effective in controlling the virus as those of regimens containing TDF/ FTC (19, 21). At that point, all of the present 71 participants were already enrolled in the study and the baseline HIV-1 RNA of 7 participants was more than 100,000 copies/ mL. Of these 7 participants, 2 had already withdrawn from the study by week 4, and the rest of participants had reached HIV-1 RNA of less than 50 copies/mL. The safety monitor board made no recommendation to amend the protocol.

As a primary endpoint, 77.8% of the EFV arm and 77.1% of the ATV/r had reached virological success, however, total cholesterol in 58.1% of the EFV arm and 46.9% of the ATV/r arm increased to more than 220 mg/dL, which required treatment. Thus, the overall proportion of participants with good viral suppression and without severe adverse events or treatment modification was 39.6% for the EFV arm and 62.3% for the ATV/r arm. Considering the reasons

for treatment modification, the neuro-psychiatric side effects required a regimen change in the EFV arm. Although several studies concluded that the neuro-psychiatric side effects are transient in nature, one study reported that treatment had to be changed in 16% of patients on EFV due to neuro-psychiatric side effects (22-24). Although there was no significant difference even with the small sample size, 5 out of 36 (13.9%) participants on EFV in our study required treatment change, compared with only 1 out of 35 (2.9%) of the ATV/r arm. This aspect of our study was similar to that reported in the Euro SIDA study (24). In the Swiss Cohort study, the treatment-limiting CNS adverse events was 3.8 (95% CI 2.7-5.2) per 100 person-years and it was clearly related to EFV (25). Considered together, these results emphasize the need for close observation of patients treated with EFV.

The incidence of hyperbilirubinemia in the present study was 77.1% in the ATV/r arm but none of these patients was above grade 4. Furthermore, none of the patients in this study developed liver function abnormality, altered renal function, renal stones, or cholelithiasis. As reported by Torti et al and Josephson et al, such clinical outcome can be used as a marker of adherence to ATV therapy (26, 27).

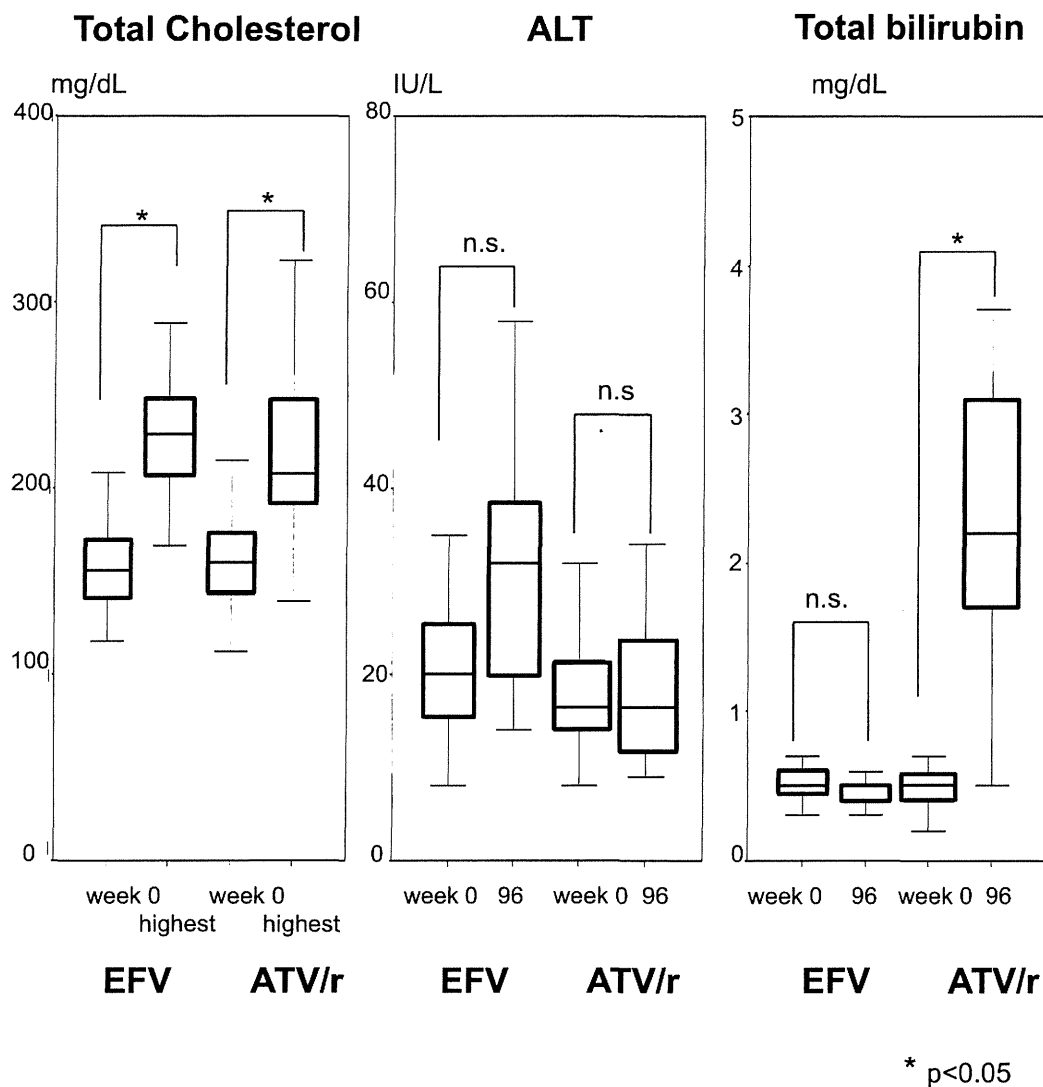Limitations of this study include a small sample size.

Figure 2.   Changes from baseline in total cholesterol, ALT and total bilirubin.
ALT and total cholesterol at week 96 were compared with the baseline values. Since participants who developed hyperlipidemia were treated with lipid-lowering agents during the study period, the highest levels registered in each participant during the follow-up were collected for analysis. There were no significant differences in total cholesterol and ALT between the two arms, while hyperbilirubinemia was significantly higher in the ATV/r arm. Modification of treatment due to hyperbilirubinemia was not required in any of the patients of the ATV/r arm. In these box-and-whisker plots, the lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively.

Considering many studies on HIV treatment held in western countries that enrolled few Asian HIV-1 patients, it is important to collect data from Asian population. The current United States Department of Health and Human Services guidelines recommend TDF/FTC as the first line regimen, while the European AIDS Clinical Society recommends 3TC and ABC addition to TDF and FTC alone (28, 29). TDF/FTC is a known potent antiretroviral agent, however, its long-term efficacy and safety remain unclear (11, 12). Considering that the combinations of NRTI are limited, the efficacy and safety of ABC in the low HLA-B*5701 population need to be evaluated for wider treatment options for HIV-1 patients (9, 10).

Conclusion

This study was designed as a selection study to compare the virologic efficacy and treatment safety of EFV and ATV/r, both with ABC/3TC, in Japanese patients. The results showed no significant differences in efficacy between the two regimens at week 48. The evaluation of safety was extended to 96 weeks, which also showed no significant difference in both arms. The results of the present study have already been applied as the basis of a follow-up study that is

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currently being conducted in Japan to compare NRTI combinations of ABC/3TC and TDF/FTC with ATV/r as key drugs.

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References

1. Lapphra K, Vanprapar N, Chearskul S, et al. Efficacy and tolerability of nevirapine-versus efavirenz-containing regimens in HIV-infected Thai Children. Int J Infect Dis 12: e33-e38, 2008.
2. Laureillard D, Prak N, Fernandez M, et al. Efavirenz replacement by immediate full-dose nevirapine is safe in HIV-1-infected patients in Cambodia. HIV Med 9: 514-518, 2008.
3. Ananworanich J, Moor Z, Siangphoe U, et al. Incidence and risk factors for rash in Thai patients randomized to regimens with nevirapine, efavirenz or both drugs. AIDS 19: 185-192, 2005.
4. Côté HC, Brumme ZL, Craib KJ, et al. Changes in mitochondrial DNA as a marker of nucleoside toxicity in HIV-infected patients. N Engl J Med 346: 811-820, 2002.
5. Chowers M, Gottesman BS, Leibovici L, Schapiro JM, Paul M. Nucleoside reverse transcriptase inhibitors in combination therapy for HIV patients: systematic review and meta-analysis. Eur J Clin Microbiol Infect Dis 29: 779-786, 2010.
6. Maggiolo F, Roat E, Pinti M, et al. Mitochondrial changes during D-drug-containing once-daily therapy in HIV-positive treatment-naive patients. Antivir Ther 15: 51-59, 2010.
7. Tanuma J, Gatanaga H, Oka S, et al. Dilated cardiomyopathy in an adult human immunodeficiency virus type 1-positive patient treated with a zidovudine-containing antiretroviral regimen. Clin Infect Dis 37: e109-e111, 2003.
8. Mallal S, Nolan D, Witt C, et al. Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir. Lancet 359: 727-732, 2002.
9. Sun HY, Hung CC, Lin PH, et al. Incidence of abacavir hypersensitivity and its relationship with HLA-B*5701 in HIV-infected patients in Taiwan. J Antimicrob Chemother 60: 599-604, 2007.
10. Park WB, Choe PG, Song KH, et al. Should HLA-B*5701 screening be performed in every ethnic group before starting abacavir? Clin Infect Dis 48: 365-367, 2009.
11. Rodriguez-Nóvoa S, Alvarez E, Labarga P, Soriano V. Renal toxicity associated with tenofovir use. Expert Opin Drug Saf 9: 545-559, 2010.
12. Wever K, van Agtmael MA, Carr A. Incomplete reversibility of tenofovir-related renal toxicity in HIV-infected men. J Acquir Immune Defic Syndr 2010 Feb 19 (Epub), DOI: 10.1097/QAI.0b013 e3181d05579
13. United States Department of Health and Human Services. DHHS Guidelines for the use of antiretroviral agents in HIV-1 infected adults and adolescents, May 2006. .
14. Gatanaga H, Tachikawa N, Kikuchi Y. Urinary beta2-microglobulin as a possible sensitive marker for renal injury caused by tenofovir disoproxil fumarate. AIDS Res Hum Retroviruses 22: 744-748, 2006.
15. Friis-Møller Nina, Reiss P, El-Sadr W, et al; D:A:D Study Group. Exposure to PI and NNRTI and risk of myocardial infarction: Results from the D:A:D Study, 13th conference on retrovirus and opportunistic infections. 2006 abstract 144.
16. Sabin CA, Worm SW, Weber R, et al; D:A:D Study Group. Use of nucleoside reverse transcriptase inhibitors and risk of myocardial infarction in HIV-infected patients enrolled in the D:A:D study: a multi-cohort collaboration. Lancet 371: 1417-1426, 2008.
17. Simon R, Wittes RE, Ellenberg SS. Randomized phase II clinical trials. Cancer Treatment Reports 69: 1375-1381, 1985.
18. Gallant JE, Rodriguez AE, Weinberg WG, et al. ESS30009 Study. Early virologic nonresponse to tenofovir, abacavir, and lamivudine in HIV-infected antiretroviral-naïve subjects. J Infect Dis 192: 1921-1930, 2005.
19. Sax PE, Tierney C, Collier AC, et al. AIDS Clinical Trials Group Study A5202 Team. Abacavir-lamivudine versus tenofovir-emtricitabine for initial HIV-1 therapy. N Engl J Med 361: 2230-2240, 2009.
20. Smith KY, Patel P, Fine D, et al. HEAT Study Team. Randomized, double-blind, placebo-matched, multicenter trial of abacavir/lamivudine or tenofovir/emtricitabine with lopinavir/ritonavir for initial HIV treatment. AIDS 23: 1547-1556, 2009.
21. BULLETIN NIAID Modifies HIV Antiretroviral treatment study combination therapy that includes ABC/3TC found less effective in subgroup of antiretroviral-naïve individuals. http://www.niaid.nih.gov/news/newsreleases/2008/Pages/actg5202bulletin.aspx (accessed on July 15 2010).
22. Clifford DB, Evans S, Yang Y, et al. Impact of efavirenz on neuropsychological performance and symptoms in HIV-infected individuals. Ann Intern Med 43: 714-721, 2005.
23. Hawkins T, Geist C, Young B, et al. Comparison of neuropsychiatric side effects in an observational cohort of efavirenz and protease inhibitor-treated patients. HIV Clin Trials 6: 187-196, 2005.
24. van Luin M, Bannister WP, Mocroft A, et al; EuroSIDA Study Group. Absence of a relation between efavirenz plasma concentrations and toxicity-driven efavirenz discontinuations in the

EuroSIDA study. Antivir Ther **14**: 75-83, 2009.

25. Elzi L, Marzolini C, Furrer H, et al; Swiss HIV Cohort Study. Treatment modification in human immunodeficiency virus-infected individuals starting combination antiretroviral therapy between 2005 and 2008. Arch Intern Med **170**: 57-65, 2010.

26. Torti C, Lapadula G, Antinori A, et al. Hyperbilirubinemia during Atazanavir Treatment in 2,404 Patients in the Italian Atazanavir Expanded Access Program and MASTER Cohorts. Infection **37**: 244-249, 2009.

27. Josephson F, Andersson MC, Flamholc L, et al. The relation between treatment outcome and efavirenz, atazanavir or lopinavir exposure in the NORTHIV trial of treatment-naïve HIV-1 infected

patients. Eur J Clin Pharmacol **66**: 349-357, 2010.

28. United States Department of Health and Human Services. DHHS Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents-December 1, 2009. http://aidsinfo.nih.gov/ contentfiles/AdultandAdolescentGL001419.pdf accessed on Jan 17 2011.

29. European AIDS Clinical Society. Guidelines of Clinical Management and Treatment of HIV infected adult in Europe. Version 5, 2009. http://www.europeanaidsclinicalsociety.org/guidelinespdf/ EACS-EuroGuidelines2009FullVersion.pdf accessed on Jan 17 2011.

PLoS one

Structural Dynamics of HIV-1 Envelope Gp120 Outer Domain with V3 Loop

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Abstract

Background: The net charge of the hypervariable V3 loop on the HIV-1 envelope gp120 outer domain plays a key role in modulating viral phenotype. However, the molecular mechanisms underlying the modulation remain poorly understood.

Methodology/Principal Findings: By combining computational and experimental approaches, we examined how V3 net charge could influence the phenotype of the gp120 interaction surface. Molecular dynamics simulations of the identical gp120 outer domain, carrying a V3 loop with net charge of +3 or +7, showed that the V3 change alone could induce global changes in fluctuation and conformation of the loops involved in binding to CD4, coreceptor and antibodies. A neutralization study using the V3 recombinant HIV-1 infectious clones showed that the virus carrying the gp120 with +3 V3, but not with +7 V3, was resistant to neutralization by anti-CD4 binding site monoclonal antibodies. An information entropy study shows that otherwise variable surface of the gp120 outer domain, such as V3 and a region around the CD4 binding loop, are less heterogeneous in the gp120 subpopulation with +3 V3.

Conclusions/Significance: These results suggest that the HIV-1 gp120 V3 loop acts as an electrostatic modulator that influences the global structure and diversity of the interaction surface of the gp120 outer domain. Our findings will provide a novel structural basis to understand how HIV-1 adjusts relative replication fitness by V3 mutations.

Introduction

The third variable (V3) element of the human immunodeficiency virus type 1 (HIV-1) envelope gp120 protein is usually composed of 35 amino acids. The element forms a protruding loop-like structure on the gp120 outer domain [1], is rich in basic amino acids, and has aromatic amino acids for the aromatic stacking interaction with proteins. The V3 loop participates in direct binding to the entry coreceptor [2] and constitutes the most critical determinant for the coreceptor use of HIV-1 [3,4,5,6]. In addition, the tip of V3 is highly immunogenic and contains neutralization epitopes for antibodies [7,8,9], although the epitopes can be inaccesible in the gp120 trimer on a virion of the HIV-1 primary isolates [10,11] or HIV-1 recombinants with less positively charged V3 [12,13]. Moreover, the V3 is reported to be the major determinant of HIV-1 sensitivity to neutralization by the soluble form of CD4 [14,15,16], a recombinant protein that binds to the cleft of the gp120 core [17]. Thus, the V3 loop plays a key role in modulating biological and immunological phenotypes of HIV-1. However, the molecular mechanisms underlying these modulations remain poorly understood.

It has been reported that the net charge of the V3 loop is tightly linked to the phenotype of HIV-1. The V3 loops of CCR5 tropic HIV-1s are usually less positively charged than those of CXCR4 tropic HIV-1s [18,19,20,21]. An increase in the V3 net charge can convert CCR5 tropic viruses into CXCR4 tropic viruses [4,22,23,24], and antibody resistant viruses into sensitive viruses [12,13]. Thus the V3 loop may be viewed as an electrostatic modulator of the structure of the gp120 interaction surface, an assumption that is largely unexamined.

Increasing evidence has indicated that the dynamics property of molecules in solution is critical for protein function and thus for many biological processes [25,26,27]. Molecular dynamic (MD) simulation is a powerful method that predicts the structural dynamics of biological molecules in solution, which is often difficult to analyze by experiments alone [28,29,30]. Recent advances in biomolecular simulation have rapidly improved the precision and application performance of this technique [28,29,30]. We have previously applied this technique to investigating the structural factors that regulate biological phenotype of viruses [13,31,32]. In this study, by combining MD simulations with antibody neutralization experiments and diversity analysis of the viral protein sequences, we studied a structural basis for the regulation of HIV-1 phenotype by V3 loop.

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Results

Molecular dynamics simulation study

To address the potential role of the V3 net charge in modulating the structure and dynamics of the gp120 surface, we performed MD simulations of the identical gp120 outer domains carrying different V3 loops with net charges of +7 or +3 (Fig. 1A). The initial structures for the simulations were constructed by homology modeling using the crystal structure of HIV-1 gp120 containing an entire V3 loop as the template. Due to the perfect identity of the outer domain sequences of the V3 recombinant gp120s, the outer domain structures of the initial models for the MD simulations were identical before the simulations. The modeling targets in this study belong to HIV-1 subtype B and had a sequence similarity of about 87.3% to the modeling template. This similarity was high enough to construct high-accuracy models with an RMSD of ~1.5 Å for the main chain between the predicted and actual structures in the tested cases with homology models and x-ray crystal structures [33]. These initial models were lacking in V1/V2 loops and glycans on the gp120. The recombinant models are therefore suitable for exploring the potency of the structural regulation that is intrinsic to the V3 loop.

Using these models as the initial structures, we analyzed the structural dynamics of the gp120 outer domains in the absence of soluble CD4 by MD simulation. It was expected that the MD simulations would eliminate initial distortions in the template crystal structure, which could be generated during crystallization, and search for the most stable structures of unliganded gp120 outer domains at 1 atm at 310 K in water. The simulations showed that the same gp120 outer domains, carrying different V3 loops with net charges of +7 or +3, exhibited marked changes in conformations and fluctuations at several functional loops at 1 atm at 310 K in water (Figs. 1 and 2).

To quantitatively monitor the overall structural dynamics of the outer domain during MD simulation, the RMSDs between the initial model and models at given times of MD simulation were measured. The RMSD sharply increased soon after heating of the initial model and then gradually reached a near plateau after 10 ns of the MD simulations (Fig. 1B). The results suggested that most of the backbone heavy atoms of the outer domain reached a thermodynamic equilibrium after 10 ns of the simulation under the conditions employed. However, fluctuations of the RMSDs were still detectable even at around 30 ns of the simulations, suggesting that some regions of the outer domains continued to fluctuate.

To map the heavily fluctuating sites in the gp120 outer domain, we calculated the RMSF of the main chains of individual amino acids during the MD simulations. The RMSFs, which provide information about the atomic fluctuations during MD simulations [34], were found to be much greater in the amino acids constituting loops than those of the structured regions, such as helixes and β-sheets (Figs. 1C and 1D). These results are consistent with the general observations of proteins in solution, and indicate that the loops of the gp120 outer domain intrinsically possess structural flexibility in water. Notably, the RMSFs in some loops were markedly different between the two V3 recombinant gp120s. For example, the RMSF in the β20–β21 loop was much greater in the Gp120$_{LAI-TH09V3}$ (Fig. 1C). Conversely, those in the D loop were greater in the Gp120$_{LAI-NH1V3}$.

HIV-1 gp120 V3 loop often has a motif for the N-linked glycosylation that is usually preferentially conserved in R5 viruses (Fig. 1A). To address potential impacts of the glycan on the MD simulations, we performed MD simulation in the presence of a high mannose oligosaccharide in the V3 loop. We observed any

significant differences in the structure and dynamics of gp120 outer domain in the presence or absence of the glycan (data not shown). This is reasonable because the glycosylation site is exposed toward an opposite direction from the gp120 core (Fig. 1D).

To clarify structural differences between the Gp120$_{LAI-NH1V3}$ and Gp120$_{LAI-TH09V3}$, we constructed their averaged structures using the 40,000 snapshots obtained from 10–30 ns of MD simulations using ptraj module in Amber 9. Superposition of the averaged structures showed that the relative configuration of the V3 loops and β20–β21 was markedly different between the two outer domains: the V3 tip protruded a greater distance from the β20–β21 loop in the Gp120$_{LAI-TH09V3}$ than in the Gp120$_{LAI-NH1V3}$ (Fig. 2A). The superposed structures also revealed differences in a region around the CD4 binding site (Fig. 2A, right panel with enlarged CD4 binding site). The relative configuration of the CD4 binding loop to the exit loop is critical for the gp120 binding to the CD4, a primary infection receptor of HIV-1 [17]. Therefore, we analyzed the distance between the CD4 binding and exit loops by measuring the distance ($D_{115-221}$) between the Cα of Gly115 and the Cα of Gly221 as an indicator (Fig. 2B). As expected from the fluctuations of the CD4 binding loop, the $D_{115-221}$ fluctuated during the MD simulations (Fig. 2C). However, the $D_{115-221}$ was significantly smaller in the Gp120$_{LAI-TH09V3}$ than in the Gp120$_{LAI-NH1V3}$ (Fig. 2D; $p < 0.001$, Student's t-test): the $D_{115-221}$ ranged from 4–15 Å with an average of ~8 Å for the Gp120$_{LAI-TH09V3}$ and from 7–17 Å with an average of ~10 Å for the Gp120$_{LAI-NH1V3}$. These data suggest that the CD4 binding loop tended to be positioned more closely to the exit loop and thus tended to be sterically less exposed in the Gp120$_{LAI-TH09V3}$ than the Gp120$_{LAI-NH1V3}$.

Neutralization study

The above structural data raised the possibility that the reduction in the V3 net charge might reduce HIV-1 neutralization sensitivity by the anti-CD4 binding site antibodies. To address this possibility, we performed a neutralization assay using the two isogenic HIV-1 recombinant viruses, HIV-1$_{LAI-NH1V3}$ and HIV-1$_{LAI-TH09V3}$ [35], which carry the Gp120$_{LAI-NH1V3}$ and Gp120$_{LAI-TH09V3}$, respectively. These viruses were pre-incubated with various human MAbs against the CD4 binding site, and the reductions in viral infectious titers were measured using a HeLa-cell-based single-round viral infectivity assay system [36].

Table 1 summarizes the results of the neutralization assay. As expected, the two viruses exhibited markedly distinct neutralization sensitivities to the three human MAbs against the CD4 binding site. HIV-1$_{LAI-NH1V3}$ was consistently neutralized with all three MAbs against the CD4 binding site (49G2, 42F6, and 0.5δ), with ND$_{50}$ values ranging between 0.224 and 0.934 μg/ml. In marked contrast, HIV-1$_{LAI-TH09V3}$ was highly resistant to neutralization by these MAbs, and 10 μg/ml of antibodies failed to block the viral infections. The two viruses were equally resistant to an anti-Gp120 antibody (4C11) that recognizes the Gp120 structure after CD4 binding. The result indicates that the CD4-induced gp120 epitope of the 4C11 are not preserved in the V3 recombinant viruses used in the present study. Conversely, they were equally sensitive to another ant-Gp120 antibody (4301 [37]) whose epitope is located outside of the CD4 binding site. A human MAb 8D11 used as a negative control had no effect on the viral infectivity in this assay.

Diversity study

Host immunity is a driving force behind the antigenic diversity of envelope proteins of the primate lentiviruses that establish persistent infection in hosts [23,38,39,40,41]. The above and