modified to sustain productive replication for a longer period. SHIV MNA in the present form does not fulfil this requirement. It is possible that animal-to-animal passage could increase the fitness of the virus in monkeys.

This study demonstrated that a CD4 mimic could modulate viral Env protein to be more susceptible to neutralization by less potent antibodies generated in the context of infection. During the early phase of infection, patients mount high titres of non-neutralizing antibodies directed against the V3 loop (Davis *et al.*, 2009a). Patients with HIV-1 clade C generate anti-Env antibodies, including anti-CD4i antibodies, with poor neutralizing activity against recent infection (Gray *et al.*, 2007). It is possible that the CD4 mimic YYA-021 causes a conformational change in SHIV MNA Env, which renders sequestered epitope(s) accessible to potentially neutralizing IgG, such as ones directed against the V3 loop and CD4i.

The current study extended the previous study by Yoshimura *et al.* (2010) and used HIV-1 MNA belonging to clade B to generate a new SHIV strain carrying Env. The neutralization sensitivity of this strain is characteristically augmented in the presence of a small-molecule CD4 mimic. Similar observations by Decker *et al.* (2005) showed that infections of a wide range of HIV-1 strains of multiple clades or circulating recombinant forms elicit high titres of anti-CD4i antibodies. These anti-CD4i antibodies neutralize viruses as divergent as HIV-2 in the presence of sCD4 (Decker *et al.*, 2005). Taking these observations into account, small-molecule CD4 mimics such as YYA-021 could potentially enhance the neutralization activity of the antibodies directed against autologous viruses belonging not only to clade B but also to multiple HIV-1 strains of various clades and possibly even HIV-2. Our results pave the way for a novel therapeutic intervention based on administration of CD4 mimics to patients with HIV to facilitate control of the virus by their own antibodies.

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See related commentary on pg 2662

Oral Administration of the CCR5 Inhibitor, Maraviroc, Blocks HIV *Ex Vivo* Infection of Langerhans Cells within the Epithelium

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TO THE EDITOR

Preexposure prophylaxis (PrEP) with oral administration of an antiretroviral is a potential method for preventing acquisition of HIV. A controlled trial in men who have sex with men (the iPrEx trial) showed that daily oral use of tenofovir disoproxil fumarate-emtricitabine (TDF-FTC; Truvada) reduced transmission rates by 44% (Grant *et al.*, 2010). In addition, the HIV Prevention Trial Network (HPTN) 052 trial recently confirmed that antiretroviral treatment leads to 96% reduction in transmission among HIV-negative heterosexual partners of HIV-positive individuals (Cohen *et al.*, 2011). Similar trials, however, with TDF-FTC (the FEM-PrEP trial) or TDF alone (the VOICE trial) were stopped because of poor outcomes (van der Straten *et al.*, 2012). Different results among various trials, which used identical antiretroviral regimens, could be explained by varying compliance with drug use and/or varying drug concentration and activity within the exposed tissue (Patterson *et al.*, 2011).

Langerhans cells (LCs) are $CCR5^+$ dendritic cells located within genital skin and mucosal epithelium (Lederman *et al.*, 2006). In female rhesus macaques exposed intravaginally to simian immunodeficiency virus, up to 90% of initially infected target cells were LCs (Hu *et al.*, 2000). *Ex vivo* experiments with human foreskin explants show that epidermal LCs are target cells for HIV, providing a likely explanation for why circumcision greatly reduces the probability of acquiring HIV (Ganor *et al.*, 2010). LCs also express CD4 and CCR5, but not CXCR4, within the tissue and demonstrate the distinctive characteristics of emigrating from tissue to draining lymph nodes in order to interact with T cells after contact with pathogens (Kawamura *et al.*, 2000). Indeed, epidermal LCs are readily infected *ex vivo* with R5 HIV, but not with X4 HIV, and promote high levels of infection upon interaction with cocultured $CD4^+$ T cells (Kawamura *et al.*, 2000; Ogawa *et al.*, 2013). Thus, LCs probably have an important role in disseminating HIV soon after exposure to virus.

Epidemiologic observations have found that the majority of HIV strains isolated from patients soon after initial infection are R5 HIV strains (i.e., they utilize CCR5; Lederman *et al.*, 2006). Not surprisingly, individuals with homozygous defects in *CCR5* are largely protected from sexually acquiring HIV (Lederman *et al.*, 2006). In addition, three different CCR5-binding topically applied compounds protected female macaques from sexually acquiring simian/human immunodeficiency virus: the N-terminally modified chemokine analog PSC-RANTES, the small-molecule inhibitor CMPD167, and maraviroc (MVC) (Lederman *et al.*, 2006; Veazey *et al.*, 2010). In addition to topical application to vaginal mucosa, oral delivery of CMPD167 protected macaques from vaginal simian/human immunodeficiency virus challenge (Veazey *et al.*, 2005). Given these data, orally administered MVC may prove to be particularly important in PrEP regimens, although its ability to prevent HIV acquisition is unknown.

In the current study, 20 healthy volunteers were randomly divided into four equal groups; they received 300 mg of MVC orally twice daily for 1, 2, 3, or 14 days. To obtain epidermal tissues, all subjects underwent suction blistering of the skin before and 2 hours after the last MVC dose. All subjects had plasma and semen collected 2 hours after their last dose. MVC concentrations in serum, semen, and epidermal tissues were determined by using the liquid chromatography– mass spectometry method, as described previously (Takahashi *et al.*, 2010). Mean concentration ± SD in the epidermis was 21.91 ± 13.80, 23.36 ± 13.28, and 31.54 ± 20.61 nM for individuals taking drug for 1, 2, or 3 days ($n = 5$ for each), respectively. MVC concentrations tended to be higher with a longer dosing period. Consistent with recent data showing high levels of MVC in genital tissue (Dumond *et al.*, 2009), these results indicate that MVC rapidly distributes into the skin at high concentrations. In addition, MVC was detected in semen of all subjects (Supplementary Figure S1 online).

To understand how HIV traverses skin and genital mucosa, an *ex vivo* model was developed whereby resident LCs within epithelial tissue explants are exposed to HIV and then allowed to emigrate from tissue, thus mimicking conditions that occur after mucosal exposure to HIV (Kawamura *et al.*, 2000; Ogawa *et al.*, 2013). In this model, although relatively few productively infected LCs are identified, these cells induce high levels of HIV infection when cocultured with resting autologous $CD4^+$ T cells (Kawamura *et al.*, 2000). In preliminary experiments, HIV infection of LCs, as well as subsequent virus transmission from emigrated LCs to cocultured $CD4^+$ T cells, was decreased in a dose-dependent manner when skin explants were pretreated with various concentrations of MVC before

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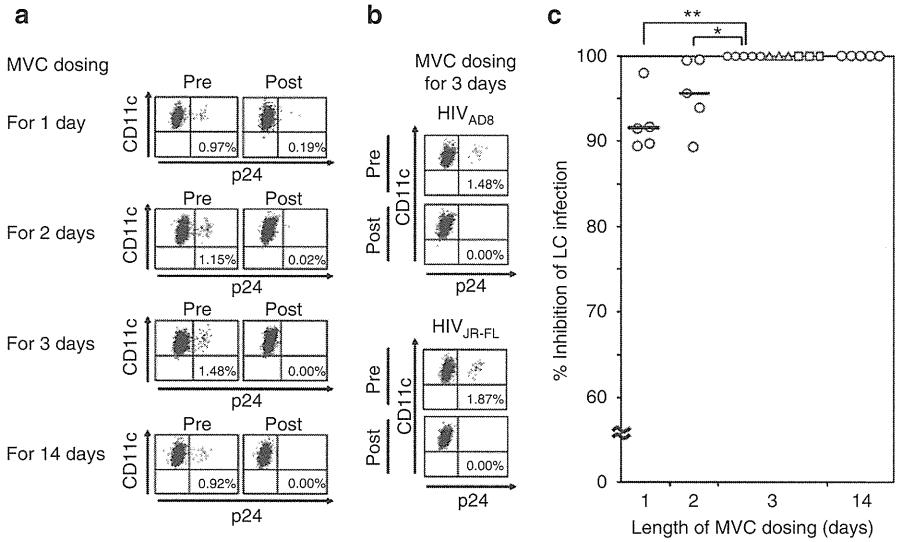

Figure 1. Oral administration of maraviroc (MVC) protects epidermal Langerhans cells (LCs) from *ex vivo* R5 HIV infection. Skin explants were isolated from healthy individuals who had received oral MVC (300 mg twice daily) for the indicated periods of time. These tissues were exposed to $HIV_{Ba\text{-}L}$ (**a**, **c**), HIV_{AD8}, or $HIV_{JR\text{-}FL}$ (**b**, **c**) and then cultured for 3 days. Emigrated LCs were collected and assessed for HIV infection by flow cytometry. Representative FACS analyses of CD11c and p24 mAb double-stained cells are shown (**a**, **b**). Percent MVC inhibition of LC infection with $HIV_{Ba\text{-}L}$ (○), HIV_{AD8} (△), or $HIV_{JR\text{-}FL}$ (□) was calculated as described in the text (**c**). $^*P<0.05$; $^{**}P<0.01$. Mean values obtained from different donors are shown as horizontal marks.

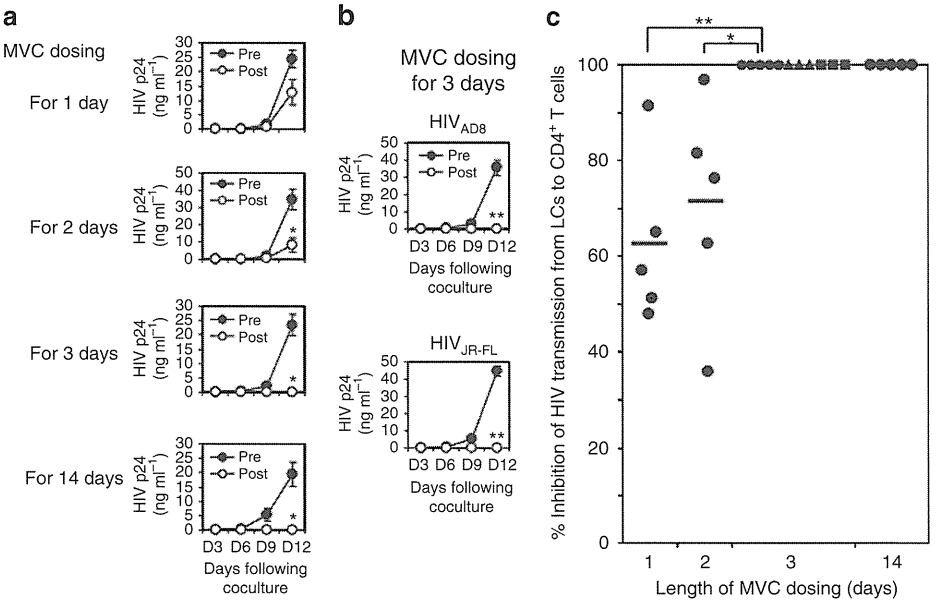

Figure 2. Oral administration of maraviroc (MVC) blocks viral transmission from HIV-exposed Langerhans cells (LCs) to cocultured $CD4^+$ T cells. Skin explants isolated from healthy individuals who had received oral MVC (300 mg twice daily) for the indicated periods of time were exposed to $HIV_{Ba\text{-}L}$ (**a**, **c**), HIV_{AD8}, or $HIV_{JR\text{-}FL}$ (**b**, **c**), as described in Figure 1. Emigrated LCs were cocultured with autologous $CD4^+$ T cells, and culture supernatants were assessed for p24 content by ELISA. Representative ELISA results are shown (**a**, **b**). Percent MVC inhibition of $HIV_{Ba\text{-}L}$ (●), HIV_{AD8} (▲), or $HIV_{JR\text{-}FL}$ (■) transmission to cocultured $CD4^+$ T cells was calculated as described in the Methods (**c**). $^*P<0.05$; $^{**}P<0.01$. Mean values obtained from different donors are shown as horizontal marks.

HIV exposure (Supplementary Figure S2 online), similar to experiments reported earlier with PSC-RANTES (Kawamura *et al.*, 2004).

Next, the epithelial tissue explants were collected from study subjects after oral treatment with MVC (Supplementary Materials and Methods online). Importantly, oral MVC pretreatment for either 1 or 2 days partially inhibited subsequent *ex vivo* $HIV_{Ba\text{-}L}$ infection of LCs within epithelial tissue, whereas MVC administration for either 3 or 14 days completely blocked LCs from *ex vivo* $HIV_{Ba\text{-}L}$ infection (Figure 1). These data demonstrate the importance of the length of MVC dosing period before HIV exposure. MVC treatment also consistently prevented $HIV_{Ba\text{-}L}$ transmission from LCs to cocultured $CD4^+$ T cells (Figure 2). Furthermore,

MVC administration for 3 days blocked *ex vivo* virus infection of LCs as well as subsequent virus transmission when different R5 HIV strains, HIV_{AD8} and HIV_{JR-FL}, were utilized for an additional six subjects ($n=3$ for each strain, Figures 1 and 2). These data demonstrate that oral administration of MVC for at least 3 days is capable of fully protecting HIV infection of LCs within epithelial tissue.

These experiments provide perhaps the best proof-of-concept test for MVC as a potential PrEP drug, as it would be unethical to expose MVC-treated volunteers to HIV *in vivo*. As proven here, orally delivered MVC rapidly distributes to skin and functionally acts to block infection of relevant target cells, LCs, supporting randomized controlled trials of MVC as a PrEP therapy for individuals at high risk of becoming infected with HIV through sexual exposure.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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JID Open

Heterozygous Mutations in *AAGAB* Cause Type 1 Punctate Palmoplantar Keratoderma with Evidence for Increased Growth Factor Signaling

Journal of Investigative Dermatology (2013) **133,** 2805–2808; doi:10.1038/jid.2013.243; published online 11 July 2013

TO THE EDITOR

Punctate palmoplantar keratoderma (punctate PPK or PPKP) is a rare autosomal dominant disorder of keratinization. Three variants of this disease have been described; PPKP1 (OMIM ♯148600, Buschke–Fischer–Brauer type) is characterized by the progressive development of discrete areas of hyperkeratosis on the palms and soles, followed by more extensive diffuse hyperkeratosis on the pressure-bearing areas of plantar skin.

Linkage analyses of families affected by PPKP1 have previously identified a

Abbreviations: AAGAB, gene encoding alpha- and gamma-adaptin-binding protein p34; bp, base pair; OMIM, Online Mendelian Inheritance in Man; PPK(P), (punctate) palmoplantar keratoderma; RTK, receptor tyrosine kinase

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EFdA, a Reverse Transcriptase Inhibitor, Potently Blocks HIV-1 *Ex Vivo* Infection of Langerhans Cells within Epithelium

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TO THE EDITOR

Despite increasing access to antiretroviral drugs, sexual transmission of HIV-1 remains a significant public health threat. A recent clinical trial, CAPRISA 004, of a vaginally administered microbicide using a nucleoside reverse transcriptase inhibitor (NRTI), tenofovir (TDF), has demonstrated that 1% TDF gel reduced HIV-1 acquisition by an estimated 39% overall (Abdool Karim *et al.*, 2010), indicating a potential utility of NRTI-based microbicides. In the VOICE study, however, a once-daily dosing regimen with TDF gel failed to demonstrate protective effects in at-risk women. These studies demonstrate the need to develop additional more potent microbicide candidates to potentially increase the activity to protect women from HIV-1 transmission.

We previously reported that a series of 4′-substituted NRTIs have excellent antiviral properties (Ohrui, 2006), and through optimization of such 4′-substituted NRTIs, 4′-ethynyl-2-fluoro-2′-deoxyadenosine (EFdA) was found to exert extremely potent activity against a wide spectrum of HIV-1 strains including highly multidrug-resistant clinical HIV-1 isolates, with favorable *in vitro* cell toxicities (Nakata *et al.*, 2007; Ohrui *et al.*, 2007). EFdA inhibited HIV-1 replication in activated peripheral blood mononuclear cells with an EC_{50} of 0.05 nM, a potency several orders of magnitude greater than any of the current clinically available NRTIs (Michailidis *et al.*, 2009). As the prevalence of new infections with drug-resistant HIV-1 variants could increase in the coming years (Nichols *et al.*, 2011), EFdA may be useful as a topical microbicide.

Langerhans cells (LCs) are dendritic cells located, among other sites, within genital skin and mucosal epithelium (Lederman *et al.*, 2006). In female rhesus macaques exposed intravaginally to simian immunodeficiency virus, up to 90% of initially infected target cells were LCs (Hu *et al.*, 2000). *Ex vivo* experiments with human foreskin explants show that epidermal LCs in inner foreskin are primary target cells for HIV-1 infection, providing a plausible explanation for why circumcision greatly reduces the probability of acquiring HIV-1 (Ganor *et al.*, 2010; Zhou *et al.*, 2011). LCs also express CD4 and CCR5, but not CXCR4, and demonstrate the distinctive characteristics of emigrating from tissue to draining lymph nodes in order to interact with T cells following contact with pathogens (Lederman *et al.*, 2006). Indeed, epidermal LCs are readily infected *ex vivo* with R5-HIV-1, but not with X4-HIV-1, and initiate and promote high levels of infection upon interactions with cocultured $CD4^+$ T cells (Kawamura *et al.*, 2000; Ogawa *et al.*, 2009, 2013), consistent with previous epidemiologic observations that the majority of HIV-1 strains isolated from newly infected patients are R5-HIV-1 strains (Zhu *et al.*, 1993). Thus, LCs likely have an important role in disseminating HIV-1 soon after exposure to the virus.

To understand how HIV-1 traverses skin and genital mucosa, an *ex vivo* model was developed in which resident LCs within epithelial tissue explants obtained from suction blisters are exposed to HIV-1 and then allowed to emigrate from the tissue, thus mimicking conditions that occur following mucosal exposure to HIV (Kawamura *et al.*, 2000; Ogawa *et al.*, 2009, 2013). In this model, although relatively few productively infected LCs are identified, these cells induce high levels of HIV-1 infection when cocultured with resting autologous $CD4^+$ T cells (Kawamura *et al.*, 2000; Ogawa *et al.*, 2013). As expected, when epidermal tissue explants were pretreated with various concentrations of TDF, EFdA, and CCR5 inhibitor, maraviroc (MVC), prior to R5-tropic $HIV\text{-}1_{Ba\text{-}L}$ exposure, HIV-1 infection of resident LCs within epidermis as well as subsequent virus transmission from emigrated LCs to cocultured $CD4^+$ T cells was decreased in a dose-dependent manner (Figure 1a and c; for detailed methods, see Supplementary Material). The blocking was confirmed by repeated experiments using skin explants from three additional randomly selected individuals (Figure 1b and d). Strikingly, although the blocking efficiency of TDF or MVC even at 5,000 nM was partial, EFdA demonstrated complete blocking of R5-HIV-1 replication in LCs as well as subsequent virus transmission from emigrated LCs to $CD4^+$ T cells at doses of 100–5,000 nM (Figure 1a–d). Furthermore, EFdA blocked *ex vivo* virus infection of LCs as well as subsequent virus transmission when two strains of R5-HIV-1, $HIV\text{-}1_{JR\text{-}FL}$ and $HIV\text{-}1_{AD8}$, were utilized in experiments ($n=3$, Supplementary Figure S1 online).

Similar to the results in epidermal LCs, preincubation of monocyte-derived LCs (mLCs) with 100–5,000 nM of EFdA

Abbreviations: EFdA, 4′-ethynyl-2-fluoro-2′-deoxyadenosine; LC, Langerhans cell; mLC, monocyte-derived LC; MVC, maraviroc; NRTI, nucleoside reverse transcriptase inhibitor; TDF, tenofovir

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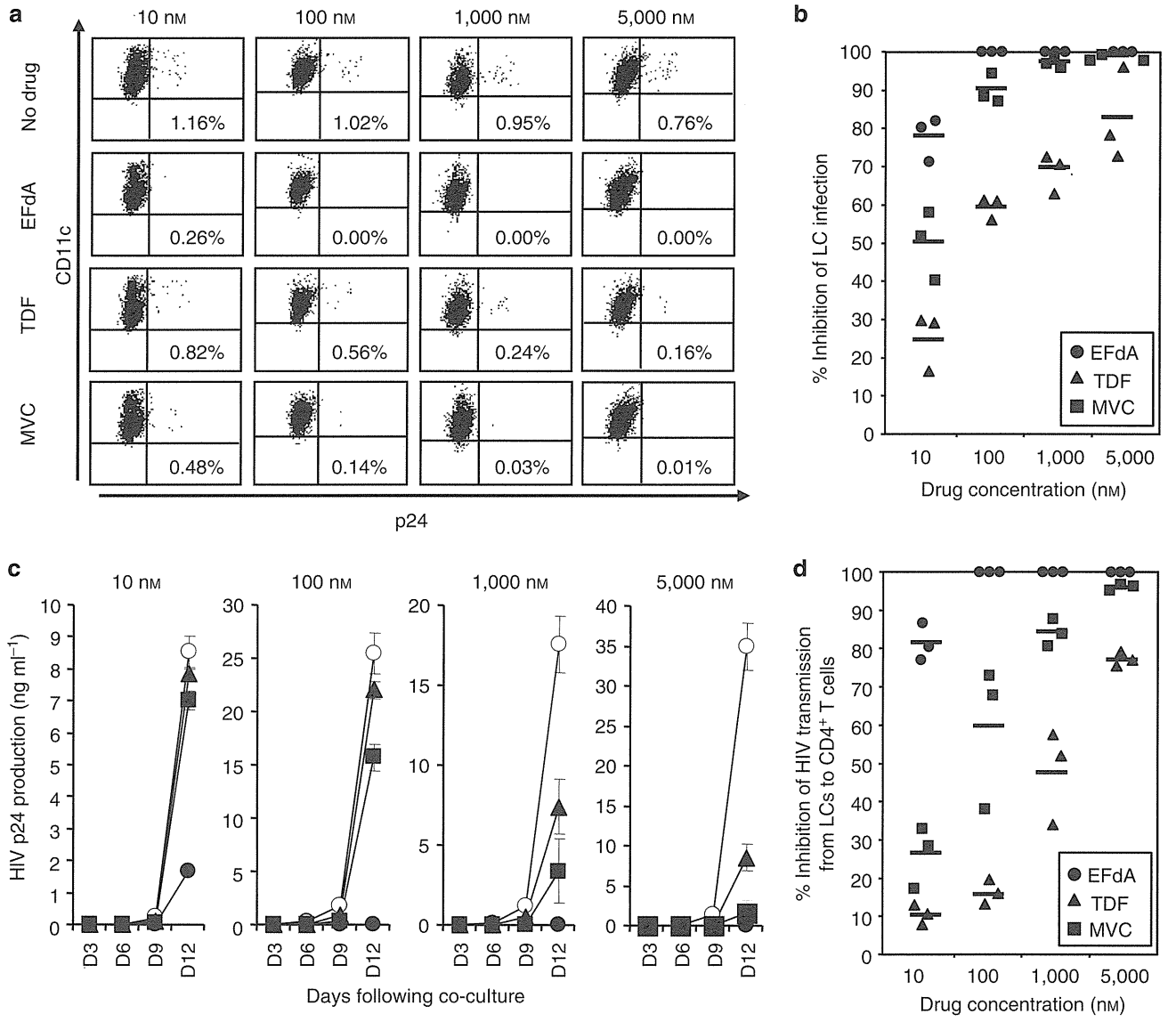

Figure 1. Preincubation of skin explants with EFdA blocks R5-HIV-1 infection in LCs and subsequent virus transmission to cocultured CD4$^+$ T cells. LCs within skin explants were preincubated with no drug (○) or the indicated concentrations of EFdA (●), TDF (▲), and MVC (■) for 30 minutes, exposed to HIV-1_{Ba-L} for 2 hours, and then floated on culture medium to allow migration of LCs from the explants. Emigrating cells from the epidermal sheets were collected 3 days following HIV-1 exposure. HIV-1-infected LCs were assessed by HIV-1 p24 intracellular staining in langerin$^+$ CD11c$^+$ LCs (**a**, **b**), or further cocultured with autologous CD4$^+$ T cells and culture supernatants were assessed for p24 content by ELISA on the indicated days (**c**, **d**). Summary of percent inhibition of LC infection (**b**) and virus transmission to CD4$^+$ T cells (**d**) of 12 experiments using skin explants from 12 individuals with the indicated each concentration of EFdA (●), TDF (▲), and MVC (■) are shown. Mean values obtained from different donors are shown as horizontal marks (**b**, **d**). EFdA, 4′-ethynyl-2-fluoro-2′-deoxyadenosine; LCs, Langerhans cells; MVC, maraviroc; TDF, tenofovir.

completely blocked HIV-1 replication in mLCs as well as subsequent virus transmission from mLCs to cocultured CD4$^+$ T cells, whereas both TDF and MVC at the same doses only partially inhibited the transmission (Figure 2a and b; for detailed methods, see Supplementary Material online). Intriguingly, even in 1–3 days following the removal of EFdA (1,000 nM), EFdA completely blocked HIV-1 infection of mLCs as well as subsequent virus transmission from mLCs to cocultured CD4$^+$ T cells, whereas TDF and MVC rapidly lost their anti-HIV-1 activity within days (Figure 2c–f). No cellular toxicity was noted for any of these drugs at the doses used in these experiments (Supplementary Figure S2 online). When similar experiments were conducted using peripheral blood mononuclear cell as target cells, virtually identical favorable persistency of EFdA in antiviral activity compared with that of TDF was observed (data not shown).

In the present work, we demonstrated that EFdA exerted extremely more potent anti-HIV-1 activity in LCs than did TDF and MVC, and the potent anti-HIV-1 activity of EFdA persisted for at least 3 days. Of note, the efficacy of TDF gel in CAPRISA 004 has been linked to its long intracellular half-life (Abdool Karim *et al.*, 2010; Rohan *et al.*, 2010). Our data strongly

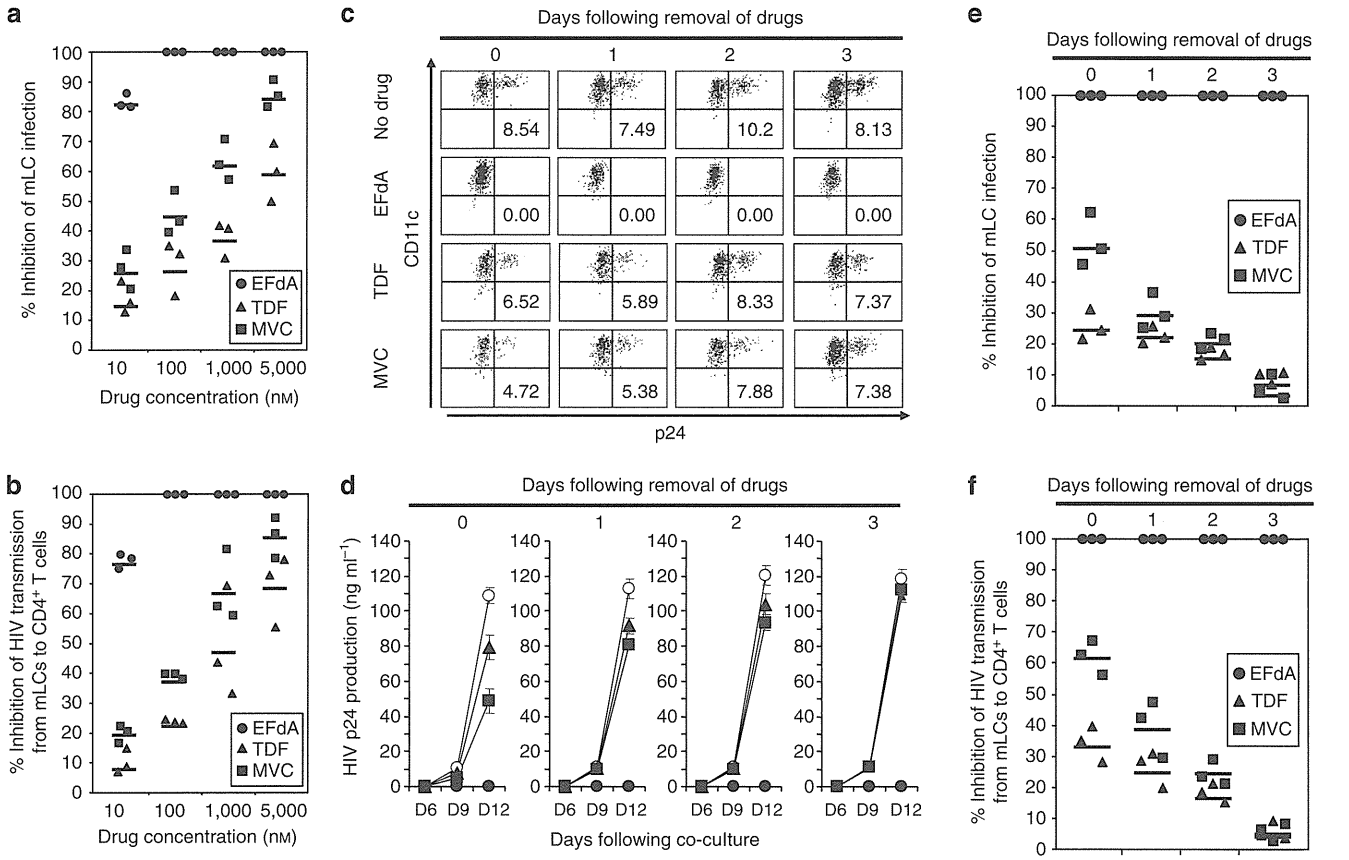

Figure 2. Preincubation of skin explants with EFdA blocks subsequent R5-HIV-1 infection in LC in a dose-dependent manner. mLCs were preincubated with no drug (○) or the indicated concentrations of EFdA (●), TDF (▲) and MVC (■) for 30 minutes, and then immediately exposed to HIV-1Ba-L for 2 hours (**a**, **b**), or thoroughly washed to remove the extracellular drug and further cultured for 1, 2, or 3 days prior to exposure to HIV-1Ba-L for 2 hours (**c**–**f**). After 7 days of HIV-1 exposure, HIV-1-infected mLCs were assessed by HIV-1 p24 intracellular staining in langerin$^+$ CD11c$^+$ mLCs (**a**, **c**, **e**), or further cocultured with autologous CD4$^+$ T cells and culture supernatants were assessed for p24 content by ELISA on the indicated days (**b**, **d**, **f**). Summary of percent inhibition of mLC infection (**a**, **e**) and virus transmission to CD4$^+$ T cells (**b**, **f**) of three independent experiments are shown. Mean values are shown as horizontal marks (**a**, **b**, **e**, **f**). EFdA, 4′-ethynyl-2-fluoro-2′-deoxyadenosine; LCs, Langerhans cells; mLCs, monocyte-derived LCs; MVC, maraviroc; TDF, tenofovir.

indicate that EFdA may serve as a promising microbicide to block sexual transmission of HIV-1 because of its potent anti-HIV-1 activity, low cytotoxicity, and superior persistence of antiviral activity against HIV-1 in LCs.

CONFLICT OF INTEREST

HM is among coinventors on a patent for EFdA; all rights, title, and interest to the patent have been assigned to Yamasa Corporation, Chiba, Japan. The other authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Mechanism of resistance to S138A substituted enfuvirtide and its application to peptide design

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ABSTRACT

T-20 (enfuvirtide) resistance is caused by the N43D primary resistance mutation at its presumed binding site at the N-terminal heptad repeat (N-HR) of gp41, accompanied by the S138A secondary mutation at the C-terminal HR of gp41 (C-HR). We have discovered that modifying T-20 to include S138A (T-20_{S138A}) allows it to efficiently block wild-type and T20-resistant viruses, by a mechanism that involves improved binding of T-20_{S138A} to the N-HR that contains the N43D primary mutation. To determine how HIV-1 in turn escapes T-20_{S138A} we used a dose escalation method to select T-20_{S138A}-resistant HIV-1 starting with either wild-type (HIV-1_{WT}) or T-20-resistant (HIV-$1_{N43D/S138A}$) virus. We found that when starting with WT background, I37N and L44M emerged in the N-HR of gp41, and N126K in the C-HR. However, when starting with HIV-$1_{N43D/S138A}$, L33S and I69L emerged in N-HR, and E137K in C-HR. T-20_{S138A}-resistant recombinant HIV-1 showed cross-resistance to other T-20 derivatives, but not to C34 derivatives, suggesting that T-20_{S138A} suppressed HIV-1 replication by a similar mechanism to T-20. Furthermore, E137K enhanced viral replication kinetics and restored binding affinity with N-HR containing N43D, indicating that it acts as a secondary, compensatory mutation. We therefore introduced E137K into T-20_{S138A} (T-$20_{E137K/S138A}$) and revealed that T-$20_{E137K/S138A}$ moderately suppressed replication of T-20_{S138A}-resistant HIV-1. T-$20_{E137K/S138A}$ retained activity to HIV-1 without L33S, which seems to be a key mutation for T-20 derivatives.

Our data demonstrate that secondary mutations can be consistently used for the design of peptide inhibitors that block replication of HIV resistant to fusion inhibitors.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) fusion to host cell membrane is mediated by formation of a six-helix bundle of the transmembrane subunit gp41 (Chan et al., 1997). Peptides corresponding to amino acid sequences of the gp41 carboxyl-terminal heptad repeat (C-HR) inhibit the HIV-1 fusion by acting as decoys and interfering with the formation of the six-helix bundle (Chan et al., 1998; Malashkevich et al., 1998). Although modified peptides such as SC34EK (Nishikawa et al., 2009), T-2635 (Dwyer et al., 2008), and D-peptides (Welch et al., 2007), and small molecules (Debnath et al., 1999) have been developed, T-20 (enfuvirtide) is the only fusion inhibitor approved for HIV therapy. It is a 36 amino acid peptide derived from the sequence of C-HR of gp41. It is thought to bind at the N-HR domain of gp41 and interfere with the C-HR-N-HR interactions required for membrane fusion and injection of virus into the host cell. T-20 has potent anti-HIV-1 activity and effectively suppresses replication of HIV-1 *in vivo* (Kilby et al., 1998; Lalezari et al., 2003; Lazzarin et al., 2003). However, HIV-1 rapidly develops resistance through mutations in the amino-terminal HR (N-HR) of gp41, especially in the region between L33 and L45, which

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is thought to be the binding site of T-20 (Aquaro et al., 2006; Cardoso et al., 2007; He et al., 2008). Among these residues, N43D in the N-HR is one of the representative mutations for resistance to T-20 (Bai et al., 2008; Cabrera et al., 2006; Oliveira et al., 2009; Izumi et al., 2009; Ueno et al., 2009). Interestingly, most variants show impaired replication fitness, and thus often go on to acquire secondary mutations, such as S138A (Xu et al., 2005), in the C-HR region of gp41 that corresponds to the sequence of T-20. We and others have recently demonstrated that S138A functions as secondary resistance mutation and enhances resistance to T-20 by restoring impaired replication kinetics of T-20-resistant variants that contain primary mutations in the N-HR region, most notably N43D (Izumi et al., 2009; Watabe et al., 2009).

To preempt this escape strategy, we have previously designed a peptide analog of T-20 with the S138A change incorporated in it (T-20_{S138A}; Fig. 1A) and showed that this peptide significantly suppresses replication of T-20-resistant HIV-1 through enhancement of binding affinity to mutated N-HR, such as N-HR_{N43D} (Izumi et al., 2009). Using circular dichroism (CD) and structural analyses, we also demonstrated that the S138A change provided increased stability to the six-helix bundle (Watabe et al., 2009). In subsequent studies, we validated our approach on another peptide-based fusion inhibitor, C34. In this case, we designed a variant of C34 carrying a secondary escape mutation, N126K, selected for the induction of C34 resistance (Nameki et al., 2005) and also present in HIV-1 isolates from T-20 experienced patients (Baldwin et al., 2004; Cabrera et al., 2006; Svicher et al., 2008). We showed that this C34 variant can effectively inhibit replication of C34-resistant HIV-1. These studies provided the proof of principle that it is possible to design improved peptide-based fusion inhibitors that are efficient against a major mechanism of drug resistance through introduction of resistance-associated mutation(s).

It remains unknown to this date how HIV-1 develops further resistance to T-20_{S138A}. Moreover, it is not known whether we can expand our strategy and modify T-20_{S138A} to include the secondary mutation(s) that emerge during the selection of T-20_{S138A}-resistant HIV, resulting in a strategy that is applicable to the design of peptides customized to address viral resistance mutations. Hence, in the current study we selected T-20_{S138A}-resistant HIV-1 *in vitro* by a dose-escalating method. We revealed that the resistance mutations that emerged during selection experiments with wild-type or T-20-resistant HIV-1 are located in both the N-HR and the C-HR regions. Furthermore, the I37N and L33S mutations appeared to act as primary mutations for wild-type and T-20-resistant HIV-1, respectively. E137K, a C-HR mutation located in the T-20 sequence, improved replication kinetics and enhanced affinity to N-HR, indicating that E137K acts as a secondary mutation. Introducing the E137K change into the T-20_{S138A} (T-$20_{E137K/S138A}$) resulted into a peptide inhibitor effective against T-20_{S138A}-resistant variants, suggesting that secondary or compensatory mutations can be widely applicable to the design of next generation peptide-based inhibitors that are active against HIV-1 resistant to earlier generation fusion-targeting drugs.

2. Materials and methods

2.1. Cells and viruses

MT-2 and 293T cells were grown in RPMI 1640 medium and Dulbecco's modified Eagle medium-based culture medium, respectively. HeLa-CD4-LTR-β-gal cells were kindly provided by Dr. M. Emerman through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease (Bethesda, MD), and used for the drug susceptibility assay, as previously described (Nameki et al., 2005; Nishikawa et al., 2009). Recombinant infectious HIV-1 clones carrying various mutations were generated through site-directed mutagenesis of the pNL4-3 plasmid, as previously described (Nameki et al., 2005; Nishikawa et al., 2009). Each molecular clone was transfected into 293T cells with *Trans*IT (Madison, WI). After 48 h, the supernatants were harvested and stored at −80 °C.

2.2. Antiviral agents

The peptides used in this study (Fig. 1A) were chemically synthesized using standard Fmoc-based solid-phase techniques, as previously described (Oishi et al., 2008; Otaka et al., 2002). An HIV-1 reverse transcriptase inhibitor, 2′,3′-dideoxycytidine (ddC) was purchased from Sigma–Aldrich Japan (Tokyo, Japan) and used as a control.

2.3. Determination of drug susceptibility

Peptide sensitivity of infectious clones was determined by the multinuclear activation of galactosidase indicator (MAGI) assay as previously described (Nameki et al., 2005; Nishikawa et al., 2009). Briefly, the target cells (HeLa-CD4-LTR-β-gal; 10^4 cells/well) were plated in flat 96-well microtiter culture plates. On the following day, the cells were inoculated with the HIV-1 clones (60 MAGI units/well, resulting into 60 blue cells after 48 h incubation) and cultured in the presence of various concentrations of drugs in fresh medium. Forty-eight hours after virus exposure, all the blue cells stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were counted in each well. The activity of test compounds was determined as the concentration that reduced HIV-1 infection by 50% (50% effective concentration [EC_{50}]).

2.4. Induction of HIV-1 variants resistant to T-20_{S138A}

MT-2 cells were exposed to HIV-1 and cultured in the presence of T-20_{S138A}. Cultures were incubated at 37 °C until an extensive cytopathic effect (CPE) was observed. The culture supernatants were used for further passages in MT-2 cells in the presence of two-fold increasing concentrations of T-20_{S138A} when massive CPEs were seen in the earlier periods. Each passage usually took 5–7 days. The timing is highly dependent on the type of specific mutations introduced, as previously reported (Nameki et al., 2005; Shimura et al., 2010). For example, a passage that follows introduction of novel mutation(s) should shorten the passage period to perhaps 4–5 days. However, there will be longer delays for passages where there are no novel mutations or when there is appearance of only secondary mutations. The dose-escalation process was repeated until resistant variants were obtained. This selection was carried out for a total of 60 passages (approximately 1 year). At the indicated passages (Fig. 1B and C), the sequence of the *env* region was determined by direct sequencing of the proviral DNA extracted from the infected MT-2 cells.

2.5. Viral replication kinetics assay

MT-2 cells (10^5 cells/1 mL) were infected with each virus preparation (500 MAGI units) for 16 h. Infected cells were then washed and cultured in a final volume of 3 mL. The culture supernatants were collected on day 2 through day 5 post-infection, and amounts of p24 antigen were determined.

2.6. CD spectroscopy

Each peptide was incubated at 37 °C for 30 min (the final concentrations of peptides were 10 μM in phosphate buffered saline [PBS];

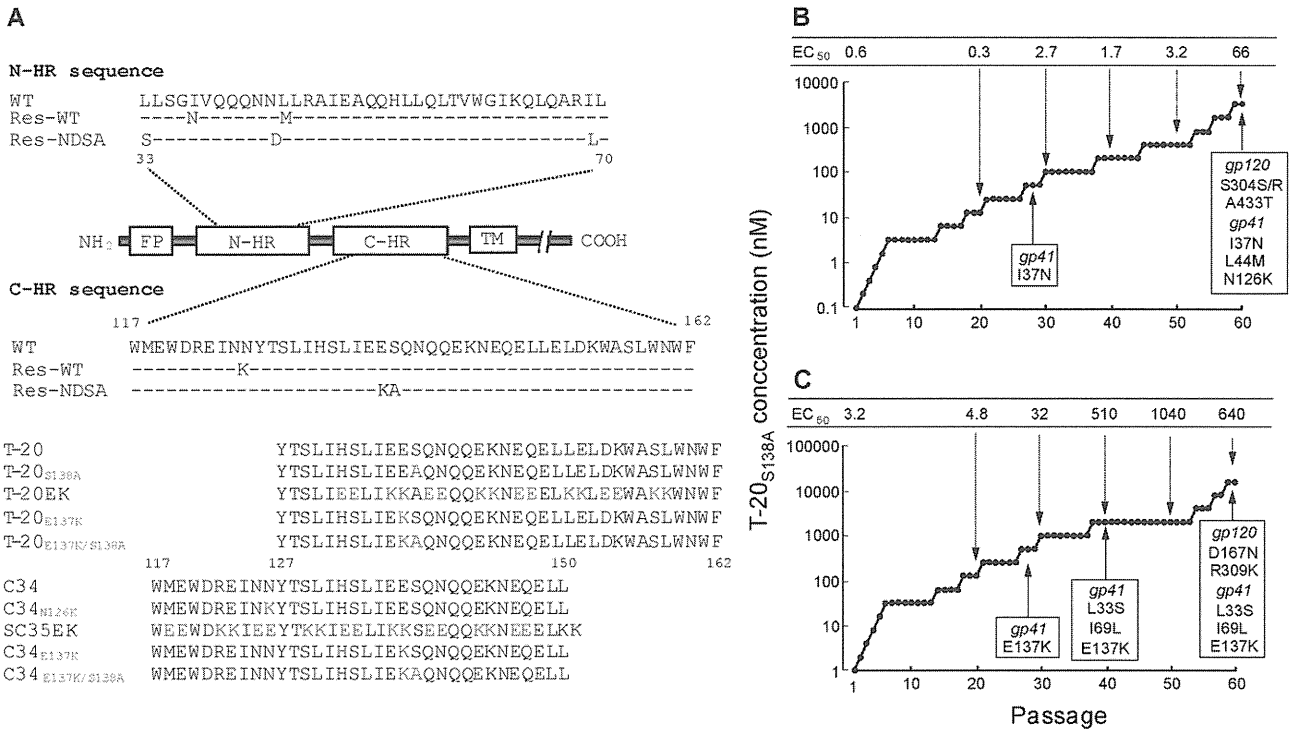


Fig. 1. Domains of gp41 and induction of T-20_{S138A}-resistant HIV-1. (A) Domains of gp41, substitutions observed during *in vitro* passage with T-20_{S138A}, and amino acid sequences of T-20- and C34-based peptides used in this study. The locations of the fusion peptide (FP), amino-terminal heptad region (N-HR), carboxyl-terminal heptad region (C-HR), transmembrane domain (TM), and C-HR-derived peptides are shown. The residue numbers of T-20 and C34 correspond to their positions in gp41. Substitutions of N- and C-HR in gp41 of wild-type (WT) and T-20_{S138A}-resistant HIV-1 are shown. Res.-WT and Res.-NDSA indicate resistant HIV-1 that were initially selected from wild-type and HIV-$1_{N43D/S138A}$, respectively. (B and C) Induction of T-20_{S138A}-resistant HIV-1 by dose-escalating selection in MT-2 cells. Induction of resistant HIV-1 was carried out for a total of 60 passages of HIV-1_{WT} (B) and HIV-$1_{N43D/S138A}$ (C), in 0.1 nM and 1 nM of T-20_{S138A}, respectively. At the indicated passages, proviral DNA was sequenced, and the EC_{50} values of the HIV-1 variants were determined using the MAGI assay. To improve the replication kinetics, substitution of D36G was introduced into the NL4-3 background used in this study (wild-type virus) (Izumi et al., 2009; Mink et al., 2005).

pH 7.4). CD spectra were recorded on an AVIV model 202 spectropolarimeter (Aviv Instruments, Proterion Corporation, Piscataway, NJ) with a 1 mm path-length cuvette at 25 °C as the average of eight scans. The thermal stability was assessed by monitoring the change in the CD signal at 222 nm. The midpoint of the thermal unfolding transition (melting temperature [T_m]) of each complex was determined as previously described (Izumi et al., 2009).

3. Results

3.1. Selection of HIV-1 resistant to T-20_{S138A}

An HIV-1_{NL4-3} strain containing a D36G substitution, which improves replication kinetics, was used as a wild-type virus (HIV-1_{WT}) and for the construction of various mutants, as described (Izumi et al., 2009; Mink et al., 2005). HIV-1_{WT} or T-20-resistant HIV-$1_{N43D/S138A}$ were used for selection of T-20_{S138A}-resistant HIV-1. MT-2 cells were infected with HIV-1_{WT} and HIV-$1_{N43D/S138A}$, and incubated in the presence of T-20_{S138A} at the initial concentrations of 0.1 nM and 1 nM, respectively. At the indicated passages, the sequence of the *env* region was determined by direct sequencing of the proviral DNA extracted from the infected MT-2 cells. During the selection, mutations in the gp41 were observed and are shown in Fig. 1B and C.

In the selection with HIV-1_{WT} (Fig. 1B), at passage 28 (P-28), when T-20_{S138A} concentration was 51.2 nM (P-28, 51.2 nM), isoleucine at position 37 in the gp41 was substituted to asparagine (I37N). At P-60 (3.3 μM), L44 M and N126K in the gp41 further emerged. On the other hand, in the selection with T-20-resistant HIV-$1_{N43D/S138A}$ (Fig. 1C), at P-28 (512 nM) and at P-40 (2 μM), E137K in the gp41, and L33S and I69L in the gp41 emerged, respectively. The emergence of the I69L mutation in diverse HIV-1 strains has been previously reported (Eshleman et al., 2007). At P-60, the resistance of selected viruses from HIV-1_{WT} and HIV-$1_{N43D/S138A}$ to T-20_{S138A}, reached approximately 110- and 200-fold, respectively. These results indicate that even though T-20_{S138A} was active against T-20 resistant variants, resistant HIV-1 emerged relatively rapidly compared with the next generation fusion inhibitors, such as SC34EK, which required 120 passages to acquire the resistance (Shimura et al., 2010).

3.2. Susceptibility of T-20_{S138A}-resistant HIV-1 to T-20 and C34 derivatives

To validate our resistance data we used site-directed mutagenesis to prepare recombinant HIV-1 with the T-20_{S138A}-resistance mutations and examined its susceptibility to T-20 and C34 derivatives with MAGI assay (Table 1). We also used as controls the modified α-helix T-20- and C34-peptide inhibitors, T-20EK (Oishi et al., 2008) and SC35EK (Nishikawa et al., 2009; Shimura et al., 2010), respectively, which are more efficient *in vitro* replication inhibitors of T-20-resistant HIV-1 than T-20 or C34. Finally, we also used as a control $C34_{N126K}$, a modified version of C34 that includes the resistance-associated N126K substitution that effectively suppress replication of C34-resistant HIV-1 *in vitro* (Izumi et al., 2009).

Selected mutations I37N and L33S provided various levels of resistance to T-20 and its derivatives, T-20_{S138A} and T-20EK, apparently acting as primary mutations to peptides with a T-20 backbone (Table 1). Other mutations, L44M, I69L, and E137K, which were

Table 1
Antiviral activity of C-HR-derived peptides against gp41 recombinant viruses.

	EC_{50} (nM)					
	T-20	$T\text{-}20_{S138A}$	T-20EK	C34	$C34_{N126K}$	SC35EK
$HIV\text{-}1_{WT}$ [a]	2.4 ± 0.6	0.6 ± 0.1	1.9 ± 0.5	2.1 ± 0.7	1.6 ± 0.5	2.4 ± 0.9
$HIV\text{-}1_{I37N}$	47 ± 6.9 (**20**)	4.3 ± 1.3 (7.2)	21 ± 2.4 (**11**)	3.3 ± 1.1(1.6)	1.9 ± 0.1 (1.2)	1.0 ± 0.4(0.4)
$HIV\text{-}1_{L44M}$	4.1 ± 1.2 (1.7)	0.7 ± 0.2 (1.2)	2.2 ± 0.6 (1.2)	1.1 ± 0.3(0.5)	0.8 ± 0.2 (0.5)	0.6 ± 0.2(0.3)
$HIV\text{-}1_{N126K}$	4.4 ± 1.3 (1.8)	1.2 ± 0.4 (2.0)	2.8 ± 0.2 (1.5)	6.3 ± 1.2(3.0)	1.5 ± 0.2 (0.9)	3.3 ± 0.2(1.4)
$HIV\text{-}1_{I37N/N126K}$	660 ± 180(**275**)	16 ± 4.8 (**27**)	14 ± 5.1 (7.4)	20 ± 4.5(9.5)	3.4 ± 0.4 (2.1)	2.9 ± 0.3(1.2)
$HIV\text{-}1_{I37N/L44M/N126K}$	>1000 (>417)	130 ± 40(220)	240 ± 95(126)	66 ± 23 (31)	4.0 ± 0.8 (2.5)	1.1 ± 0.1(0.5)
$HIV\text{-}1_{L33S}$	23 ± 5.5 (9.6)	3.1 ± 0.6 (5.2)	13 ± 2.6 (6.8)	3.2 ± 1.1(1.5)	2.1 ± 0.1 (1.3)	3.0 ± 0.8(1.2)
$HIV\text{-}1_{N43D}$	49 ± 10 (**20**)	3.5 ± 0.9 (5.8)	4.1 ± 1.2 (2.2)	4.4 ± 0.4(2.1)	1.4 ± 0.1 (0.8)	0.4 ± 0.2(0.2)
$HIV\text{-}1_{I69L}$	2.1 ± 0.5 (0.9)	0.5 ± 0.2 (0.8)	2.2 ± 0.4 (1.2)	2.7 ± 0.2(1.3)	2.2 ± 0.5 (1.4)	2.7 ± 0.5(1.1)
$HIV\text{-}1_{E137K}$	2.0 ± 0.3 (0.8)	0.7 ± 0.1 (1.2)	2.5 ± 0.4 (1.3)	2.6 ± 0.2(1.2)	2.3 ± 0.7 (1.4)	3.1 ± 0.8(1.3)
$HIV\text{-}1_{N43D/S138A}$	84 ± 16 (**35**)	3.2 ± 1.0 (5.3)	3.4 ± 1.1 (1.8)	2.7 ± 0.2(1.3)	1.6 ± 0.5 (1.0)	0.3 ± 0.1(0.1)
$HIV\text{-}1_{L33S/N43D/S138A}$	>1000 (**>417**)	550 ± 72(**174**)	330 ± 94 (14)	30 ± 9.2 (2.6)	4.2 ± 1.2 (0.4)	0.9 ± 0.3(0.4)
$HIV\text{-}1_{N43D/E137K/S138A}$	110 ± 31 (**46**)	14 ± 4.7 (**23**)	7.0 ± 2.4 (3.7)	7.4 ± 1.9(3.5)	2.1 ± 0.7 (1.3)	1.9 ± 0.6(0.8)
$HIV\text{-}1_{L33S/N43D/E137K/S138A}$	>1000 (**>417**)	>1000(**>1667**)	>1000 (**>526**)	31 ± 5.0 (**15**)	6.7 ± 1.7 (4.2)	1.2 ± 0.2(0.5)
$HIV\text{-}1_{L33S/N43D/I69L/E137K/S138A}$	>1000 (**>417**)	>1000(**>1667**)	>1000 (**>526**)	50 ± 12 (**24**)	28 ± 7.1(**17.5**)	1.0 ± 0.9(0.4)

Anti-HIV activity was determined using the MAGI assay. Fifty percent effective concentration (EC_{50}) values and SD were obtained from the results of at least three independent experiments. Shown in parentheses are the fold-increases in resistance (increase in EC_{50} value) calculated by comparison to a wild-type virus ($HIV\text{-}1_{WT}$). Increases of over 10-fold are indicated in bold.

[a] To improve the replication kinetics, substitution of D36G, observed in majority of HIV-1 strains, was introduced into the NL4-3 background used in this study (wild-type virus; $HIV\text{-}1_{WT}$) (Izumi et al., 2009; Mink et al., 2005).

observed in wild-type HIV-1 as polymorphisms (Kuiken et al., 2010; Loutfy et al., 2007), conferred little resistance to all peptide fusion inhibitors tested. However, introduction of L44M to $HIV\text{-}1_{I37N/N126K}$ ($HIV\text{-}1_{I37N/L44M/N126K}$) remarkably enhanced resistance to T-20 derivatives. This was consistent with previous studies that also reported a resistance enhancement (1.8-fold) by L44M to T-20 (Loutfy et al., 2007). Collectively, these data suggest that L44M has as a role in HIV-1 resistance as a secondary mutation. All peptides sufficiently suppressed $HIV\text{-}1_{I69L}$, suggesting that I69L may be a secondary mutation or a polymorphism. N126K conferred only marginal resistance (<3-fold) to all peptide fusion inhibitors, but in the background of I37N ($HIV\text{-}1_{I37N/N126K}$) it enhanced resistance to T-20, $T\text{-}20_{S138A}$, and C34. L33S, which was originally reported as a C34 resistance associated mutation (Armand-Ugon et al., 2003), significantly enhanced resistance in the background of N43D/S138A mutations ($HIV\text{-}1_{L33S/N43D/S138A}$). Similar to the N126K mutation, E137K also enhanced resistance by N43D/S138A ($HIV\text{-}1_{N43D/E137K/S138A}$) and L33S/N43D/S138A ($HIV\text{-}1_{L33S/N43D/E137K/S138A}$) to $T\text{-}20_{S138A}$, T-20, and T-20EK. These results indicate that L33S and I37N appear to be primary mutations for T-20 derivatives.

3.3. *Effect of substitutions in the gp120 on peptide susceptibility*

Polymorphisms in the gp120 that influence co-receptor usage may influence T-20 susceptibility (Labrosse et al., 2003; Reeves et al., 2002). Meanwhile, others reported that T-20 susceptibility was not influenced by co-receptor usage (Cilliers et al., 2004; Melby et al., 2006). Resistance induction experiments performed in this study revealed that most laboratory strains with *in vitro* resistance to fusion inhibitors acquired substitutions in both the gp120 and the gp41 (Armand-Ugon et al., 2003; Eggink et al., 2011; Fikkert et al., 2002; Izumi et al., 2010; Nameki et al., 2005; Shimura et al., 2010). However, most substitutions showed little impact on resistance, and only contributed to a small enhancement of replication capacity (Eggink et al., 2011; Izumi et al., 2010; Nameki et al., 2005; Shimura et al., 2010). In the present study, we examined peptide susceptibility of cloned viruses that contain all Env substitutions observed in the selection (both gp120 and gp41). Most substitutions in the gp120 attenuated resistance to fusion inhibitors (Table 3). Therefore, *in vitro* experiments showed that substitutions in the gp120 are not likely associated with resistance.

3.4. *Influence of mutations in the gp41 on HIV-1 replication*

To address the effects of mutations on HIV-1 replication, we examined the replication kinetics of $T\text{-}20_{S138A}$-resistant $HIV\text{-}1_{N43D/S138A}$ variants. Consistent with a previous report (Lohrengel et al., 2005), the L33S mutation did not significantly affect the replication kinetics and infectivity compared with those of $HIV\text{-}1_{WT}$ (Fig. 2A). The S138A mutation restored the replication

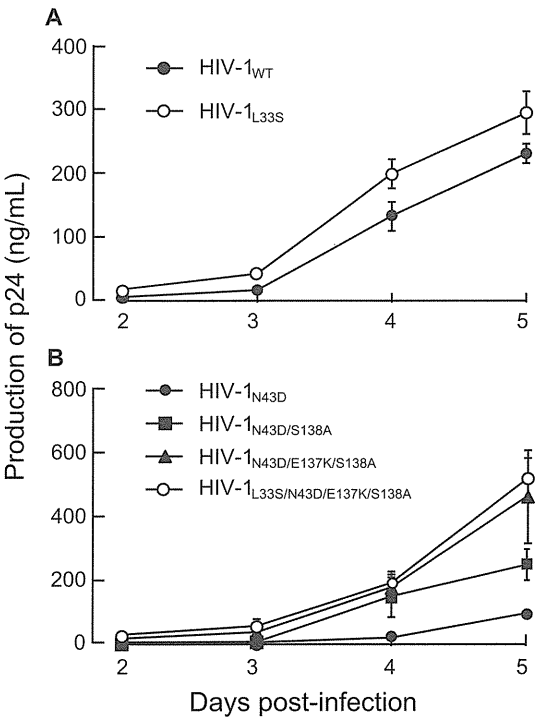


Fig. 2. Replication kinetics of $T\text{-}20_{S138A}$-resistant variants. Replication kinetics of $T\text{-}20_{S138A}$-resistant recombinant variants that introduced L33S mutation (A), or combinations of L33S, E137K, and S138A mutations in $HIV\text{-}1_{N43D}$ (B). To improve replication kinetics, the D36G polymorphism was introduced into the NL4-3 background used in this study ($HIV\text{-}1_{WT}$). Supernatants from infected MT-2 cells were collected on days 2–7 and the amount of p24 produced was determined. Representative results are shown as mean values with standard deviations estimated from three independent experiments.

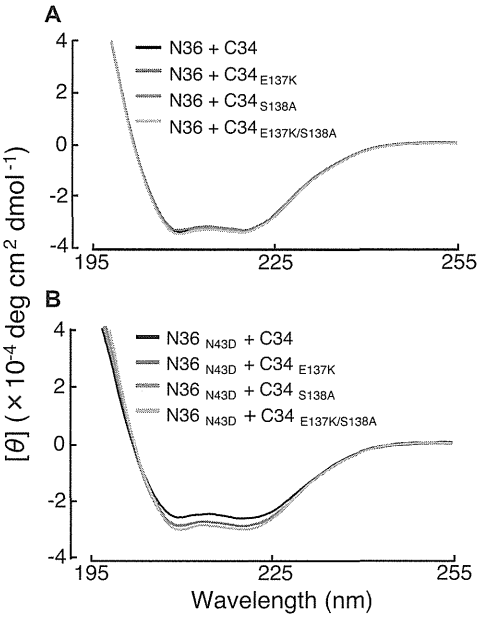
kinetics of HIV-1_{N43D} (Fig. 2B), as previously described (Izumi et al., 2009). E137K was also associated with N43D mutation *in vivo* (Svicher et al., 2008), and restored infectivity impaired by N43D (Tolstrup et al., 2007). Introduction of E137K into N43D/S138A enhanced the replication kinetics, and further addition of L33S to N43D/E137K/S138A resulted in equivalent replication kinetics compared with HIV-$1_{N43D/E137K/S138A}$ (Fig. 2B) as observed in HIV-1_{WT} based mutants. During the passage of HIV-$1_{N43D/S138A}$, a synonymous mutation at amino acid position L44, TTG to CTG, was observed. Interestingly, $L_{TTG}44L_{CTG}$ enhanced viral replication kinetics through enhanced stability of the Rev-responsive element (RRE) secondary structure (Ueno et al., 2009). Therefore, we examined the viral replication kinetics of mutants with $L_{TTG}44L_{CTG}$, and compared HIV-1_{WT}, with HIV-$1_{L44L\text{-}CTG}$, and HIV-$1_{L33S/N43D/L44L\text{-}CTG/E137K/S138A}$ with HIV-$1_{L33S/N43D/L44L\text{-}CTG/I69L/E137K/S138A}$. As expected, the presence of $L_{TTG}44L_{CTG}$ enhanced replication in all viruses. Surprisingly, mutants with resistance mutations showed enhanced replication kinetics as determined by the p24 production assay of culture supernatants (Fig. 4A). Therefore, we further examined infectivity using the MAGI assay and determined that the infectivity of resistance variants containing $L_{TTG}44L_{CTG}$ was reduced compared with HIV-1_{WT} (Fig. 4B). These results indicate that the primary mutation, L33S, possesses less ability to attenuate HIV-1 replication, while I69L, S138A, and E137K enhance replication kinetics of T-20-resistant HIV-1 to a comparable level of HIV-1_{WT}.

3.5. Circular dichroism

To clarify the effect of E137K substitutions on peptide binding, we examined the binding affinities of E137K-containing C-HR peptides to N-HR using CD analysis. CD spectra reveal the presence of stable α-helical structures of six-helix bundles that are required for biological activity and are thought to mechanistically and thermodynamically correlate with HIV-1 fusion (Bianchi et al., 2005). Since *in vitro* T-20 does not interact with the N36 peptide (amino acid positions 35–70 of the N-HR), we used instead peptide C34 with E137K and/or S138A substitutions (Fig. 1A). We found that mixtures of $C34_{E137K}$, $C34_{S138A}$, or $C34_{E137K/S138A}$ with N36 or $N36_{N43D}$ showed sufficient and comparable α-helicity at 25 °C (Fig. 3A and B). We also determined the thermal stability of the helical complexes formed by the N36 and C34 peptides, which is also an indication of the binding affinity of these peptides. Hence, we measured and compared the melting temperatures (T_m) of various complexes, which indicates the 50% disruption of the six-helix bundle (Fig. 3C). Complexes of N36 and C34 containing the S138A and E137K/S138A substitutions (N36/$C34_{S138A}$ and N36/$C34_{E137K/S138A}$, respectively), showed higher thermal stability than N36/C34. Similarly, S138A and E137K/S138A restored the binding affinity of C34 to $N36_{N43D}$. These results indicate that E137K acts as a compensatory mutation for the T-20_{S138A}-resistance primary mutation, causing enhancement of replication kinetics.

3.6. Antiviral activity of E137K-modified peptides

Recently, we demonstrated that introduction of the S138A secondary mutation to T-20 (T-20_{S138A}) enhanced binding to mutated N-HR and suppresses resistance of T-20-resistance variants (Izumi et al., 2009). Similarly, as shown in Fig. 3, E137K enhanced binding affinity with N-HR, suggesting that introduction of E137K to T-20 may enhance the antiviral activity of T-20. We synthesized T-20 and T-20_{S138A} variants containing the E137K change (T-20_{E137K} and T-$20_{E137K/S138A}$) (Fig. 1A) and examined their anti-HIV activity against T-20_{S138A}-resistant HIV-1 (Table 2). All peptides exhibited potent antiviral activity against HIV-1_{WT}. HIV-$1_{L33S/N43D/S138A}$ and HIV-$1_{I37N/L44M/N126K}$ showed high resistance to T-20_{E137K},



C

	T_m (°C)	
	N36	$N36_{N43D}$
C34	67.6	53.2
$C34_{E137K}$	68.9	56.7
$C34_{S138A}$	73.2	58.0
$C34_{E137K/S138A}$	74.6	63.3

Fig. 3. CD spectra (A and B) and thermal stability (C) of N36/C34 complexes. Peptide sequences used in this study are shown in FIG 1A and have also been previously described (Izumi et al., 2009). CD spectra of $C34_{E137K}$, $C34_{S138A}$, and $C34_{E137K/S138A}$ complexes with N36 (A) and $N36_{N43D}$ (B) are shown. Equimolar amounts (10 μM) of the N- and C-HR peptides were incubated at 37 °C for 30 min in PBS. The CD spectra of each mixture were then collected at 25 °C using a Jasco (Model J-710) spectropolarimeter. (C) Thermal stabilities, defined as the midpoint of the thermal unfolding transition (T_m) values, of the potential six-helix bundles of N- and C-HR peptides, were determined.

indicating that the resistance mechanism of T-20_{E137K} is similar to that of T-20_{S138A}. On the other hand, T-$20_{E137K/S138A}$ (Table 2) maintained some antiviral activity against HIV-$1_{L33S/N43D/S138A}$, HIV-$1_{L33S/N43D/E137K/S138A}$, and HIV-$1_{I37N/L44M/N126K}$ compared with other T-20 derivatives including electrostatically constrained T-20EK (Table 1 and Fig. 1). $C34_{E137K}$ and $C34_{E137K/S138A}$ significantly suppressed all HIV-1 variants tested except for HIV-$1_{I37N/L44M/N126K}$ by $C34_{E137K}$. These results indicate that peptides with resistant mutations may sustain their activity against particular resistant variants.

4. Discussion

The current study describes the introduction of resistance changes into the original and modified (T-20_{S138A}) versions of the T-20 peptide-fusion inhibitor. We analyzed the new T-20 derivatives using both wild-type and T-20-resistant strains. We also identified through dose escalation experiments, T-20_{S138A}-resistants. We found that T-20_{S138A}-resistant HIV-1 showed cross-resistance only to the T-20 derivatives, but not to C34 derivatives. Through the CD analysis, the N126K and E137K mutations in the C-HR may act as compensatory mutations for impaired interaction by a primary mutation, I37N and N43D in the N-HR, respectively. Since E137K is located within the T-20 sequence, we synthesized and characterized the activity of novel T-20-based peptides containing E137K (T-20_{E137K}). Here we demonstrate that

Table 2
Antiviral activity of E137K-induced C-HR peptides against T-20_{S138A}-resistant variants.

	EC_{50} (nM)			
	T-20_{E137K}	T-$20_{E137K/S138A}$	$C34_{E137K}$	$C34_{E137K/S138A}$
HIV-1_{WT} [a]	0.8 ± 0.2	0.5 ± 0.1	1.0 ± 0.3	0.7 ± 0.2
HIV-1_{L33S}	13 ± 3.2 (**16**)	2.2 ± 0.4 (4.5)	0.7 ± 0.2(0.7)	0.5 ± 0.1(0.7)
HIV-$1_{N43D/S138A}$	4.2 ± 0.7 (5.3)	0.7 ± 0.2 (1.4)	0.3 ± 0.1(0.3)	0.4 ± 0.1(0.6)
HIV-$1_{L33S/N43D/S138A}$	700 ± 150 (**880**)	45 ± 9.9 (**90**)	2.3 ± 0.4(2.3)	0.5 ± 0.2(0.7)
HIV-$1_{N43D/E137K/S138A}$	12 ± 3.6 (**15**)	2.4 ± 0.8 (4.8)	0.2 ± 0.1(0.2)	0.4 ± 0.1(0.6)
HIV-$1_{L33S/N43D/E137K/S138A}$	480 ± 47 (**600**)	36 ± 3.1 (**72**)	3.8 ± 1.3(3.8)	1.0 ± 0.4(1.4)
HIV-$1_{L33S/N43D/I69L/E137K/S138A}$	1808 ± 852(2260)	157 ± 83(314)	4 ± 2 (4)	1.0 ± 0.4(1.4)
HIV-$1_{I37N/L44M/N126K}$	200 ± 24 (**250**)	30 ± 8.7 (**60**)	17 ± 3.8 (**17**)	2.2 ± 0.3(3.1)

Anti-HIV activity was determined using the MAGI assay. Fifty percent effective concentration (EC_{50}) values and SD were obtained from the results of at least three independent experiments. Shown in parentheses are the fold-increases in resistance (increase in EC_{50} value) calculated by comparison to a wild-type virus (HIV-1_{WT}). Increases of over 10-fold are indicated in bold.

[a] To improve the replication kinetics, substitution of D36G, observed in majority of HIV-1 strains, was introduced into the NL4-3 background used in this study (wild-type virus; HIV-1_{WT}) (Izumi et al., 2009; Mink et al., 2005).

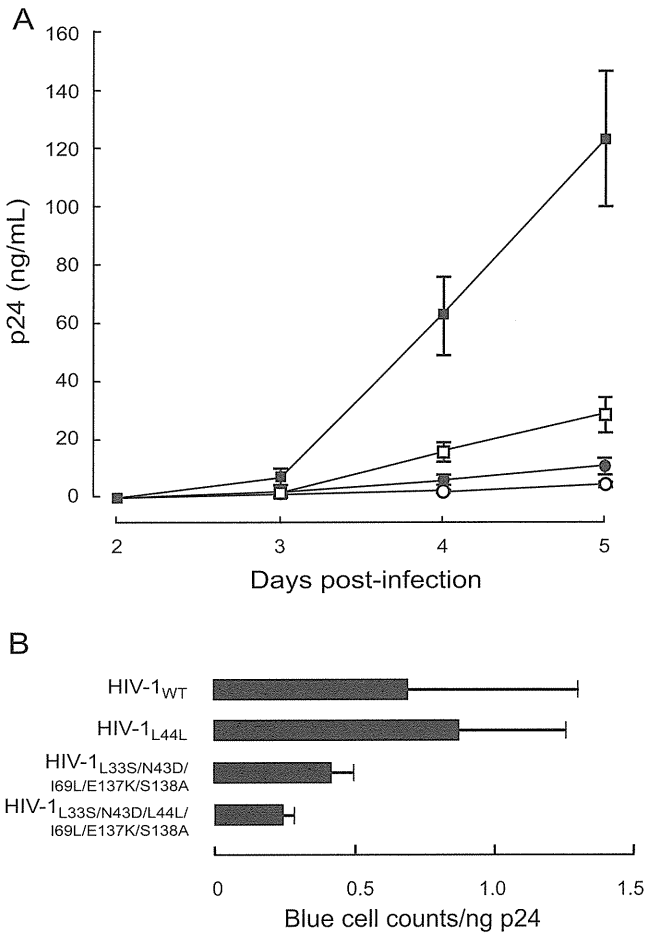


Fig. 4. Effect of secondary mutations in the N-HR on (A) replication kinetics and (B) infectivity. $L_{TTG}44L_{CTG}$ was introduced into HIV-1_{WT} and T-20_{S138A} resistant HIV-1 (HIV-$1_{L33S/N43D/L44L\text{-}CTG/I69L/E137K/S138A}$). Replication kinetics were determined by measuring p24 production in culture supernatants. HIV-1_{WT} (open circles), HIV-1_{L44L} (closed circles) HIV-$1_{L33S/N43D/I69L/E137K/S138A}$ (open squares), and HIV-$1_{L33S/N43D/L44L\text{-}CTG/I69L/E137K/S138A}$ (closed squares). $L_{TTG}44L_{CTG}$ introduction statistically enhanced both replication of HIV-1_{WT} and HIV-$1_{L33S/N43D/L44L\text{-}CTG/I69L/E137K/S138A}$ (Student's *t*-test, $p < 0.01$ on day 4 and 5). Relative infectivity (blue cell counts in MAGI cells divided by amount of p24) was calculated (B). Error bars indicate SD of three determinations. Decrease of infectivity between HIV-$1_{L33S/N43D/I69L/E137K/S138A}$ and HIV-$1_{L33S/N43D/L44L\text{-}CTG/I69L/E137K/S138A}$ were statistically significant (Student's *t*-test, $p < 0.05$).

the introduction of a secondary resistance mutation (E137K) in the backbone of a peptide fusion inhibitor is a useful change that results into more potent fusion inhibitors, even for HIV-1 strains that are resistant to peptide fusion inhibitors.

Selection of T-20_{S138A}-resistance starting with wild-type HIV-1 resulted in the emergence of I37N and L44M substitutions, which were located in the N-HR region that is thought to interact with T-20. Other substitutions at position 37 (I37T or I37K) also conferred resistance to T-20 and C34 (Nameki et al., 2005), suggesting that I37 in N-HR is critical for the attachment of C-HR-derived peptide fusion inhibitors. The L44M mutation has only been observed in subtype B HIV-1-infected patients treated with T-20 (Carmona et al., 2005), and conferred weak resistance to T-20 (Loutfy et al., 2007). In this study, L44M did not confer resistance to all peptide inhibitors; however, L44M in combination with other mutations (I37N/N126K) remarkably enhanced resistance to T-20_{S138A}, suggesting that L44M serves as a secondary mutation to enhance resistance to T-20_{S138A}. N126K also enhances resistance to some fusion inhibitors (Baldwin et al., 2004; Nameki et al., 2005; Eggink et al., 2008) by helping recover losses in intra-gp41 interactions that were caused by primary mutations, such as N43D.

When we selected T-20_{S138A}-resistant HIV-1 (HIV-$1_{N43D/S138A}$) we obtained a somehow different set of mutations that included L33S, which is located at the presumed T-20 binding site at N-HR, as well as I37N, N43D, and L44M. L33S was previously reported in HIV-1 variants resistant to T-20 (Fikkert et al., 2002), C34 (Armand-Ugon

Table 3
Antiviral activity of C-HR-derived peptides against gp160 recombinant viruses.

Compound	EC_{50} (nM)	
ddC	771 ± 272	
T-20 derivatives		
T20	>10,000	(NA)
T20EK	2729 ± 1113	(NA)
$T20_{S138A}$	3126 ± 453	(NA)
$T20_{E137K}$	2761 ± 1477	(NA)
$T20_{E137K/S138A}$	203 ± 54	(0.6)
C34 derivatives		
C34	171.0 ± 106	(3.4)
$C34_{N126K}$	25.9 ± 4.6	(NA)
SC34EK	1.0 ± 0.8	(1)
$C34_{E137K}$	7.0 ± 4.4	(0.4)
$C34_{E137K/S138A}$	0.3 ± 0.1	(0.3)

Anti-HIV activity was determined using the MAGI assay. Fifty percent effective concentration (EC_{50}) values and SD were obtained from the results of at least three independent experiments. Shown in parentheses are the fold-increases in resistance (increase in EC_{50} value) calculated by comparison to the resistant clone with mutations only in gp41 (HIV-$1_{L33S/N43D/I69L/E137K/S138A}$). To improve the replication kinetics, substitution of D36G, observed in majority of HIV-1 strains, was introduced into the NL4-3 background used in this study (Izumi et al., 2009; Mink et al., 2005). NA, not available; ddC, dideoxycytidine.

et al., 2003), and a membrane-anchored C-HR-derived peptide, M87 (Lohrengel et al., 2005). Although our work clearly demonstrates that L33S is involved in resistance to T-20 derivatives, it was not possible to discern whether L33S affected binding affinity to C-HR in the CD analyses because L33 was not in the sequence of the N36 N-HR peptide that we had to use in this study. As shown in Fig. 2, L33S did not significantly affect replication kinetics compared with HIV-1_{WT}, suggesting that L33S might sustain binding affinity with C-HR to form a stable six-helix bundle. The L33S mutation is located in the loop of stem IIc of the RRE (Ueno et al., 2009). Hence, nucleotide changes for L33S do not require compensatory mutations to maintain secondary structure of the RRE. Therefore, it is likely that L33S has little effect on replication kinetics. In this study, L33S conferred little resistance to C34 in this study, while it was previously reported to confer up to 10-fold resistance (Armand-Ugon et al., 2003), suggesting that some other viral background might affect the resistance, since Armand-Ugon et al. (2003) examined bulk virus samples obtained from the selection.

A prevalent polymorphism, E137K, which was associated with N43D *in vivo* (Svicher et al., 2008), has been proven to restore infectivity that has been impaired by N43D (Tolstrup et al., 2007). E137K did not affect susceptibility to all peptide fusion inhibitors by itself, but in combination with primary mutations, it remarkably enhanced resistance to T-20_{S138A}. Moreover, introduction of the E137K change into N43D/S138A enhanced the viral replication kinetics as shown in Fig. 2. A possible hydrogen bond between K137 and D43 may partially restore the reported loss in six-helix bundle stability conferred by the N43D mutation (Bai et al., 2008), suggesting that E137K can compensate for losses in the interactions between N-HR_{N43D} and C-HR. This hypothesis is consistent with our CD results presented in Fig. 3.

Because E137K restored binding affinity with N-HR similar to the S138A mutation, we expected that introduction of E137K into T-20 would effectively suppresses replication of T-20-resistant HIV-1. We examined the antiviral activity of E137K- and E137K/S138A-containing T-20 and C34 to T-20_{S138A}-resistant HIV-1. We found that T-20_{E137K} had similar antiviral activity with other T-20 derivatives such as T-20_{S138A} and T-$20_{E137K/S138A}$. Hence, we believe that the combination of few substitution secondary mutations can enhance the antiviral activity of peptide fusion inhibitors. Therefore, it is possible to design peptides that include the secondary mutations in the C-HR and use them by themselves and/or in combinations to block fusion inhibitor resistant viruses. Importantly, we have successfully applied this strategy to suppress HIV-1 resistance to next generation fusion inhibitor SC34EK (Shimura et al., 2010).

In this study, we identified two distinct pathways to escape pressure of T-20_{S138A}. Emergence of drug resistance mutants under drug pressure involves a stochastic selection. Nonetheless, the makeup of the final population depends on both the ability of specific populations to evade the drug, as well as their fitness that determines their representation in the escape population. There are several examples in the literature where HIV becomes resistant to the same drug by different mechanisms. For example, in the case of the most commonly used drugs that target HIV reverse transcriptase (RT), the virus can develop multidrug resistance by either the Q151M complex pathway (Kavlick et al., 1998; Shirasaka et al., 1995) or by accumulation of thymidine associated mutations (TAMs) (Hachiya et al., 2008; Kosalaraksa et al., 1999). We recently report some of background polymorphisms can also influence resistance pathways, such 172R/K in the RT region (Hachiya et al., 2012). In the case of the T-20S138A inhibitor, the N43D/S138A may also act as such polymorphisms despite the presence of primary mutations (Izumi et al., 2009) and preferentially affect the emergence of specific mutations.

5. Conclusion

As previously discussed (Izumi et al., 2009), although other developed peptide-based fusion inhibitors need many amino acid additions and/or substitutions for the enhancement of their antiviral activity (Chinnadurai et al., 2007; Eggink et al., 2008; Dwyer et al., 2007; Otaka et al., 2002), application of secondary mutations similar to T-20_{S138A} and T-$20_{E137K/S138A}$ is straightforward. It is based on information from viral evolution studies under drug pressure that help design improved inhibitors.

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HIV-1 Resistance Mechanism to an Electrostatically Constrained Peptide Fusion Inhibitor That Is Active against T-20-Resistant Strains

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T-20EK is a novel fusion inhibitor designed to have enhanced α-helicity over T-20 (enfuvirtide) through engineered electrostatic interactions between glutamic acid (E) and lysine (K) substitutions. T-20EK efficiently suppresses wild-type and T-20-resistant variants. Here, we selected T-20EK-resistant variants. A combination of L33S and N43K substitutions in gp41 were required for high resistance to T-20EK. While these substitutions also caused resistance to T-20, they did not cause cross-resistance to other known fusion inhibitors.

Enfuvirtide (T-20), a 36-amino-acid peptide derived from the C-terminal heptad repeat (C-HR) of HIV-1 gp41, has been approved as the first fusion inhibitor of HIV-1 entry. T-20 inhibits HIV-1 replication by interfering with the formation of the fusion intermediate six-helix bundle, which is composed of three N-terminal heptad repeats (N-HRs) and three C-HRs arranged in an antiparallel orientation (1). Because of its unique mechanism of action, T-20 effectively suppresses replication of HIV-1 resistant to inhibitors targeting the reverse transcriptase and protease (2, 3). However, long-term therapy with a T-20-containing regimen can result in the emergence of T-20-resistant strains (4, 5). These strains contain substitutions at the N-HR region of gp41, including G36D, V38A, and N43K/D, both *in vitro* and *in vivo*, and exhibit reduced susceptibility to T-20 through decreased binding of T-20 to the mutated N-HR (6–14). To suppress replication of such variants and obtain durable efficacy in HIV-1-infected patients, new fusion inhibitors are needed.

To date, several novel fusion inhibitors have been developed, including tifuvirtide (T-1249) (15), sifuvirtide (SFT) (16), and T-2635 (TRI-1144) (17), that potently suppress replication of T-20-resistant variants (Fig. 1A), as well as D-peptide-based (18) or small-molecule inhibitors (19). We recently developed the electrostatically constrained fusion inhibitors SC35EK and its 29-residue shorter form, SC29EK, which also inhibit replication of T-20-resistant HIV-1 (20, 21). These are peptides with electrostatic interactions between glutamic acid (E) and lysine (K) substitutions placed at the *i* and *i* + 4 positions in the solvent-interacting site (EK motif) and are designed to enhance the α-helicity of the peptides (22). The enhancement in α-helicity correlates well with an enhancement in binding affinity for the targeted region and appears to be a key determinant for inhibition of T-20-resistant HIV-1. In addition to C34 (Fig. 1A), we have also applied the EK modification to T-20, termed T-20EK, that shows sustained activity to T-20-resistant variants and HIV-2 strains (23). Moreover, T-20EK showed activity in an animal model (24). To address the mechanism of HIV-1 resistance to T-20EK *in vitro*, we selected T-20EK-resistant HIV-1 strains by using a dose escalation method, identified the primary substitutions that caused resistance to this inhibitor, and evaluated susceptibility of the T-20EK-resistant strains to other fusion inhibitors.

Selection passages were carried out in MT-2 cells using HIV-1_{NL4-3} as the starting wild-type virus (25, 26). The first HIV-1 mutants with enhanced susceptibility to T-20EK emerged at passage 22 (P-22) and were A314T in gp120 and D36G in gp41 (Fig. 1B). The D36G substitution has been widely observed in HIV-1 strains and is thought to contribute to efficient replication rather than causing resistance by decreasing binding to the inhibitor (10, 27, 28). At P-44, we observed the K63N change and a mixture of asparagine and lysine at residue 43 (N43N/K) in gp41. Substitutions in gp120 (see Fig. S1 in the supplemental material) appear to be polymorphisms, because these substitutions were not directly involved in resistance (see Table S1 in the supplemental material). Moreover, S128N and S162N are reported as polymorphisms in the Los Alamos Database (Los Alamos National Library, HIV Sequence Database; http://www.hiv.lanl.gov) and are observed as mixed viruses over a relatively long period of time. We (21, 25, 26) and others (29) have previously reported that substitutions in gp120 can enhance fusion kinetics (30, 31) but do not significantly affect susceptibility to fusion inhibitors. Finally, HIV-1 acquired L33S, N43K, and cytoplasmic tail (CT) substitutions, resulting in viruses that replicated efficiently even in the presence of 1,000 nM T-20EK.

We prepared HIV-1 recombinant clones with the substitutions discovered during our passages and determined the antiviral activities of T-20EK and other peptides against the T-20EK-resistant variants (Fig. 1B) and clones by using a MAGI (multinuclear ac-

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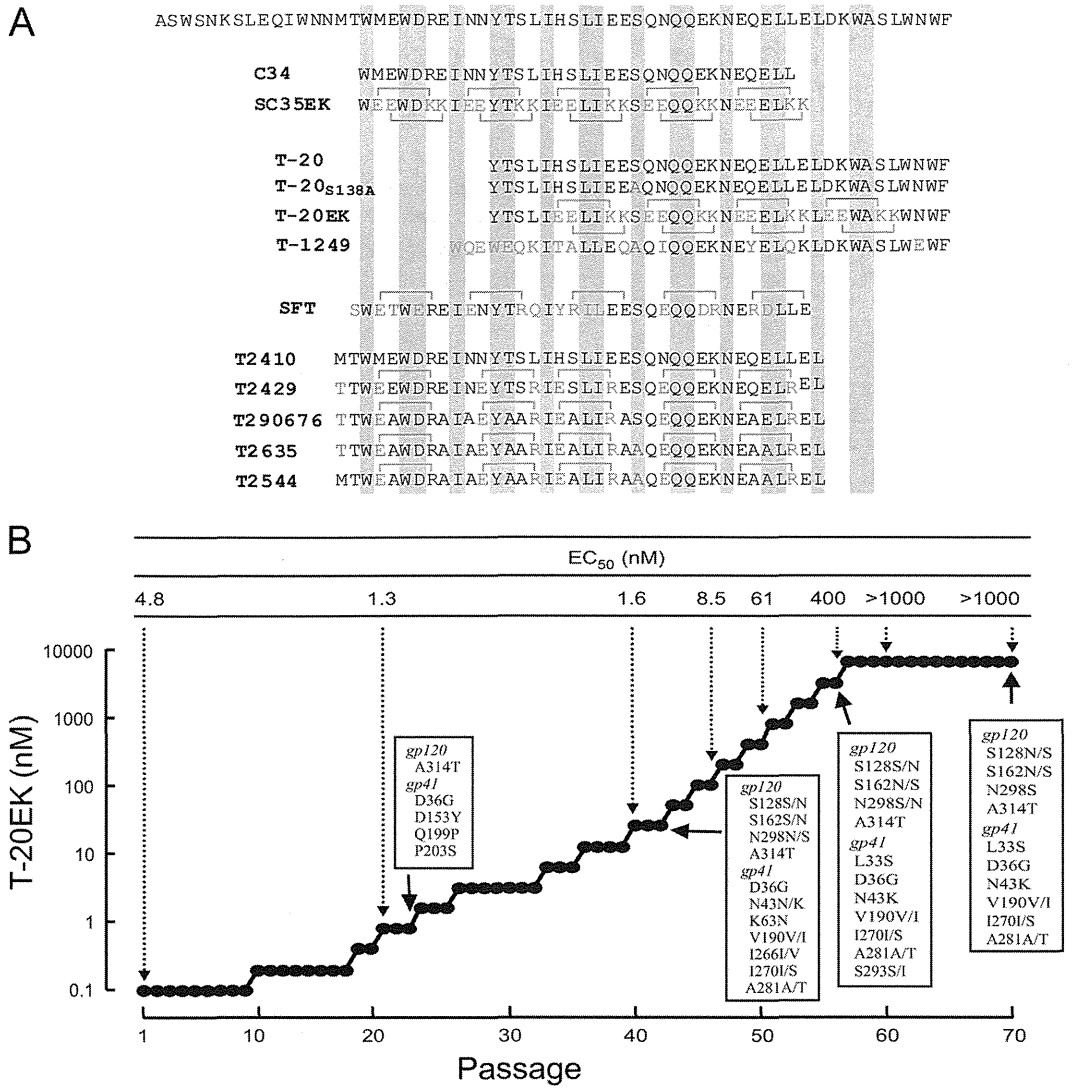

FIG 1 (A) Amino acid sequences of fusion-inhibitory peptides used in this study. The HIV-1 C-HR amino acid sequence is shown in the first row. Electrostatic interactions are indicated in pink and light blue for acidic (glutamic acid [E]) and basic (lysine [K]) residues, respectively. Modified amino acids are indicated in orange. A resistance-associated substitution, S138A, is indicated in red. Amino acids for the interactive site are shaded gray. (B) The dose escalation method for selection of T-20EK resistance through passage in MT-2 cells. Induction of resistant HIV-1 was performed over a total of 70 passages from 0.1 nM T-20EK. At the indicated passage, proviral DNAs were sequenced, and the 50% effective concentrations (EC_{50}s) of the HIV-1 variants were determined in a MAGI assay. All substitutions shown in boxes were observed in combination.

tivation of a β-galactosidase indicator) assay (10, 25, 26). Our data revealed that L33S and N43K are major primary substitutions for T-20EK resistance (Table 1). Substitutions in the CT domain weakly enhanced the resistance induced by L33S/N43K. We previously showed that the S138A substitution in T-20 ($T\text{-}20_{S138A}$) leads to substantial inhibition of the T-20-resistant variant HIV-$1_{L33S/N43K}$ (10, 14, 23). Interestingly, $T\text{-}20EK_{S138A}$, a variant of T-20EK that is expected to exert strong activity to resistant variants, did not inhibit efficiently HIV-$1_{L33S/N43K}$ (Table 1). We found cross-resistance between T-20EK and other T-20-based fusion inhibitors, except for T1249, which has an amino acid sequence that overlaps with T-20 (Fig. 1A). In contrast, C34 and its derivatives maintained their activity to T-20EK-resistant variants. These results indicated that T-20EK may show cross-resistance only with T-20-derived peptides and that the mechanisms of resistance to T-20 and C34 derivatives are different.

During the selection, we observed one substitution in the gp41 transmembrane domain, V190I, and two in the cytoplasmic domain (intravirion), I270S and A281T (Fig. 1B). These three substitutions are also observed in T-20-naive isolates (Los Alamos National Library, HIV Sequence Database; http://www.hiv.lanl.gov). Although these substitutions contributed little to the resistance (Table 1), I270S/A281T substitutions in gp41 cytoplasmic tail restored significantly reduced replication kinetics by substitutions in the ectodomain and transmembrane domain (see Fig. S2 in the supplemental material). Other substitutions, K63N, D153Y, Q199P, P203S, I266V, and S293I, were transiently observed but later disappeared during the selection (see Fig. S1 in the supplemental material). K63N is located adjacent to Q64, which was previously shown to be a resistance-associated substitution (32). The synonymous change in V72 (GTG to GTA) may influence the RRE structural stability, as we have previously described (28).

TABLE 1 Susceptibilities of HIV-1 recombinant clones with substitutions in gp41 to peptide fusion inhibitors[a]

Peptide	EC_{50} (nM) of HIV-1 clone				
	HIV-1_{WT}[b]	HIV-1_{L33S}	HIV-1_{N43K}	HIV-$1_{L33S/N43K}$	HIV-$1_{L33S/N43K/T\text{-}CT}$[c]
Dideoxycytosine	356 ± 89	347 ± 129 (1.0)	489 ± 37 (1.4)	431 ± 75 (1.2)	474 ± 273 (1.3)
T-20-based peptides					
T-20	2.5 ± 0.2	66 ± 9 (**26**)	31 ± 4 (**12**)	>1,000 (>**400**)	>1,000 (>**400**)
$T\text{-}20_{S138A}$	0.55 ± 0.14	3.2 ± 0.7 (5.8)	0.45 ± 0.07 (0.8)	30 ± 10 (**55**)	73.3 ± 23 (**133**)
T-20EK	1.2 ± 0.3	12 ± 2 (**10**)	12 ± 1 (**10**)	>1,000 (>**833**)	>1,000 (>**833**)
$T\text{-}20EK_{S138A}$	0.43 ± 0.06	2.2 ± 0.4 (5.1)	2.3 ± 0.7 (5.4)	112 ± 20 (**260**)	301 ± 110 (**700**)
T1249	0.29 ± 0.07	0.31 ± 0.10 (1.1)	0.35 ± 0.10 (1.2)	0.60 ± 0.16 (2.2)	ND
C34-based peptides					
C34	1.1 ± 0.2	2.9 ± 0.4 (2.6)	2.6 ± 0.7 (2.4)	4.1 ± 1.6 (3.7)	9 ± 5.2 (8)
SC35EK	1.7 ± 0.4	1.8 ± 0.3 (1.1)	2.4 ± 0.5 (1.4)	2.5 ± 0.6 (1.5)	3.4 ± 1.9 (2)
Sifvirtide	2.1 ± 0.4	2.5 ± 0.2 (1.2)	1.5 ± 0.3 (0.7)	2.2 ± 0.5 (1.0)	ND
T2410	1.1 ± 0.3	0.64 ± 0.23 (0.6)	1.8 ± 0.5 (1.6)	1.4 ± 0.5 (1.3)	ND
T2429	1.9 ± 0.3	1.9 ± 0.5 (1.0)	2.5 ± 0.8 (1.3)	1.8 ± 0.1 (0.9)	ND
T290676	0.46 ± 0.14	0.54 ± 0.12 (1.2)	0.46 ± 0.10 (1.0)	1.1 ± 0.5 (2.4)	ND
T2635	0.43 ± 0.02	0.55 ± 0.22 (1.3)	1.4 ± 0.6 (3.3)	0.57 ± 0.22 (1.3)	1.7 ± 0.4 (4)
T2544	0.46 ± 0.08	1.3 ± 0.3 (2.8)	2.4 ± 0.4 (5.2)	1.1 ± 0.4 (2.4)	ND

[a] Drug susceptibilities were determined in a MAGI assay and are reported here as means ± standard deviations from at least three independent assays. Values in parentheses are the fold change in resistance compared with HIV-1_{WT}. Peptide fusion inhibitors that caused more than 10-fold resistance are indicated in bold. ND, not determined.
[b] $NL4.3_{D36G}$ was defined as wild-type HIV-1 (HIV-1_{WT}).
[c] T-CT (transmembrane and cytoplasmic tail domains) indicates the substitutions V190I, I270S, and A281T, which were only observed in a mixture.

With the exception of D153Y, Q199P, and P203S in gp41, all substitutions were observed in the vast majority of T-20-naive patients, indicating that they are natural polymorphisms. P203S was also selected as a low-level SC34EK resistance-associated substitution (26). This is consistent with our data showing that combinations of CT substitutions only enhance resistance by 2- to 3-fold (Table 1). Moreover, most substitutions coexisted with variants containing the wild-type sequence, strongly indicating that they, as well as those in gp120, exerted only a modest effect on resistance.

We previously demonstrated that T-20EK inhibited T-20-resistant variants harboring G36D, V38A, or N43D/K substitutions (28) and that it maintains its strong antiviral effect against HIV-2 (23). T-20EK-resistant variants showed very limited cross-resistance to other novel fusion inhibitors, with the exception of T-20-based peptides, indicating that the combination of T-20EK with other new fusion inhibitors may be suitable for therapy. The enhanced hydrophilicity of T-20EK by the engineered hydrophilic amino acids (Glu and Lys) is a favorable property for solubility, which is expected to reduce some of the adverse effects of T-20, such as skin reactions at the injection site (33). Interestingly, our experiments did not result in the selection of any secondary substitutions in the C-HR, such as N126K and S138A, that are frequently observed in variants that are resistant to novel fusion inhibitors (26, 34, 35). In contrast, one of the primary substitutions was L33S, a substitution at a site outside the N-HR. Notably, the replication kinetics of L33S are comparable to those of wild-type HIV-1 (unpublished data). Therefore, L33S seems to be a T-20-specific substitution that does not require the presence of secondary substitutions. Importantly, use of T-20EK does not lead to the appearance of substitutions that confer cross-resistance to other novel fusion inhibitors. Thus, our study establishes that T-20EK can become an efficient new fusion inhibitor.

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