type of KAY-2-41^r strains. We finally showed, in our TK competition assays, that KAY-2-41 competed with the dThd substrate of VACV TK. These findings demonstrate that the mutations found in the viral TK most probably occurred due to KAY-2-41 selection pressure and that viral TK may contribute to KAY-2-41 sensitivity, although it may not be the sole determinant. As a consequence, additional studies are still required (i) to determine whether the identified mutations or mutations in other genes contribute to drug resistance, (ii) to identify the metabolites of the compound, and (iii) to investigate whether cellular partners can be implicated in KAY-2-41 antiviral activity.

Both the KAY-2-41^r viruses and viruses with TK deletions showed hypersensitivity to BVDU. While we and others have shown that VACV TK and hTK1 do not measurably phosphorylate BVDU in enzymatic assays (30), the mitochondrial homodimer hTK2 was efficient in this activity (Table 4). Also, the secondary phosphorylation of BVDU has been shown to rely on the viral TMPK (33), and we observed that KAY-2-41 still potently inhibited TMPK OPV mutants resistant to BVDU (our unpublished data). Although these two molecules seem to have a distinct mode of activation, how can we explain this hypersensitivity? The TK deletion could affect pyrimidine metabolism and, consequently, the pool of available nucleotides (i.e., dTTP) needed for viral replication. For these reasons, more BVDU triphosphate might then be accepted as a substrate for incorporation by the DNA polymerase (E9L), potentiating BVDU antiviral activity. Alternatively, it cannot be excluded that the mutations in the viral TK may make BVDU a better substrate for further phosphorylation.

We can further speculate about the impact of the identified mutations on TK enzymatic activity, albeit we did not prove, through marker rescue experiments, that such changes were responsible for KAY-2-41 resistance. In KAY-2-41-resistant VACV-WR, a single nucleotide deletion caused a codon frameshift starting at amino acid 15 (S15Q) and finishing at position 21 with a stop codon, and in the CPXV-BR strain, the double nucleotide deletion changed the valine codon at position 25 into a stop codon. These deletion mutations occurred in the amino-terminal region of the gene and presumably led to the production of a truncated TK (Fig. 2 and 6). This region is critical for TK enzymatic activity, as it includes the site where ATP binds to act as a γ-phosphoryl donor required to produce dThd monophosphate. In VACV, this region consists of a P loop (amino acids 11 to 16) preceded by a β sheet (β1, amino acids 4 to 10) and followed by an α helix (α1, amino acids 17 to 29) (44, 45). A similar organization is found in structurally related TKs, including the human cytosolic TK (hTK1) and the TK of *Thermotoga maritima* (*Tm*TK) (45, 46).

The amino acid residue mutated in drug-resistant CML1 (D110Y) mapped to a highly conserved residue in the type II TK family (D110) that is localized close to phenylalanine 113 (F113), which has been shown to interact with the substrate via its main-chain amide nitrogen (Fig. 2) (44). The substrate binding region has been well characterized in the model of VACV TK bound to dTTP (44), as well as in related hTK1 and *Tm*TK bound to a bisubstrate analogue, TP4A {P^1-(5′-adenosyl)P^4-[5′-(2′-deoxythymidyl)]tetraphosphate} (45). While no direct interactions between D110 and the nucleoside substrate have been described, we observed that changing a negatively charged amino acid (i.e., aspartic acid [D]) into a residue with a hydrophobic aromatic side chain (i.e., tyrosine [Y]) may disturb the loop localized between the β5 sheet (amino acids 106 to 110) and the α4 helix (amino acids 121 to 128) and, as a consequence, the binding of the nucleoside substrate (Fig. 6). This mutation may have a second impact on VACV TK enzymatic activity, since this enzyme is catalytically active as a homotetramer. Based on the model of El Omari and colleagues (44), we observed that the D110 residue is located at the interface of two monomers (Fig. 6). The mutation D110Y may thus create a steric hindrance that can disrupt monomer assembly, explaining a loss of enzymatic activity.

KAY-2-41 was found to be cytostatic for growing cells, although no apparent morphological changes were seen on confluent (monolayer) cells. Also, the KAY-2-41^r virus strains did not show high levels of resistance to the drug (from 2.7- to 6.0-fold increases in EC_{50}s), and they remained partially sensitive to the inhibitory effect of KAY-2-41. These observations suggest that other cellular enzymes may be involved in the mode of action of the molecule as well. Similar observations have been made with 4′-thioIDU (27). Animal experiments, however, showed that a repetitive dose of 50 mg/kg once daily for 5 consecutive days did not visually affect the naive animals. However, toxicity studies in animals are required to investigate this in more detail. Additionally, the fact that the KAY-2-41^r virus strains did not display high levels of drug resistance suggests that KAY-2-41-based antiviral treatment might still be efficacious if resistant mutants would emerge. Also, such TK-altered viruses might show a decreased pathogenicity, as noticed before in mice challenged with VACV with a TK deletion (47), albeit this property was not assessed here.

Finally, we demonstrated the potency of KAY-2-41 in protecting mice from disease and subsequent death after a lethal challenge with VACV-WR. This was observed with a daily treatment regimen of 50 mg/kg for 5 consecutive days, while reducing the drug concentration to 5 mg/kg protected against mortality but not weight loss. After i.p. administration of KAY-2-41 (50 mg/kg), virus replication was completely abolished in the primary target organs, i.e., lungs, as well as in the liver, spleen, and kidneys. Our results also showed that the levels of viral DNA in serum or in several organs were markedly reduced or even abolished. In contrast, oral administration of 4′-thioIDU (15 mg/kg twice daily for 5 consecutive days beginning at 24 h after infection) reduced lung virus titers by only 10-fold, as reported by Kern and colleagues (27), although differences in the doses and routes of administration of the compounds and/or the volume of virus inoculated might be at the origin of the discrepancy in the results. Histology of the lungs of VACV-WR-infected mice revealed signs only of inflammation and not of pneumonia, which could be due to (i) the time point of sacrifice of the animals, i.e., at day 5 p.i., or (ii) the use of a relatively small volume for virus inoculation, as discussed by Smee and colleagues (48).

The rationale of using viral TKs in the selective activation of nucleoside analogues mainly comes from research done with herpesviruses, which possess a type 1 TK that can phosphorylate a variety of substrates. Although OPVs encode a TK, its narrower substrate specificity and its resemblance to hTK1 have hampered the development of TK-specific drugs for OPVs. However, VACV TK might have a substrate specificity broader than was initially thought (44, 49). Indeed, the structure of VACV TK and the demonstration that a bulky nucleoside analogue can be OPV-TK specific, as shown by Fan et al. (49), have provided new evidence that conformational differences between OPV TK and hTK1 may be exploited to design selective poxvirus inhibitors (44, 49). Altogether, our study proves that nucleoside analogues such as KAY-2-41 and possibly also other 1′-substituted 4′-thiothymidine derivatives should not be neglected as potential antiviral agents, as

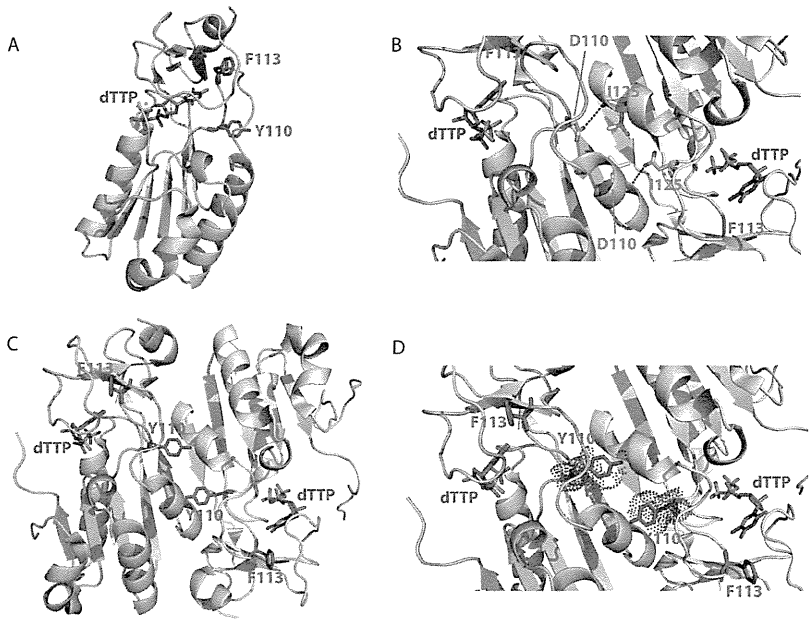

FIG 6 Potential impact of D110Y mutation on substrate binding and thymidine kinase monomer assembly. (A) Tridimensional structure of one monomer of VACV TK in complex with dTTP (Protein Data Bank accession number 2J87). Residue F113, localized in the loop between the β5 sheet and α4 helix and required for substrate binding, is highlighted (see also Fig. 2). On the same loop is also shown the tyrosine residue (red sticks) found at position 110 and identified as the D110Y mutation. The presence of the mutated residue may disturb the loop and, as a consequence, substrate recognition. (B to D) Two monomers in complex with dTTP are shown in green and yellow. The phenylalanine at position 113 is depicted in red on each monomer. The aspartic acid at position 110 of one monomer establishes a direct interaction with the isoleucine 125 of the other monomer (B). Modification of the aspartic acid 110 into a tyrosine provokes a steric hindrance that may destabilize the assembly of monomers (C and D). The figures were generated using PyMol software (version 0.99; DeLano Scientific LLC).

they may show promise for future development of broad-spectrum therapy against both pox- and herpesviruses.

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REFERENCES

1. **Vogel S, Sardy M, Glos K, Korting HC, Ruzicka T, Wollenberg A.** 2012. The Munich outbreak of cutaneous cowpox infection: transmission by infected pet rats. Acta Derm. Venereol. **92:**126–131. http://dx.doi.org/10.2340/00015555-1227.
2. **Silva DC, Moreira-Silva EA, Gomes JA, Fonseca FG, Correa-Oliveira R.** 2010. Clinical signs, diagnosis, and case reports of Vaccinia virus infections. Braz. J. Infect. Dis. **14:**129–134. http://dx.doi.org/10.1590/S1413-86702010000200003.
3. **Bhanuprakash V, Venkatesan G, Balamurugan V, Hosamani M, Yogisharadhya R, Gandhale P, Reddy KV, Damle AS, Kher HN, Chandel BS, Chauhan HC, Singh RK.** 2010. Zoonotic infections of buffalopox in India. Zoonoses Public Health **57:**e149–e155. http://dx.doi.org/10.1111/j.1863-2378.2009.01314.x.
4. **Reynolds MG, Damon IK.** 2012. Outbreaks of human monkeypox after cessation of smallpox vaccination. Trends Microbiol. **20:**80–87. http://dx.doi.org/10.1016/j.tim.2011.12.001.
5. **Centers for Disease Control and Prevention.** 2012. Human orf virus infection from household exposures—United States, 2009-2011. MMWR Morb. Mortal. Wkly. Rep. **61:**245–248.
6. **Al-Qattan MM.** 2011. Orf infection of the hand. J. Hand Surg. Am. **36:**1855–1858. http://dx.doi.org/10.1016/j.jhsa.2011.08.019.
7. **de Carvalho CH, de Andrade AL, de Oliveira DH, Lima E, da Silveira EJ, de Medeiros AM.** 2012. Intraoral molluscum contagiosum in a young immunocompetent patient. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. **114:**e57–e60. http://dx.doi.org/10.1016/j.oooo.2011.10.009.
8. **Fiandeiro PT, Attard N.** 2013. Koebnerized molluscum contagiosum. QJM **106:**1043. http://dx.doi.org/10.1093/qjmed/hcs183.
9. **Elsendoorn A, Agius G, Le Moal G, Aajaji F, Favier AL, Wierzbicka-Hainault E, Beraud G, Flusin O, Crance JM, Roblot F.** 2011. Severe ear chondritis due to cowpox virus transmitted by a pet rat. J. Infect. **63:**391–393. http://dx.doi.org/10.1016/j.jinf.2011.06.004.
10. **Haase O, Moser A, Rose C, Kurth A, Zillikens D, Schmidt E.** 2011. Generalized cowpox infection in a patient with Darier disease. Br. J. Dermatol. **164:**1116–1118. http://dx.doi.org/10.1111/j.1365-2133.2011.10226.x.
11. **Lin HY, Linn G, Liu CB, Chen CJ, Yu KJ.** 2010. An immunocompromised woman with severe molluscum contagiosum that responded well to topical imiquimod: a case report and literature review. J. Low Genit. Tract Dis. **14:**134–135. http://dx.doi.org/10.1097/LGT.0b013e3181bf1a50.
12. **Pelkonen PM, Tarvainen K, Hynninen A, Kallio ER, Henttonen K, Palva A, Vaheri A, Vapalahti O.** 2003. Cowpox with severe generalized eruption, Finland. Emerg. Infect. Dis. **9:**1458–1461. http://dx.doi.org/10.3201/eid0911.020814.
13. **Andrei G, Snoeck R.** 2010. Cidofovir activity against poxvirus infections. Viruses **2:**2803–2830. http://dx.doi.org/10.3390/v2122803.
14. **Hostetler KY.** 2010. Synthesis and early development of hexadecyloxypropylcidofovir: an oral antipoxvirus nucleoside phosphonate. Viruses **2:**2213–2225. http://dx.doi.org/10.3390/v2102213.
15. **Centers for Disease Control and Prevention.** 2009. Progressive vaccinia in a military smallpox vaccinee—United States, 2009. MMWR Morb. Mortal. Wkly. Rep. **58:**532–536.
16. **Kaiser J.** 2007. Smallpox vaccine. A tame virus runs amok. Science **316:**1418–1419. http://dx.doi.org/10.1126/science.316.5830.1418.
17. **Lanier R, Trost L, Tippin T, Lampert B, Robertson A, Foster S, Rose M, Painter W, O'Mahony R, Almond M, Painter G.** 2010. Development of CMX001 for the treatment of poxvirus infections. Viruses **2:**2740–2762. http://dx.doi.org/10.3390/v2122740.
18. **Papanicolaou G, Kurtzberg J, Grimley M, Storch GA, Goyal R, Anderson M, Mommeja-Marin H, Painter W.** 2011. CMX001 is not nephro-

toxic or myelosuppressive in 183 patients with life threatening dsDNA infections including refractory cytomegalovirus, adenovirus, and BK virus, abstr V-499b. Abstr. 51st Intersci. Conf. Antimicrob. Agents Chemother. American Society for Microbiology, Washington, DC.

19. **Gammon DB, Snoeck R, Fiten P, Krecmerova M, Holy A, De Clercq E, Opdenakker G, Evans DH, Andrei G.** 2008. Mechanism of antiviral drug resistance of vaccinia virus: identification of residues in the viral DNA polymerase conferring differential resistance to antipoxvirus drugs. J. Virol. **82:**12520–12534. http://dx.doi.org/10.1128/JVI.01528-08.
20. **Stittelaar KJ, Neyts J, Naesens L, van Amerongen G, van Lavieren RF, Holy A, De Clercq E, Niesters HG, Fries E, Maas C, Mulder PG, van der Zeijst BA, Osterhaus AD.** 2006. Antiviral treatment is more effective than smallpox vaccination upon lethal monkeypox virus infection. Nature **439:** 745–748. http://dx.doi.org/10.1038/nature04295.
21. **Duraffour S, Snoeck R, de Vos R, van Den Oord JJ, Crance JM, Garin D, Hruby DE, Jordan R, De Clercq E, Andrei G.** 2007. Activity of the anti-orthopoxvirus compound ST-246 against vaccinia, cowpox and camelpox viruses in cell monolayers and organotypic raft cultures. Antivir. Ther. **12:**1205–1216.
22. **Yang G, Pevear DC, Davies MH, Collett MS, Bailey T, Rippen S, Barone L, Burns C, Rhodes G, Tohan S, Huggins JW, Baker RO, Buller RL, Touchette E, Waller K, Schriewer J, Neyts J, DeClercq E, Jones K, Hruby D, Jordan R.** 2005. An orally bioavailable antipoxvirus compound (ST-246) inhibits extracellular virus formation and protects mice from lethal orthopoxvirus challenge. J. Virol. **79:**13139–13149. http://dx.doi.org/10.1128/JVI.79.20.13139-13149.2005.
23. **Chinsangaram J, Honeychurch KM, Tyavanagimatt SR, Leeds JM, Bolken TC, Jones KF, Jordan R, Marbury T, Ruckle J, Mee-Lee D, Ross E, Lichtenstein I, Pickens M, Corrado M, Clarke JM, Frimm AM, Hruby DE.** 2012. Safety and pharmacokinetics of the anti-orthopoxvirus compound ST-246 following a single daily oral dose for 14 days in human volunteers. Antimicrob. Agents Chemother. **56:**4900–4905. http://dx.doi.org/10.1128/AAC.00904-12.
24. **Srivastav NC, Rai D, Tse C, Agrawal B, Kunimoto DY, Kumar R.** 2010. Inhibition of mycobacterial replication by pyrimidines possessing various C-5 functionalities and related 2′-deoxynucleoside analogues using in vitro and in vivo models. J. Med. Chem. **53:**6180–6187. http://dx.doi.org/10.1021/jm100568q.
25. **Secrist JA, III, Tiwari KN, Riordan JM, Montgomery JA.** 1991. Synthesis and biological activity of 2′-deoxy-4′-thio pyrimidine nucleosides. J. Med. Chem. **34:**2361–2366. http://dx.doi.org/10.1021/jm00112a007.
26. **Dyson MR, Coe PL, Walker RT.** 1991. The synthesis and antiviral activity of some 4′-thio-2′-deoxy nucleoside analogues. J. Med. Chem. **34:**2782–2786. http://dx.doi.org/10.1021/jm00113a016.
27. **Kern ER, Prichard MN, Quenelle DC, Keith KA, Tiwari KN, Maddry JA, Secrist JA, III.** 2009. Activities of certain 5-substituted 4′-thiopyrimidine nucleosides against orthopoxvirus infections. Antimicrob. Agents Chemother. **53:**572–579. http://dx.doi.org/10.1128/AAC.01257-08.
28. **Rahim SG, Trivedi N, Bogunovic-Batchelor MV, Hardy GW, Mills G, Selway JW, Snowden W, Littler E, Coe PL, Basnak I, Whale RF, Walker RT.** 1996. Synthesis and anti-herpes virus activity of 2′-deoxy-4′-thiopyrimidine nucleosides. J. Med. Chem. **39:**789–795. http://dx.doi.org/10.1021/jm950029r.
29. **Prichard MN, Quenelle DC, Hartline CB, Harden EA, Jefferson G, Frederick SL, Daily SL, Whitley RJ, Tiwari KN, Maddry JA, Secrist JA, III, Kern ER.** 2009. Inhibition of herpesvirus replication by 5-substituted 4′-thiopyrimidine nucleosides. Antimicrob. Agents Chemother. **53:**5251–5258. http://dx.doi.org/10.1128/AAC.00417-09.
30. **Prichard MN, Williams AD, Keith KA, Harden EA, Kern ER.** 2006. Distinct thymidine kinases encoded by cowpox virus and herpes simplex virus contribute significantly to the differential antiviral activity of nucleoside analogs. Antiviral Res. **71:**1–6. http://dx.doi.org/10.1016/j.antiviral.2006.01.013.
31. **Solaroli N, Johansson M, Balzarini J, Karlsson A.** 2006. Substrate specificity of three viral thymidine kinases (TK): vaccinia virus TK, feline herpesvirus TK, and canine herpesvirus TK. Nucleosides Nucleotides Nucleic Acids **25:**1189–1192. http://dx.doi.org/10.1080/15257770600894451.
32. **Caillat C, Topalis D, Agrofoglio LA, Pochet S, Balzarini J, Deville-Bonne D, Meyer P.** 2008. Crystal structure of poxvirus thymidylate kinase: an unexpected dimerization has implications for antiviral therapy. Proc. Natl. Acad. Sci. U. S. A. **105:**16900–16905. http://dx.doi.org/10.1073/pnas.0804525105.
33. **Topalis D, Collinet B, Gasse C, Dugue L, Balzarini J, Pochet S, Deville-Bonne D.** 2005. Substrate specificity of vaccinia virus thymidylate kinase. FEBS J. **272:** 6254–6265. http://dx.doi.org/10.1111/j.1742-4658.2005.05006.x.
34. **Haraguchi K, Takahashi H, Tanaka H, Hayakawa H, Ashida N, Nitanda T, Baba M.** 2004. Synthesis and antiviral activities of 1′-carbon-substituted 4′-thiothymidines. Bioorg. Med. Chem. **12:**5309–5316. http://dx.doi.org/10.1016/j.bmc.2004.07.057.
35. **Duraffour S, Andrei G, Topalis D, Krecmerova M, Crance JM, Garin D, Snoeck R.** 2012. Mutations conferring resistance to viral DNA polymerase inhibitors in camelpox virus give different drug-susceptibility profiles in vaccinia virus. J. Virol. **86:**7310–7325. http://dx.doi.org/10.1128/JVI.00355-12.
36. **Carroll DS, Emerson GL, Li Y, Sammons S, Olson V, Frace M, Nakazawa Y, Czerny CP, Tryland M, Kolodziejek J, Nowotny N, Olsen-Rasmussen M, Khristova M, Govil D, Karem K, Damon IK, Meyer H.** 2011. Chasing Jenner's vaccine: revisiting cowpox virus classification. PLoS One **6:**e23086. http://dx.doi.org/10.1371/journal.pone.0023086.
37. **Pfeffer M, Meyer H, Wernery U, Kaaden OR.** 1996. Comparison of camelpox viruses isolated in Dubai. Vet. Microbiol. **49:**135–146. http://dx.doi.org/10.1016/0378-1135(95)00181-6.
38. **Ramyar H, Hessami M.** 1972. Isolation, cultivation and characterization of camel pox virus. Zentralbl. Veterinarmed. B **19:**182–189.
39. **Gammon DB, Gowrishankar B, Duraffour S, Andrei G, Upton C, Evans DH.** 2010. Vaccinia virus-encoded ribonucleotide reductase subunits are differentially required for replication and pathogenesis. PLoS Pathog. **6:**e1000984. http://dx.doi.org/10.1371/journal.ppat.1000984.
40. **Duraffour S, Snoeck R, Krecmerova M, Van den Oord J, de Vos R, Holy A, Crance JM, Garin D, De Clercq E, Andrei G.** 2007. Activities of several classes of acyclic nucleoside phosphonates against camelpox virus replication in different cell culture models. Antimicrob. Agents Chemother. **51:** 4410–4419. http://dx.doi.org/10.1128/AAC.00838-07.
41. **Duraffour S, Matthys P, van den Oord JJ, De Schutter T, Mitera T, Snoeck R, Andrei G.** 2011. Study of camelpox virus pathogenesis in athymic nude mice. PLoS One **6:**e21561. http://dx.doi.org/10.1371/journal.pone.0021561.
42. **Schwarzer H, Kurth A, Hermel M, Plange N.** 2013. Severe ulcerative keratitis in ocular cowpox infection. Graefes Arch. Clin. Exp. Ophthalmol. **251:**1451–1452. http://dx.doi.org/10.1007/s00417-012-2138-x.
43. **Delhon G, Tulman ER, Afonso CL, Lu Z, Concha-Bermejillo A, Lehmkuhl HD, Piccone ME, Kutish GF, Rock DL.** 2004. Genomes of the parapoxviruses ORF virus and bovine papular stomatitis virus. J. Virol. **78:**168–177. http://dx.doi.org/10.1128/JVI.78.1.168-177.2004.
44. **El Omari K, Solaroli N, Karlsson A, Balzarini J, Stammers DK.** 2006. Structure of vaccinia virus thymidine kinase in complex with dTTP: insights for drug design. BMC Struct. Biol. **6:**22. http://dx.doi.org/10.1186/1472-6807-6-22.
45. **Segura-Pena D, Lutz S, Monnerjahn C, Konrad M, Lavie A.** 2007. Binding of ATP to TK1-like enzymes is associated with a conformational change in the quaternary structure. J. Mol. Biol. **369:**129–141. http://dx.doi.org/10.1016/j.jmb.2007.02.104.
46. **Segura-Pena D, Lichter J, Trani M, Konrad M, Lavie A, Lutz S.** 2007. Quaternary structure change as a mechanism for the regulation of thymidine kinase 1-like enzymes. Structure **15:**1555–1566. http://dx.doi.org/10.1016/j.str.2007.09.025.
47. **Puhlmann M, Brown CK, Gnant M, Huang J, Libutti SK, Alexander HR, Bartlett DL.** 2000. Vaccinia as a vector for tumor-directed gene therapy: biodistribution of a thymidine kinase-deleted mutant. Cancer Gene Ther. **7:**66–73. http://dx.doi.org/10.1038/sj.cgt.7700075.
48. **Smee DF, Gowen BB, Wandersee MK, Wong MH, Skirpstunas RT, Baldwin TJ, Hoopes JD, Sidwell RW.** 2008. Differential pathogenesis of cowpox virus intranasal infections in mice induced by low and high inoculum volumes and effects of cidofovir treatment. Int. J. Antimicrob. Agents **31:**352–359. http://dx.doi.org/10.1016/j.ijantimicag.2007.11.013.
49. **Fan X, Zhang X, Zhou L, Keith KA, Prichard MN, Kern ER, Torrence PF.** 2006. Toward orthopoxvirus countermeasures: a novel heteromorphic nucleoside of unusual structure. J. Med. Chem. **49:**4052–4054. http://dx.doi.org/10.1021/jm060404n.

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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 gp41and 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 ($T\text{-}20_{S138A}$; 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 $N\text{-}HR_{N43D}$ (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 $T\text{-}20_{S138A}$. Moreover, it is not known whether we can expand our strategy and modify $T\text{-}20_{S138A}$ to include the secondary mutation(s) that emerge during the selection of $T\text{-}20_{S138A}$-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 $T\text{-}20_{S138A}$-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 $T\text{-}20_{S138A}$ ($T\text{-}20_{E137K/S138A}$) resulted into a peptide inhibitor effective against $T\text{-}20_{S138A}$-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 $T\text{-}20_{S138A}$

MT-2 cells were exposed to HIV-1 and cultured in the presence of $T\text{-}20_{S138A}$. 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 $T\text{-}20_{S138A}$ 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];

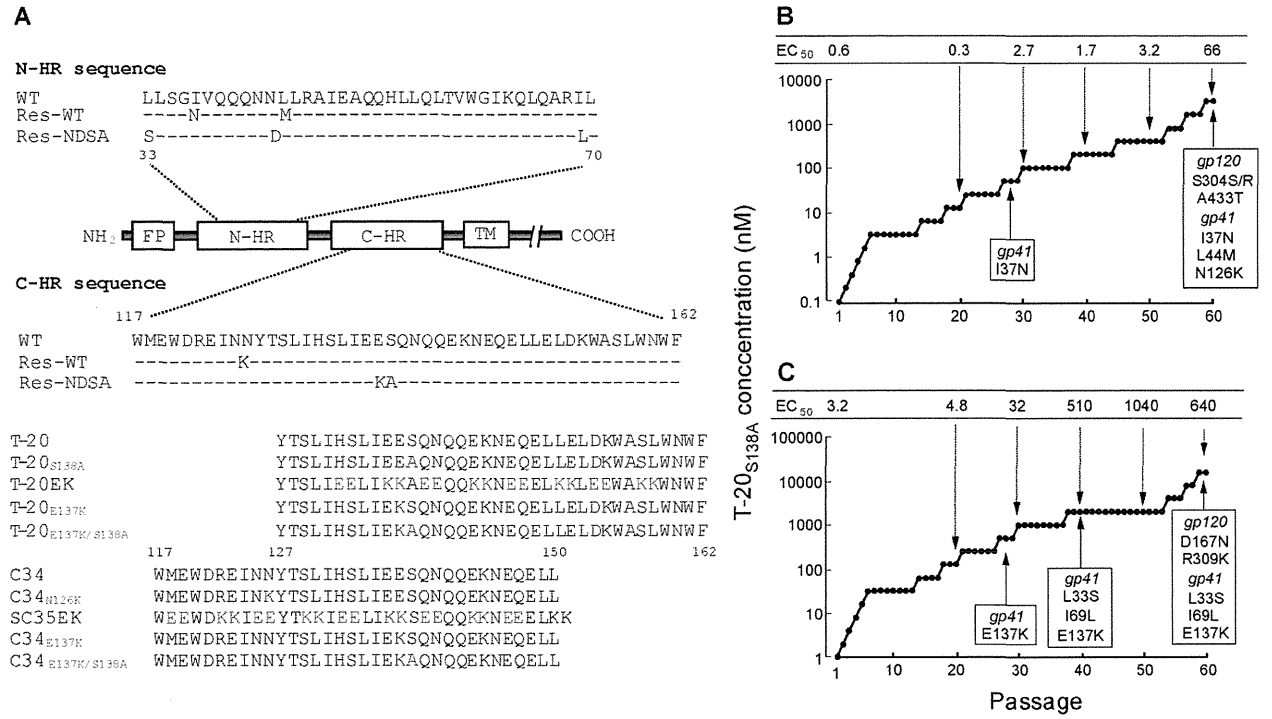


Fig. 1. Domains of gp41 and induction of T-20_{S138A}-resistant HIV-1. (A) Domains of gp41, substitutions observed during *in vitro* passage with T-20_{S138A}, and amino acid sequences of T-20- and C34-based peptides used in this study. The locations of the fusion peptide (FP), amino-terminal heptad 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), L44M 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-20_{S138A}	T-20EK	C34	$C34_{N126K}$	SC35EK
HIV-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-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-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-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-$1_{I37N/N126K}$	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-$1_{I37N/L44M/N126K}$	>1000 (>417)	130±40(220)	240±95(126)	66±23 (31)	4.0±0.8 (2.5)	1.1±0.1(0.5)
HIV-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-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-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-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-$1_{N43D/S138A}$	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-$1_{L33S/N43D/S138A}$	>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-$1_{N43D/E137K/S138A}$	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-$1_{L33S/N43D/E137K/S138A}$	>1000 (**>417**)	>1000(**>1667**)	>1000 (**>526**)	31±5.0 (**15**)	6.7±1.7 (4.2)	1.2±0.2(0.5)
HIV-$1_{L33S/N43D/I69L/E137K/S138A}$	>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-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).

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-$1_{I37N/N126K}$ (HIV-$1_{I37N/L44M/N126K}$) 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-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-$1_{I37N/N126K}$) it enhanced resistance to T-20, T-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-$1_{L33S/N43D/S138A}$). Similar to the N126K mutation, E137K also enhanced resistance by N43D/S138A (HIV-$1_{N43D/E137K/S138A}$) and L33S/N43D/S138A (HIV-$1_{L33S/N43D/E137K/S138A}$) to T-$20_{S138A}$, 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-20_{S138A}-resistant HIV-$1_{N43D/S138A}$ 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-1_{WT} (Fig. 2A). The S138A mutation restored the replication

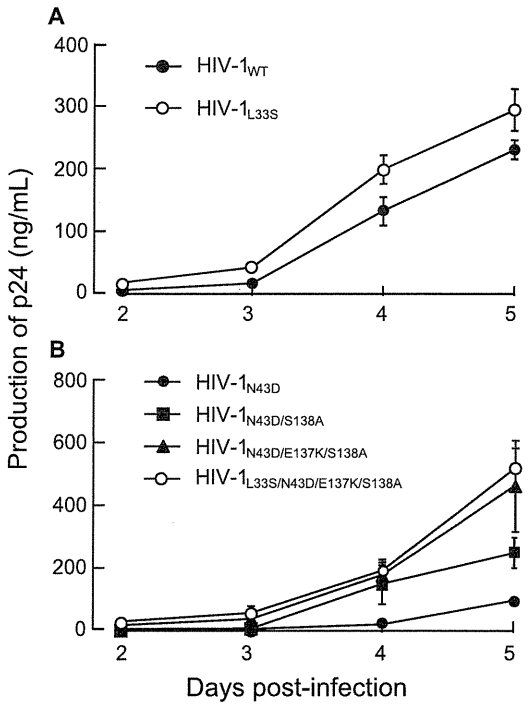


Fig. 2. Replication kinetics of T-20_{S138A}-resistant variants. Replication kinetics of T-20_{S138A}-resistant recombinant variants that introduced L33S mutation (A), or combinations of L33S, E137K, and S138A mutations in HIV-1_{N43D} (B). To improve replication kinetics, the D36G polymorphism was introduced into the NL4-3 background used in this study (HIV-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.

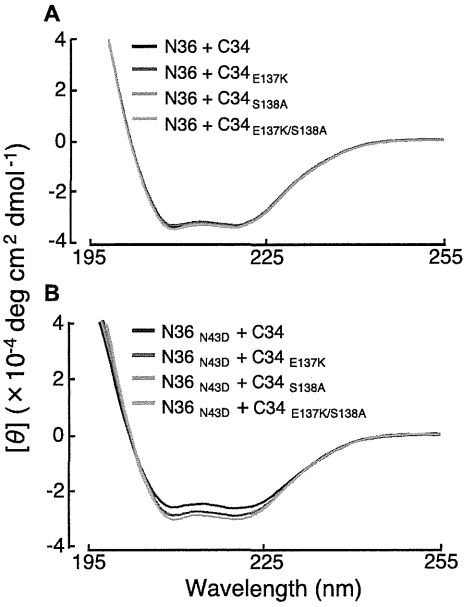
kinetics of HIV-1_{N43D} (Fig. 2B), as previously described (Izumi et al., 2009). E137K was also associated with N43D mutation *in vivo* (Svicher et al., 2008), and restored infectivity impaired by N43D (Tolstrup et al., 2007). Introduction of E137K into N43D/S138A enhanced the replication kinetics, 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).

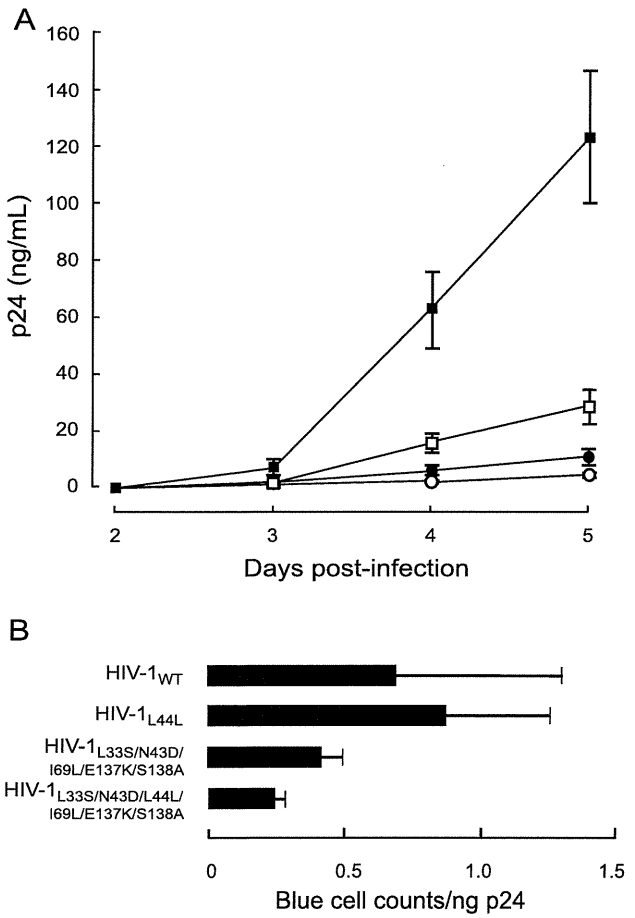


Fig. 4. Effect of secondary mutations in the N-HR on (A) replication kinetics and (B) infectivity. $L_{TTG}44L_{CTG}$ was introduced into HIV-1_{WT} and T-20_{S138A} resistant HIV-1 (HIV-$1_{L33S/N43D/L44L\text{-}CTG/I69L/E137K/S138A}$). Replication kinetics were determined by measuring p24 production 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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References

Aquaro S, D'Arrigo R, Svicher V, Perri GD, Caputo SL, Visco-Comandini U, et al. Specific mutations in HIV-1 gp41 are associated with immunological success in HIV-1-infected patients receiving enfuvirtide treatment. Journal of Antimicrobial Chemotherapy 2006;58:714–22.

Armand-Ugon M, Gutierrez A, Clotet B, Este JA. HIV-1 resistance to the gp41-dependent fusion inhibitor C-34. Antiviral Research 2003;59:137–42.

Bai X, Wilson KL, Seedorff JE, Ahrens D, Green J, Davison DK, et al. Impact of the enfuvirtide resistance mutation N43D and the associated baseline polymorphism E137K on peptide sensitivity and six-helix bundle structure. Biochemistry 2008;47:6662–70.

Baldwin CE, Sanders RW, Deng Y, Jurriaans S, Lange JM, Lu M, et al. Emergence of a drug-dependent human immunodeficiency virus type 1 variant during therapy with the T20 fusion inhibitor. Journal of Virology 2004;78:12428–37.

Bianchi E, Finotto M, Ingallinella P, Hrin R, Carella AV, Hou XS, et al. Covalent stabilization of coiled coils of the HIV gp41N region yields extremely potent and broad inhibitors of viral infection. Proceedings of the National Academy of Sciences of the United States of America 2005;102:12903–8.

Cabrera C, Marfil S, Garcia E, Martinez-Picado J, Bonjoch A, Bofill M, et al. Genetic evolution of gp41 reveals a highly exclusive relationship between codons 36, 38 and 43 in gp41 under long-term enfuvirtide-containing salvage regimen. AIDS 2006;20:2075–80.

Cardoso RM, Brunel FM, Ferguson S, Zwick M, Burton DR, Dawson PE, et al. Structural basis of enhanced binding of extended and helically constrained peptide epitopes of the broadly neutralizing HIV-1 antibody 4E10. Journal of Molecular Biology 2007;365:1533–44.

Carmona R, Perez-Alvarez L, Munoz M, Casado G, Delgado E, Sierra M, et al. Natural resistance-associated mutations to enfuvirtide (T20) and polymorphisms in the gp41 region of different HIV-1 genetic forms from T20 naive patients. Journal of Clinical Virology 2005;32:248–53.

Chan DC, Chutkowski CT, Kim PS. Evidence that a prominent cavity in the coiled coil of HIV type 1 gp41 is an attractive drug target. Proceedings of the National Academy of Sciences of the United States of America 1998;95:15613–7.

Chan DC, Fass D, Berger JM, Kim PS. Core structure of gp41 from the HIV envelope glycoprotein. Cell 1997;89:263–73.

Chinnadurai R, Rajan D, Munch J, Kirchhoff F. Human immunodeficiency virus type 1 variants resistant to first- and second-version fusion inhibitors and cytopathic in ex vivo human lymphoid tissue. Journal of Virology 2007;81:6563–72.

Cilliers T, Patience T, Pillay C, Papathanasopoulos M, Morris L. Sensitivity of HIV type 1 subtype C isolates to the entry inhibitor T-20. AIDS Research and Human Retroviruses 2004;20:477–82.

Debnath AK, Radigan L, Jiang S. Structure-based identification of small molecule antiviral compounds targeted to the gp41 core structure of the human immunodeficiency virus type 1. Journal of Medicinal Chemistry 1999;42:3203–9.

Dwyer JJ, Wilson KL, Davison DK, Freel SA, Seedorff JE, Wring SA, et al. Design of helical, oligomeric HIV-1 fusion inhibitor peptides with potent activity against enfuvirtide-resistant virus. Proceedings of the National Academy of Sciences of the United States of America 2007;104:12772–7.

Dwyer JJ, Wilson KL, Martin K, Seedorff JE, Hasan A, Medinas RJ, et al. Design of an engineered N-terminal HIV-1 gp41 trimer with enhanced stability and potency. Protein Science 2008;17:633–43.

Eggink D, Baldwin CE, Deng Y, Langedijk JP, Lu M, Sanders RW, et al. Selection of T1249-resistant human immunodeficiency virus type 1 variants. Journal of Virology 2008;82:6678–88.

Eggink D, Bontjer I, Langedijk JP, Berkhout B, Sanders RW. 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. Journal of Virology 2011;85:10785–97.
Eshleman SH, Hudelson SE, Bruce R, Lee T, Owens MR, Hackett J, et al. Analysis of HIV type 1 gp41 sequences in diverse HIV type 1 strains. AIDS Research and Human Retroviruses 2007;23:1593–8.
Fikkert V, Cherepanov P, Van Laethem K, Hantson A, Van Remoortel B, Pannecouque C, et al. env chimeric virus technology for evaluating human immunodeficiency virus susceptibility to entry inhibitors. Antimicrobial Agents and Chemotherapy 2002;46:3954–62.
Hachiya A, Kodama EN, Sarafianos SG, Schuckmann MM, Sakagami Y, Matsuoka M, Takiguchi M, Gatanaga H, Oka S. Amino acid mutation N348I in the connection subdomain of human immunodeficiency virus type 1 reverse transcriptase confers multiclass resistance to nucleoside and nonnucleoside reverse transcriptase inhibitors. Jouranl of Virology 2008;82:3261–70.
Hachiya A, Marchand B, Kirby KA, Michailidis E, Tu X, Palczewski K, Ong YT, Li Z, Griffin DT, Schuckmann MM, Tanuma J, Oka S, Singh K, Kodama EN, Sarafianos SG. HIV-1 reverse transcriptase (RT) polymorphism 172K suppresses the effect of clinically relevant drug resistance mutations to both nucleoside and non-nucleoside RT inhibitors. Journal of Biological Chemistry 2012;287:29988–99.
He Y, Xiao Y, Song H, Liang Q, Ju D, Chen X, et al. Design and evaluation of sifuvirtide, a novel HIV-1 fusion inhibitor. Journal of Biological Chemistry 2008;283:11126–34.
Izumi K, Kodama E, Shimura K, Sakagami Y, Watanabe K, Ito S, et al. Design of peptide-based inhibitors for human immunodeficiency virus type 1 strains resistant to T-20. Journal of Biological Chemistry 2009;284:4914–20.
Izumi K, Nakamura S, Nakano H, Shimura K, Sakagami Y, Oishi S, et al. Characterization of HIV-1 resistance to a fusion inhibitor, N36, derived from the gp41 amino-terminal heptad repeat. Antiviral Research 2010;87:179–86.
Kavlick MF, Wyvill K, Yarchoan R, Mitsuya H. Emergence of multi-dideoxynucleoside-resistant human immunodeficiency virus type 1 variants, viral sequence variation, and disease progression in patients receiving antiretroviral chemotherapy. Journal of Infectious Diseases 1998;177: 1506–13.
Kilby JM, Hopkins S, Venetta TM, DiMassimo B, Cloud GA, Lee JY, et al. Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. Nature Medicine 1998;4:1302–7.
Kosalaraksa P, Kavlick MF, Maroun V, Le R, Mitsuya H. Comparative fitness of multi-dideoxynucleoside-resistant human immunodeficiency virus type 1 (HIV-1) in an In vitro competitive HIV-1 replication assay. Jouranl of Virology 1999;73:5356–63.
Kuiken C, Foley B, Leitner T, Apetrei C, Hahn B, Mizrachi I, et al. HIV sequence compendium 2010. Los Alamos, NM: Los Alamos National Laboratory, Theoretical Biology and Biophysics; 2010.
Labrosse B, Labernardiere JL, Dam E, Trouplin V, Skrabal K, Clavel F, et al. Baseline susceptibility of primary human immunodeficiency virus type 1 to entry inhibitors. Journal of Virology 2003;77:1610–3.
Lalezari JP, Henry K, O'Hearn M, Montaner JS, Piliero PJ, Trottier B, et al. Enfuvirtide, an HIV-1 fusion inhibitor, for drug-resistant HIV infection in North and South America. New England Journal of Medicine 2003;348:2175–85.
Lazzarin A, Clotet B, Cooper D, Reynes J, Arasteh K, Nelson M, et al. Efficacy of enfuvirtide in patients infected with drug-resistant HIV-1 in Europe and Australia. New England Journal of Medicine 2003;348:2186–95.
Lohrengel S, Hermann F, Hagmann I, Oberwinkler H, Scrivano L, Hoffmann C, et al. Determinants of human immunodeficiency virus type 1 resistance to membrane-anchored gp41-derived peptides. Journal of Virology 2005;79:10237–46.
Loutfy MR, Raboud JM, Montaner JS, Antoniou T, Wynhoven B, Smaill F, et al. Assay of HIV gp41 amino acid sequence to identify baseline variation and mutation development in patients with virologic failure on enfuvirtide. Antiviral Research 2007;75:58–63.
Malashkevich VN, Chan DC, Chutkowski CT, Kim PS. Crystal structure of the simian immunodeficiency virus (SIV) gp41 core: conserved helical interactions underlie the broad inhibitory activity of gp41 peptides. Proceedings of the National Academy of Sciences of the United States of America 1998;95: 9134–9.
Melby T, Sista P, DeMasi R, Kirkland T, Roberts N, Salgo M, et al. Characterization of envelope glycoprotein gp41 genotype and phenotypic susceptibility to enfuvirtide at baseline and on treatment in the phase III clinical trials TORO-1 and TORO-2. AIDS Research and Human Retroviruses 2006;22:375–85.
Mink M, Mosier SM, Janumpalli S, Davison D, Jin L, Melby T, et al. 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. Journal of Virology 2005;79:12447–54.
Nameki D, Kodama E, Ikeuchi M, Mabuchi N, Otaka A, Tamamura H, et al. Mutations conferring resistance to human immunodeficiency virus type 1 fusion inhibitors are restricted by gp41 and Rev-responsive element functions. Journal of Virology 2005;79:764–70.
Nishikawa H, Nakamura S, Kodama E, Ito S, Kajiwara K, Izumi K, et al. Electrostatically constrained alpha-helical peptide inhibits replication of HIV-1 resistant to enfuvirtide. International Journal of Biochemistry and Cell Biology 2009;41:891–9.
Oishi S, Ito S, Nishikawa H, Watanabe K, Tanaka M, Ohno H, et al. Design of a novel HIV-1 fusion inhibitor that displays a minimal interface for binding affinity. Journal of Medicinal Chemistry 2008;51:388–91.
Oliveira AC, Martins AN, Pires AF, Arruda MB, Tanuri A, Pereira HS, et al. Enfuvirtide (T-20) resistance-related mutations in HIV type 1 subtypes B, C, and F isolates from Brazilian patients failing HAART. AIDS Research and Human Retroviruses 2009;25:193–8.
Otaka A, Nakamura M, Nameki D, Kodama E, Uchiyama S, Nakamura S, et al. Remodeling of gp41-C34 peptide leads to highly effective inhibitors of the fusion of HIV-1 with target cells. Angewandte Chemie International Edition in English 2002;41:2937–40.
Reeves JD, Gallo SA, Ahmad N, Miamidian JL, Harvey PE, Sharron M, et al. Sensitivity of HIV-1 to entry inhibitors correlates with envelope/coreceptor affinity, receptor density, and fusion kinetics. Proceedings of the National Academy of Sciences of the United States of America 2002;99:16249–54.
Shimura K, Nameki D, Kajiwara K, Watanabe K, Sakagami Y, Oishi S, et al. Resistance profiles of novel electrostatically constrained HIV-1 fusion inhibitors. Journal of Biological Chemistry 2010;285:39471–80.
Shirasaka T, Kavlick MF, Ueno T, Gao WY, Kojima E, Alcaide ML, Chokekijchai S, Roy BM, Arnold E, Yarchoan R, et al. Emergence of human immunodeficiency virus type 1 variants with resistance to multiple dideoxynucleosides in patients receiving therapy with dideoxynucleosides. Proceedings of the National Academy of Sciences of the United States of America 1995;92:2398–402.
Svicher V, Aquaro S, D'Arrigo R, Artese A, Dimonte S, Alcaro S, et al. Specific enfuvirtide-associated mutational pathways in HIV-1 Gp41 are significantly correlated with an increase in CD4(+) cell count, despite virological failure. Journal of Infectious Diseases 2008;197:1408–18.
Tolstrup M, Selzer-Plon J, Laursen AL, Bertelsen L, Gerstoft J, Duch M, et al. Full fusion competence rescue of the enfuvirtide resistant HIV-1 gp41 genotype (43D) by a prevalent polymorphism (137 K). AIDS 2007;21:519–21.
Ueno M, Kodama EN, Shimura K, Sakurai Y, Kajiwara K, Sakagami Y, et al. Synonymous mutations in stem-loop III of Rev responsive elements enhance HIV-1 replication impaired by primary mutations for resistance to enfuvirtide. Antiviral Research 2009;82:67–72.
Watabe T, Terakawa Y, Watanabe K, Ohno H, Nakano H, Nakatsu T, et al. X-ray crystallographic study of an HIV-1 fusion inhibitor with the gp41 S138A substitution. Journal of Molecular Biology 2009;392:657–65.
Welch BD, VanDemark AP, Heroux A, Hill CP, Kay MS. Potent D-peptide inhibitors of HIV-1 entry. Proceedings of the National Academy of Sciences of the United States of America 2007;104:16828–33.
Xu L, Pozniak A, Wildfire A, Stanfield-Oakley SA, Mosier SM, Ratcliffe D, et al. Emergence and evolution of enfuvirtide resistance following long-term therapy involves heptad repeat 2 mutations within gp41. Antimicrobial Agents and Chemotherapy 2005;49:1113–9.

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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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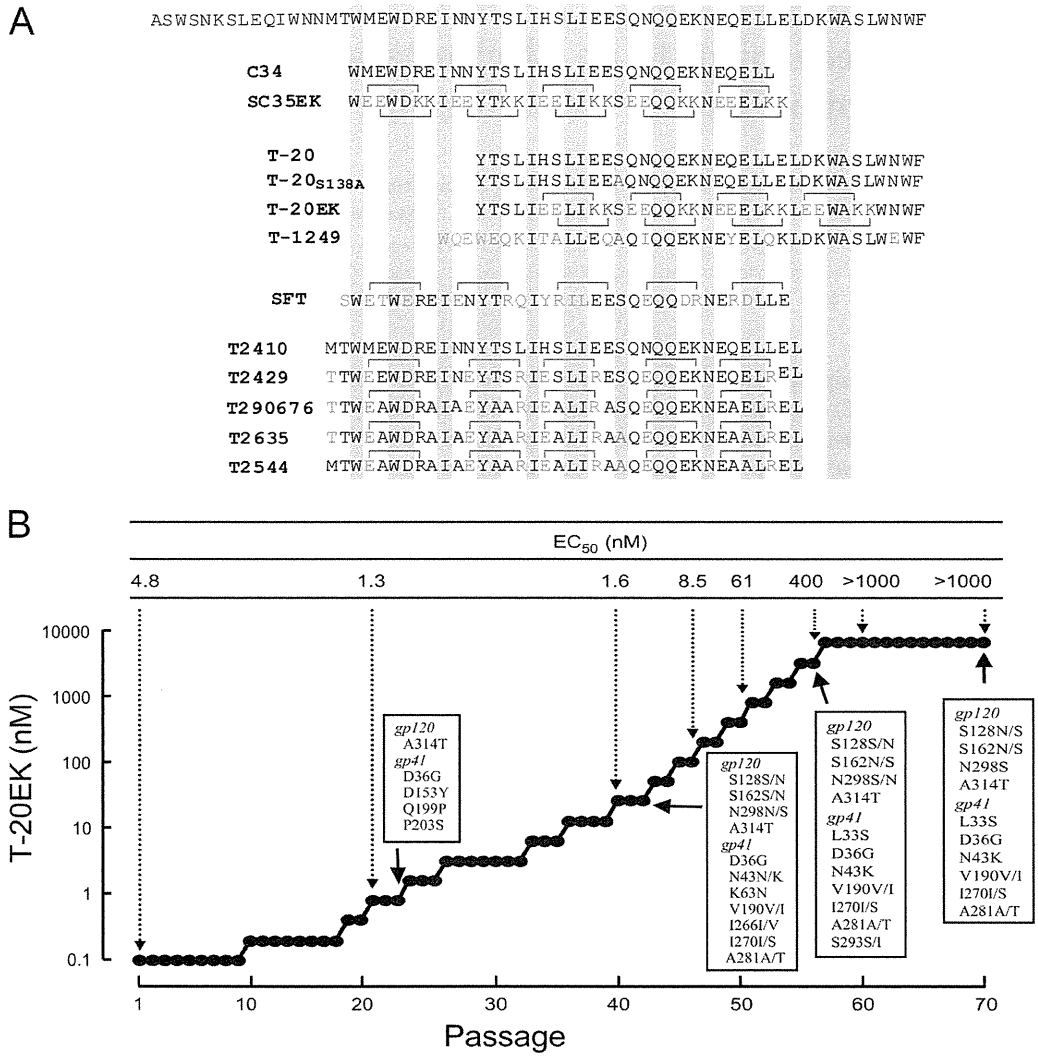

FIG 1 (A) Amino acid sequences of fusion-inhibitory peptides used in this study. The HIV-1 C-HR amino acid sequence is shown in the first row. Electrostatic interactions are indicated in pink and light blue for acidic (glutamic acid [E]) and basic (lysine [K]) residues, respectively. Modified amino acids 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 $HIV\text{-}1_{L33S/N43K}$ (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 $HIV\text{-}1_{L33S/N43K}$ (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-$1_{L33S/N43K/T-CT}$[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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REFERENCES

1. **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.
2. **Lalezari JP, Henry K, O'Hearn M, Montaner JS, Piliero PJ, Trottier B, Walmsley S, Cohen C, Kuritzkes DR, Eron JJ, Jr, Chung J, DeMasi R, Donatacci L, Drobnes C, Delehanty J, Salgo M.** 2003. Enfuvirtide, an HIV-1 fusion inhibitor, for drug-resistant HIV infection in North and South America. N. Engl. J. Med. **348**:2175–2185.
3. **Lazzarin A, Clotet B, Cooper D, Reynes J, Arasteh K, Nelson M, Katlama C, Stellbrink HJ, Delfraissy JF, Lange J, Huson L, DeMasi R, Wat C, Delehanty J, Drobnes C, Salgo M.** 2003. Efficacy of enfuvirtide in patients infected with drug-resistant HIV-1 in Europe and Australia. N. Engl. J. Med. **348**:2186–2195.
4. **Mink M, Mosier SM, Janumpalli S, Davison D, Jin L, Melby T, Sista P, Erickson J, Lambert D, Stanfield-Oakley SA, Salgo M, Cammack N, Matthews T, Greenberg ML.** 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.
5. **Xu L, Pozniak A, Wildfire A, Stanfield-Oakley SA, Mosier SM, Ratcliffe D, Workman J, Joall A, Myers R, Smit E, Cane PA, Greenberg ML, Pillay D.** 2005. Emergence and evolution of enfuvirtide resistance following long-term therapy involves heptad repeat 2 mutations within gp41. Antimicrob. Agents Chemother. **49**:1113–1119.
6. **Bai X, Wilson KL, Seedorff JE, Ahrens D, Green J, Davison DK, Jin L, Stanfield-Oakley SA, Mosier SM, Melby TE, Cammack N, Wang Z, Greenberg ML, Dwyer JJ.** 2008. Impact of the enfuvirtide resistance

mutation N43D and the associated baseline polymorphism E137K on peptide sensitivity and six-helix bundle structure. Biochemistry 47:6662–6670.
7. Baldwin CE, Sanders RW, Deng Y, Jurriaans S, Lange JM, Lu M, Berkhout B. 2004. Emergence of a drug-dependent human immunodeficiency virus type 1 variant during therapy with the T20 fusion inhibitor. J. Virol. 78:12428–12437.
8. Cabrera C, Marfil S, Garcia E, Martinez-Picado J, Bonjoch A, Bofill M, Moreno S, Ribera E, Domingo P, Clotet B, Ruiz L. 2006. Genetic evolution of gp41 reveals a highly exclusive relationship between codons 36, 38 and 43 in gp41 under long-term enfuvirtide-containing salvage regimen. AIDS 20:2075–2080.
9. Champagne K, Shishido A, Root MJ. 2009. Interactions of HIV-1 inhibitory peptide T20 with the gp41 N-HR coiled coil. J. Biol. Chem. 284: 3619–3627.
10. Izumi K, Kodama E, Shimura K, Sakagami Y, Watanabe K, Ito S, Watabe T, Terakawa Y, Nishikawa H, Sarafianos SG, Kitaura K, Oishi S, Fujii N, Matsuoka M. 2009. Design of Peptide-based inhibitors for human immunodeficiency virus type 1 strains resistant to T-20. J. Biol. Chem. 284:4914–4920.
11. Lu J, Deeks SG, Hoh R, Beatty G, Kuritzkes BA, Martin JN, Kuritzkes DR. 2006. Rapid emergence of enfuvirtide resistance in HIV-1-infected patients: results of a clonal analysis. J. Acquir. Immune Defic. Syndr. 43: 60–64.
12. Oliveira AC, Martins AN, Pires AF, Arruda MB, Tanuri A, Pereira HS, Brindeiro RM. 2009. Enfuvirtide (T-20) resistance-related mutations in HIV type 1 subtypes B, C, and F isolates from Brazilian patients failing HAART. AIDS Res. Hum. Retroviruses 25:193–198.
13. Reeves JD, Lee FH, Miamidian JL, Jabara CB, Juntilla MM, Doms RW. 2005. Enfuvirtide resistance mutations: impact on human immunodeficiency virus envelope function, entry inhibitor sensitivity, and virus neutralization. J. Virol. 79:4991–4999.
14. Watabe T, Terakawa Y, Watanabe K, Ohno H, Nakano H, Nakatsu T, Kato H, Izumi K, Kodama E, Matsuoka M, Kitaura K, Oishi S, Fujii N. 2009. X-ray crystallographic study of an HIV-1 fusion inhibitor with the gp41 S138A substitution. J. Mol. Biol. 392:657–665.
15. Lalezari JP, Bellos NC, Sathasivam K, Richmond GJ, Cohen CJ, Myers RA, Jr, Henry DH, Raskino C, Melby T, Murchison H, Zhang Y, Spence R, Greenberg ML, Demasi RA, Miralles GD. 2005. T-1249 retains potent antiretroviral activity in patients who had experienced virological failure while on an enfuvirtide-containing treatment regimen. J. Infect. Dis. 191: 1155–1163.
16. He Y, Xiao Y, Song H, Liang Q, Ju D, Chen X, Lu H, Jing W, Jiang S, Zhang L. 2008. Design and evaluation of sifuvirtide, a novel HIV-1 fusion inhibitor. J. Biol. Chem. 283:11126–11134.
17. Dwyer JJ, Wilson KL, Davison DK, Freel SA, Seedorff JE, Wring SA, Tvermoes NA, Matthews TJ, Greenberg ML, Delmedico MK. 2007. Design of helical, oligomeric HIV-1 fusion inhibitor peptides with potent activity against enfuvirtide-resistant virus. Proc. Natl. Acad. Sci. U. S. A. 104:12772–12777.
18. Welch BD, VanDemark AP, Heroux A, Hill CP, Kay MS. 2007. Potent D-peptide inhibitors of HIV-1 entry. Proc. Natl. Acad. Sci. U. S. A. 104: 16828–16833.
19. Wang H, Qi Z, Guo A, Mao Q, Lu H, An X, Xia C, Li X, Debnath AK, Wu S, Liu S, Jiang S. 2009. ADS-J1 inhibits human immunodeficiency virus type 1 entry by interacting with the gp41 pocket region and blocking fusion-active gp41 core formation. Antimicrob. Agents Chemother. 53: 4987–4998.
20. Naito T, Izumi K, Kodama E, Sakagami Y, Kajiwara K, Nishikawa H, Watanabe K, Sarafianos SG, Oishi S, Fujii N, Matsuoka M. 2009. SC29EK, a peptide fusion inhibitor with enhanced alpha-helicity, inhibits replication of human immunodeficiency virus type 1 mutants resistant to enfuvirtide. Antimicrob. Agents Chemother. 53:1013–1018.
21. Nishikawa H, Nakamura S, Kodama E, Ito S, Kajiwara K, Izumi K, Sakagami Y, Oishi S, Ohkubo T, Kobayashi Y, Otaka A, Fujii N, Matsuoka M. 2009. Electrostatically constrained alpha-helical peptide inhibits replication of HIV-1 resistant to enfuvirtide. Int. J. Biochem. Cell Biol. 41:891–899.
22. Marqusee S, Baldwin RL. 1987. Helix stabilization by Glu-Lys$^+$ salt bridges in short peptides of de novo design. Proc. Natl. Acad. Sci. U. S. A. 84:8898–8902.
23. Oishi S, Ito S, Nishikawa H, Watanabe K, Tanaka M, Ohno H, Izumi K, Sakagami Y, Kodama E, Matsuoka M, Fujii N. 2008. Design of a novel HIV-1 fusion inhibitor that displays a minimal interface for binding affinity. J. Med. Chem. 51:388–391.
24. Li X, Qian H, Miyamoto F, Naito T, Kawaji K, Kajiwara K, Hattori T, Matsuoka M, Watanabe K, Oishi S, Fujii N, Kodama EN. 2012. A simple, rapid, and sensitive system for the evaluation of anti-viral drugs in rats. Biochem. Biophys. Res. Commun. 424:257–261.
25. Nameki D, Kodama E, Ikeuchi M, Mabuchi N, Otaka A, Tamamura H, Ohno M, Fujii N, Matsuoka M. 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.
26. Shimura K, Nameki D, Kajiwara K, Watanabe K, Sakagami Y, Oishi S, Fujii N, Matsuoka M, Sarafianos SG, Kodama EN. 2010. Resistance profiles of novel electrostatically constrained HIV-1 fusion inhibitors. J. Biol. Chem. 285:39471–39480.
27. Izumi K, Nakamura S, Nakano H, Shimura K, Sakagami Y, Oishi S, Uchiyama S, Ohkubo T, Kobayashi Y, Fujii N, Matsuoka M, Kodama EN. 2010. Characterization of HIV-1 resistance to a fusion inhibitor, N36, derived from the gp41 amino-terminal heptad repeat. Antiviral Res. 87: 179–186.
28. Ueno M, Kodama EN, Shimura K, Sakurai Y, Kajiwara K, Sakagami Y, Oishi S, Fujii N, Matsuoka M. 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.
29. 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. 85:10785–10797.
30. Reeves JD, Gallo SA, Ahmad N, Miamidian JL, Harvey PE, Sharron M, Pohlmann S, Sfakianos JN, Derdeyn CA, Blumenthal R, Hunter E, Doms RW. 2002. Sensitivity of HIV-1 to entry inhibitors correlates with envelope/coreceptor affinity, receptor density, and fusion kinetics. Proc. Natl. Acad. Sci. U. S. A. 99:16249–16254.
31. Reeves JD, Miamidian JL, Biscone MJ, Lee FH, Ahmad N, Pierson TC, Doms RW. 2004. Impact of mutations in the coreceptor binding site on human immunodeficiency virus type 1 fusion, infection, and entry inhibitor sensitivity. J. Virol. 78:5476–5485.
32. Yu X, Lu L, Cai L, Tong P, Tan S, Zou P, Meng F, Chen YH, Jiang S. 2012. Mutations of Gln64 in the HIV-1 gp41 N-terminal heptad repeat render viruses resistant to peptide HIV fusion inhibitors targeting the gp41 pocket. J. Virol. 86:589–593.
33. Borras-Blasco J, Navarro-Ruiz A, Borras C, Castera E. 2008. Adverse cutaneous reactions associated with the newest antiretroviral drugs in patients with human immunodeficiency virus infection. J. Antimicrob. Chemother. 62:879–888.
34. Liu Z, Shan M, Li L, Lu L, Meng S, Chen C, He Y, Jiang S, Zhang L. 2011. In vitro selection and characterization of HIV-1 variants with increased resistance to sifuvirtide, a novel HIV-1 fusion inhibitor. J. Biol. Chem. 286:3277–3287.
35. Melby T, Demasi R, Cammack N, Miralles GD, Greenberg ML. 2007. Evolution of genotypic and phenotypic resistance during chronic treatment with the fusion inhibitor T-1249. AIDS Res. Hum. Retroviruses 23:1366–1373.

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Hypersusceptibility mechanism of Tenofovir-resistant HIV to EFdA

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Abstract

Background: The K65R substitution in human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is the major resistance mutation selected in patients treated with first-line antiretroviral tenofovir disoproxil fumarate (TDF). 4'-ethynyl-2-fluoro-2'-deoxyadenosine (EFdA), is the most potent nucleoside analog RT inhibitor (NRTI) that unlike all approved NRTIs retains a 3'-hydroxyl group and has remarkable potency against wild-type (WT) and drug-resistant HIVs. EFdA acts primarily as a chain terminator by blocking translocation following its incorporation into the nascent DNA chain. EFdA is in preclinical development and its effect on clinically relevant drug resistant HIV strains is critically important for the design of optimal regimens prior to initiation of clinical trials.

Results: Here we report that the K65R RT mutation causes hypersusceptibility to EFdA. Specifically, in single replication cycle experiments we found that EFdA blocks WT HIV ten times more efficiently than TDF. Under the same conditions K65R HIV was inhibited over 70 times more efficiently by EFdA than TDF. We determined the molecular mechanism of this hypersensitivity using enzymatic studies with WT and K65R RT. This substitution causes minor changes in the efficiency of EFdA incorporation with respect to the natural dATP substrate and also in the efficiency of RT translocation following incorporation of the inhibitor into the nascent DNA. However, a significant decrease in the excision efficiency of EFdA-MP from the 3' primer terminus appears to be the primary cause of increased susceptibility to the inhibitor. Notably, the effects of the mutation are DNA-sequence dependent.

Conclusion: We have elucidated the mechanism of K65R HIV hypersusceptibility to EFdA. Our findings highlight the potential of EFdA to improve combination strategies against TDF-resistant HIV-1 strains.

Keywords: HIV-1, RT, EFdA, K65R

Background

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is the major target of antiretroviral drug treatments. RT inhibitors constitute the largest class of HIV-1 drugs and are grouped in two separate categories. The first category consists of the nucleos(t)ide RT inhibitors (NRTIs), which are analogs of the natural nucleosides. Most NRTIs lack a 3'-OH and act as chain terminators by blocking DNA polymerization [1-8]. The other group includes the nonnucleoside RT inhibitors (NNRTIs), which are non-competitive RT inhibitors with respect to either dNTP or nucleic acid substrates and block DNA synthesis by binding to a hydrophobic pocket of RT [9-15]. Highly Active Antiretroviral Therapies (HAART) are based on combinations of antiretrovirals and have helped extend the lives of HIV-1 patients. However, the efficacy of combination therapies is being challenged by the selection of drug-resistant variants of HIV-1.

There are two major mechanisms of NRTI resistance [16,17]. The first is the discrimination mechanism, which is based on decreased incorporation of the nucleotide analog into the elongating DNA over the canonical dNTP substrate [16,18-21]. An example of this

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type of resistance is conferred by the M184V mutation, which decreases HIV susceptibility to lamivudine (3TC) and emtricitabine (FTC) [20-24]. The second mechanism is the excision mechanism, which is based on the enhanced ability of the mutant RT to remove the chain-terminating inhibitor from the DNA terminus [25-28] through a phosphorolytic reaction that uses primarily adenosine triphosphate (ATP) as a substrate. Upon removal of the inhibitor DNA synthesis resumes. The excision reaction is facilitated by Excision Enhancement Mutations (EEMs), typically M41L, D67N, K70R, T215Y/F, L210W, and K219E/Q, which are also known as Thymidine Associated Mutations (TAMs) because they were historically linked to resistance to thymidine analogs AZT and d4T [29,30].

Tenofovir disoproxil fumarate (TDF) is one of the most prescribed anti-HIV drugs, and is described as a key component of all first-line regimens in the DHHS HIV guidelines (http://aidsinfo.nih.gov/contentfiles/lvguidelines/adultandadolescentgl.pdf). The K65R mutation in HIV-1 RT is the signature mutation selected during tenofovir-based therapy. Viruses carrying K65R have reduced susceptibility to tenofovir and other NRTIs, but remain susceptible to zidovudine (AZT) [31-36]. This mutation has also been associated with a reduction in viral replication capacity, NRTI excision, NRTI incorporation, and dNTP incorporation [37-43]. Recent crystallographic data suggest that the K65R mutation disrupts the interaction between the side chains of 65R and 72R resulting in structural changes that lead to NRTI resistance [44].

We have previously shown that a series of NRTIs with 4'-substitutions and a 3'-OH group are very potent inhibitors of WT and multi-drug resistant HIV-1. The most effective of these compounds is the adenosine analog 4'-ethynyl-2-fluoro-2'-deoxyadenosine (EFdA) [45,46]. We have demonstrated that EFdA acts in a DNA-sequence specific manner, primarily inhibiting DNA synthesis as an immediate chain terminator, but less often, at some DNA sequences can also act as a delayed chain terminator [46]. Compounds that exhibit this novel mechanism of inhibition have been dubbed Translocation Defective Reverse Transcriptase Inhibitors (TDRTIs) [46].

In an effort to investigate the effect of EFdA against drug-resistant strains of HIV-1 we found that RT mutation K65R confers hypersusceptibility to EFdA. We carried out a series of biochemical experiments to elucidate the mechanism of this phenomenon and we propose here that K65R increases the susceptibility to EFdA mainly by suppressing the ATP- or PPi-dependent repair of EFdA-MP-terminated DNA. Understanding the molecular basis of K65R hypersusceptibility to EFdA may lead to new and more effective combination therapies.

Results

The K65R RT mutation enhances susceptibility of HIV to EFdA

In order to determine the susceptibility of HIV-1 to EFdA we performed single infectivity viral replication assays according to the experimental procedures described in Methods section. We used as a positive control of resistance to K65R HIV-1 the nucleotide analog TDF. Table 1 shows that K65R-containing viruses are at least 2.5-fold more susceptible to EFdA than WT viruses. In contrast, there was a 3-fold resistance to tenofovir caused by K65R RT mutation.

The K65R mutation enhances susceptibility of RT to EFdA-TP

In order to recapitulate the HIV hypersusceptibility to EFdA observed in cell-based assays and determine the biochemical mechanism of this phenomenon we carried out a series of biochemical experiments. We used a primer extension assay to compare the effect of EFdA-TP on DNA-dependent DNA polymerization by WT and K65R RTs. In order to assess the effect of ATP-based excision on the susceptibility of RT to EFdA-TP we performed the reactions in the absence (Figure 1A) and in the presence of ATP (Figure 1B). In the absence of ATP, any changes in the susceptibility to the inhibitor would be caused by the "decreased incorporation" mechanism as there are no NRTI excision events under these conditions. However, changes in inhibitor susceptibility in the presence of ATP could be caused either by "changes in inhibitor incorporation" or "changes in inhibitor excision", or both. Figure 1 and Table 2 show that RT mutation K65R causes hypersusceptibility to EFdA-TP. In the presence of ATP the K65R mutation caused a 2.5-fold increase in susceptibility to EFdA-TP (Table 2). These data are consistent with the results from the cell-based assays shown in Table 1. The ~2-fold effect of ATP-based excision on the hypersusceptibility to EFdA suggests that excision is the major mechanism of this phenomenon and is further characterized in the subsequent experiments.

Table 1 EC_{50} determination of EFdA and TDF in single cycle cell-based assays

Virus	EC_{50} ± SD (nM) (Fold change)	
	EFdA	TDF
WT	3.2 ± 0.7	32 ± 6
	(1)	(1)
K65R	1.3 ± 0.4	96 ± 3
	(0.4)	(3)

The data show "mean value ± standard deviation" obtained from the results of at least three independent experiments ($P < 0.013$). The relative increase in EC_{50} values against K65R virus compared with WT virus is given in parentheses.

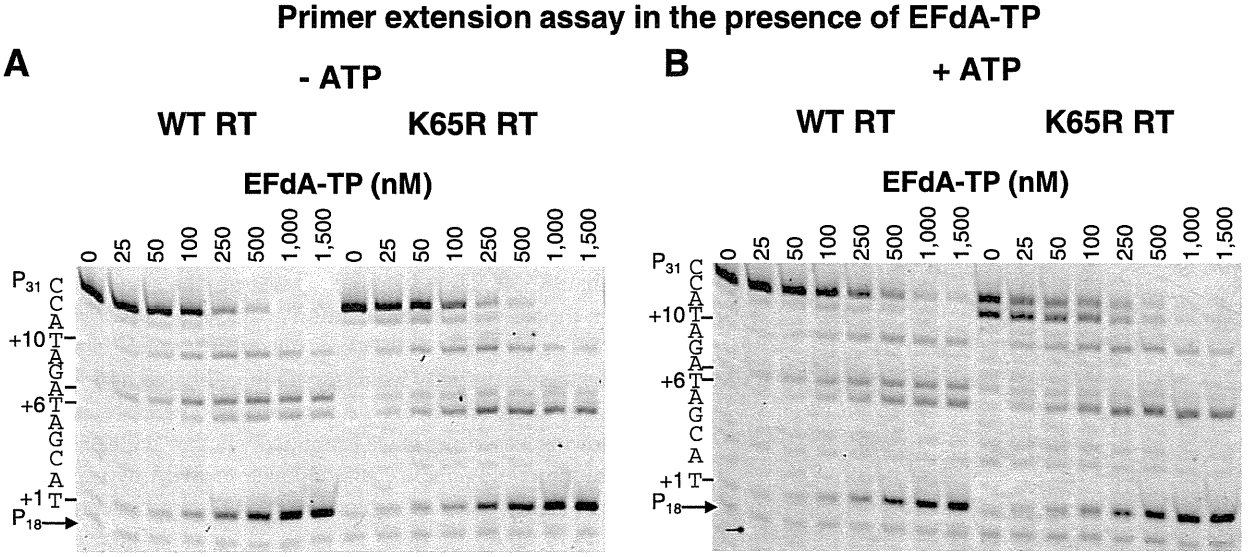

Figure 1 Inhibition of WT and K65R RT-catalyzed DNA synthesis by EFdA-TP. $T_{d31}/P_{d18\text{-}P0}$ was incubated with WT or K65R HIV-1 RT for 50 minutes in the presence of 1 μM dNTPs, $MgCl_2$ and increasing concentrations of EFdA-TP (0–1,500 nM). The experiment was carried out in the (**A**) absence or (**B**) presence of 3.5 mM ATP. The template sequence is shown next to the gels and the numbers indicate the points of EFdA-TP incorporation (+1, +6 and +10).

The $T_{d31}/P_{d18\text{-}P0}$ DNA substrate (Table 3) allows incorporation of dATP analogs opposite template dTs at positions "1", "6" and "10" (Figure 1). The stopping pattern of the gels showed that EFdA-TP caused major pauses at all possible points of incorporation (positions 1, 6 and 10), suggesting that EFdA-TP inhibits RT mainly as an immediate chain terminator at the point of incorporation. Interestingly, EFdA-TP caused an additional strong stop of WT RT at position "+7", which is one nucleotide after its incorporation (Figure 1), thereby acting as delayed chain terminator at this site. Hence, this appears to be a sequence-dependent phenomenon, as we did not observe delayed chain extension at positions +2 and +11 and there was no pause at this site in the absence of inhibitor (Figure 1).

The K65R RT mutation does not enhance susceptibility to EFdA by significantly affecting incorporation of the inhibitor

To determine the biochemical mechanism of increased K65R HIV inhibition by EFdA we examined several possible mechanisms. The first hypothesis was that the K65R RT mutation selectively enhances incorporation of the EFdA-TP inhibitor into DNA because of changes in kinetic parameters such as binding or turnover rate of inhibitor incorporation. To evaluate this hypothesis we performed single nucleotide incorporation assays under steady state conditions. In order to eliminate a sequence-dependent bias we used three different template/primers (T/P) ($T_{d26}/P_{d18\text{-}P5}$, $T_{d31}/P_{d18\text{-}P0}$, and T_{d31A}/P_{d21} [Table 3]). Our results showed that under these conditions the ratio of the incorporation efficiency (k_{cat}/K_m) of EFdA-TP and the incorporation efficiency of dATP by K65R RT was between 0.8 and 1. These results suggest that the K65R mutation does not have a significant effect on the binding and incorporation of EFdA-TP (Table 4).

The K65R RT mutation does not enhance susceptibility to EFdA by significantly affecting enzyme translocation on EFdA-MP-terminated template/primers

We have previously shown that the inability of RT to form a stable ternary complex with $T/P_{EFdA\text{-}MP}$ and the next

Table 2 Enhancement of hypersusceptibility to EFdA-TP under ATP-based excision conditions

Enzyme	IC_{50} (nM) of EFdA-TP ± SD (Fold change)		Hypersusceptibility enhancement in the presence of ATP
	Without ATP	With 3.5 mM ATP	
WT RT	186 ± 40[a] (1)[b]	318 ± 99[a] (1)[b]	1[c]
K65R RT	125 ± 28[a] (0.7)[b]	131 ± 28[a] (0.4)[b]	1.8[c]

[a]The data show "mean value ± standard deviation" obtained from the results of at least four independent experiments ($P < 0.011$).
[b]The relative increase in IC_{50} value in K65R RT compared with WT RT without, or with ATP is given in parentheses.
[c]The effect of ATP-based excision on hypersusceptibility is calculated by the relative change in IC_{50} for K65R compared to WT RT without ATP/the relative change in IC_{50} for K65R compared to WT RT with ATP.

Table 3 Sequences of oligonucleotides used in this study

Polymerization assays	
T_{d31}	5'CCA TAG ATA GCA TTG GTG CTC GAA CAG TGA C
$P_{d18\text{-}P0}$	5'Cy3 GTC ACT GTT CGA GCA CCA
T_{d26}	5'CCA TAG ATA GCA TTG GTG CTC GAA CA
$P_{d18\text{-}P5}$	5'Cy3 TGT TCG AGC ACC AAT GCT
T_{d31A}	5'AAA AAA AAA TGG ATA CAT ATG GTT AAA GTA T
P_{d21}	5'Cy3 ATA CTT TAA CCA TAT GTA TCC
Footprinting assays	
T_{d43}	5'Cy3 CCA TAG ATA GCA T TG GTG CTC GAA CAG TGA CAA TCA GTG TAG A
P_{d30}	5'TCT ACA CTG ATT GTC ACT GTT CGA GCA CCA

complementary dNTP was due to the inability of the 3'-terminal EFdA-MP primer to efficiently translocate from the nucleotide binding site (N site, which is also the pre-translocation site) to the post-translocation primer site (P site or the post-translocation site) [46]. Hence, another possible mechanism by which the K65R mutation could enhance susceptibility to EFdA is by further suppressing translocation of RT on the EFdA-MP-terminated T/P. To evaluate this hypothesis we used the site-specific Fe^{2+} footprinting assay [46,47] to assess the translocation state of WT and K65R RT·T/$P_{EFdA\text{-}MP}$ complexes in the absence, and in the presence of varying concentrations of the next incoming dNTP. Figure 2 shows that EFdA-TP blocked translocation and acted as a strong TDRTI against both WT and K65R RTs. At physiological dNTP concentrations (1-25 μM) we observed a 1.5-fold decrease in the translocation efficiency of K65R compared to WT RT under these conditions. To determine whether the lower amount of observed translocated K65R RT·T/$P_{EFdA\text{-}MP}$ complex (Figure 2) is due to a decreased affinity of K65R RT for EFdA-MP-terminated T/P we studied the effect of K65R on the formation of RT·T/$P_{EFdA\text{-}MP}$ binary complex using gel-shift assays. Data in Additional file 1: Figure S1 show that WT RT binds EFdA-MP-terminated T/P only slightly stronger than K65R RT (~1.3-fold). Therefore, the small differences in the selectivity, translocation activity, and DNA binding of WT and K65R RTs were not sufficient to explain the hypersusceptibility we observed in cell-based and RT assays with EFdA and EFdA-TP respectively. Therefore, in the following experiments we examined whether the K65R substitution could enhance susceptibility to EFdA by suppressing the ability of RT to unblock EFdA-MP-terminated primers.

ATP- and PPi-dependent Excision/Rescue of EFdA-MP

We have previously demonstrated that using simple pyrophosphorolysis reactions (in the absence of concurrent DNA polymerization) is not an effective way to monitor unblocking of EFdA-MP-terminated primers. This is because the net phosphorolysis is limited from the apparently facile reincorporation of the newly excised EFdA-TP [46]. Hence, to better study the potential role of the excision mechanism in EFdA resistance we employed rescue assays, where in addition to the ATP or PPi which are used as unblocking reagents, we also include dNTPs that compete with and prevent reincorporation of EFdA-TP, and also allow further DNA synthesis. For these experiments we used as a substrate

Table 4 Steady state kinetic parameters for EFdA-TP and dATP incorporation by WT and K65R HIV-1 RTs

Enzyme	dNTP	K_m (μM)	k_{cat} (min^{-1})	k_{cat} / K_m ($min^{-1} \cdot \mu M^{-1}$)	Selectivity[a]	Fold change[b]
$T_{d26}/P_{d18\text{-}P5}$						
WT RT	dATP	2.38 ± 0.27	1.58 ± 0.12	0.66	1	1
	EFdA-TP	0.66 ± 0.04	3.26 ± 0.42	4.94	7.5	
K65R RT	dATP	5.99 ± 0.81	0.92 ± 0.14	0.15	1	0.8
	EFdA-TP	1.83 ± 0.34	1.74 ± 0.42	0.95	6.3	
$T_{d31}/P_{d18\text{-}P0}$						
WT RT	dATP	0.33 ± 0.07	6.32 ± 0.14	19.15	1	1
	EFdA-TP	0.23 ± 0.01	4.73 ± 0.33	20.57	1.1	
K65R RT	dATP	0.42 ± 0.01	3.62 ± 0.71	8.62	1	1
	EFdA-TP	0.31 ± 0.02	3.07 ± 0.15	9.90	1.1	
T_{d31A}/P_{d21}						
WT RT	dATP	0.37 ± 0.08	3.64 ± 0.57	9.84	1	1
	EFdA-TP	0.19 ± 0.06	3.54 ± 0.44	18.63	1.9	
K65R RT	dATP	1.06 ± 0.11	3.68 ± 0.28	3.47	1	0.9
	EFdA-TP	0.53 ± 0.05	3.38 ± 0.29	6.38	1.8	

Values are mean ± S.D. of two to four independent experiments and were determined from Michaelis-Menten equation using GraphPad Prism 4.
[a]Selectivity is the ratio of the incorporation efficiency (k_{cat}/K_m) of EFdA-TP over that of dATP ($[k_{cat}/K_m]_{EFdA\text{-}TP}$ / $[k_{cat}/K_m]_{dATP}$).
[b]Fold Change is the ratio of the selectivity in K65R over the selectivity in WT RT.

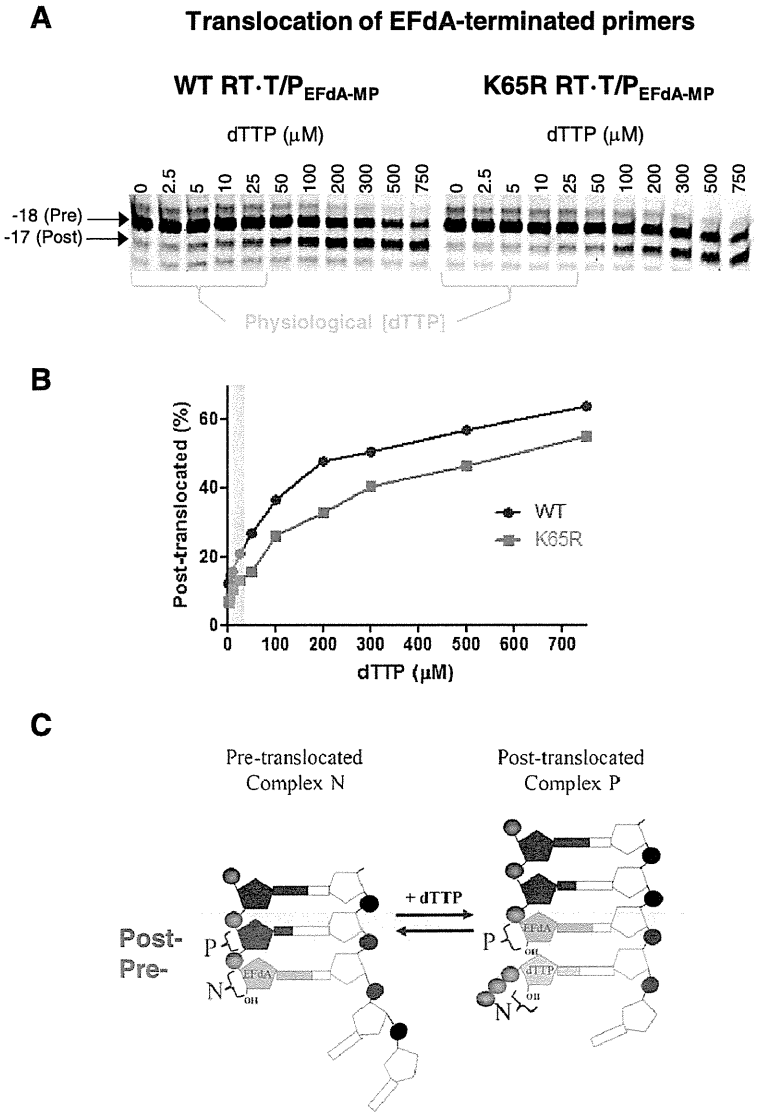

Figure 2 Effect of K65R mutation on the translocation state of RT bound to T/P$_{EFdA-MP}$. (**A**) The translocation state of HIV-1 RT after EFdA-MP incorporation was determined using site-specific Fe^{2+} footprinting. $T_{d43}/P_{d30\text{-}EFdA\text{-}MP}$ (100 nM) with 5'-Cy3-label on the DNA template was incubated with WT or K65R HIV-1 RT (600 nM) and various concentrations of the next incoming nucleotide (dTTP). The complexes were treated for 5 minutes with ammonium iron sulphate (1 mM) and resolved on a polyacrylamide 7 M urea gel. An excision at position −18 indicates a pre-translocation complex, while the one at position −17 represents a post-translocation complex. (**B**) The post-translocated complexes were determined from the gels and plotted using GraphPad Prism. Light blue indicates the physiological dNTP concentrations. (**C**) Schematic representation of the position of EFdA-MP-terminated primers at the pre- and post-translocated sites.

nucleic acid having at the 3'-primer terminus EFdA-MP ($T_{d31}/P_{d18\text{-}P0\text{-}EFdA\text{-}MP}$). Using ATP as the pyrophosphate donor, we found that the initial rates of the rescue reactions were 2.8-fold slower by K65R than by WT RT (Figure 3A). A 6.5-fold decrease was also observed in PPi-based rescue (Figure 3B). As previously reported [48] the PPi-rescue was faster than the ATP-based rescue assay. Whereas the PPi-based hydrolysis is exactly the opposite of DNA synthesis in reverse, ATP-based hydrolysis has some differences, as we have also structurally demonstrated in the crystal structure of RT in complex with DNA and tetraphosphate excision product [49]. The above experiments provide strong evidence that K65R mutation confers hypersusceptibility to EFdA mainly through decreased excision.

Discussion

Tenofovir is a major component of current antiviral therapies (http://aidsinfo.nih.gov/contentfiles/lvguidelines/adultandadolescentgl.pdf) and new HIV drugs are likely to be used in patients that have failed tenofovir-based treatment. Hence, the ability of novel HIV inhibitors to efficiently block tenofovir-resistant viruses is critical for their potential utility