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Short communication

HIV-1 resistance to the gp41-dependent fusion inhibitor C-34

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Abstract

The gp41 subunit of HIV-1 has been recently recognized as a target for antiviral therapy. C-34 is a peptide that mimics the heptad repeat 2 in the ectodomain of gp41. Here, we describe two HIV-1 strains selected after 5 and 17 passages in culture with increasing concentrations of C-34 (breakthrough concentration of 10 μg/ml). The HXB2-derived strain was more than 1000-fold resistant and contained a V38E mutation in the gp41 coding DNA sequence. The NL4-3-derived strain was more than 500-fold resistant and contained a L33S mutation in gp41. No cross-resistance to the RT inhibitor AZT, the HIV binding inhibitor dextran sulfate (DS), or the chemokine receptor antagonist ALX-40-4C was detected. These data indicate that HIV-1 can overcome C-34 inhibition through mutations at residues not involved in the formation of the hydrophobic cavity of gp41.

Keywords: HIV-1; gp41; Heptad repeat; Fusion inhibitor; Resistance

The HIV-1 envelope glycoproteins consist of a trimeric complex of two non-covalently associated subunits, gp120 and gp41 that mediate binding to and fusion with the target cell membrane. Binding of the surface subunit gp120 to the specific receptors on the target cell (Freed and Martin, 1995) promotes the fusion-active conformation of the transmembrane subunit gp41. In this conformation, gp41 mediates the attachment and fusion of viral and cellular membranes (Chan and Kim, 1998; Lu et al., 1995). The gp41 ectodomain residue sequence predicts the presence of a fusion peptide followed by two α-helical heptad repeats (HR1 and HR2). In the fusogenic state of gp41, the fusion peptide is inserted into the cellular membrane and HR2 helixes fold in an antiparallel way on a central HR1 coiled coil, forming a trimer of hairpins. On the surface of the central coiled coil, three particularly deep hydrophobic grooves are formed between adjacent helices. The HR1 residues that delimit these grooves are highly conserved among SIV and HIV, as well as concrete residues of HR2 that are predicted to project into them.

HIV is able to overcome the inhibitory capacity of antiviral drugs by developing mutations that make the emerging HIV strain resistant to the compounds. The mutations associated with resistance can potentially affect the function of the HIV molecules in which they appear. Moreover, the study of resistance-conferring mutations can help to understand the mechanism through which the drug acts (Esté et al., 1997; Esté, 2001; Barretina et al., 2003) and it may be a useful tool for therapeutic advice (Meynard et al., 2002). A new generation of anti-HIV agents are fusion inhibitors that are synthetic peptides homologous to the HR2 region that associate with the viral HR1 complexes. The first gp41-mediated fusion inhibitor that has been brought to clinical trials is DP-178 (T-20, enfuvirtide), a 36-amino acid peptide that comprises residues 127–162 of gp41 (Kilby et al., 1998). C-34 is a 34-amino acid peptide that contains the residues 117–150 of gp41. C-34 inhibitory capacity seems more related to its ability to prevent the formation of the fusogenic hairpin. In contrast, the inhibitory capacity of DP-178 is more strongly due to its ability of preventing pore formation through the interaction with the membrane (Kliger et al., 2001). Within the non-overlapping residues of C-34 with T-20, there are three hydrophobic residues (W117, W120, and I124) predicted to project into the hydrophobic grooves of the internal HR1 trimer (Chan et al., 1997, 1998; Eckert and Kim, 2001). Apart from being highly conserved among HIV-1, amino acids W117, W120, and I124 play a critical role in the stability of the envelope glycoprotein complex (Chan et al., 1997). Taken together, these observations suggest that HIV-1 resistance to gp41-mediated fusion inhibitors containing the residues that project into the hydrophobic cavities would be more difficult to appear.

We were interested in studying the evolution of laboratory adapted HIV-1 strains when cultured under the selective

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pressure of C-34. NL4-3 and HXB2 HIV-1 stocks were titrated in MT-4 cells and normalized to cell culture infective dose 50% ($CCID_{50}$)/ml using the MTT colorimetric assay as described before (Pauwels et al., 1988). With this method a relative value of the extent of cell death after a 5-day infection can be calculated. The 50% effective concentration or concentration required to inhibit 50% HIV-induced cell death (EC_{50}) for C-34 was evaluated with the same method for each virus using a multiplicity of infection (moi) of 0.03. The EC_{50} for NL4-3 and HXB2 was estimated as 4 and 0.1 ng/ml, respectively. Then, MT-4 cells were infected as described before (Cabrera et al., 1999) with HIV-1 in medium containing an inhibitor concentration 2–4 times the EC_{50}. Briefly, cell cultures were incubated at 37 °C until an extensive cytopathic effect was observed (3–4 days). The culture supernatants were used for further passage in MT-4 cells in the presence of two- to five-fold increasing concentrations of C-34. During the course of the experiment, a wild-type control of each virus was maintained in a parallel series of passages without C-34. After five passages (18 days) of NL4-3 and 17 passages (36 days) of HXB2 in the presence of C-34, we recovered viruses (NC10 and HC43, respectively) that were able to grow at a C-34 concentration of 10 μg/ml. Viral stocks were titrated, and sensitivity to C-34 and control compounds was re-evaluated. Cell viability and p24 antigen production (Innogenetics, Barcelona, Spain) as a measure of virus replication in the presence of C-34 are shown in Fig. 1A and B. A high decrease in sensitivity to C-34 was observed for NC10 (500-fold lower) and HC43 (>1000-fold lower), compared to the corresponding wild-type virus (Table 1). No cross-resistance was observed when the reverse transcriptase inhibitor azidothymidine (AZT), the HIV-1 binding inhibitor dextran sulfate (DS) (both compounds purchased from Sigma, Madrid, Spain), or the CXCR4 antagonist ALX-40-4C (Doranz et al., 1997) were evaluated against the C-34-resistant virus. The results show that selective pressure with C-34 conferred HIV-1 resistance to this agent without affecting the sensitivity to compounds that act at a previous step in the viral entry process or at a later intracellular step of the HIV life cycle.

To further confirm C-34 resistance of the selected viruses, we evaluated HIV-1 cell entry in the presence or absence of C-34. Wild-type or C-34-resistant HIV were titered in U87-CD4-CXCR4 (Bjorndal et al., 1997) cells by a quantitative-PCR (Q-PCR) assay. U87-CD4-CXCR4 cells (2×10^5) were infected with a moi of 0.001–0.003 or the concentration required to generate a detectable Q-PCR signal of the corresponding HIV-1 strain. Eight hours post infection, cells were recovered, washed in PBS and total DNA from wild-type or C-34-resistant infected cells was purified with the QIAamp DNA Blood Mini kit (Qiagen, Valencia, CA). Total proviral DNA was amplified in an ABI-7000 Sequence Detection System (PE Biosystems, Warringtong, England), using primers CCTAGCATTTCATCACGTGGC and TTCTTGAAGTACTCCGGATGCAG corresponding to the HIV-1 LTR and SYBR Green detection (Applied Biosystems). A standard curve was generated using dilutions of 8E5 cells (Folks et al., 1986) diluted in an equivalent amount of uninfected cellular DNA. As seen in Fig. 1C, C-34 was able to block proviral DNA detection of wild-type NL4-3 and HXB2-infected cells at a concentration of 0.04 μg/ml. Conversely, proviral DNA could be detected in cells infected with the C-34-resistant strains even at a significantly higher (25-fold) drug concentration (1 μg/ml).

Then, the envelope region of wild-type and C-34 resistant viruses was amplified from proviral DNA of infected cells and recombined into a HXB2 backbone by a procedure described before (Blanco et al., 2001). Recovered virus was tested for sensitivity to C-34. As seen in Table 2, recombination of the HIV envelope from C-34-resistant HIV in the HXB2 background recovered the HIV-resistance to C-34, while recombinant wild-type virus remained sensitive to the compound.

To evaluate genotypic differences, DNA from C-34-resistant or wild-type-infected cells was purified with the QIAamp DNA Blood Mini kit (Qiagen). The entire gp41 gene was first amplified with primers 5′-AATCTTTAAGCAATCCTCAG-3′ (7290–7309) and 5′-GCCACTCCCCAGTCCCGCCC-3′ (9465–9485) and then a nested PCR with primers 5′-GGTGGTAATAACAACAATGGGTCCG-3′ (7596–7620) and 5′-TTCTAGGTCTCGAGATACTGCT-3′ (8840–

Table 1
Anti-HIV activity of different compounds tested against wild-type and C-34-resistant viruses

Compound	EC_{50} (μg/ml)[a] [fold resistance[c]]				CC_{50} (μg/ml)[b]
	HIV-1 NL4-3		HIV-1 HXB2		
	Wild-type	NC10	Wild-type	HC43	
C-34	0.0002 ± 0.0001	0.1 ± 0.1 [500]	0.002 ± 0.001	3.45 ± 0.4 [1725]	>100
AZT	0.001 ± 0.0004	0.0025 ± 0.002 [3]	0.001 ± 0.001	0.0007 ± 0.0003 [1]	>2
DS	0.09 ± 0.04	0.11 ± 0.003 [1]	0.39 ± 0.39	0.05 ± 0.03 [0.13]	>125
ALX-40-4C	0.34 ± 0.04	0.37 ± 0.01 [1]	0.18 ± 0.11	0.06 ± 0.02 [0.3]	21

Values are mean for at least two independent assays done in triplicate.

[a] EC_{50}: 50% effective concentration, or concentration needed to inhibit 50% HIV-induced cell death, evaluated with the MTT method in MT-4 cells.

[b] CC_{50}: 50% cytotoxic concentration, or concentration needed to induce 50% death of non-infected cells, evaluated with the MTT method in MT-4 cells.

[c] Fold resistance: mean EC_{50} C-34-resistant virus/mean EC_{50} wild-type virus.

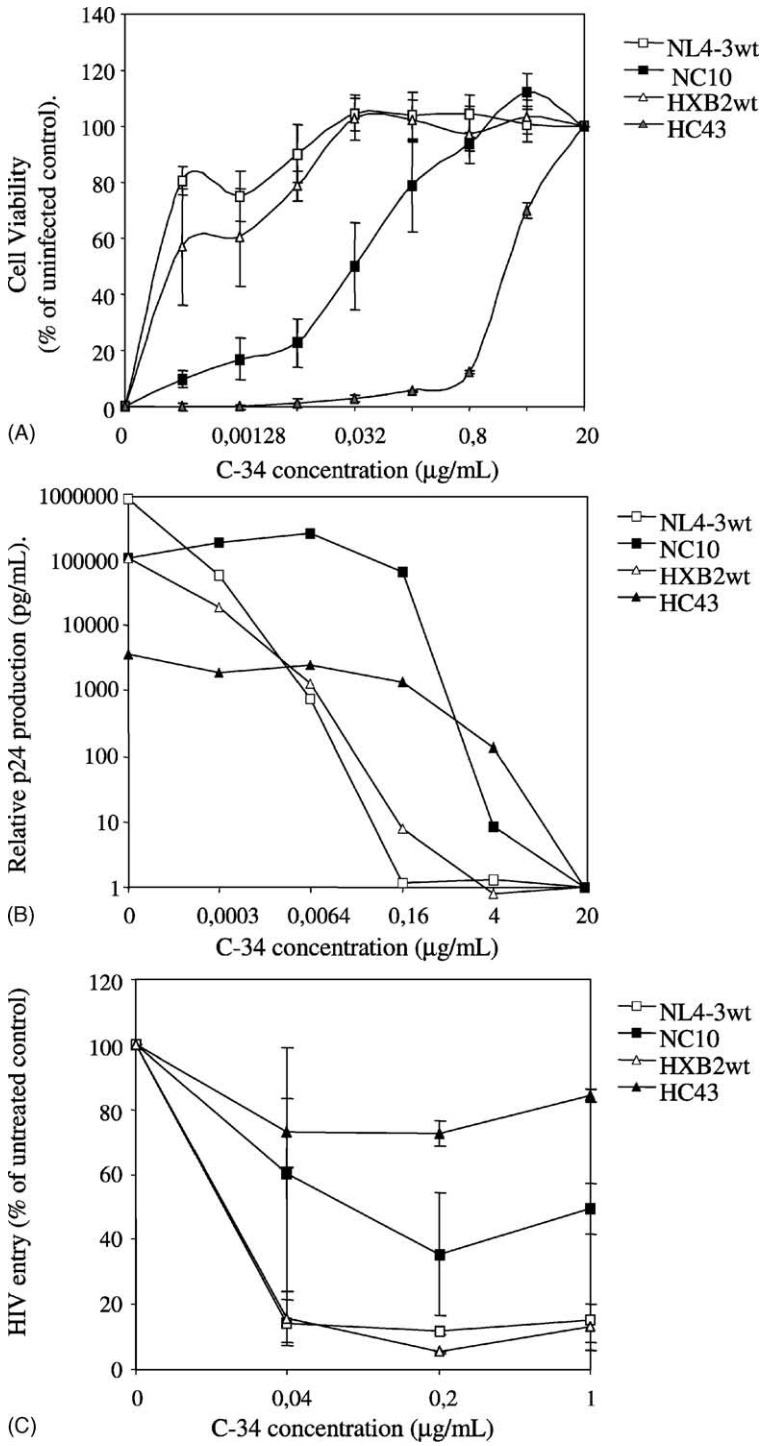

Fig. 1. Sensitivity of the HIV-1 wild-type (NL4-3 and HXB2 (wt)) and the C-34-passage-derived viruses (NC10 and HC43) to different concentrations of C-34. (A) HIV-induced cell death as measured by the MTT method in MT-4 cells. Values represent the mean of two experiments. (B) Inhibition of p24 antigen production by MT-4 cells at 5 days post-infection with a moi of 0.003. Values of one experiment representative of three experiments. (C) Inhibition of HIV-1 entry in U87-CD-CXCR4 cells. Quantitative PCR was used to detect integrated HIV-1 DNA at 8-h post infection.

8860) was performed (primer positions correspond to the NY5/BRU (LAV-1) recombinant clone (GeneBank accession no. M19921)). Both strands were sequenced by an ABI Prism 377 Genetic Analyzer (PE Biosystems) using the dRhodamine Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems, Warringtong, England). Fig. 2 shows the gp41 amino acid sequence of the C-34-resistant and wild-type virus stocks obtained from the last passage of the experiment. Comparison of the wild-type and the C-34-resistant strain revealed the presence of mutation

Table 2
Anti-HIV activity of different compounds tested against env-recombinant viruses

Compound	EC_{50} (μg/ml)[a] [fold resistance[c]]				CC_{50} (μg/ml)[b]
	HIV-1 NL4-3 env		HIV-1 HXB2 env		
	Wild-type	NC10	Wild-type	HC43	
C-34	0.0006 ± 0.0	0.035 ± 0.01 [60]	0.0003 ± 0.0	2.955 ± 0.96 [9850]	>20
AZT	0.002 ± 0.0004	0.002 ± 0.0 [1]	0.003 ± 0.0	0.003 ± 0.0004 [1]	>1
DS	0.20 ± 0.049	0.25 ± 0.021 [1.3]	0.07 ± 0.018	0.07 ± 0.007 [1]	>125
ALX-40-4C	0.38 ± 0.01	0.40 ± 0.0 [1.1]	1.34 ± 0.06	1.02 ± 0.29 [0.8]	21

Values are mean for two independent assays done in triplicate.
[a] EC_{50}: 50% effective concentration, or concentration needed to inhibit 50% HIV-induced cell death, evaluated with the MTT method in MT-4 cells.
[b] CC_{50}: 50% cytotoxic concentration, or concentration needed to induce 50% death of non-infected cells, evaluated with the MTT method in MT-4 cells.
[c] Fold resistance: mean EC_{50} C-34-resistant virus/mean EC_{50} wild-type virus.

L33S mutation in NC10 and V38E in HC43. The L33S mutation that was found in the NL4-3-derived C-34-resistant virus has not been previously associated with resistance to gp41-mediated fusion inhibitors. Similarly, this is also the first report of the mutation V38E conferring high level (>1000-fold) resistance to C-34. No other mutations were found in the gp120 or gp41 coding regions of the C-34-resistant virus (data not shown). Positions 36–38 form the GIV motif in which mutations confer resistance to the DP-178 peptide but not to T-649 (Derdeyn et al., 2001; Rimsky et al., 1998; Wei et al., 2002). C-34 and T-649 peptides differ only in that C-34 is two amino acids shorter than T-649 at the C-terminus. We expected to find few differences in the way that both peptides exert their inhibitory activity.

Thus, our results indicated that mutations in 36GIV38 alter the sensitivity to C-34. These conflicting results could be explained by the nature of the amino acid change (V–E) at position 38 as compared to those reported to confer resistance to DP-178, i.e. V38A, V38M. Alternatively, our findings could be explained by the sensitivity of the drug-resistance testing. Previous reports have suggested that changes in GIV conferred up to 10-fold resistance to DP-178 (Rimsky et al., 1998), which means that smaller changes in sensitivity may be overlooked. In turn, our assay allowed for the evaluation of >1000-fold changes in sensitivity. DP-178 was not available for evaluation against the C-34-resistant HIV strains. However, we would expect that the mutation found in the GIV motif of HXB2 gp41 and most probably the L33S found in the NL4-3 strain would affect the sensitivity to DP-178 but this needs to be confirmed.

It may be intriguing that the development of C-34 resistance to NL4-3 occurred though the selection of a different mutation than that for HXB2 despite few differences in the gp41 amino acid sequence of these two strains (Fig. 1).

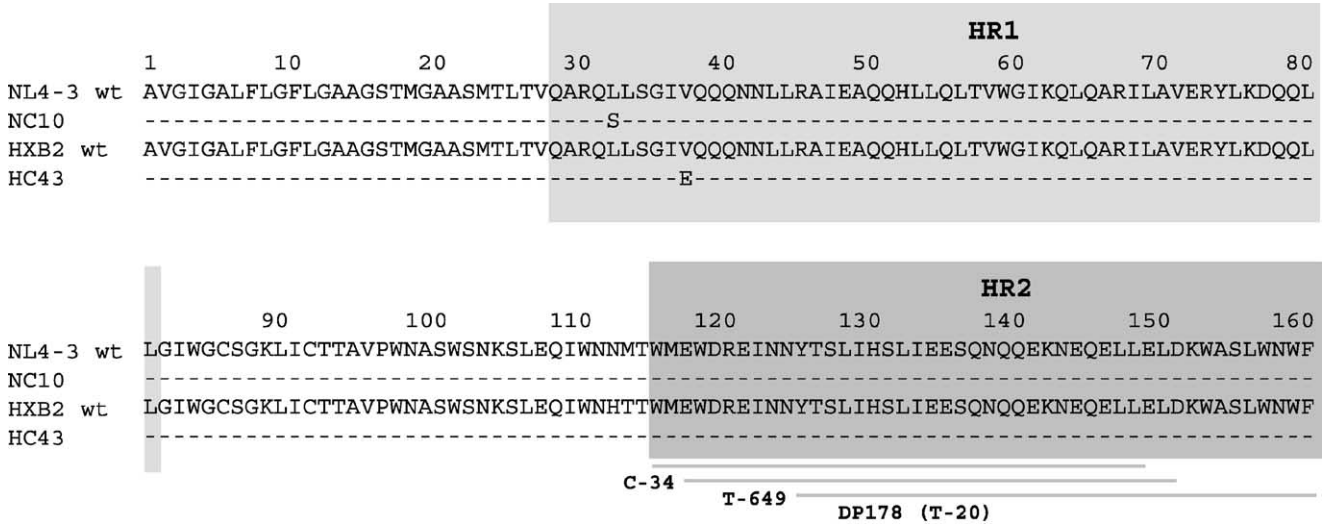

Fig. 2. Sequence analysis of gp41 from NL4-3 wild-type (wt), NC10, HXB2 (wt), and HC43. After 5 and 17 passages of NL4-3 and HXB2 in culture with or without C-34, respectively, DNA from the infected cells was extracted and the envelope sequence was analyzed. In this figure the HR1 and HR2 amino acid sequence of all four stocks is represented. HR1 and HR2 regions are shaded at their corresponding residues. The residues of C-34 (117–150), T-649 (119–152), and DP-178 (127–162) peptides are underlined. Amino acid changes with respect to the corresponding wild-type strain are indicated. Amino acid differences between the two wild-type strains are indicated by "∗" (positions 114 and 115).

Selection of drug-resistance is an stochastic event in which mutations that confer a strong selective advantage (500- and 1000-fold resistance) could be equally favored. Phenotypic resistance to DP-178 has been shown to appear after the emergence of different patterns of mutations in the same viral strain or clinical isolates (Rimsky et al., 1998; Sista et al., 2002).

Our results also raise doubts on how difficult it is for HIV-1 to become resistant to peptides that contain the cavity-binding residues if these peptides also contain amino acids that interact with other regions of HR1. Residues of C-34 that bind to the hydrophobic cavity (residues 54–66 of gp41) have been shown to play a critical role on its inhibitory capacity (Chan et al., 1997, 1998). Our results show that the inhibitory capacity of C-34 is also determined by its interaction with more N-terminal residues of the HR1 cavity and support the idea that the hydrophobic cavity of gp41 may be a good target for the inhibition of HIV-1 replication as changes within this cavity were not found. The fusion-mediated by gp41 must be further investigated in order to re-address gp41 HR2-based inhibitors. C-34-resistant HIV will help in delineating the role of C-34-resistant mutations in the activity profile of other gp41-dependent inhibitors such as DP-178 and ADS-J1 (Jiang et al., 2002) and their relevance in virus replication capacity as evaluated for other HIV fusion inhibitors (Armand-Ugon et al., 2003). Shortened peptides with their binding activity centered in the hydrophobic cavity of gp41 should be designed for evaluation as inhibitors of HIV-l replication.

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