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Inhibition of HIV-1 group M and O isolates by fusion inhibitors

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We examined the susceptibility of HIV-1 group M and O isolates to the fusion inhibitors T-20 and T-1249. Unexpectedly, HIV-1 O isolates were as sensitive as group M viruses to inhibition by T-20 but were usually less susceptible to T-1249. Our data suggest that T-20 has broad antiretroviral activity and would be effective in individuals with HIV-1 O infection. However, polymorphisms in gp41 might affect the sensitivity of HIV-1 O to second-generation fusion inhibitors.

The fusion inhibitor T-20 (enfuvirtide, fuzeon) is the first of a new class of antiretroviral drugs that are active against HIV-1 variants resistant to protease and reverse transcriptase inhibitors [1,2]. T-20 is a 36-amino acid peptide corresponding to the C-helix structure (HR-2) of the HIV-1Lai subtype B gp41 sequence [3,4]. T-1249 is a second-generation fusion inhibitor, which shows greater antiviral potency than T-20 and is active against most T-20-resistant HIV-1 isolates [5–7]. It encompasses 39 amino acids and is composed of gp41 HR-2 sequences derived from HIV-1, HIV-2, and SIV. Both T-20 and T-1249 inhibit HIV-1 entry by competitive binding of HR-1 and preventing the formation of the fusion-active six-helix hairpin structure [8]. Changes in the 36–45 amino acid domain of the HR-1 region, particularly in a conserved 3 amino acid sequence (GIV) of gp41, can confer drug resistance to T-20 [9–11].

T-20 was optimized to block HIV-1 M subtype B strains and T-1249 efficacy has mainly been demonstrated in HIV-1 M infections [6,7]. It is not well known whether fusion inhibitors are active against highly divergent HIV-1 O strains. Group O is mainly restricted to central Africa but sporadic infections have been reported in Europe and the USA [12,13]. Altogether, group O viruses have infected several tens of thousands of individuals. On the basis of gp41 sequence alignments it has been proposed that HIV-1 O might be resistant to T-20 but sensitive to T-1249 [14]. However, no phenotypic data have been presented to validate this assumption.

To study their susceptibility to T-20 and T-1249 we analysed six primary HIV-1 O isolates [15] and six group M viruses. The non-B 92UG029, 98IN022, 92UG024 and 93BR020 isolates [16] and the subtype B pYU-2 [17] and pLAI.2 [18] molecular clones have been obtained through the AIDS Research and Reference Reagent Program from Drs Beatrice Hahn, George Shaw and Keith Peden. To determine the diversity within the HR-1 and HR-2 regions we polymerase chain reaction amplified and sequenced the gp41 region from HIV-1 O-infected cells. All HIV-1 M and O variants except NL4-3 contain the GIV motif in HR-1 (Fig. 1a) [19]. Residues 36–45 of HR-1, important for T-20 inhibition [9–11], were highly conserved except for changes of N42S in the subtype A (92UG029) and C (98IN022) HIV-1 as well as N42D in the group O HR-1 sequences. Notably, group M and O gp41 sequences differ by changes of Q56R and T58S in the deep hydrophobic groove of HR-1, which is important for HR-2 binding [20], and is targeted by T-1249 but not by T-20 [6] (Fig. 1a). Compared with

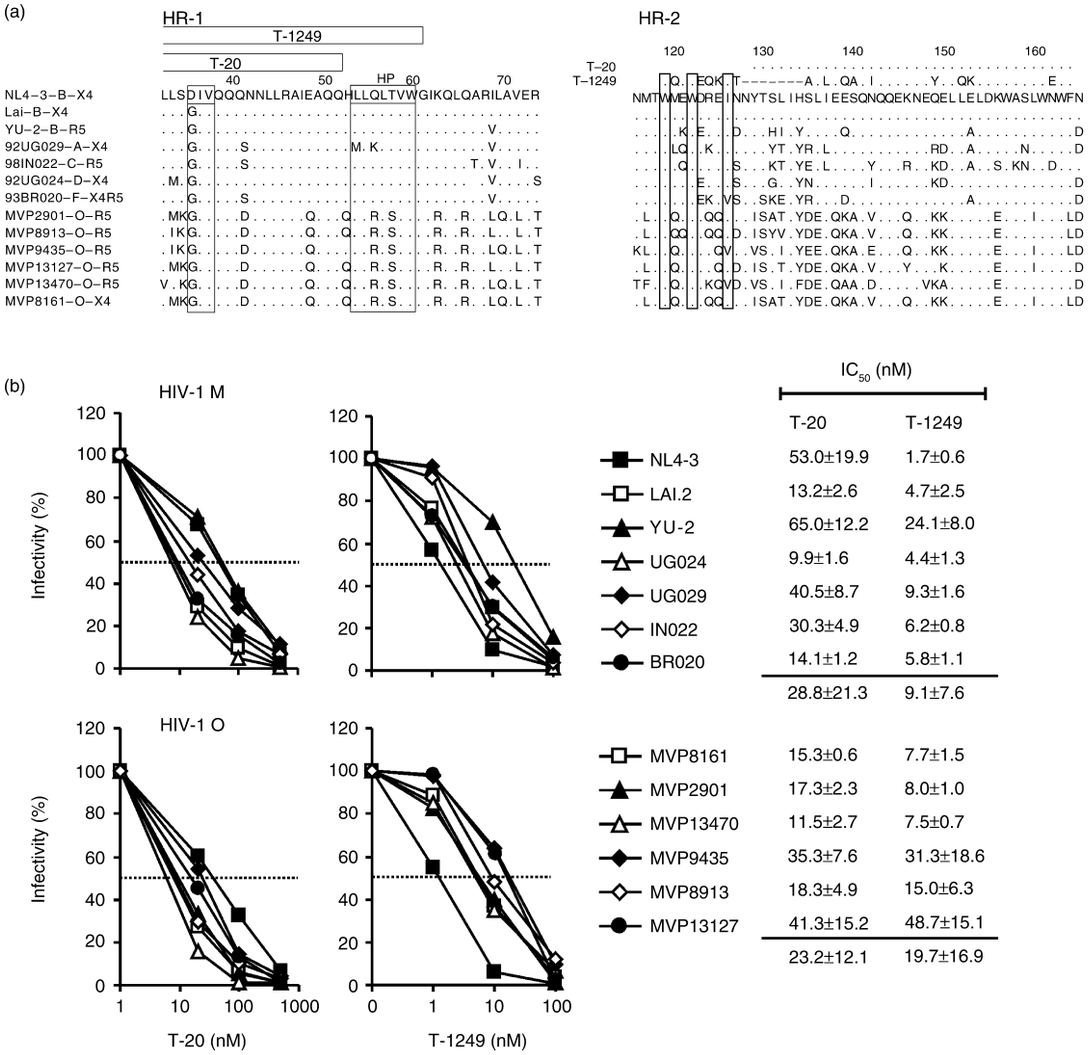

Fig. 1. Sequence diversity within the HR-1 and HR-2 regions and inhibition of highly divergent HIV-1 group M and O variants by T-20 and T-1249. (a) Alignment of the HIV-1 M and O HR-1 (left) and HR-2 (right) gp41 sequences. Dots indicate identity with the NL4-3 sequence and dashes the gaps introduced to optimize the alignments. The GIV motif (DIV in NL4-3) and the sequence of the hydrophobic pocket (HP) in HR-1 and the hydrophobic anchor residues in HR-2 are boxed. The HIV-1 group M sequences were derived from the Genbank database (accession numbers AAF69304, AAK31042, AAT67532 and AAT67533). Numbering refers to the HXB2 gp41 sequence. The letters and numbers following the names of the HIV-1 isolates or molecular clones specify the subtype or group, respectively, and the co-receptor tropism. (b) TZM-bl indicator cells were infected in triplicate with HIV-1 group M or O variants in the presence of the indicated concentrations of T-20 (left) or T-1249 (middle) as described previously [19]. Shown are average values of triplicate measurements for each drug concentration. The IC_{50} values (right) were derived from two independent experiments each performed in triplicate.

HR-1, the HR-2 amino acid sequences showed a higher degree of sequence diversity (Fig. 1a). Notably, only 20 of 36 (56%) and 21 of 39 (54%) of the T-20 and T-1249 amino acid residues, respectively, were preserved in the HIV-1 O HR-2 consensus sequence. However, the three hydrophobic anchor residues in HR-2 (W117, W120 and I124), proposed to bind in to the HR-1 hydrophobic pocket [20], were conserved.

Next, we analysed the sensitivity of the HIV-1 group B NL4-3, LAI.2 and YU-2 molecular clones, four HIV-1 M (clades A, C, D and F) and six HIV-1 O isolates to T-20 and T-1249-mediated inhibition as described [19]. All HIV-1 variants were inhibited by T-20 and T-1249, albeit with differential efficiency. The IC_{50} of LAI.2 (13.2 ± 2.6 nM) for T-20 was fourfold lower than that of NL4-3 (53.0 ± 19.9 nM; Fig. 1b) indicating that the G36D change in the 'GIV' motif reduces the susceptibility of NL4-3 to inhibition by T-20. In contrast, NL4-3 was highly sensitive to T-1249 (Fig. 1b). A mutation of N42D in the NL4-3 HR-1 region did not significantly affect HIV-1 sensitivity to inhibition by T-20 and T-1249

(data not shown). It has previously been proposed that the N42D variations might render group O viruses resistant to T-20 [14]. Unexpectedly, the average IC_{50} of T-20 obtained for the six HIV-1 O isolates (23.2 ± 12.1 nM; Fig. 1b, lower panel) was even lower than that of group M isolates (28.8 ± 21.3 nM; Fig. 1b, upper panel). Therefore, despite its high genetic diversity HIV-1 O is sensitive to T-20.

On average, HIV-1 M isolates were approximately threefold more sensitive to T-1249 than to T-20 ($P < 0.05$). In contrast to HIV-1 M, the group O isolates showed no significant differences in their susceptibility to T-20 and T-1249 (Fig. 1b). The T-1249 IC_{50} values of the HIV-1 O isolates ranged from 7.7 ± 1.5 to 48.7 ± 15.1 nM and were on average 2.2-fold higher than those of HIV-1 M variants (Fig. 1b). It is noteworthy that the susceptibility of the six HIV-1 O isolates analysed to both T-20 and T-1249 inhibition correlated significantly ($R^2 = 0.96$; $P = 0.001$). The HIV-1 O MVP9435 and MVP13127 isolates were less sensitive to T-20 and T-1249 than the remaining group O isolates (Fig. 1b), but did not contain any HR-1 sequence variations explaining their reduced susceptibility to fusion inhibitors. Therefore, changes outside of the HR-1 region might also modulate the sensitivity of HIV-1 to fusion inhibitors.

In conclusion, our data show that T-20 efficiently inhibits HIV-1 O entry, suggesting that this fusion inhibitor would be effective in individuals infected with highly divergent group O strains. Consistent with our results it has recently been documented that T-20 reduced the viral load in a patient with HIV-1 O infection [21]. On average, group O isolates were less efficiently inhibited by T-1249 than HIV-1 M isolates. This result was unexpected because T-1249 is composed of sequences derived from HIV-1, HIV-2 and SIV and targets the hydrophobic cavity in HR-1. However, the HIV-1 M and O deep pocket regions differ by changes of Q56R and T58S and the HR-2 region is also highly diverse (Fig. 1a). It has been reported that variations in HR-2 might impact the sensitivity of HIV-1 to entry inhibitors targeting HR-1 [22]. Peptides related to T-1249 might become the next generation of fusion inhibitors. Therefore, further studies are warranted on the impact of sequence variations in the HR-1 hydrophobic cavity and in the HR-2 region on the susceptibility of HIV-1 to fusion inhibitors and on viral fitness.

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A new insertion in the HIV-1 reverse transcriptase gene inducing major resistance to non-nucleoside reverse transcriptase inhibitors

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We identified an HIV-1 isolate with a 3 base pairs insertion in the 100–105 region of the reverse transcriptase gene (RT) along with a G190E and a V75A mutation. Virus carrying the insertion alone or in association with G190A was not infectious. The association of G190E and the 100–105 insertion displayed a high level of resistance to non-nucleoside reverse transcriptase inhibitors; the addition of the insertion to G190E may increase the activity of RT.

Non-nucleoside reverse transcriptase inhibitors (NNRTI) directly bind reverse transcriptase (RT), at a hydrophobic pocket near the catalytic site. The binding site is formed by amino acids from codons 100–110, 180–190 and 220–240 [1]. The most common mutations in viruses isolated from patients treated with NNRTI are Y181C and K103N. Other mutations include L100I, K101E, V106A, V179D, Y188H/C, G190A/C/Q/S/V/E/T, P225H, P236L and Y318F [2,3]. Mutations of residue 190 (mostly G190A/S) represent approximately 15% of NNRTI-resistant variants and confer variable levels of drug resistance and fitness [4,5]. Variants carrying the G190E mutation are linked to reduced susceptibility to NNRTI, but show impaired replication with significantly reduced polymerase, RNase H and protease activities [2–4,6].

We isolated a virus that simultaneously developed the triple association G190E mutation, the V75A mutation and a 3 base pairs insertion in the 100–105 region (LKKKKS → LKKKKKS) of the RT encoding region. To assess the impact on drug resistance of this new insertion, alone or in combination with other substitutions, we used overlap extension polymerase chain reaction mutagenesis and then conducted phenotypic testing using a recombinant virus assay (Phenoscript; Viralliance, Paris, France).

Briefly, these three mutations (V75A/insertion/G190E) appeared in a background of other mutations (RT gene: T69D, M184V, T215Y; protease gene: M46I, I54V, V82T, I884V) in a patient heavily pretreated, 6 weeks after the introduction of the combination abacavir, efavirenz and nelfinavir. The insertion initially encoded a lysine (LKKKKKS) and subsequently an arginine (LKKRKKS). Efavirenz was withdrawn after 14 months, and within 7 months the mutations V75A and G190E and the 3 bp insertion were no longer detected in classical genotypic resistance tests. When efavirenz was reintroduced 16 months later, V75A, G190E and the insertion reappeared within 2 weeks. No viral fitness variation was observed *in vivo* as judged on the plasma viral load and CD4 cell count, which were stable over time.

Site directed mutagenesis and phenotypic testing provided the following information (Table 1): the G190E mutation alone or in association with the V75A mutation conferred high-level resistance to efavirenz and nevirapine and a much reduced level of resistance to delavirdine. When combined with the insertion (K or R), G190E conferred high-level resistance to delavirdine, in addition to efavirenz and nevirapine. Surprisingly, the insertion (K or R) without the 190E mutation, whatever the codon at position 75, was not infectious in the Phenoscript assay. This may be the result of a marked conformational change in the RT protein, resulting in a loss of enzyme function. The G190E mutation could thus compensate for the insertion, restoring polymerase activity.

Mutation G190E is seen in only 1% of NNRTI-treated patients [2]. We found for the past 5 years in our laboratory nine G190E (in association with a L74V mutation in one case) compared with 278 G190A (ratio 1 : 29) (data not shown). Huang *et al.* [4] reported that G190 substitutions resulted in reduced infectivity, reduced RT protein synthesis, and low RT and protease activities. But clinical isolates carrying an amino acid substitution at position 190 replicated significantly more efficiently than their respective site-directed mutants in the context of other mutations or polymorphisms in the RT and the protease region, in a complex compensatory mechanism [4]. *In vitro*, G190E is selected only under