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The rare HIV-1 gp41 mutations 43T and 50V elevate enfuvirtide resistance levels of common enfuvirtide resistance mutations that did not impact susceptibility to sifuvirtide

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ABSTRACT

Mutations that are selected at low frequency and/or reside outside the enfuvirtide target region, amino acid 36–45 of gp41, might still be important determinants for drug resistance. This study aimed to investigate the phenotypic impact against enfuvirtide and sifuvirtide of uncharacterized gp41 mutations 42G, 43T and 50V, selected in patients failing enfuvirtide-containing regimens. As single mutations, neither 42G, 43T nor 50V conferred resistance to enfuvirtide. However, 50V increased slightly resistance levels for 36D, 38M, 43D or 43T as did 43T for 38M. All mutants displayed a reduced replication capacity, except 42S, 50V and 36D + 50V. None of the mutants displayed resistance to the next-generation fusion inhibitor sifuvirtide. This study highlights the necessity to confirm the in vitro effect of infrequently selected mutations as 42G was not associated with enfuvirtide resistance whereas 43T and 50V should be considered as secondary enfuvirtide resistance mutations.

1. Introduction

Historically, highly active antiretroviral therapy against HIV-1 infection consisted solely of reverse transcriptase (RT) and protease (PR) inhibitors. Despite being successful at suppressing viral replication for prolonged time, selection of resistant strains was observed and novel drugs inhibiting other viral targets were needed. Enfuvirtide was the first drug licensed for clinical use that did not interfere with HIV-1 RT and PR (Kilgore et al., 2003). This fusion inhibitor demonstrated to be very effective in treating patients with multidrug-resistant HIV-1. In subsequent large clinical trials, it was often an essential factor to achieve long-term successes with regimens containing new investigational drugs (Hicks et al., 2006; Clotet et al., 2007; Grinsztejn et al., 2007).

However, due to the patient's reluctance to subcutaneous injections, a switch from enfuvirtide to raltegravir in multidrug-resistant HIV-1 patients who were receiving a successful enfuvirtide-containing regimen was recently investigated (Harris et al., 2008; De Castro et al., 2009; Grant et al., 2009). As it demonstrated to be virologically non-inferior to the maintenance of enfuvirtide, this switch has also been implemented in clinical practice in most patients on enfuvirtide. Nevertheless, targeting the fusion process with antiviral drugs is still a promising approach, even in patients that failed enfuvirtide-containing regimens before (Lalezari et al., 2005; Cossarini et al., 2009). Therefore, extensive efforts are being made in developing novel fusion inhibitors with a longer half-life and a higher potency (Davison et al., 2006; Dwyer et al., 2007; He et al., 2008a; He et al., 2008c; Huet et al., 2009; Naito et al., 2009). The combination of these next-generation fusion inhibitors with enfuvirtide exhibit a highly synergistic effect, which could lead to a new therapeutic strategy. Despite in vitro synergistic effects even for enfuvirtide-resistant strains (Pan et al., 2009b; Pan et al., 2009a), it is likely that such strategy will be more successful against enfuvirtide-sensitive isolates. The major enfuvirtide resistance mutations were mapped to its target region, amino acids 36–45 within gp41 (Sista et al., 2004). Thus far, most enfuvirtide-resistant isolates remained susceptible to next-generation fusion inhibitors (Chinnadurai et al., 2007; He et al., 2008c; Eggink et al., 2009), but some degree of cross-resistance has recently been observed (Wang et al., 2009). In addition, in a clinical trial with the second generation fusion inhibitor T-1249 in enfuvirtide-experienced patients, mostly additional enfuvirtide resistance mutations were selected, suggesting a decreased genetic barrier to resistance due to prior enfuvirtide resistance (Melby et al., 2007). A thorough understanding of how specific enfuvirtide

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resistance mutations impact the susceptibility and development of resistance to other fusion inhibitors is thus necessary.

The clinical trials investigating the effect of enfuvirtide were performed in treatment-experienced patients harboring multidrug-resistant virus that at that time was mainly found in the western world (Lalezari et al., 2003; Lazzarin et al., 2003). As its HIV-1 epidemic has historically been dominated by subtype B viruses, research on enfuvirtide resistance was mostly restricted to this subtype. It remains uncertain if and to what extent the variable env genetic background influences resistance development to enfuvirtide and other entry inhibitors. In recent work, including ours, similar mutations have been observed for different subtypes within HR1 (D'Arrigo et al., 2007; Covens et al., 2009b). However, we did observe one novel mutation, S42G, within a subtype A strain. This mutation was previously only described as occurring at very low frequency in enfuvirtide-naïve virus strains (2 subtype G and 2 CRF02_AG strains) (Aghokeng et al., 2005). We also observed the concomitant selection within a subtype G strain of the frequently observed V38M with a phenotypically yet uncharacterized mutation N43T that by itself is very rarely selected. In addition, we observed twice the presence of a mutation that resides outside of gp41 aa 36–45, A50V, that was previously reported in vitro (Cilliers et al., 2005) and in vivo (Bienvenu et al., 2006) but of which the effect was not characterized. This mutation was concomitantly selected with a N43D and was already present at baseline in the strain that selected for V38M + N43T. Their selection under treatment with enfuvirtide, as well as their reversion after cessation of enfuvirtide, suggested that they may play a role in resistance.

The goal of this study was to examine the impact of S42G, N43T and A50V on replication capacity, susceptibility to enfuvirtide and cross-resistance against one of the next-generation fusion inhibitors, sifuvirtide.

2. Materials and methods

2.1. Cells and compounds

Human embryo kidney cells (293T) were purchased from the ATCC (LGC Standards, Teddington, UK) and cultivated in DMEM (Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal calf serum (FCS) (Perbio Science, Erembodegem, Belgium), 20 μg/ml gentamicin (Invitrogen) and 75 mM $NaHCO_3$ (Invitrogen). U87.CD4.CXCR4.CCR5 cells (Princen et al., 2004) were cultured in DMEM containing 10% FCS supplemented with 0.2 mg/ml geneticin (Invitrogen) and 1 μg/ml puromycin (Sigma–Aldrich, Bornem, Belgium). Subcultivation was performed every 2–3 days by digestion with trypsin/EDTA (Invitrogen). MT-4 cells (Miyoshi et al., 1981) and CEMX174 cells (Salter et al., 1985) were grown in RPMI 1640 (Invitrogen) supplemented with 10% FCS, 2 mM glutamine (Invitrogen) and 20 μg/ml gentamicin. All cell cultures were maintained in a humidified CO_2-controlled atmosphere. Enfuvirtide and AZT were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Sifuvirtide was kindly provided by Dr. Michaela Mack (FusoGen Pharmaceuticals, Inc, Chaoyang District, Beijing, China). AMD3100 was obtained from Merck & Co (Rahway, NJ).

2.2. Plasmids

The plasmid p83-10 (Gibbs et al., 1994) containing the 3′-half of the HIV-1-NL4.3-genome was obtained through the Research and Reference Reagent Program (Division of AIDS, NIAID, NIH) from Dr. Ronald Desrosiers. The plasmid p83-10D36G has been described previously (Covens et al., 2009a) and was used as template for site directed mutagenesis unless mentioned otherwise. The molecular clone pNL4.3-Δenv-EGFP, containing a deletion between nucleotide 6404 and 8458 and the gene encoding an enhanced green fluorescent protein (EGFP) between env and nef without affecting expression of any HIV-gene, was provided by M. Quiñones-Mateu of The Cleveland Clinic Foundation (Cleveland, OH) (Weber et al., 2006). All rights, title, and interest in pNL4.3-Δenv-EGFP are owned by The Cleveland Clinic Foundation.

2.3. Site directed mutagenesis

All mutations were introduced in the plasmid p83-10D36G (Covens et al., 2009a) by extending primers containing the desired mutations with PfuUltra (Stratagene, Amsterdam, The Netherlands) through thermocycling, followed by digestion of the template with DpnI (Fermentas, St. Leon-Rot, Germany) and subsequent transformation in *Escherichia coli* DH5α (Invitrogen). Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen, Venlo, The Netherlands). Presence of the desired mutation and absence of other mutations was confirmed by sequencing the entire gp160 gene as previously described (Van Laethem et al., 2005).

2.4. Generation of recombinant viruses

Env-recombinant viruses containing the EGFP gene were generated as previously described (Auwerx et al., 2008; Covens et al., 2009a). Briefly, the day before transfection 700,000 293T cells were subcultivated in 5 cm dishes containing 5 ml DMEM. Two micrograms of purified env-PCR product (QIAquick PCR Purification Kit, Qiagen) and 10 μg of purified XbaI-digested pNL4.3-Δenv-EGFP (EndoFree Plasmid Maxi Kit, Qiagen) were co-transfected in these 293T cells using the standard Calcium–Phosphate method. After overnight incubation, medium was refreshed and 48 h later, the supernatant was transferred to freshly plated U87.CD4.CXCR4.CCR5. Cell cultures were monitored for EGFP-expression using fluorescence microscopy. Virus supernatant were harvested by low speed centrifugation and stored in 1 ml aliquots at −80 °C for further use. The env sequence of the recombinant viruses was confirmed as previously described (Van Laethem et al., 2005; Covens et al., 2009a).

2.5. Susceptibility testing

The day before infection, MT-4 cells were subcultivated to a density of 600,000 cells/ml to ensure exponential growth at the time of infection. Within the single-cycle format, 30,000 exponentially growing MT-4 cells were added to 5 fold serial dilutions of enfuvirtide in 96-well plates and subsequently infected with EGFP-tagged virus in a total volume of 200 μl. Twenty-four hours after infection, cells were fixed by addition of 100 μl 6% PFA in PBS, reaching a final concentration of 2% PFA. Within the multiple-cycle format, 50,000 exponentially growing MT-4 cells were added to 5 fold serial dilutions of drugs in 96-well plates and were subsequently infected with EGFP-tagged virus in a total volume of 200 μl. Seventy-two hours after infection cells were fixed by the addition of 100 μl 6% PFA in PBS, reaching a final concentration of 2% PFA.

The percentage of EGFP-expressing cells was determined using a FACSCantoII flow cytometer (BD Biosciences, Erembodegem, Belgium) equipped with a High Throughput Sampler. The amount of virus used in the different assays was always based on viral titration experiments to yield 10% of EGFP-expressing cells under identical conditions. EC_{50} defined as the concentration of the inhibitor needed to achieve 50% inhibition of viral replication relative to a control experiment in absence of the inhibitor was determined for wild-type and mutant viruses in triplicate. For each experiment, the mean EC_{50} value of the mutant virus was compared to the mean EC_{50} value of the wild-type virus, generating the fold change (FC).

The mean fold change (mFC) and the standard deviation (SD) were obtained from at least three independent experiments. To enable the comparison of the degree of variation from different data series, the coefficient of variation (CV = SD/mFC) was also determined and expressed as %. Statistical analysis was performed in Microsoft Excel, *P* values for a two-sided Student's *t*-test were calculated.

2.6. Analysis of replication capacity

For the analysis of the replication capacity of the different mutant viruses, 5,000,000 exponentially growing CEMX174 cells were infected with virus (pre-diluted to achieve 0.01% of EGFP-positive cells after 72 h) in a total volume of 1 ml. After 3 h, the remaining virus was washed away with PBS and cells were cultured in 10 ml medium. Seventy-two hours after infection 5 ml of the culture were removed and these cells were fixed, and 5 ml of fresh medium was added to the culture. This procedure was repeated every 24 h for the following 72 h (i.e. 96, 120 and 144 h). The percentage of EGFP-expressing cells was determined on the FACSCantoII flow cytometer. The amount of infected cells was normalized to the percentage of infected cells at 72 h. The curves were fitted exponentially and the resulting *k*-values ($y = \exp(kt)$) were used as a measure of replication capacity (Brockman et al., 2006; Auwerx et al., 2008).

2.7. Selective advantage profile

To estimate the total impact on replication offered by a mutation within a range of drug concentrations, the relative infection of mutant virus (% EGFP + cells/% EGFP + cells in absence of drug as determined within the multiple-cycle MT-4 assay) was divided by the relative infection of the wild-type virus at different concentrations of enfuvirtide and plotted in function of the concentration of enfuvirtide expressed relative to the EC_{50} value of the wild-type.

2.8. Determination of EGFP-expression by flow cytometry

EGFP was excited using a 488-nm argon-ion laser and its expression was detected using a 530/30 nm-band-pass filter. Data were analyzed using FACSDiva v5.0.2 software (BD Biosciences). Forward- versus side-scatter plots were used to exclude dead cells and debris from analysis. For susceptibility testing, acquisition was stopped when 10,000 gated events were counted. For the growth curves, acquisition was stopped when 200,000 gated events were counted. For susceptibility testing, analytical gates were set in such a manner that fewer than 0.1% of cells in an uninfected control experiment were within the EGFP-positive region. For determination of the replication capacity of the recombinant viruses, the gates were set such that less than 0.001% of cells in an uninfected control experiment were observed within the EGFP-positive region.

3. Results

3.1. Construction of gp41 mutants

We previously described the selection of some uncommon mutations or mutational patterns under selective pressure of enfuvirtide in clinical isolates not belonging to subtype B (Fig. 1) (Covens et al., 2009b). To investigate the effect on enfuvirtide susceptibility of the mutations N42G, N43T, A50V, and the addition of A50V to N43D, V38M, N43T and V38M + N43T, the single mutations and combinations thereof were introduced into the NL4.3-D36G backbone. As the natural backbone of NL4.3 contains the enfuvirtide resistance mutation G36D, this mutant ± 50V was also investigated. In addition, as the 42G mutation was selected in a virus that bore the 42S polymorphism at baseline, known to increase susceptibility to enfuvirtide (Mink et al., 2005), virus bearing N42S was also generated.

3.2. Drug susceptibility of gp41 mutants

Susceptibility to enfuvirtide was tested in a multiple-cycle assay on MT-4 cells (Table 1 and Fig. 2). The viruses bearing N42S or N42G were significantly more susceptible to enfuvirtide than wild-type (0.07–0.38 fold) ($P < 0.05$). The single mutants N43T and A50V displayed similar values as the wild-type (1.21–1.60 fold). An additive effect of A50V and N43T on single mutants was observed, although not significantly. High fold changes were observed for the known enfuvirtide mutants D36G, V38M, N43D with and without A50V, although most were not significantly different from 1 due to large inter-assay variation. When tested for susceptibility to the next-generation fusion inhibitor sifuvirtide, none of the mutant viruses displayed any cross-resistance (Table 1). All viruses were apparently more susceptible (0.28–0.83 fold), yet only significantly for the viruses bearing N42S, D36G, D36G + A50V, N43T, V38M + N43T or V38M + N43T + A50V ($P < 0.05$). None of the viruses displayed significant increases or reductions in susceptibility to the controls, i.e. CXCR4 antagonist AMD3100 and NRTI AZT (Table 1).

As the observed fold changes for the A50V and N43T mutations were varying around 1 and failed to reach statistical significance, we wanted to investigate whether modifying assay conditions would confirm their phenotypic effect. In the single-cycle MT-4 assay (Table 2 and Fig. 2), these single mutants displayed again similar values as the wild-type (1.06–1.26 fold). However, the enfuvirtide susceptibility for all the other mutants were significantly different from wild-type ($P < 0.05$, with a trend for N43D + A50V, P 0.073). Most mutants were resistant to enfuvirtide whereas an increased susceptibility of N42S and N42G (0.29 and 0.42 fold) was observed. The A50V mutation increased slightly the susceptibility to enfuvirtide of the G36D, V38M, N43T, N43D and V38M + N43T mutations (1.07–3.18×), again not significantly. Overall, the observed mean FC were larger in the multiple-cycle assay but also the inter-assay variability, evidenced by higher CV values.

It thus appeared that the N42G mutation yielded a virus that was more susceptible to enfuvirtide than the parental virus, yet no significant difference was observed with the more common wild-type virus displaying N42S. As the phenotypic effect of resistance mutations can depend on the genetic background (Goubard et al., 2009), we tried to investigate the impact of 42G within the context of the original clinical strain. Although this mutant was present as a major variant within the plasma-derived virus, as determined by population sequencing, and obtained at a rather high viral load, we were not able to grow any clone bearing 42G despite successful transfection of 293T cells (as monitored by fluorescence microscopy). To our surprise we were able to grow a clone in which the 42G was back-mutated to 42S within a time-span comparable with laboratory adapted strains carrying resistance mutations (data not shown).

3.3. Replication capacity of the gp41 mutants upon normalization of infectious viral particles

To investigate how the described mutations and combinations of mutations affected the replication capacity, growth kinetic experiments were performed in a drug-free environment. Results for the different viruses are shown in Table 2. In agreement with a previously published study (Ueno et al., 2009) the G36D mutation did not result in a decreased replication of the NL4.3 virus. The impact of the N42S and A50V single mutations and of A50V on other investigated mutations appeared to be minimal. As observed in other studies, the single V38M and N43D mutations negatively

Strain	Week	HR1 sequence							
		30	40	50	60	70	80		
Ancestor M		QA RQLLS	**GIVQQ QSNLL**	RAIEA QQHLL	QLTVW GIKQL	QARVL AVERY	LKDQQ LL		
Subtype A	0	-- -----	----- -----	K---- -----	K---- -----	---L- -----	-R--- --		
	+48	-- -----	----- **-G**---	----- -----	K---- -----	----- -----	-R--- --		
Subtype D	0	-- ----F	----- **-N**---	----- -----	----- -----	---I- -----	----- --		
	+41	-- -----	----- **-ND**--	----v -----	----- -----	---I- -----	----- --		
Subtype G	0	-V -----	----- -----	K---V -----	K---- -----	----- ---C-	----- --		
	+52	-V -----	--**M**-- --**T**--	K---V -----	K---- -----	----- ---C-	----- --		
	+108	-V -----	--**M**-- --**T**--	K---V -----	K---- -----	----- ---C-	----- --		
	+155	-V -----	--**M**-- --**T**--	K---V -----	K---- -----	----- ---C-	----- --		
	+174	-V -----	--**M**-- --**T**--	K-m-V -----	R---- -----	----- ---C-	----- --		

Fig. 1. Sequence analysis of the HIV-1 HR1 domain (gp41 position 29–82) from patients failing enfuvirtide-containing therapy displaying the selection of N42G, N43D + A50V, V38M + N43T (the latter within A50V background) (Covens et al., 2009b). The amino acid sequence 36–45, previously associated with enfuvirtide resistance, is displayed in bold. Samples are annotated with their isolation date, as weeks following enfuvirtide initiation (+). Genetic forms were determined by submitting the sequences to the Rega HIV-1 subtyping tool (de Oliveira et al., 2005) (http://jose.med.kuleuven.be/subtypetool/html/index.html) and were subsequently confirmed by a manual phylogenetic analysis. Only the differences between the ancestor group M sequence and the respective patient-derived sequences are displayed in one letter symbols. Mixtures of wild-type and mutant are displayed in lower case.

Table 1
Drug susceptibility of recombinant viruses containing gp41 mutations in multiple-cycle assay.

Mutations[a]	Drug susceptibility[b]															
	Enfuvirtide				Sifuvirtide				AZT				AMD3100			
	mFC	SD	CV	*P*-value	mFC	SD	CV	*P*-value	mFC	SD	CV	*P*-value	mFC	SD	CV	*P*-value
N42S	0.38	0.17	43.40	0.024	0.28	0.15	54.30	0.014	1.75	0.47	27.14	0.246	1.48	0.98	65.78	0.453
N42G	0.07	0.02	22.53	0.000	0.63	0.22	34.52	0.100	1.61	1.45	90.31	0.500	0.79	0.53	66.21	0.547
A50V	1.21	0.36	30.02	0.419	0.83	0.34	40.59	0.478	1.79	0.83	46.38	0.200	0.78	0.14	17.86	0.076
D36G	8.11	3.6	44.37	0.076	0.40	0.12	28.50	0.013	1.39	0.58	41.57	0.328	1.23	0.34	27.28	0.314
D36G + A50V	10.88	4.36	40.13	0.058	0.32	0.12	36.82	0.010	1.15	0.46	39.90	0.601	0.88	0.07	8.44	0.071
V38M	10.74	5.51	51.29	0.092	0.63	0.33	51.84	0.192	1.34	0.26	19.05	0.102	1.23	0.23	18.98	0.182
V38M + A50V	7.4	1.34	18.14	0.014	0.50	0.28	56.53	0.091	1.51	0.84	55.70	0.373	1.35	0.21	15.26	0.062
N43T	1.6	0.54	33.50	0.194	0.32	0.15	46.81	0.016	1.15	0.49	42.31	0.632	1.29	0.40	31.12	0.298
N43T + A50V	3.1	0.54	17.33	0.021	0.37	0.90	246.17	0.349	1.03	0.79	77.09	0.960	1.17	0.49	42.01	0.662
N43D	31.43	8.08	25.72	0.023	0.76	0.30	39.26	0.300	0.73	0.30	41.47	0.288	0.75	0.19	25.13	0.142
N43D + A50V	41.56	10.8	25.98	0.023	0.36	0.30	82.81	0.066	1.76	0.63	35.78	0.128	1.04	0.35	33.20	0.784
V38M + N43T	34.81	23.14	66.48	0.127	0.35	0.05	13.75	0.002	1.52	0.74	48.44	0.307	1.01	0.07	6.61	0.866
V38M + N43T + A50V	84.59	50.31	59.48	0.103	0.40	0.08	19.41	0.006	1.45	0.97	67.33	0.484	1.12	0.56	50.24	0.744

[a] Mutations generated by site directed mutagenesis within the parental sequence NL4.3-D36G.
[b] For each independent experiment, the mean EC_{50} value of the mutant virus was compared to the mean EC_{50} value of the wild-type virus from triplicates, generating the fold change (FC). The mean fold change (mFC) was calculated as the mean of the FC values obtained from at least three independent experiments. Standard deviation (SD) of the mFC. To enable the comparison of the degree of variation from different data series, the coefficient of variation (CV = SD/mFC) was also determined and expressed as %. *P* value obtained with two-sided student's *t* test.

impacted viral replication (Lu et al., 2004; Tolstrup et al., 2007). The impact of the N42G and N43T singles was smaller but also noticeable. The negative impact of the 42G on viral replication helps to explain our inability to grow any env-recombinant virus derived from the clinical isolate bearing this mutation whereas a strain in which it was back-mutated to 42S was grown successfully. Finally, viruses containing the V38M + N43T mutations (either with or without A50V) displayed the most decreased replication.

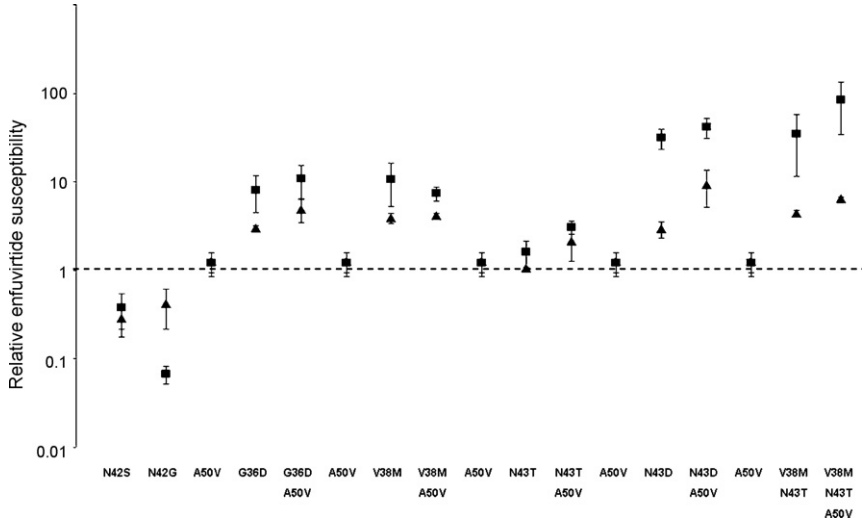

Fig. 2. Influences of single- versus multiple-cycle format on enfuvirtide susceptibility. Relative enfuvirtide susceptibility (fold change in EC_{50}) of recombinant viruses containing specific mutations within gp41 in a background of the parental strain NL4.3-D36G are shown. Enfuvirtide susceptibility was tested in MT4 cells in (▲) single-cycle and (■) multiple-cycle assays respectively. Mean fold changes in EC_{50} and standard deviations of 3 independent experiments are shown.

Table 2
Single-cycle susceptibility to enfuvirtide and replication capacity of recombinant viruses containing gp41 mutations.

Mutations[a]	Enfuvirtide susceptibility[b]				Replication capacity[c]	
	mFC	SD	CV	*P*-value	*k*	R^2
N42S	0.29	0.11	38.44	0.008	2.41	0.989
N42G	0.42	0.20	47.80	0.037	2.05	0.996
A50V	1.26	0.31	24.76	0.283	2.38	0.998
D36G	3.03	0.14	4.70	0.002	2.42	0.996
D36G + A50V	4.87	1.43	29.46	0.043	2.38	0.995
V38M	3.87	0.51	13.17	0.010	1.79	0.987
V38M + A50V	4.15	0.26	6.21	0.002	1.85	0.988
N43T	1.06	0.02	1.71	0.035	1.98	0.995
N43T + A50V	2.13	0.85	39.99	0.148	2.10	0.997
N43D	2.94	0.62	20.90	0.032	1.94	0.968
N43D + A50V	9.34	4.13	44.27	0.073	1.84	0.976
V38M + N43T	4.41	0.38	8.64	0.004	1.63	0.978
V38M + N43T + A50V	6.40	0.30	4.70	0.001	1.60	0.972

[a] Mutations generated by site directed mutagenesis within the parental sequence NL4.3-D36G.
[b] For each independent experiment, the mean EC_{50} value of the mutant virus was compared to the mean EC_{50} value of the wild-type from triplicates, generating the fold change (FC). The mean fold change (mFC) was calculated as the mean of the FC values obtained from at least three independent experiments. Standard deviation (SD) of the mFC. To enable the comparison of the degree of variation from different data series, the coefficient of variation (CV = SD/mFC) was also determined and expressed as %. *P* value obtained with two-sided student's *t* test.
[c] Slope *k* of regression curve log10 (fold replication capacity) = e^{kt}, time *t* as intervals of 24 h after 72 h post-inoculation. For parental virus NL4.3-D36G, $k = 2.42$ and $R^2 = 0.997$.

3.4. Selective advantage profile of the gp41 mutants

The selective advantage (relative infection of mutant divided by the relative infection of wild-type virus) was calculated for each mutant virus as described in the materials and methods section for all concentrations of enfuvirtide tested in the multiple-cycle assay. The average of two independent experiments plotted in function of enfuvirtide concentration is shown in Fig. 3 and consistent results were obtained with repeated testing. The profiles revealed that no mutation had a selective advantage in comparison to wild-type at concentrations lower than the EC_{50} for wild-type. The single mutations N42S, N42G and A50V offered no benefit at any concentration. The peak selective advantage of N43T was very small, but when A50 V was added an increased selective advantage was observed. This effect of A50V was observed for all investigated mutations. The third in line was V38M, followed by V38M + A50V, G36D, G36D + A50V, V38M + N43T, V38M + N43T + A50V, N43D and finally, N43D + A50V (Fig. 3). The peak selective advantage was often observed at lower concentrations than its respective EC_{50}. The concentration ranges for which the resistance mutations offered a benefit in respect to the wild-type were largely overlapping (extrapolated values between 1 and 75× EC_{50} wild-type, measured values between 3 and 15× EC_{50} wild-type), with the exception of V38M + N43T (±A50V) and N43D (±A50V) who also displayed a selective advantage at enfuvirtide concentrations above 75× wild-type EC_{50}. For N43D and N43T, the addition of A50V had only a remarkable impact on the peak of the selective advantage profile. For G36D, V38M and V38M + N43T, it broadened the range of enfuvirtide concentrations for which a selective advantage was observed.

4. Discussion

Rare HIV-1 mutations can be important determinants for drug resistance, as is e.g. proven for the rare T69-ins and Q151M reverse transcriptase mutations against nucleoside reverse transcriptase inhibitors. We therefore wanted to investigate the phenotypic impact of the S42G, N43T and A50V gp41 mutations selected in non-B subtypes under pressure of enfuvirtide (Covens et al., 2009b). Such investigations could provide additional information on the residual activity of enfuvirtide when recycling would be considered and are important for understanding the mechanism of action of enfuvirtide and the next-generation fusion inhibitor sifuvirtide.

Our results suggest that the rarely selected N43T and A50V mutations, observed by us in non-B subtypes, have no effect on enfuvirtide susceptibility by themselves. As they displayed an additive effect on the resistance levels of known primary enfuvirtide resistance mutations, they should however be considered as secondary resistance mutations. In contrast and as already known for N42S, N42G did not confer resistance to enfuvirtide. We used a two-sided Student's *t*-test to determine statistical significance of the observed fold changes in drug susceptibility (Clark et al., 2006). Considering the multiple testing problem (i.e. that using a *P* value of <0.05 as the limit for statistical significance resulting in 1 out of 20 insignificant FC wrongly assigned significant), the obtained *P* values should be cautiously interpreted. However, as none of the viruses displayed any significant changes in susceptibility to AZT or AMD3100 it could indicate that our observed effects for enfuvirtide and sifuvirtide with a *P* value <0.05 could be considered significant. Nevertheless, extrapolation of phenotypic resistance testing to clinical practice is not straight forward and requires the determination of clinical cut-off values which is beyond the scope of this study.

The reduced replication capacity observed for N42G is in agreement with 42S being the most prevalent polymorphism in the majority of HIV-1 group M subtypes (Kuiken et al., 2009). This was further supported by our failure to grow a recombinant virus derived from clinical clones containing the 42G mutation and the successful culturing of a back-mutated clone. This showed that the 42G mutation, and not the genetic background of the investigated on-treatment clones, accounted for the low replication capacity. The V38M, N43D and N43T mutations also showed a reduced replication capacity. Consistent with its potential role as a resistance-associated mutation, the addition of N43T to V38M ± A50V further decreased their replication capacity. The A50V mutation on the other hand hardly impacted replication of either wild-type or mutant virus.

The total impact of any mutation is a combination of its influence on viral replication capacity and antiviral susceptibility. The selective advantage profiles, previously used for resistance against protease and integrase inhibitors (Mammano et al., 2000; Perrin and Mammano, 2003; Quercia et al., 2009) revealed that A50V offered to the other gp41 mutants an increased benefit at enfuvirtide concentrations higher than the wild-type EC_{50}. In some occasions, it even resulted in a broadening of the concentration range in which the mutant viruses had a selective advantage to the wild-type.

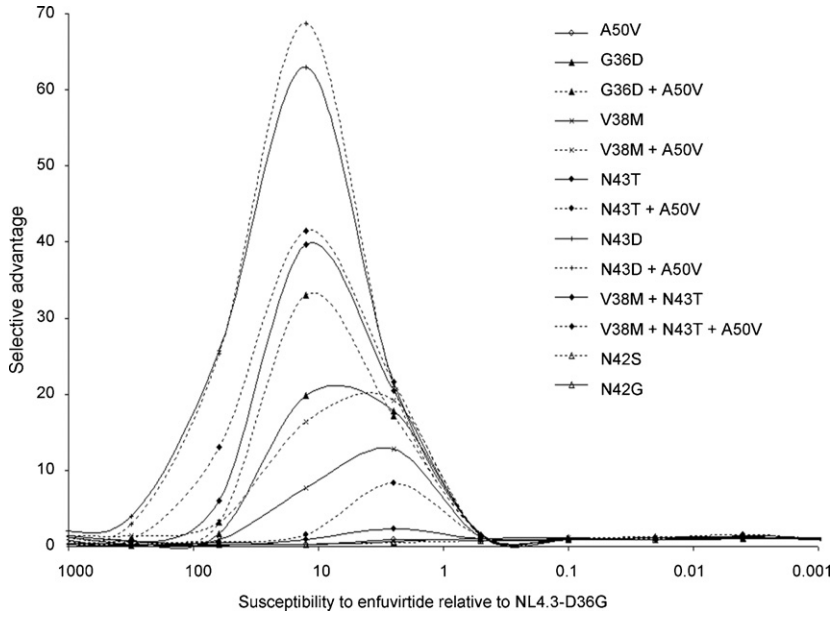

Fig. 3. The selective advantage profiles of viruses containing gp41 mutations N42S, N42G, G36D, V38 M, N43D, N43T, A50V and combinations thereof. The infectivity of mutant viruses relative to wild-type virus in function of enfuvirtide concentrations was determined.

All investigated mutants were characterized with an increased susceptibility to the next-generation fusion inhibitor as already published for other mutants (Eggink et al., 2008; Naito et al., 2009; Wang et al., 2009). This is not caused by a reduced replication capacity because increased susceptibility was also observed for mutants with normal replication capacity. Additionally, wild-type susceptibilities were obtained with the NRTI AZT and AMD3100, an entry inhibitor not targeting fusion but the co-receptor CXCR4.

Although the positioning of enfuvirtide and sifuvirtide in gp41 HR2 is largely overlapping and both drugs inhibit the fusion process between viral and cellular membranes, recent studies suggest that they might have a different mechanism of action (Liu et al., 2005; Liu et al., 2007; He et al., 2008b). Sifuvirtide (He et al., 2008c) and many other next-generation fusion inhibitors are shifted more to the N-terminus of the HR2 and contain the pocket-binding domain that is essential for their stable packing on HR1 coiled-coil and antiviral activity (Fig. 4). Enfuvirtide lacks this domain but extends to the gp41 membrane proximal external region (MPER). Although enfuvirtide's N-terminus substantially influences the affinity for the HR1 coiled-coil, it is the tryptophan-rich region at its C-terminus that is critical for its antiviral activity through the interaction with the cellular lipid membrane (Champagne et al., 2009). Additionally, it seems that enfuvirtide cannot make a stable 6-helix bundle in the presence of HR1-peptides, in contrast with the next-generation fusion peptides characterized by an increased helical structure in comparison to enfuvirtide. This might explain why single mutations within HR1 aa 36–45 that reduce the antiviral activity of enfuvirtide, do not influence the activity of next-generation peptides such as sifuvirtide (He et al., 2008a; He et al., 2008b). Additionally, it has been reported that enfuvirtide resistance mutations generally slow fusion kinetics (Reeves et al., 2005), which could increase the window of opportunity for a fusion inhibitor. In case of enfuvirtide, a decreased affinity for the mutant apparently has more impact than the increased lifespan of the targeted intermediate rendering the described mutants resistant. For the next-generation inhibitors the relative loss of binding affinity for enfuvirtide selected mutants is probably not as big and hence the prolonged lifetime of the sensitive intermediate conformation could result in increased susceptibilities. However, this does not necessarily mean that the next-generation HR2-peptides have a higher genetic barrier to resistance. Instead they could be more susceptible to helix-disrupting amino acid changes, whether induced by multiple mutations or specific structural and physicochemical changes at a certain position (Eggink et al., 2008; Eggink et al., 2009).

In conclusion, our study highlights the necessity to confirm the in vitro effect of infrequently in vivo selected mutations. We showed that S42G was not associated with enfuvirtide resistance despite its presence after therapy failure with enfuvirtide. However, N43T and A50V should be considered as secondary mutations

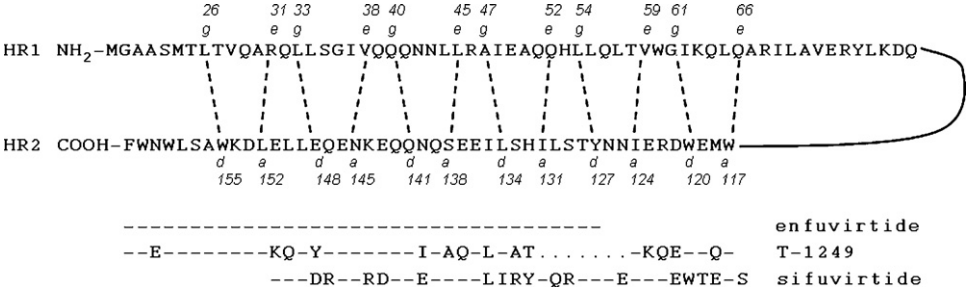

Fig. 4. Interaction between the HR1 and HR2 of gp41 of the NL4.3-D36G strain, with fusion inhibitors enfuvirtide, T-1249 and sifuvirtide aligned to HR2. The dashed lines between the HR1 and HR2 indicate interaction between the residues located at the (e and g) and the (a and d) positions in HR1 and HR2, respectively. Amino acids are numbered according to the NL4.3-D36G gp41 sequence. Dashes in the aligned fusion inhibitor peptides indicate that identical amino acids are present in the fusion inhibitor relative to HR2 of NL4.3-D36G and dots indicate absence of these amino acids in the fusion inhibitor.

that elevate resistance levels of primary resistance mutations, such as G36D, V38M and N43D. The next-generation fusion inhibitor sifuvirtide retained its activity against all investigated mutants regardless of their effect on enfuvirtide.

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