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Evolution of Genotypic and Phenotypic Resistance during Chronic Treatment with the Fusion Inhibitor T-1249

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ABSTRACT

T-1249 is a peptide HIV fusion inhibitor (FI) previously under development for use in FI-naive and experienced patients. Here we present prospectively planned longitudinal analyses of FI resistance during 48 weeks of T-1249 dosing in patients with extensive prior FI exposure. T1249-105 was a single-arm rollover study in patients with prior resistance to enfuvirtide (ENF) and 10 days of T-1249 functional monotherapy exposure. The phenotype and genotype of plasma virus envelopes were analyzed at baseline and at study weeks 8, 16, and 48. At study entry, viruses had a geometric mean decrease in susceptibility to ENF of 51.8-fold but to T-1249 of 1.8-fold; extensive genotypic resistance to ENF was observed. A median viral load response of −1.5 $\log_{10}$ copies/ml was observed at week 2 that was partially sustained (−0.5 $\log_{10}$ copies/ml) through 48 weeks. Resistance to T-1249 gradually increased to a geometric mean 92.7-fold decrease from FI-naive baseline; this occurred concomitant with further evolution of gp41 amino acids 36–45, most commonly the G36D ($n = 6$, 16%) or N43K ($n = 9$, 24%) substitutions. A novel substitution, A50V ($n = 12$, 32%), was also common, as were the N126K and S138A substitutions in heptad-repeat 2 (HR-2). These data point toward a primary role for the gp41 36–45 locus in modulating FI binding and suggest that residues in HR-2 may contribute in a more limited manner to development of peptide FI resistance. These data also point toward a substantial genetic barrier and fitness cost to development of resistance to next-generation fusion inhibitors.

INTRODUCTION

HIV ENTRY INTO HOST TARGET CELLS is mediated by the viral envelope glycoproteins gp120 and gp41, which mediate host receptor binding and membrane fusion, respectively. The first inhibitor of viral entry approved for clinical use was enfuvirtide (ENF), a peptide inhibitor of virus and host cell membrane fusion.[1] T-1249 is a peptide fusion inhibitor (FI) engineered from HIV-1 and -2 and SIV sequences to retain activity against ENF-resistant viruses. T-1249 demonstrated potent anti-HIV activity in FI-naive patients and in patients with demonstrated resistance to ENF;[2–4] however, clinical development of T-1249 was discontinued due to formulation difficulties. T-1249 continues to be studied as a potential topical microbicide and promising results have been reported from an exploratory SHIV vaginal challenge study (presented by J.P. Moore, XVI International AIDS Conference, Toronto, Canada, 2006; Entry inhibition as models for microbicide development).

ENF acts by binding to the heptad repeat (HR) 1 region of gp41 and preventing the association of HR2, which is required for HIV envelope mediated fusion.[5] Emergence of resistance to ENF has been closely linked to substitutions in HR1 in the region of gp41 amino acids (aa) 36–45, part of the predicted HR1 binding region for ENF.[6–12] ENF resistance mutations have also been observed at HR2 positions 126 and 138, which lie across from the ENF HR1 binding domain.[11,13] T-1249 binds to a partially overlapping region of HR1 and, additionally, binds to part of the structurally important "deep pocket" domain.[14] Genotypic resistance to T-1249 has been described for five fusion inhibitor-naive patients receiving T-1249 in dose-ranging monotherapy studies; these all carried mutations at gp41 positions 38 and 40.[3] Here we present prospectively planned, lon-

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gitudinal analyses of the development of resistance to T-1249 through 48 weeks in a single-arm chronic dosing rollover study, T1249-105.

MATERIALS AND METHODS

The present study was an open-label, single-arm, multicenter trial assessing the safety and tolerability of long-term T-1249 dosing in combination with an optimized antiretroviral regimen.[4] Patients enrolling in T1249-105 had previous experience in one of several ENF clinical protocols and had also participated in the T1249-102 study, which assessed response to T-1249 over 10 days when used to replace ENF in subjects harboring ENF-resistant viruses.[2,4] Approximately half of all patients rolled directly from the earlier T1249-102 study into the chronic dosing study without an interruption in T-1249 dosing. However, 23 subjects experienced an interruption in T-1249 dosing prior to entry; of these, 11 resumed dosing with ENF during that period while the other 12 temporarily discontinued FIs. Plasma virus envelopes from several patients who interrupted both T-1249 and ENF dosing showed substantial reductions in FI resistance at entry into T1249-105; therefore baseline data were analyzed by FI interruption, no FI interruption, and for all patients. On-treatment data were comparable between patients with and without interruption and thus were analyzed for all patients only.

Changes in non-FI antiretrovirals at entry into T1249-105 occurred simultaneously with initiation of chronic T-1249 dosing. However, due to extensive prior resistance only 20/49 (41%) subjects changed any non-FI antiretroviral agent. In both the T1249-102 and -105 studies, T-1249 was dosed at 192 mg/day, either as two injections once daily or one injection twice daily, depending on patient preference.

Envelope genotype and phenotype testing

Envelope susceptibility to T-1249 and ENF were measured using the PhenoSense Entry assay (Monogram Biosciences, South San Francisco, CA).[15] To allow comparisons between viruses with differing coreceptor tropism (defined here based on reporting of IC_{50}s on U87 cell lines expressing CD4 and either the CCR5 or CXCR4 coreceptor because specific coreceptor tropism test results were not obtained) data were normalized by multiplying the sample IC_{50} by the ratio of the standard reference value to the assay run reference value where the standard reference values were determined from multiple assay runs by Monogram Biosciences and the assay run reference value was the IC_{50} of the reference strain run concurrently with the patient sample; this was designated as nIC_{50}. Standard IC_{50} values to ENF for JRCSF (CCR5) and HXB2 (CXCR4) were 0.0286 and 0.00834 μg/ml, respectively, and to T-1249 were 0.0201 and 0.0071 μg/ml, respectively. For viruses with IC_{50} values reported for both cell lines, the average of the values was used; formal coreceptor use data were not collected. Genotypic data were obtained for gp41 amino acids 1–345 and were analyzed for the gp41 ectodomain residues 1–177. In cases where patients had two or more on-treatment visits, data were summarized from the visit with the highest viral nIC_{50} to T-1249. Resistance testing was attempted (when plasma viral load levels were greater than 1000 copies/ml) at baseline and weeks 8, 16, and 48 of treatment and data were compared to FI-naive baseline.

RESULTS

Study population and response to treatment

Forty-nine patients participated in the T1249-105 study with 40 (82%) completing 48 weeks of treatment; for all patients the median baseline plasma HIV-1 load was 5.0 $\log_{10}$ copies/ml and the median $CD4^+$ cell count was 96 cells/mm^3. Patients had extensive prior antiretroviral treatment experience; correspondingly, of patients with reverse transcriptase (RT)/protease phenotypes available at entry, 26/37 (70%) had no background antiretrovirals (ARVs) with predicted activity at screening and T-1249 was thus given to more than half of all patients as functional monotherapy. The median period of continued ENF dosing between virological rebound and entry into this study was 65 weeks.

Following an initial median drop in HIV RNA at 2 weeks of -1.5 $\log_{10}$ copies/ml, most patients experienced a partial virological rebound such that by week 8 the median decrease from baseline was -0.6 $\log_{10}$ copies/ml. This level of response was, however, maintained throughout the study period (range, -0.5 to -0.6 $\log_{10}$ copies/ml) and at week 48 50% of patients preserved at least a -0.5 $\log_{10}$ decrease in HIV RNA from baseline.[4] $CD4^+$ cell counts had increased by a median of 63 and 43 cells/mm^3 at weeks 24 and 48, respectively. Informal analyses found no discernible relationship between maintenance of virological response through 48 weeks and the degree of resistance or types of mutations found at either baseline or on treatment.

Baseline susceptibility to T-1249 and ENF and changes in susceptibility on treatment

Among patients with continuous FI treatment at baseline, the geometric mean (GM) fold-losses in susceptibility to T-1249 and ENF were 2.0-fold and 112-fold, respectively ($n = 28$). This compared to GM losses of susceptibility to T-1249 and ENF for patients who had interrupted FI treatment at baseline ($n = 12$, median 112 days) of 1.4- and 8.6-fold, respectively. Thus substantial losses of ENF resistance were observed among patients who interrupted FI therapy for a median of 16 weeks prior to entry (Table 1).

For patients demonstrating resistance to ENF at baseline, a modest correlation ($R^2 = 0.59$) was seen between the degree of resistance to ENF and that for T-1249; however, the degree of baseline resistance was not predictive of the development of subsequent resistance to T-1249 or ENF. One notable exception was for two patients with no resistance at baseline due to possible nonadherence (discussed below); neither of them developed resistance to T-1249 during subsequent study.

After study initiation, susceptibility to T-1249 decreased steadily to a GM of 17.9-fold at week 8 and 92.7-fold at week 48 (Table 2). In general, measurable remaining susceptibility to ENF was rapidly lost such that as early as week 8 the majority of patients had IC_{50}s to ENF that exceeded the maximum levels tested (20/31, 65%; data not shown).

Table 1. Baseline Susceptibility to T-1249 and Enfuvirtide[a]

Patient population	*T1249-105 baseline nIC$_{50}$ (μg/ml)*		*Fold change from FI naive (fold)*	
	T-1249	*ENF*	*T-1249*	*ENF*
FI interrupt				
N	12		12	
GM	0.016	0.279	1.4	8.6
Median	0.017	1.482	1.3	7.5
IQR	0.009–0.029	0.048–1.5	0.7–1.9	1.5–43.3
Non-FI interrupt				
N	35		28	
GM	0.033	4.470	2.0	111.7
Median	0.035	6.015	1.8	135.5
IQR	0.020–0.053	1.8–13.0	1.3–2.8	78.8–247.2
All patients				
N	47		40	
GM	0.027	2.201	1.8	51.8
Median	0.031	3.497	1.8	102.3
IQR	0.017–0.050	1.3–12.3	1.2–2.2	13.2–22.3

[a]Normalized IC$_{50}$ or fold change from baseline to T-1249 and enfuvirtide at entry into T1249-105. Patients who interrupted fusion inhibitors did so for a median of 112 days. Patients in FI-interrupt and noninterrupt groups had comparable levels of resistance prior to interruption (data not shown).

Genotypic substitutions in the gp41 ectodomain at baseline and emerging on-treatment

At entry into T1249-105, ENF resistance mutations were observed in virus envelopes from 41/47 patients (87%). Four of the six remaining patients had interrupted FI treatment prior to entry while two other patients showed no evidence of genotypic or phenotypic resistance, suggesting possible nonadherence at entry. Genotypic substitutions during T-1249 dosing were commonly observed in the region of gp41 aa 36–45; these increased from an average of 1.9 mutations at baseline to 2.6 following T-1249 treatment ($n = 37$ patients with paired data). The most common changes in plasma virus population genotype on treatment were the loss of wild-type residues at positions 36 (G) and 43 (N), which occurred in 12 (32%) and 10 (27%) patients, respectively (Table 3 and Fig. 1A). At position 36, complete or partial replacement by D, S, or R was observed in two or more patients each (5%) while at position 43, N was at least partially replaced by D or K in plasma virus sequences for three (8%) and nine (24%) patients, respectively. Emergence of the N43K substitution was particularly notable as only one example each of N43K or N43N/K was observed at baseline. Other relatively common changes from study entry included what appeared to be purifying selection at position 38 (as evidenced by a decrease in V38V/A mixture and increase of both wild-type and V38A genotypes), a shift away from H toward other substitutions at position 40, an increase in prevalence of N42T, the emergence of L44M, and the emergence of a novel resistance substitution, L45Q.

In addition to substitutions at positions in the region of primary ENF resistance, a novel locus for substitutions was observed at or near gp41 position 50 in the HR-2 region. The most

Table 2. Susceptibility to T-1249 by Study Week[a]

	Week 8		*Week 16*		*Week 48*	
	Fold change[b]	*nIC$_{50}$*	*Fold change*[b]	*nIC$_{50}$*	*Fold change*[b]	*nIC$_{50}$*
N	28	31	28	31	26	28
GM	17.9	0.296	24.8	0.418	92.7	1.190
Median	24.7	0.353	48.1	0.499	149.2	2.019
IQR	7.2–38.8	0.163–0.753	7.6–73.9	0.219–1.780	54.6–279.7	0.700–3.624

[a]Susceptibility to T-1249 by study week. Note the steady increase in resistance to T-1249 over 48 weeks. Susceptibility to ENF is not shown as it exceeded the range measured in the assay in the majority of patients at weeks studied. However, as an example, at week 48 the GM fold decrease from baseline was >899-fold with a median of >1839-fold and interquartile range of 1233 to 4530-fold.

[b]Calculation of fold change requires baseline data that may be missing for some patients.

TABLE 3. AMINO ACID CHANGES IN gp41 aa 36–45 AT THE TIMEPOINT OF HIGHEST T1249 nIC_{50} ON TREATMENT[a]

		Baseline (n = 37)		*On treatment*		*Gain on treatment*	
Position	*Amino acid*	n	%	n	%	n	%
36	G[b]	31	83.8%	19	51.4%	−12	−32.4%
	D	2	5.4%	5	13.5%	3	8.1%
	G/D	0	0.0%	3	8.1%	3	8.1%
	S	1	2.7%	3	8.1%	2	5.4%
	R (n = 2) or C	0	0.0%	3	8.1%	3	8.1%
38	V[b]	24	64.9%	26	70.3%	2	5.4%
	V/A	5	13.5%	1	2.7%	−4	−10.8%
	A	3	8.1%	6	16.2%	3	8.1%
40	Q[b]	30	81.1%	31	83.8%	1	2.7%
	T, H/Y or	0	0.0%	3	8.1%	3	8.1%
	H	5	13.5%	3	8.1%	−2	−5.4%
	Q/H	2	5.4%	0	0.0%	−2	−5.4%
41	Q[b]	37	100.0%	35	94.6%	−2	−5.4%
	Q/H	0	0.0%	1	2.7%	1	2.7%
	K	0	0.0%	1	2.7%	1	2.7%
42	N[b]	20	54.1%	19	51.4%	−1	−2.7%
	T	6	16.2%	10	27.0%	4	10.8%
	N/T	3	8.1%	1	2.7%	−2	−5.4%
	S[c] or S/T	5	13.5%	3	8.1%	−2	−5.4%
43	N[b]	26	70.3%	16	43.2%	−10	−27.0%
	D	2	5.4%	5	13.5%	3	8.1%
	K	1	2.7%	7	18.9%	6	16.2%
	K/R or R/S	0	0.0%	2	5.4%	2	5.4%
	N/K	1	2.7%	4	10.8%	3	8.1%
44	L[b]	36	97.3%	32	86.5%	−4	−10.8%
	M	0	0.0%	2	5.4%	2	5.4%
	L/M	1	2.7%	3	8.1%	2	5.4%
45	L[b]	25	67.6%	24	64.9%	−1	−2.7%
	Q	0	0.0%	4	10.8%	4	10.8%
	M	6	16.2%	7	18.9%	1	2.7%
	L/M	3	8.1%	1	2.7%	−2	−5.4%
	L/W or	2	5.4%	0	0.0%	−2	−5.4%

[a]Amino acid changes on treatment are summarized from the time point of each patients' highest nIC_{50} to T-1249 by position in gp41 aa 36–45. Note that the total N at each position may vary due to exclusion of genotypes seen in only one patient. Where multiple genotypes are shown in a single row, the incidence was $n = 1$ unless otherwise noted.

[b]Consensus baseline wild-type amino acid.

[c]Common polymorphism present in approximately 17% of isolates in the TORO studies.

common change there, A50V (or A/V), was observed at a frequency similar to changes at positions 36 or 43 (n = 12, 32%; Fig. 1B). Substitutions at adjacent gp41 residues 51–54 were also observed, albeit less frequently (total n = 4, 10%). In the HR-2 region, sequence changes were most common at positions 126 and 138 (each n = 9, 24%), and primarily involved the same N126K and S138A changes previously associated with ENF resistance. Of note, the N126K and S138A substitutions were detected in the same population sequence only once, and then both were present as mixtures; they thus appeared to be largely mutually exclusive. An increase in the prevalence of 140I and decrease in prevalence of 157S were the most frequent other changes in HR-2 (Fig. 1B).

Correlation between change in viral Env susceptibility to T-1249 and patterns of amino acid substitutions

To assess the genotypic changes associated with the development of substantial resistance to T-1249, we examined substitutions in viruses with nIC_{50}s to T-1249 above an arbitrarily chosen cutoff of 0.5 μg/ml (n = 26 or 70% of patients); for any patient with multiple on treatment samples, the sample with the highest nIC_{50} to T-1249 was characterized. In all envelopes meeting these criteria, changes were observed in gp41 aa 36–45 that included the accumulation of additional ENF-associated substitutions and/or the development of novel amino acid substitutions (Table 4, substitution patterns 1 and 2, respectively). In addition, changes at position 50 (Table 4, substitution pattern 3) were relatively common in this group but were rare in envelopes associated with lower nIC_{50}s (11/26, 42% versus 1/11, 9%). Substitutions in HR-2 relative to FI-naive baseline were also common, particularly at positions 126 and 138. In many cases these were present prior to T-1249 therapy, but in other instances they emerged or became more dominant (i.e., N126N/K to N126K) during the study. One intriguing observation was that the S138A mutation was observed in nearly all cases where a mutation at position 50 was detected. These two mutations were also always observed in conjunction with one

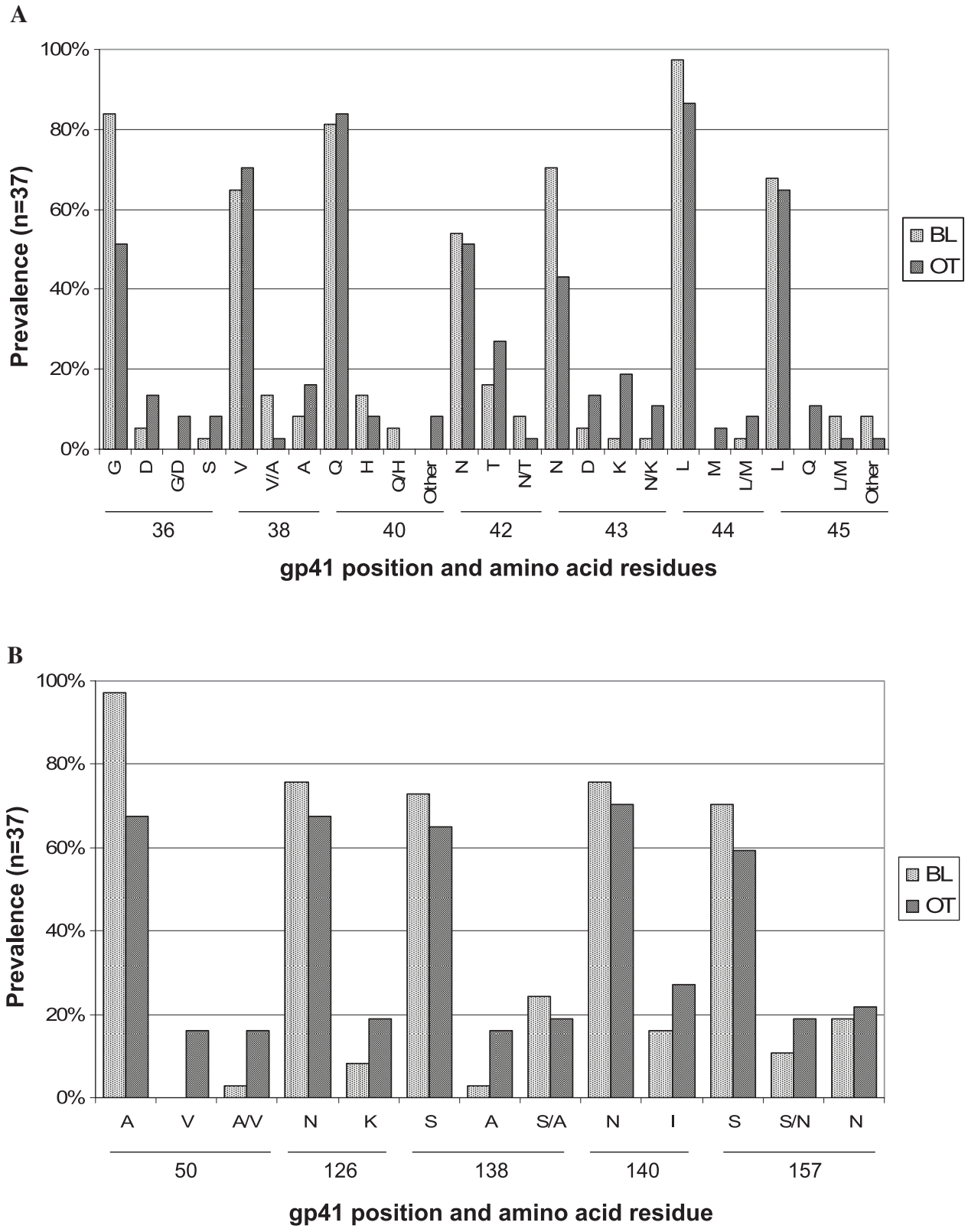

FIG. 1. Amino acid prevalence at baseline and on treatment, positions with most frequent changes. Positions are included that showed a particular change in virus envelopes from at least three patients; the most prevalent residue and any residues with changes in samples from at least two patients are shown at T1249-105 baseline and time of highest nIC_{50} to T-1249 during treatment. (**A**) Primary enfuvirtide resistance region of gp41 residues 36–45. (**B**) All other positions in the gp41 ectodomain (aa 1–177).

TABLE 4. MUTATIONAL PATTERNS FOR PATIENTS WITH MAX T-1249 $nIC_{50} > 0.5$ μG/ML[a]

Patient	*Cohort*	*Max nIC_{50} T1249*	*Max visit week*	*Substitution pattern*	*Substitutions at 126 or 138 (nearby positions)*
02	1	0.650	8	2	None
03	1	0.662	16	2, 3	S138S/A
43	2	0.833	8	1	N126K
04	1	0.866	16	1,3	S138A
53	2	0.947	48	1, 3	S138S/A
08	1	1.028	48	2, 3	S138S/A
14	1	1.386	48	1	None
40	2	1.515	16	1, 2	N126K
34	2	1.784	48	1,2	None
30	2	1.884	48	2	S138A
31	2	1.932	48	1	N126K
26	1	2.107	48	1, 3	N126N/H, S138S/A
28	1	2.254	48	1, 3	N126K
39	2	2.394	48	1, 3	S138A
47	2	2.959	16	1, 2	N126K
15	1	3.442	48	1, 3	S138A
06	1	3.445	48	1, 2	N126N/K
13	1	3.555	48	1	S138A
38	2	3.831	48	1,2	None[b]
54	2	5.841	16	1, 2	None (Q141L)
20	1	6.268	48	1, 2	None (E148K)
46	2	6.322	48	2	N126N/K
41	2	7.895	48	2	N126K
42	2	8.671	48	1, 2, 3	None (I135L)
16	1	13.543	48	1, 3	S138A
07	1	23.355	48	1, 2, 3	None (Q141L)

[a]Patterns of mutations observed in viruses with high nIC_{50} to T-1249 (>0.5 μg/ml). Patterns of mutations were defined as follows: pattern 1, additional enfuvirtide resistance mutations in gp41 aa 36–45; pattern 2, novel mutations in residues 36–45; pattern 3, additional mutations at or near position 50.

[b]The consensus wild-type N126 and S138 were present at week 48; however, no pretreatment sample was available for comparison.

or more resistance mutations in gp41 residues 36–45; however, neither appeared to be associated with a specific mutational pattern in residues 36–45. Of note, changes at positions 126 and 138 were absent in five of the nine samples (33%) with the highest nIC_{50}s to T-1249.

DISCUSSION

This study characterizes for the first time the genotypic and phenotypic characteristics of virus populations emerging *in vivo* during chronic treatment with the peptide fusion inhibitor T-1249. We found that despite extensive genotypic and phenotypic resistance to ENF at baseline, viruses remained largely susceptible to T-1249. Furthermore, although T-1249 was used as functional monotherapy in over 50% of patients, a median viral load response at week 2 of -1.5 $\log_{10}$ copies/ml was observed and partially sustained through 48 weeks at the level of -0.5 $\log_{10}$ copies/ml. Loss of susceptibility to T-1249 was gradual and was associated with continued evolution in gp41 residues 36–45 as well as other loci in the gp41 HR-1 and HR-2 domains. These data suggest that a relatively high barrier to resistance exists for T-1249, even in patients with prior long-term failing ENF treatment.

At baseline of this study, patients had major reductions in susceptibility to ENF but only minor losses for T-1249; these did, however, show a modest correlation with one another in patients with evidence of resistance to ENF. Consistent with other reports, resistance to ENF had waned substantially for patients interrupting fusion inhibitor therapy prior to study baseline.[16,17] However, resistance rapidly emerged upon resuming treatment with T-1249, suggesting that ENF-resistant strains, while unfit, are probably archived in the manner of strains resistant to other antiretrovirals. It also implies that ENF-resistant strains are more fit to replicate in the presence of T-1249 than are wild-type strains.[17–19] During this study, development of resistance to T-1249 was gradual and was generally accompanied by further loss of susceptibility to ENF. This indicates that T-1249 exerted additional selective pressure on viruses with high-level resistance to ENF. When taken in the context of the continued virological benefit observed through 48 weeks for the majority of patients, these data demonstrate a substantial barrier to the development of T-1249 resistance *in vivo* and therefore corroborate and extend previous *in vitro* findings (and unpublished data).[20,21]

In all samples demonstrating substantially reduced susceptibility to T-1249, additional substitutions were observed in gp41 residues 36–45. This raises an important issue in interpreting

these data: that prior ENF therapy will have selected for virus populations dramatically different from those present prior to FI treatment. The impact of such differences on the pathways of resistance observed during this study is difficult to assess, but will likely have been profound. Nonetheless, the notion that the gp41 aa 36–45 region has general relevance to T-1249 resistance is supported by observations for five FI-naive patients who developed resistance to T-1249 in an earlier study; in each case mutations were observed at gp41 positions 38 or 40.[3] If mutations in gp41 aa 36–45 are indeed a common denominator in resistance to HR-2 peptide FIs, it might point to a role for these residues in the nucleation of HR-2 peptide binding.

In addition to changes in gp41 residues 36–45, novel substitutions at or near gp41 amino acid position 50, particularly A50V, were common; this mutation has also been observed in some viruses following *in vitro* selection experiments with T-1249 (unpublished observations). Substitutions were also frequently observed in the HR-2 region; interestingly, these were largely the same as those observed during development of ENF resistance, N126K and S138A, although changes at other positions in HR-2 were also observed. Given the relatively long duration of partial virological suppression documented here, these data suggest that the HR-2 region may have a limited ability to confer resistance to T-1249 or to compensate for defects in fitness associated with resistance mutations in gp41 aa 36–45. However, similar to observations previously made for ENF, a wide range of IC_{50}s to T-1249 was observed for similar patterns of mutations, indicating that other regions of envelope are likely to contribute to determining susceptibility to ENF.

One novel observation was that the A50V and S138A mutations were observed almost exclusively in combination with one another. Given that these residues would not be expected to be in close proximity in the six-helix bundle, the nature of this relationship is unclear. Experiments to assess the impact of various combinations of these and other mutations on resistance to T-1249 and on viral fitness and fusion kinetics will be needed in order to address this question.

The data presented here have two major areas of relevance. First, they demonstrate the feasibility of the development of later generation peptide fusion inhibitors exhibiting potent activity against viruses with resistance to current compounds. Taken together with the higher durability of T-1249 and particularly of next-generation peptides during *in vitro* selection experiments, these data point to what may be substantial barriers to the development of resistance to future-generation HR-2 peptides. Second, the observation of substitutions largely at overlapping positions in the gp41 ectodomain during T-1249 and ENF therapies highlights the functional importance of this region to the entry process. Further studies will be necessary to understand the exact role in the viral entry process of positions associated with resistance to ENF and T-1249.

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