Poster No. 56

XII International HIV Drug Resistance Workshop, Cabo Del Sol, Los Cabos, Mexico, June 10-14, 2003

Determinants of Susceptibility to Enfuvirtide (ENF) map to gp41 in ENF-naïve HIV-1

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Introduction

Enfuvirtide (ENF, formerly T-20) is the first fusion inhibitor to demonstrate efficacy in controlled Phase III trials and was recently approved by the U.S. FDA, the European EMEA and the Swiss Health Authority. We previously reported that 118 fusion inhibitor (FI) naïve HIV-1 isolates from patients enrolled in phase II studies of ENF exhibited a range of in vitro susceptibility to ENF using a cMAGI based assay [1]. We also reported a similar spectrum of *in vitro* susceptibilities to ENF using a reporter virus assay employing viruses pseudotyped with HIV-1 envelopes from patient plasma pools derived from 612 fusion inhibitor naïve patients participating in phase III trials of ENF [2, see Fig. 1]. Several studies have suggested that HIV-1 envelope coreceptor tropism [3, 4] or affinity [5] may contribute to this observed range of *in vitro* sensitivities, although other investigations have not been able to demonstrate that coreceptor tropism is a determinant of sensitivity to ENF [6, 7]. Coreceptor tropism and affinity are properties conferred by the gp120 subunit of the HIV-1 envelope glycoprotein, thus, studies suggesting a role for coreceptor tropism or affinity imply that determinants of susceptibility to ENF lie within gp120. To further investigate where within the viral envelope gp160 determinants of ENF susceptibility were located and assess a possible role for the gp120 subunit, we employed an approach that used chimeric envelope constructs and site directed mutagenesis to examine the envelope gp120 and gp41 subunits as loci for determinants of ENF susceptibility. We used a set of low passage primary isolates from fusion inhibitor naïve patients that exhibited a 3 $\log_{10}$ spread in *in vitro* susceptibility to ENF as starting reagents to clone virus envelopes for further study (Table 1). Both R5 and X4 isolates were employed in these studies. Further studies are ongoing to better define the properties of these and additional envelope chimeras.

Methods

Preparation and Characterization of Virus Stocks

Low passage primary HIV-1 isolates were prepared from PBMC of FI naïve patients by coculture with activated normal donor PBMC or CD8-depleted PBMC. Donor cells were activated with PHA or a combination of soluble anti-CD3 and anti-CD28 antibodies. Sensitivity of HIV-1 isolates and virus stocks to ENF was determined in a cMAGI infectious center assay using the method of Reed and Muensh [8] to calculate IC_{50}. Tropism of virus stocks was inferred by either a) the ability to replicate on MAGI versus cMAGI cells [6] or b) the ability to form syncytia on MT-2 cells versus replication in activated PBMC.

Preparation and Characterization of Pseudotyped Reporter Viruses and Chimeric Envelopes

HIV-1 env genes from R5- and X4-tropic isolates exhibiting a range of ENF sensitivities (Table 1) were amplified by PCR from proviral DNA or by RT-PCR from virus RNA extracts. In addition, the envelope of an NL4-3 molecular clone modified by site directed mutagenesis (SDM) to contain the consensus glycine at gp41 amino acid position 36 was also cloned into an expression vector and used in these studies. The sensitivity to ENF of this NL4-3 molecular clone has been previous reported [9]. Full length envelope amplicons were cloned into an expression vector and used for production of pseudotyped reporter viruses [10, 11] and served as the starting point for gp120 and gp41 chimeric envelope constructs. We inserted an Mlu I site by SDM near the C-terminus of gp120 to facilitate construction of the gp120 and gp41 chimeras. Insertion of the Mlu I site did not alter amino acid residues in any of the starting envelopes. The Mlu I site was used to construct chimeric envelopes where the last 8 amino acids of gp120 and the entire gp41 subunit were derived from one donor envelope and the gp120 subunit (minus the C-terminal 8 amino acids) were derived from the other donor envelope (Fig 3). To construct chimeric envelopes where the HR1 region was contributed by the same donor envelope as the gp120 subunit (Fig. 4) we inserted a Sac II site in gp41. Insertion of this site allowed chimeras to be constructed where the gp120 subunit through the first 96 amino acids of gp41 (containing the HR1 region) were contributed by one donor envelope and the remainder of the gp41 subunit (containing the HR2 region through the cytoplasmic tail) were contributed by the other donor envelope (Fig. 4). Insertion of the Sac II site resulted in an S96A substitution in the MSL 20.4.21 envelope clone and a T96A substitution in the SC13 4.1.1 envelope clone. No amino acid alterations occurred in any of the other envelopes and the S96A and T96A substitutions did not alter the susceptibility to ENF of the MSL 20.4.21 or SC13 4.1.1 envelopes. Both the Mlu I and Sac II sites were used to construct chimeras where the gp120 subunit and gp41 regions downstream of gp41 amino acid 96 (containing HR2 through the cytoplasmic tail) were contributed by one donor envelope and the last 8 amino acids of gp120 and the first 96 amino acids of gp41 (containing the HR1 region) were contributed by the other donor envelope (Figure 5).

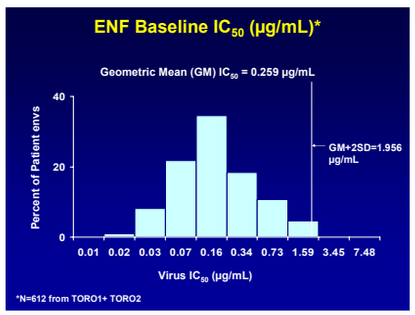
Coreceptor tropism and sensitivity to ENF inhibition of reporter viruses pseudotyped with envelope clones (and chimeric envelopes) were determined from reporter viruses produced following cotransfection of envelope expression vectors and an env-deficient NL4-3 based reporter virus construct into 293T cells (Fig. 2, Tables 2, 3, and 4). The pseudotyped reporter viruses were evaluated on U87 cells expressing CD4 and either CCR5 or CXCR4 [10, 11].

Sequences of all cloned and chimeric envelope constructs were determined from both strands by dideoxy sequencing chemistries using a Beckman Coulter CEQ 2000XL system and DNAStar software.

Site-directed mutagenesis was performed on parental envelopes at gp41 amino acids 45 (within HR1) and 135 (within HR2) as indicated (Table 4), to further investigate the role of these specific residues and the HR1 and HR2 regions as possible contributors to baseline susceptibility to ENF.

Results

Figure 1: In vitro sensitivities at baseline for HIV envelopes from 612 patients participating in the TORO1 and TORO2 Phase III trials.



- Full-length functional envelopes were cloned from five R5-tropic and two X4 tropic clade B primary virus isolates of HIV-1 (Table 1). The cloned envelopes exhibited ENF $IC_{50's}$ ranging from 0.04 µg/ml to 13.5 µg/ml in the U87-based pseudotyped reporter virus assay. The cloned envelopes retained the same tropism characteristics noted with the parental virus isolates and included envelopes with sensitivities that reflected both ends of the range observed in Fig. 1.

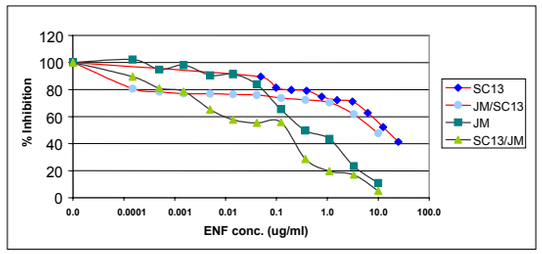
Results

Table 1: Fusion Inhibitor naïve primary isolates used for envelope cloning and their associated ENF IC_{50s}

	cMagi			U87
Virus Isolate	ENF IC_{50} (µg/ml)	Phenotype	Clone	ENF IC_{50} (µg/ml)
584.000084	0.001	CCR5	JM 8.2.22	0.261
589.000041	0.002	CXCR4	DEH 35.1.21	0.339
QH0707	0.005	CCR5	SC32 #5	0.036
QH0956	0.011	CCR5	SC47 #7	0.180
589.000033	0.480	CCR5	MMM 8.3.21	2.50
208.000092	0.168	CXCR4	MSL 20.4.21	3.72
QH0065	5.75	CCR5	SC13 4.1.1	13.5

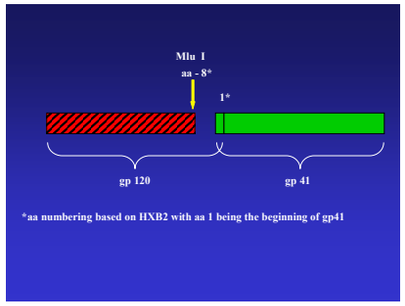
- Figure 2 shows representative data for sensitivity to ENF determined for 2 parental envelopes (SC13 and JM) and 2 gp120-gp41 chimeric envelope constructs (JM/SC13 and SC13/JM).

Figure 2: Sensitivity of cloned Envs and gp120/gp41 chimeras to ENF



- Figure 3 depicts the design of gp120-gp41 chimeric envelopes where the red and green portions are used to highlight the regions derived from the two differing envelope donors. The chimeric envelopes were examined for their susceptibility to ENF inhibition and the IC_{50} values obtained for the chimeric envelopes were compared to the starting gp120 and gp41 envelope donors to ascertain which, if either, subunit donor most closely resembled the sensitivity of the chimera (Tables 2a and 2b).

Figure 3: Schematic of gp120-gp41 chimeric envelopes



- Table 2a shows results with chimeric envelope constructs where the gp120 donor was held constant and the gp41 donor was varied. As seen in the column showing the ENF IC_{50s} of the chimeras, the observed ENF IC_{50s} of the chimeras varied considerably (over a range of 2 $\log_{10}$) and more closely followed that of the gp41 donor than the constant gp120 donor.

Table 2a: ENF sensitivity of chimeric envelopes with gp120 constant

gp120 donor		gp 41 donor		Chimera	
	ENF IC_{50} (µg/ml)		ENF IC_{50} (µg/ml)		ENF IC_{50} (µg/ml)
JM	0.261	GIV	0.196	JM/GIV	0.137
JM	0.261	DEH	0.339	JM/DEH	0.081
JM	0.261	MMM	2.50	JM/MMM	1.87
JM	0.261	MSL	3.70	JM/MSL	2.04
JM	0.261	SC13	13.5	JM/SC13	9.44

- Table 2b shows the results obtained with chimeric envelopes where the gp41 donor was constant and the gp120 donor was varied. Although the sensitivity to ENF inhibition of the gp120 donors varied by almost 15-fold, the ENF susceptibility of the chimeric envelopes varied only by about 2-fold and more closely resembled that of the gp41 donor.

Table 2b: ENF sensitivity of chimeric envelopes with gp120 constant

gp120 donor		gp41 donor		Chimera	
	ENF IC_{50} (µg/ml)		ENF IC_{50} (µg/ml)		ENF IC_{50} (µg/ml)
SC47	0.180	SC13	13.5	SC47/SC13	5.38
GIV	0.196	SC13	13.5	GIV/SC13	3.20
JM	0.261	SC13	13.5	JM/SC13	9.44
MMM	2.50	SC13	13.5	MMM/SC13	>10

Results

- The gp120-gp41 chimeras in Table 2a and 2b exhibited tropism specificity of the gp120 parental envelope. On the other hand, the major determinants of sensitivity to ENF tracked with the gp41 donor.
- We further constructed a series of chimeric envelopes exchanging either the N-terminal ectodomain of gp41 (containing HR1) or the C-terminal ectodomain of gp41 through the cytoplasmic tail (containing HR2) to examine whether we could discern if either or both of these regions were contributors to ENF susceptibility. Two sets of chimeras (depicted in Fig 4 and Table 3a) were made where the gp120 and gp41 N-terminal ectodomain donor were kept constant (designated JM or SC13 in Table 3a) and the gp41 C-terminal ectodomain through cytoplasmic tail donor was varied. As seen in Table 3a, although the HR2 donor envelope IC_{50s} for ENF varied by nearly 2 $\log_{10}$ in the set where the JM donor was constant, the ENF IC_{50s} for the chimeras varied only about 3-fold and approximated that of the JM donor. These results suggest the HR1 donor can contribute to ENF susceptibility. The data with SC13 as the donor suggests that for some constructs (i.e. with GIV or JM as the donor) the chimera ENF IC_{50} either falls in between the HR1 donor and HR2 donor values or nearer that of the HR2 donor, suggesting that HR2 can contribute to ENF susceptibility. The outlier value obtained for the SC13-MSL chimera in Table 3a points out some of the limitations and caveats of this approach. Some envelope chimeras can display ENF susceptibility values that fall outside the range for the parental envelope donors, other chimeras (not shown) fail to yield functional Envs. Caution should be used in interpreting results of chimeras as important interactions required for proper envelope function may be disrupted or altered. Studies are ongoing to further address these issues.

Figure 4: Schematic of gp120:HR1/HR2 chimeras

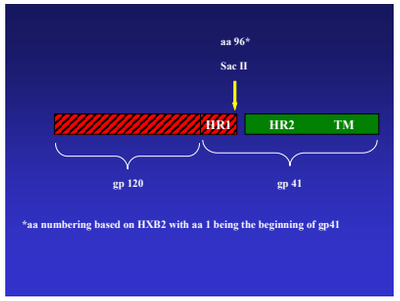
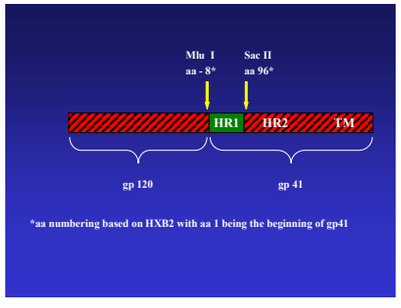


Table 3a: ENF sensitivity of chimeric envelopes with gp120/HR1 constant

gp120/HR1 donor		HR2/CT donor		HR1/HR2 chimera	
	ENF IC_{50} (µg/ml)		ENF IC_{50} (µg/ml)		ENF IC_{50} (µg/ml)
JM	0.261	GIV	0.196		0.103
JM	0.261	MMM	2.50		0.093
JM	0.261	MSL	3.70		0.262
JM	0.261	SC13	13.5		0.306
SC13	13.5	GIV	0.196		0.392
SC13	13.5	JM	0.261		1.63
SC13	13.5	MMM	2.50		>10
SC13	13.5	MSL	3.70		0.842

- Additional chimeric envelopes were constructed where the gp120 and C-terminal gp41 ectodomain through cytoplasmic tail were contributed by one donor and the N-terminal gp41 ectodomain contributed by another envelope donor (Fig. 5 and Table 3b). In Table 3b the $IC_{50's}$ of the chimeric envelopes more closely resembles that of the HR2 donor envelope than the HR1 donor envelope, suggesting that the gp41 C-terminal ectodomain through the cytoplasmic tail region can contribute to ENF susceptibility.

Figure 5: Schematic of gp120:HR2/HR1 chimeras



Results

Table 3b: ENF sensitivity of chimeric envelopes with gp120/HR2 constant

gp120/HR2/CT donor		HR1 donor		HR1/HR2 chimera	
	ENF IC_{50} (µg/ml)		ENF IC_{50} (µg/ml)		ENF IC_{50} (µg/ml)
JM	0.261	MMM	2.50		0.176
JM	0.261	MSL	3.70		0.550
JM	0.261	SC13	13.5		0.300

- Overall, we interpret the results with chimeric Env constructs exchanging either the N-terminal ectodomain (containing HR1) or the C-terminal ectodomain through the end of gp41 (containing HR2) as suggesting that both regions can contribute to baseline susceptibility to ENF.
- Inspection of the amino acid sequences for the HR1 and HR2 region of the SC13 envelope clone (and virus isolate) did not identify an obvious basis for the unusual ENF suceptibility of this virus isolate. However, we noted an L45M substitution in the HR1 region and a conservative (though uncommon) I135L substitution in the HR2 region of the SC13 virus envelope. Molecular modeling of the HR1-HR2 six helix bundle [12] suggested that these two residues were brought into close proximity within this structure (Fig.6), and therefore we performed site directed mutagenesis to examine the effects of these amino acids on the ENF susceptibility of the SC13 virus envelope and extended these studies to several other envelopes (Table 4).

Figure 6: Model of HR1/HR2 highlighting positions of aa45 and aa135.

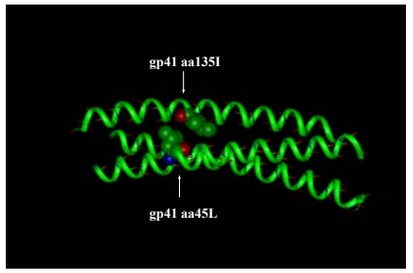


Table 4: ENF sensitivities of SDM clones

	gp41aa 45,135			
	ENF IC_{50} (µg/ml)			
Parental envelope clone	M,L	L,L	M,I	L,I
SC13 4.1.1	13.5	1.61	6.98	0.824
MMM 8.3.21	6.52	2.50	3.40	1.35
MSL 20.4.21	7.81	3.72	5.01	2.75
DEH 35.1.21	0.258	0.227	0.35	0.339
JM 8.2.22	1.47	0.120	1.03	0.261

- The results shown in Table 4 demonstrate that ENF sensitivity can be modulated by particular residues at gp41 amino acids 45 (within HR1) and 135 (within HR2) with amino acids observed in rare FI-naïve isolates or those more commonly found. In addition, these results provide additional support for the assertion that both the HR1 and HR2 regions can contribute to susceptibility to ENF

Conclusions

- Previous studies have demonstrated that the HR1 region of the HIV-1 gp41 is the target for ENF.
- Results from Phase III clinical studies of ENF have shown that this same region is the primary locus for development of ENF resistance.
- Our current results suggest that gp41 also contains the major determinants for baseline sensitivity to ENF of clade B FI-naïve virus.
- Both HR1 and HR2 regions of gp41 contribute to baseline susceptibility to ENF.
- ENF sensitivity can be modulated by SDM of gp41 amino acids 45 (within HR1) and 135 (within HR2) with amino acids observed in rare FI-naïve isolates or those more commonly found.
- Although our data support the conclusion that gp41 is the major influence on ENF susceptibility, they do not rule out a more modest role for other envelope regions such as gp120.

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Poster Number 56 – XII International HIV Drug Resistance Workshop, Cabo Del Sol, Los Cabos, Mexico, June 10-14, 2003