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The human immunodeficiency virus type 1 reverse transcriptase E138A natural polymorphism decreases the genetic barrier to resistance to etravirine *in vitro*

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Running title: HIV-1 RT 138A polymorphism decreases the genetic barrier to resistance to etravirine

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Abstract

Objectives. The HIV-1 reverse transcriptase (RT) natural polymorphism E138A is included among the mutations with a minor impact on response to etravirine. However, the interpretation of E138A on etravirine susceptibility is not consistent across different genotypic resistance algorithms. The aim of the study was to investigate the effect of E138A on the genetic barrier to resistance to etravirine *in vitro*.

Methods. A panel of 20 clinically derived recombinant viruses (10 with wild type 138E and 10 with 138A, all without any other resistance mutation) were cultured in the presence of increasing etravirine concentration and analysed for genotypic changes at virus breakthrough. Parallel experiments were conducted with 138E/A/G/K/Q NL4-3 based clones.

Results. In the NL4-3 background, codon 138 changes increased etravirine resistance in the following order: Q>K>A>G>E. The 138A viruses were less susceptible to etravirine compared with the 138E viruses (median [IQR] fold-change, 1.8 [1.5-2.8] versus 1.3 [0.8-1.8]; P = 0.026), overcame etravirine pressure earlier (HR [95% CI] for viral outgrowth with 138A, 5.48 [2.95-28.24]; P < 0.001) and grew at higher drug concentrations (median [IQR], 1350 [1350-1350] versus 0 [0-1350] nM; P = 0.005). A variety of etravirine resistance related mutations and changes in the RT connection and RNase H domain accumulated without any consistent pattern depending on baseline codon 138.

Conclusions. E138A can contribute to reduced response to etravirine through a decreased genetic barrier to resistance. *In vitro* drug resistance selection is a valuable complement to define the full potential of low-level resistance mutations.

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Introduction

Etravirine is a second generation NNRTI indicated for the treatment of HIV type 1 (HIV-1) infection in treatment experienced patients both in Europe and US.[1,2] *In vitro* experiments demonstrated that etravirine has a higher genetic barrier to resistance and a different resistance profile compared to the first generation NNRTIs nevirapine and efavirenz.[3,4] The efficacy and safety of etravirine were originally investigated in treatment-experienced patients in the DUET-1 and DUET-2 clinical trials, where the addition of etravirine to background regimen showed significantly higher rates of viral suppression compared to the placebo plus background regimen up to week 96.[5] Accordingly, etravirine has then been used in the clinical setting primarily in heavily treatment experienced patients.[6,7] Based on favourable data on durability, tolerability and genetic barrier, etravirine has been also evaluated as a component of two-drug regimens in several studies in patients with either viral suppression or virologic failure.[8-10] These studies concluded that etravirine based dual regimens were effective both in maintaining undetectable viremia in suppressed patients and in contributing to achievement of virological control in highly treated patients with limited therapy options.

Analysis of the correlation between baseline HIV-1 genotype and virological response in the DUET studies led to the identification of 17 etravirine resistance associated mutations (RAMs) in HIV-1 reverse transcriptase (RT) including V90I, A98G, L100I, K101E/H/P, V106I, E138A, V179D/F/T, Y181C/I/V, G190A/S, and M230L,[11] while treatment emergent mutations were most often detected at codons 101, 138, 179 and 181.[12] Variants emerging at codon 138 were highly heterogeneous and most often accompanied by other etravirine RAMs, prompting to further investigation through site directed mutagenesis and resulting in the addition of E138G, K, and Q to the existing etravirine weighted genotypic score.[13] Of note, the etravirine RAM E138A occurs as a natural polymorphism and is more prevalent in subtype C than B in different databases with frequencies ranging from 5.9 to 7.5% versus 0 to 2.3% in treatment naïve patients and 5.9 to 6.1% versus 2.0 to 2.5% in treatment experienced individuals, respectively.[14] However, subtype specificity is subject to geographic

variation. For example, E138A is found in 5.2% (270/5209) of RT sequences from treatment naïve patients stored in the Italian Antiviral Response Cohort Analysis database (www.dbarca.net), with comparable frequency in subtype B (6.0%) and C (5.3%). In addition, E138A is interpreted differently by the most widely used genotype interpretation systems, with Stanford HIVdb, REGA and ANRS scoring this variant as potential low-level resistance, full susceptibility and possible resistance to etravirine, respectively. Based on these data, we were interested in assessing the impact of E138A in the genetic barrier to resistance to etravirine. In this study, we evaluated the development of resistance to etravirine by *in vitro* selection experiments in two panels of clinically derived recombinant strains carrying the wild type 138E or the 138A variant in the absence of any other etravirine and NNRTI RAMs. Site-directed mutant infectious plasmids were also used to evaluate the effect of different amino acids at codon 138 in a syngeneic background.

Materials and methods

Clinical samples and ethics

Twenty plasma samples were selected from HIV-1 positive untreated patients previously tested for routine drug resistance genotyping at baseline, as recommended by European guidelines [EACS 2017]. Samples choice was based on the presence of the 138E wild type codon (n = 10) or the 138A polymorphism (n = 10) within the RT coding region and no other NNRTI RAMs, according to the IAS-USA drug resistance mutations list,[15] as well as no RT mutation (except for E138A itself) conferring any level of etravirine resistance in the Stanford HIVdb algorithm, version 8.6 (A98G, L100I/V, K101E/H/P, E138G/K/Q/R, V179D/E/F/L, Y181C/F/G/I/S/V, Y188L, G190A/C/E/Q/S/T/V, H221Y, F227C, and M230I/L). The use of residual, anonymized clinical samples for research studies was regulated by patient informed consent, as approved by the South-East Tuscany Ethical Committee. Clinical and laboratory data of the patients were stored in the ARCA database (www.dbarca.net).

Construction of recombinant viruses and phenotypic determination of susceptibility to etravirine

104 The method used for the creation and titration of recombinant viruses carrying patient derived RT-RNaseH
105 coding region has been previously described.[16] The PCR primers and the protocol for amplification of the RT-
106 RNaseH region from viral RNA are included in the Supplementary Data. The HIV-1 subtype B NL4-3 laboratory
107 strain (harbouring the RT 138E codon) was used as the wild-type virus. In addition, pNL4-3 based plasmids
108 carrying RT 138A, 138G, 138K and 138Q were constructed through site-directed mutagenesis by using the
109 QuikChange® Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA), according to
110 manufacturer's instructions. Baseline etravirine susceptibility was evaluated by quantifying luciferase activity
111 after infection of TZM-bl cells with recombinant viruses in the presence of serial dilutions of the drug. To
112 determine the IC_{50} of each recombinant virus, TZM-bl cells were seeded in a 96-well plate at 15,000 cells per
113 well and infected at a multiplicity of infection (MOI) of 0.01 in the presence of 5-fold serial dilutions of
114 etravirine (range 10,000 – 5.12 nM). After 48 hours, the cells were lysed by adding 50 µL/well of Glo-Lysis
115 Buffer (Promega, Madison, WI, USA) and the lysates were transferred to a luminescence plate. Fifty microliters
116 of Bright-Glo Luciferase Reagent (Promega) were added to each well and the luminescence was measured with
117 the GloMax® Discover Multimode Microplate Reader (Promega). Relative Luminescence Units (RLU) detected in
118 each well were elaborated with GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA) to calculate
119 the IC_{50} values of recombinant and wild-type viruses.
120
121 *In vitro resistance selection (IVRS)*
122 The recombinant viruses, as well as the reference wild type and mutant NL4-3 strains were used to infect 10^6
123 MT-2 cells at a MOI of 0.1 in the presence of an initial concentration of 50 nM etravirine corresponding to 4-
124 fold the IC_{50} of wild type 138E. Viral replication was monitored every 48-72 hours by microscopic inspection of
125 syncytia. When advanced cytopathic effect was observed, the supernatant was harvested, cleared by
126 centrifugation for 10 minutes at 300 g and stored at -80°C until genotypic analysis. Subsequent passages were
127 set up using the harvested virus to infect a new culture of 10^6 MT-2 cells in the presence of a 3-fold higher

concentration of etravirine (150, 450 and 1350 nM). IVRS cultures were interrupted after viral breakthrough at 1350 nM etravirine or after 108 days from the start of the experiment, whichever occurred first.

RT-RNaseH sequencing

The whole RT-RNaseH coding region sequence (nucleotides 2550-4229 in HIV-1 HXB2 reference strain) was obtained from each recombinant virus by Sanger sequencing at baseline and at each viral breakthrough to detect emergent mutations. Viral RNA was extracted using the EZ1 Advanced XL system (Qiagen, Hilden, Germany) with the EZ1 DSP Virus Kit (Qiagen), reverse-transcribed and amplified as described in the Supplementary Data.

Statistical analysis

The probability of virus breakthrough in cell culture under etravirine pressure was computed by survival analysis and the difference between 138E and 138A viruses was examined by Mantel-Cox log rank analysis. The Mann-Whitney U test was used to analyse the differences in baseline etravirine susceptibility and in the maximum etravirine concentration at which virus growth occurred with 138E and 138A. All tests were done by GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA).

Results

Based on phylogenetics analysis of the whole RT-RNaseH coding region, the clinical variants carrying the wild type 138E codon included six subtypes B, two subtypes F1 and two B/F recombinants close to CRF12_BF while the clinical variants carrying the mutant 138A codon included nine subtypes B and one CRF02_AG. Following recombination, the chimeric viruses were confirmed to retain the original sequence as determined at the time of routine drug resistance genotyping, without any NNRTI resistance mutation. NRTI and major PI resistance

mutations were also not present in any isolate. Natural polymorphisms are shown in Supplementary table 1 and the baseline sequences have been made available through GenBank at accession codes MH682065-MH682084.

At baseline, the fold-change etravirine susceptibility with respect to the reference NL4-3 was significantly higher with 138A compared with 138E carrying viruses (median and IQR 1.8, 1.5-2.8 versus 1.3, 0.8-1.8; P = 0.026) (Figure 1). All site-directed NL4-3 mutants carrying different amino acids at codon 138 were less susceptible to etravirine compared to the wild type 138E reference virus (Table 1). However, the 138K NL4-3 virus replicated poorly in drug resistance selection experiments yielding cytopathic effect only at 50 nM etravirine in the absence of any RT mutation. The 138A NL4-3 virus also was not able to grow at etravirine concentration higher than 150 nM, despite initial selection of one etravirine resistance mutation (M230I). By contrast, the wild type 138E and the mutant 138G and 138Q NL4-3 viruses all grew up to the highest etravirine concentration used but selecting different combinations of mutations at codons involved in resistance to etravirine, i.e. 100, 106, 179, 181, 227, 230.

All of the ten clinically derived 138A viruses replicated up to 450 nM etravirine and all but one replicated up to the final highest 1350 nM etravirine concentration. By contrast, only three of the clinically derived 138E viruses were able to replicate at the highest concentration, one grew up to 450 nM etravirine and the remaining six did not even grow at the lowest 50 nM etravirine concentration used. The difference between the 138E and 138A groups was statistically significant, both in terms of time to first virus breakthrough (hazard ratio for viral outgrowth with 138A, 5.48, 95% CI 2.95-28.24; P < 0.001; Figure 2) and in terms of maximum etravirine concentration overcome by virus growth (median and IQR: 0 and 0-1350 nM for 138E versus 1350 and 1350-1350 for 138A; P = 0.005; Figure 3).

Table 2 shows the RT amino acid changes detected in the breakthrough virus for all clinical isolates at the different etravirine concentration steps, with respect to the individual baseline sequence. Mutations emerging with at least three isolates at any time point included V179D/E/F (7 cases), Y181C (5), M230I (4), and T240I (3). Of these, T240I was the only mutation exclusively emerging with baseline 138A viruses (the three clinically derived recombinants and the 138A NL4-3 reference virus). Of the 14 viruses replicating at least up to the 450 nM etravirine concentration, 12 eventually carried at least one of the mutations included in the etravirine resistance score derived from the DUET studies and/or conferring at least low-level resistance to etravirine according to the Stanford HIVdb algorithm. One of the two cases without any of these mutations showed the V179E change conferring only potential low-level resistance to etravirine in the Stanford HIVdb algorithm (sample 138185) while sample 146102, which was the 138E virus with the largest decrease in baseline etravirine susceptibility, selected mutations not known to be involved in any NNRTI resistance and did not grow at the highest etravirine concentration used.

Interestingly, mutations in the RT connection domain (amino acids 320 to 440) emerged in two 138E (143026, 146473) and seven 138A (60154, 72009, 81958, 100442, 127757, 138185, 141163) clinically derived viruses. Likewise, mutations in the RNase H domain (amino acids 441 to 560) also emerged in two 138E (143035, 146473) and seven 138A (52420, 60154, 81958, 95551, 100442, 127757, 138185) clinically derived viruses. Overall, as many as 31 distinct connection or RNase H domain mutations emerged in 12 clinically derived viruses, appearing before than or concomitantly with etravirine resistance mutations in 5 and 5 cases, respectively. However, mutational patterns were highly diversified, with only few mutations selected with two different viruses (K451R, R461K, Q464R, K527R, V531I) and no mutation selected in more than two. By contrast, the only mutation selected outside the RT domain with the NL4-3 viruses was A400T (also detected with sample 141163) emerging in the wild type 138E virus following selection of the etravirine resistance mutation L100I (Table 1).

Discussion

Etravirine was originally approved as a component of salvage regimens in patients harbouring multidrug resistant virus. Indeed, etravirine retains activity against several mutants selected at failure of first generation NNRTI and can be a key drug even in deep salvage.[6] Both *in vitro* and *in vivo* data soon revealed that resistance to etravirine is much more complex than resistance to first generation NNRTI and genotypic etravirine susceptibility scoring systems have included up to 49 mutations.[17] Although different systems mostly agree on mutations conferring high resistance, there is much less consensus on the role of minor mutations. Among these, E138A is particularly interesting because it occurs in nature in up to 1.8% to 7.8% of isolates, depending on HIV-1 subtype (Stanford HIVdb, https://hivdb.stanford.edu/cgi-bin/MutPrevBySubtypeRx.cgi), and it is interpreted differently by the most common genotype algorithms. In addition, codon 138 can accommodate different polymorphisms in nature (mainly A and Q) and selects for different variants following etravirine pressure.[13]

The 2.8-fold decrease in etravirine susceptibility measured in our study for the 138A clone is remarkably similar to the 2.9-fold and the 2.5-fold previously obtained with subtype B and C clones, respectively.[13,18] The same applies to the 138G clone while in our experiments the 138K and 138Q constructs showed a fold-change closer to the data obtained in a subtype C, rather than B, background.[13,18] The lower clinical cutoff used for etravirine in the Phenosense assay from Monogram is 2.9-fold,[19] confirming that the impact of E138A on etravirine susceptibility is difficult to define. Interestingly, codon 138 appeared to dictate different resistance pathways within the same NL4-3 backbone, with L100I, Y181C and M230I as the initial etravirine resistance mutation emerging from 138E, 138G and 138A/Q, respectively. However, the choice of different major etravirine mutations may have been partly stochastic as different changes have been documented in multiple *in vitro* resistance selection studies, all starting from the wild type 138E virus.[4,20-22] The 138 A, K and Q variants replicated at the lowest etravirine concentration used without selecting for any mutation, a finding compatible

with their lower baseline susceptibility to etravirine. Accordingly, the most resistant K and Q clones generated a cytopathic effect significantly earlier than the other variants. However, the 138K clone was lost at the first increase in etravirine concentration (150 nM), despite baseline resistance, suggesting reduced fitness as previously documented.[23] Similarly, the 138A clone did not grow at 450 nM etravirine, irrespective of previous acquisition of M230I. On the other hand, the wild type 138E and the 138G and 138Q variants accumulated further etravirine resistance mutations at higher drug concentration. This indicates a delicate balance between resistance and fitness for different variants at codon 138.

With the panel of 20 clinically derived recombinant viruses, E138A was confirmed to confer a low but significant level of resistance to etravirine. The median fold-change in etravirine susceptibility (1.8) was lower than that of the 138A NL4-3 clone (2.8), possibly reflecting better adaptation of the laboratory NL4-3 virus to cell culture. However, two patient derived 138A viruses had fold-change values larger than the 138A NL4-3, suggesting a role for background polymorphisms. Accordingly, among isolates without any other NNRTI mutation included in the Monogram database, E138A was present in 71.4% of 49 clinically derived viruses with >2.9 (median 3.5) etravirine susceptibility fold-change as compared with 3.3% of another 9,409 samples with fold-change <2.9.[24] The most interesting and novel data shown in this study is that E138A significantly lowers HIV-1 genetic barrier to resistance to etravirine as indicated by both a shorter time to virus breakthrough and the ability to grow at higher etravirine concentration compared to the wild type 138E. Of note, six of the ten 138E viruses did not even grow at the lowest etravirine concentration used indicating that the experimental condition was challenging in the absence of the E138A mutation. The resistance pathway was highly diversified in the other 14 viruses and did not allow to define any preferential pattern for 138A versus 138E at baseline. This highlights that etravirine has a complex resistance pattern but also that there are multiple options for resistance to emerge, likely guided by natural polymorphisms. One 138A and one 138E accumulated only non etravirine related mutations and grew up to the last and penultimate drug concentration used, respectively,

suggesting that alternative resistance patterns may go undetected by currently used genotypic interpretation systems.

RT evolution also involved the RT connection and/or RNase H domain in 12 of the 14 clinically derived viruses overcoming drug pressure. Again, the pattern of emergent mutations was highly heterogeneous and no specific mutation was significantly associated with either 138E or 138A. A number of RT connection and RNase H domain have been reported to enhance NRTI resistance and, in a few cases, to contribute to NNRTI resistance likely through decreased RNase activity leading to increased time for NRTI excision or NNRTI dissociation from RT.[25] C-terminal RT mutations of this kind emerging in this etravirine resistance selection study include N348I, G359S, A360T, A376S, A400T, A554T and K558R. Among these, a possible role in NNRTI resistance has been suggested for N348I,[26-31] A376S,[28,30,32] and A400T,[33] although the impact on etravirine activity could be modest.[30] A negative interaction between N348I and E138K has also been reported.[34] It must be noted that under our experimental conditions several other C-terminal mutations emerged, possibly reflecting adaptation of the different virus backbones to cell culture. Indeed, with the laboratory adapted NL4-3 viruses only one C-terminal RT mutation emerged, A400T in the wild type 138E virus as also reported in one previous etravirine resistance selection experiment.[22]

In summary, this study suggests that the main contribution of E138A to reduced response to etravirine may result from an impact on the genetic barrier to resistance rather than from, or in addition to, low-level resistance. This data supports inclusion of E138A as an etravirine resistance mutation in the REGA genotype interpretation algorithm, similar to HIVdb and ANRS. However, the naturally occurring E138A variant may still have higher genetic barrier to resistance to etravirine compared to first generation low-barrier NNRTIs such as efavirenz and nevirapine. A role for E138A in response to etravirine based therapy in treatment-experienced viremic patients has been shown both in clinical trials and observational cohorts.[13,35] By contrast, limited data suggest that naturally occurring E138A does not impact virological response to rilpivirine.[36] It remains to be

established whether such role remains when using etravirine as a component of treatment switch in patients under suppressive therapy.[37] As a more general model, it may be advisable to examine the potential of low-level resistance mutations to decrease the genetic barrier to resistance to specific antiretrovirals, particularly those being used in dual regimens.

Transparency Declarations

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395 Table 1. Changes in RT genotype detected at virus breakthrough with increasing etravirine concentration in the five NL4-3 variants differing at codon

396 138.

Codon 138	Etravirine susceptibility fold-change[a]	Breakthrough at increasing etravirine concentration[b]							
		50 nM		150 nM		450 nM		1350 nM	
		Day	Mutations	Day	Mutations	Day	Mutations	Day	Mutations
E	1.0 (reference)	28	**L100I**	31	**L100I**	45	**L100I** V106A S400T	49	**L100I** V106A **F227C** A400T
A	2.8	33	No mutation	47	**M230I** Q269H	No viral growth		Not tested	
G	2.4	31	**Y181C**	40	**Y181C**	73	**Y181C**	76	**V179F 181C**
K	9.3	14	No mutation	No viral growth		Not tested		Not tested	
Q	14.1	14	No	33	**M230I**	47	**M230I**	76	**F227C**

			mutation						**M230I** V241A

[a]Fold-change with respect to the reference 138E wild type NL4-3 virus.

[b]The day at which virus breakthrough occurred and the list of mutations with respect to the individual baseline sequence are shown. Mutations included in the etravirine resistance score derived from the DUET studies are underlined (E138A itself is also included in this list).[11] Mutations conferring at least low-level resistance to etravirine according to the Stanford HIVdb 8.6 algorithm are in bold.

403 Table 2. Changes in RT genotype detected at virus breakthrough with increasing etravirine concentration in the 20 clinically derived recombinant

404 viruses.

Sample code, subtype	Codon 138	Etravirine susceptibility fold-change[a]	Breakthrough at increasing etravirine concentration[b]							
			50 nM		150 nM		450 nM		1350 nM	
			Day	Mutations	Day	Mutations	Day	Mutations	Day	Mutations
52420 (B)	A	1.5	33	No mutation	52	T240I	59	T240I	91	H221Y L228H **M230I** K275R T477N A554FS
60154 (B)	A	2.0	19	R356K A376S K527R	40	K102R **M230I** K527R	66	V8I V179D **M230I** G359S A376S	83	V8I V179D **M230I** G359S A376S

								K527R		K527R
72009 (CRF02_AG)	A	1.5	80	T107A G333V	95	T107A V179E **Y181C** G333V	105	T107A **Y181C** G333V	No viral growth	
81958 (B)	A	3.0	19	Q197K E370A	59	T240I E370A K558R	66	T240I E370A K558R	80	**L100I** T240I E370A K558R
95551 (B)	A	1.7	45	E6D V35I A554T	59	E6D E29K I47N A554T	66	E6D **G190E** A554T	73	E6D **G190E** A554T
100442 (B)	A	2.5	40	P294A H483Y	52	P294A H483Y	59	**G190E** P294A H483Y	66	**G190E** P294A N348I E413D T477I H483Y
127757 (B)	A	1.7	40	**M230I** K451R	52	**M230I** R461K	73	**M230I** P420L	83	V179D **M230I**

				Q464R L517Q V531I		L517Q		K451R Q464R L517Q V531I V536G		K451R Q464R L517Q V531I
138185 (B)	A	3.9	19	V179E R461K S468F	52	V179E V381I K451R Q464R V531I	59	V179E T240I R461K	66	L120F V179E T240I R461K
138992 (B)	A	1.3	40	V10I	59	**M230I**	73	**F227C** **M230I**	108	**Y181C** **F227C** **M230I**
141163 (B)	A	1.8	59	I135T V179D A360T	66	I135T Y144F V179D Q336H R358I	80	I135T V179D A360T	83	I135T V179D **Y181C**NS A360T A400T

						A360T				
146473 (F1)	E	1.3	52	**L100I** E169G I178L K281R R358S E370G K530R	59	**L100I** E169G I178L K281R R358S E370G K530R	73	**L100I** E169G I178L K281R R358S E370G K530R	76	**L100I** E169G I178L K281R R358S E370G K530R
146446 (CRF12_BF)	E	0.8	No viral growth		Not tested		Not tested		Not tested	
146440 (CRF12_BF)	E	1.6	No viral growth		Not tested		Not tested		Not tested	
146356 (B)	E	0.4	No viral growth		Not tested		Not tested		Not tested	
146352 (B)	E	0.5	No viral growth		Not tested		Not tested		Not tested	
146213 (B)	E	2.4	No viral growth		Not tested		Not tested		Not tested	

146150 (F1)	E	1.3	No viral growth		Not tested		Not tested		Not tested	
143035 (B)	E	1.3	47	V35I K64R **Y181C** K527R	52	V35I K64R **Y181C** K527R	59	V35I K64R **Y181C** K527R	73	V35I K64R V179F **Y181C** K527R
143026 (B)	E	1.8	59	**Y181C**	66	**Y181C** V372L	76	**V179F** **Y181C**	108	**V179F** **Y181C**
146102 (B)	E	3.4	47	T165K	66	R78K T165K G273E	80	T165K G273E	No viral growth	

[a]Fold-change with respect to the reference 138E wild type NL4-3 virus.

[b]The day at which virus breakthrough occurred and the list of mutations with respect to the individual baseline sequence are shown. Mutations included in the etravirine resistance score derived from the DUET studies are underlined (E138A itself is also included in this list).[11] Mutations conferring at least low-level resistance to etravirine according to the Stanford HIVdb 8.6 algorithm are in bold.

Figure 1. Baseline fold-change etravirine susceptibility with respect to the reference NL4-3 138E virus for the panel of 138E and 138A viruses.

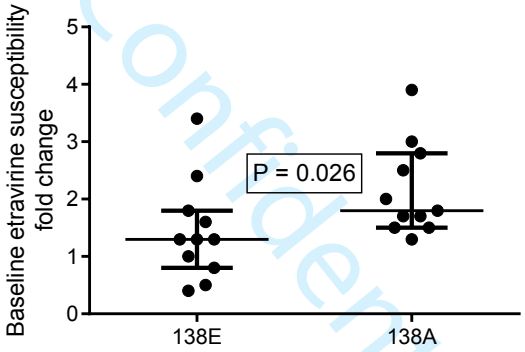


Figure 2. Kaplan-Meier curve showing the probability of cell cultures remaining free from virus breakthrough at the initial etravirine concentration (50 nM) starting with wild type 138E (n = 10) and mutant 138A (n = 10) virus.

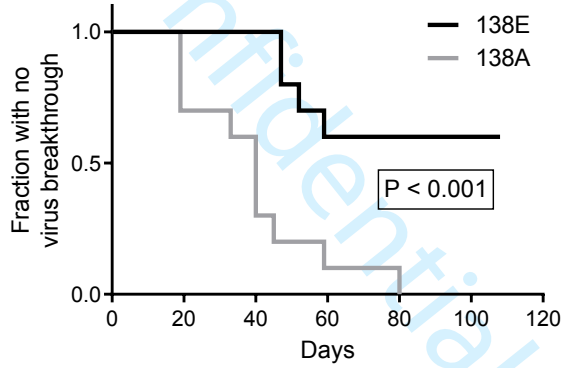
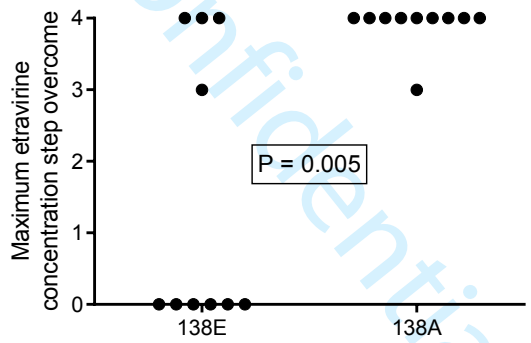


Figure 3. Comparison between the maximum etravirine concentration step overcome by virus growth in the 138E (n = 10) and 138A (n = 10) virus group. The 0 to 4 steps correspond to 0, 50, 150, 450 and 1350 nM etravirine, with the cultures terminated at day 108.



The human immunodeficiency virus type 1 reverse transcriptase E138A natural polymorphism decreases the genetic barrier to resistance to etravirine in vitro

Supplementary Material

- *Amplification and sequencing of RT-RNAseH coding region from plasma viral RNA*

Viral RNA from plasma was extracted using the EZ1 Advanced XL system (Qiagen, Hilden, Germany) with the EZ1 DSP Virus Kit (Qiagen). Twenty microliters of eluted viral RNA were firstly denatured at 70°C for 5 minutes, then mixed with a solution containing 6 µl of ImProm-II™ 5X Reaction Buffer (Promega), 1.5 mM of $MgCl_2$, 0.17 mM of each dNTP, 50 ng of random primers (Promega), 20 U of Recombinant RNasin® Ribonuclease Inhibitor (Promega), 1 µl of ImProm-II™ Reverse Transcriptase (Promega) in a total volume of 30 µl. Reactions were incubated for 5 minutes at 25°C, followed by 45 minutes at 37°C and 5 minutes at 80°C.

Five microliters of cDNA were used as template for the amplification of the whole RT and RNAseH coding region in a reaction including 10 µl of 5X Q5 Reaction Buffer (NEB), 0.5 mM of each dNTP, 5 pmol of each primer (P535, forward, 5'-GARAGRCAGGCTAATTTTTTAGGGA-3', coordinates on HIV-1 HXB2 reference strain 2071-2095; P216, reverse, 5'-TGTCCTGTTTCTGCTGGRATAACYTCTGC-3', HXB2 4485-4513), 1 U of Q5 High-Fidelity DNA Polymerase (NEB), and nuclease free water in a final volume of 50 µl. PCR conditions included an initial denaturation at 98°C for 30 seconds, followed by 35 cycles of 63°C for 30 seconds, 72°C for 1 minute and 30 seconds, 98°C for 10 seconds, and a final cycle of 63°C for 1 minute and 72°C for 3 minutes. The outer PCR was followed by an inner PCR consisting in 2 µl of the outer PCR, 6 µl of 5X Q5 Reaction Buffer (NEB), 0.5 mM of each dNTP, 3 pmol of each primer (P189, forward, 5'- TTCAGAGCAGACCAGAGCCAACAGC-3', HXB2 2135-2159; P215, reverse, 5'-CCTTCTAAATGTGTACAATCTARTTGCCA-3', HXB2 4410-4438), 1 U of Q5 High-Fidelity DNA Polymerase (NEB), and nuclease free water in a final volume of 30 µl. PCR conditions included an initial denaturation at 98°C for 30 seconds, followed by 35 cycles of 62°C for 30 seconds, 72°C for 1 minute and 30 seconds, 98°C for 10 seconds, and a final cycle of 62°C for 1 minute and 72°C for 3 minutes. Inner PCR was

loaded on a 1.5% Seakem agarose gel and ran at 6 V/cm for 50 minutes, then the presence of expected bands was checked in a transilluminator after gel staining in a solution with GelRed dye (Biotium).

Sequencing reactions were performed on a 3130 XL Genetic Analyzer (Applied Biosystems). PCR products were diluted to a final concentration of about 1-3 ng/µl, then 10 µl were purified adding 2 µl of ExoSAP-IT For PCR Product Clean-Up (Affimetrix) incubating at 37°C for 15 minutes, followed by an inactivation step at 80°C for 15 minutes. Each sequencing reactions included 3 µl of purified PCR product, 3.2 pmol of sequencing primer, 2 µl of BigDye® Terminator v1.1 Ready Reaction Mix (Life Technologies), 1 µl of 5x Sequencing Buffer and bi-distilled sterile water in a total volume of 10 µl. Thermal cycler profile for this reaction was: initial denaturation step at 94° for 4 minutes, followed by 25 cycles of 50°C for 1 minute, 68°C for 4 minutes, 94°C for 1 minute. The primer used were P214 (forward, 5'- TTTGCCAGGAAAATGGAAACCAAAAATGAT-3', HXB2 2363-2392), P192 (forward, 5'- GGGATTTACCACACCAGACAAAAAACATC-3', HXB2 3185-3213), P220 (reverse, 5'- TTCTGCTATTAAGTCTTTTGMTGGGTCRTA-3', HXB2 3504-3533), and P215. Sequencing reactions were treated with X-Terminator® Purification kit (Applied Biosystems) in a 96-wells plate as suggested by manufacturer, then loaded in the capillary electrophoresis sequencer. Chromatograms were assembled and edited with the DNAStar 7.1.0 SeqMan module.

- *Supplementary Table 1. Natural polymorphisms of samples included in the study according to the Stanford HIVdb algorithm*

Sample (subtype)	Codon 138	Polymorphisms compared to consensus subtype B reference sequence
52420 (B)	A	R83K, D123E, S162C, E248D, D250N, A272P, P294Q, L301LF, E302EK, E328EK, R356K, M357ML, G359S, A376S, T377S, E378EK, K390R, A400S, F416FL, R448K, D460N, S519N, K527S, A554S
60154 (B)	A	E6D, K20R, V35VI, D123E, I135IT, S162C, D177DE, E248D, A272P, K277R,

		I293V, P345Q, A360T, A376AS, A400S, T450S, L452I, V467I, S468P, H483Y, K512Q, S519N, K527KR, A554T, K558R
72009 (CRF02_AG)	A	P4T, V35T, S162A, K173T, Q174K, D177E, I178M, T200A, Q207E, R211K, F214L, P243T, V245Q, K281R, T286A, E291D, I293V, P294T, K311R, S322T, I326V, G335D, R356K, M357R, G359S, T369A, A371V, I375V, K390R, A400T, T403M, E432D, L469I, D471E, Q480H, L491S, K512R, S519N, Q524K, K527E, E529D, A534S, V548I, A554S
81958 (B)	A	E6D, K20R, D123E, S162C, T200I, E248D, A272P, K277R, L283I, I293V, E344EK, A360T, A376V, T386A, A446AG, T450S, L452M, V467I, S468P, T477TS, H483Y, E492EQ, K512Q, S519N, A554S, K558KR
95551 (B)	A	R83K, K104R, K122E, I135M, T139I, A272P, P294T, E297D, T369A, A376T, T386I, K390R, E399D, A400T, R461K, S468C, L517V, A554N, V559I
100442 (B)	A	V35I, V60I, R83K, D121H, K122E, I135T, K173E, R211RK, A272P, P294AS, E297D, T369A, A376T, K390R, E399D, A400T, R461K, S468C, H483HY, A554N, V559VI
127757 (B)	A	K102Q, I135T, I142V, K173E, Q174QK, D177E, V245I, A272P, K277R, Q278H, R356K, G359S, A360T, S379G, V381I, T386I, K390R, K451KR, L452LI, S468SP, H483Y, L517LQ, A554N
138185 (B)	A	K102Q, D123E, I135T, I142V, K173E, Q174R, D177E, V245I, A272P, K277R, Q278H, R356K, G359S, A360T, S379G, T386I, K390R, L452I, L469I, H483Y, L517Q, A554N
138992 (B)	A	K122KE, K166R, F171Y, R199T, I202V, R211K, V245T, K277R, L283I, E297K, E312Q, I329V, S379G, T386TI, K390R, T403M, K431T, V435A, T477TA, H483HY, L491P, I495V, Q524L, V531T

141163 (B)	A	D123E, I135IT, I142IV, K173E, D177E, V245I, A272P, K277KR, Q278H, E297A, R356K, G359S, S379G, T386I, K390R, L452I, H483Y, L517Q, A554N
146473 (F1)	E	V35T, K49R, K122E, I135T, S162C, E169D, K173A, Q174K, I178IL, Q207E, R211K, V245Q, E248D, A272P, T286A, L289LI, V292I, I293V, E297V, D324E, Q334S, R356K, R358K, G359S, E370ED, A371AV, I375V, T377Q, Q394L, A400T, K431T, I434V, V435P, G436R, A446S, L452Q, V466VA, V467VI, S468P, D471E, T477A, H483L, G490E, L491S, Q507H, K512R, L517I, S519N, Q524K, K527Q, K530KR, A534S
146446 (CRF12_BF)	E	V35IT, D123E, I142T, P176K, I178M, G196E, K249Q, A272P, K277KR, I293V, E297A, V317A, I329V, G359S, A360AT, A376S, V381VM, E432D, V435E, A446S, R461K, A508G, L517I, S519N, K530KR, A554S
146440 (CRF12_BF)	E	V35T, D123E, P176K, I178M, G196E, Q207QE, K249Q, I293V, E297A, V317A, I329V, G359S, A360T, A376S, E432D, V435E, A446S, R461K, A508G, L517I, S519N, A554S
146356 (B)	E	K49R, E53D, K122E, D123S, I135T, K166KR, E169D, I202V, Q207K, R211K, A272S, K277R, E297K, K311R, I329V, Q334Y, T338S, A360AT, A376T, T386I, K390R, T403M, V435I, S468P, H483Y, V548I, A554D
146352 (B)	E	K49R, E53D, K122E, D123S, I135T, T165I, E169D, T200A, I202V, Q207R, R211K, A272S, K277R, E297K, K311R, Q334Y, T338S, G359GS, A376AT, T386I, K390R, T403V, S468P, H483Y, V548I
146213 (B)	E	K20R, K122E, D123N, I135T, I244V, V245T, A272P, I293V, V317A, I326V, Q334Y, A355T, R356K, G359S, T362TS, T369V, Q373N, K390R, T403V, V435E, R461K, H483HY, V559I
146150 (F1)	E	I2IV, K22R, V35T, T39A, E40N, K46Q, D123E, I135L, K173E, Q174R, I178L,

		I195L, Q207E, R211K, V245Q, A272P, K277R, T286A, E291D, V292I, I293V, E297A, I329L, F346C, M357I, G359S, Q367E, E370D, I375V, T377R, S379C, K390R, E404D, K431T, V435I, A446S, L452K, V467T, S468T, L469LQ, T470L, D471KR, T472TP, T473TP, T477A, L491S, Q509K, S519N, Q520K, Q524E, K527Q, K530R, A534S, A554S
143035 (B)	E	V35VL, K64KR, A98S, D123E, K166R, I180V, T200A, R211K, A272S, P294Q, K311R, E312T, M357S, K366R, A376V, T386A, A400T, V435A, D460N, R461K, K476Q, H483Y, L517I, S519SN, K527GR
143026 (B)	E	K20R, D123E, I135T, T165I, T200A, I202V, F214L, V245E, A272P, V276I, L283I, A288T, V292I, I293V, E297A, R356K, K366R, A376T, T386I, K390R, E399EG, A400T, V435I, R461RK, S468SP, T470N, H483Y, L491S, L517I, I522V, V548I, A554D
146102 (B)	E	K20R, V60I, D123E, I142V, T165I, I178L, A272P, K277R, T286A, E291D, V292I, M357R, A360T, K390R, A400T, T403M, V435I, V466I, V467I, S468P, H483N, L491V, K512E, V548I, A554T