Background: HIV type-1 (HIV-1) protease (PR), reverse transcriptase (RT) and integrase (IN) share the same precursor polyprotein and there is much evidence to suggest functional interactions between IN and RT. We aimed to elucidate whether long-term highly active antiretroviral therapy (HAART) targeting PR and RT could influence raltegravir susceptibility and the fitness of IN.

Methods: HIV-1 IN sequences from 45 heavily antiretroviral-experienced patients with longitudinal samples separated by a median of 10 years were obtained to estimate the rate of nucleotide substitution. IN recombinant viruses were generated from five selected patients. Phenotypic susceptibility to raltegravir was tested in vitro. Changes in viral replication capacity were assayed by growth kinetics and competition of intrapatient IN recombinant viruses.

Results: The amino acid substitution rate within IN was 0.06% per year during long-term antiretroviral treatment. Some substitutions had previously been associated with resistance to different IN inhibitors. Despite this, neither the early- nor late-derived IN recombinant viruses showed an increase in phenotypic susceptibility to raltegravir. Moreover, IN recombinant viruses corresponding to IN samples after 10 years of HAART had a replication capacity that was similar to or better than IN recombinant viruses from baseline samples.

Conclusions: HIV-1 IN from longitudinal samples taken from patients treated with IN inhibitor-sparing regimens showed no evidence of genotypic or phenotypic resistance to raltegravir. Additionally, long-term pressure with PR and RT inhibitors did not impair the fitness of HIV-1 IN. These data suggest that current antiretroviral regimens do not diminish the fitness of IN or influence raltegravir efficacy.

Introduction

Most drugs approved during the past 20 years to inhibit the replication of HIV type-1 (HIV-1) have targeted viral protease (PR) and reverse transcriptase (RT). Inhibitors of the HIV-1 integrase (IN) have only recently been used in highly active antiretroviral therapy (HAART) with promising results. The IN of HIV-1 is responsible for the integration of proviral DNA into the host genome [1,2], an indispensable step in productive HIV-1 infection of human cells. The protein HIV-1 IN is 288 amino acids long and folds into three functional domains: an N-terminal zinc finger (amino acids 1–50) that contains an HHCC motif [3,4], the central catalytic core domain (amino acids 51–212) that contains the DDE triad (Asp64, Asp116 and Glu152) and requires a divalent metal cation for activity, and a C-terminal DNA-binding domain (amino acids 213–288). To be fully functional in vivo, IN also requires cellular cofactors of which the most widely described is the human lens epithelium-derived growth factor (LEDGF/p75) that is required during chromosomal tethering [5]. The IN is encoded at the 3′ end of the pol gene, in frame with the PR and RT coding regions. Thus, the three major drug-targeted HIV-1 enzymes (PR, RT and IN) mature from the same large Gag–Pol precursor polyprotein (Pr160Gag–Pol). Moreover, the pre-integration complex includes RT, IN and viral DNA.
Many compounds with very diverse chemical structures have been reported to inhibit IN activity [2], but the development of IN inhibitors has been plagued by difficulties with identifying compounds that target the IN [11]. The only IN inhibitor currently approved for clinical use is raltegravir (MK-0518, chemical formula N-(4-fluorophenyl)methyl)-1,6-dihydro-5-hydroxy-1-methyl-2-[[1-methyl-1-[[5-methyl-1,3,4-oxadiazol-2-yl]carbonyl]amino]ethyl]-6-oxo-4-pyrimidine carboxamide; Merck, Whitehouse Station, NJ, USA) [12], a hydroxypyrimidine derivative unrelated to diketo acid inhibitors. This drug blocks the integration of HIV-1 complementary DNA through the inhibition of DNA strand transfer. A 24-week analysis showed that raltegravir plus optimized background therapy had a higher antiretroviral efficacy in patients with triple-class resistance, whose antiretroviral therapy failed, than those taking placebo plus optimized background therapy [13,14]. The potential toxicity of raltegravir is thought to be low as there is no human homologue of HIV-1 IN [15]. However, as with all other antiretrovirals, resistance has been shown to occur with IN inhibitors [16–18]. Thus, HIV-1 variants with raltegravir or elvitegravir resistance mutations have been identified in vivo using increasing concentrations of these drugs [19,20] and have been identified in vitro in most virological failures with raltegravir- or elvitegravir-containing regimens [19,21]. Moreover, raltegravir resistance mutations are suspected of reducing replication capacity relative to the wild type, suggesting an association between drug resistance mutations, fitness cost and decreased enzymatic efficiency of IN [19].

An understanding of the differential prevalence rate of mutations in viral strains from HAART-experienced IN inhibitor-naive patients might help to identify whether clinically relevant viral mutations or natural polymorphisms occur and whether the sequence diversity of the IN gene has important implications in the clinical response to IN inhibitors. In this study, we explored intrapatient longitudinal evolution of the HIV-1 clade B IN-coding region over a median of 10 years of heavy antiretroviral therapy in IN inhibitor-naive patients. We also explored changes in phenotypic susceptibility to raltegravir and replication capacity in samples that had accumulated the highest number of amino acid substitutions during the study period.

Methods

Study patients

We selected 45 highly antiretroviral-experienced HIV-1-infected patients with longitudinal samples separated by a median of 10 years (range 4–13) and for whom the first sample was taken either before they started antiretroviral treatment or soon after they started monotherapy/bitherapy. Demographic, antiretroviral treatment, CD4+ T-cell counts and viral load data were available. A median of three plasma samples (range 2–4) per patient were taken between 1993 and 2007. All sequences were subtype using the REGA HIV-1 automated subtyping tool [22].

Population-based sequencing of the HIV-1 IN-coding region

Viral RNA was extracted from plasma samples using the QIAamp Viral RNA kit (Qiagen, Barcelona, Spain) and the full-length IN-coding region was amplified. The RT-PCR (SuperScript One-Step RT-PCR kit; Invitrogen, Barcelona, Spain) was performed using primers EinteF and EinteR (nucleotides 2997–3019 and 5534–5574 of the HIVHXB2 numbering system, respectively). A nested PCR (Platinum® Taq DNA Polymerase High Fidelity; Invitrogen) was then carried out with primers NinteF and NinteR (nucleotides 3111–3134 and 5251–5270, respectively). Population-based sequencing was carried out with primers NinteF (nucleotides 4540–4560), NinteR (4142–4165) and 1seqF, 2seqF, 3seqF (4744–4764), using the Big-Dye Terminator Cycle Sequencing kit and the ABI 3100 sequencer (Applied Biosystems, Barcelona, Spain). All sequences were assembled, aligned and edited using the Sequencer (GeneCodes, version 4.6, Ann Arbor, MI, USA) and GeneDoc software. GenBank accession numbers for the IN sequences from EU883794 to EU883928. Codon-aligned sequences were submitted to the Synonymous/Non-synonymous Analysis Program server for codon-specific ratio of non-synonymous to synonymous substitution rate (dN/dS) analysis [23,24]. The evolution rate was calculated using the formula below with _S_ being the number of amino acid substitutions between the last and first sequence, _m_ the number of amino acid positions, _t_ the time (in years) between the last and first sequence and _n_ the number of patients analysed.

\[
\sum \left( \frac{S_i - S_0}{t - t_0} \right) \times 100
\]

Generation of IN recombinant viruses

For further detailed phenotypic analysis, we constructed IN recombinant viruses from five selected patients whose HIV-1 IN sequences accumulated between 3 and 14 amino acid substitution differences during the study period. Two IN recombinant viruses were constructed for each patient selected. These contained IN from the first sample, obtained either before antiretroviral treatment was started or soon after monotherapy/bitherapy.
pairs (bp) fragment was then ligated into a viral 5′ electroporation of MT4 cells and cotransfecting the recombinant clones were obtained and their IN genotypes of IN. After transformation diketo acid drug family [28].

by introducing a T66I substitution within the IN gene. The second was constructed using site-directed mutagenesis who harboured the mutations G140S and Q148H. The whose raltegravir-containing therapy had failed and HIV-1 promoter [27].

which contain a luciferase gene under the control of the titrations within the mutagenized plasmid. The IN-coding region between the restriction site KpnI at position 4158 and the restriction site NdeI at position 5123 was then replaced by a polylinker.

PCR-amplified fragments of the HIV-1 IN, generated using plasma HIV-1 RNA from infected patients, were digested with KpnI and NdeI. The resulting 863 base pairs (bp) fragment was then ligated into a KpnI/NdeI predigested pJM30ΔIN. After transformation of Escherichia coli-competent cells, individual recombinant clones were obtained and their IN genotypes were verified by DNA sequencing. For each patient and sample, a representative clone of the previously obtained HIV-1 IN population-based sequence was chosen to generate the IN recombinant virus.

Infectious HIV-1 molecular clones were generated by electroporation of MT4 cells and cotransfecting the proviral 5′-half genome plasmid (pJM30ΔIN reconstructed with patient-derived HIV-1 IN) and the 3′-half genome plasmid (p83-10), previously cut with EcoRI. Two different p83-10 plasmids (p83-10–green fluorescent protein [GFP] and p83-10–discosoma red fluorescent protein [RFP]) [26] were used to identify viruses from the first and last samples during competition assays. Titration of recombinant viruses was performed using TZM-bl cells, which contain a luciferase gene under the control of the HIV-1 promoter [27].

Two additional recombinant viruses were constructed. One was an HIV-1 IN recombinant virus from a patient whose raltegravir-containing therapy had failed and who harboured the mutations G140S and Q148H. The second was constructed using site-directed mutagenesis by introducing a T66I substitution within the IN gene. T66I confers resistance to the IN inhibitors from the diketo acid drug family [28].

Raltegravir susceptibility assay
A total of 200 median tissue culture infective doses (TCID_{50}) of each viral stock were used to infect 10,000 TZM-bl cells (multiplicity of infection [MOI]=0.02) in quadruplicate in 96-well optical bottom plates containing 32.5 μg/ml diethylaminoethyl-dextran and fourfold serial dilutions ranging from 0.06 to 4 mM of raltegravir. Replication was monitored by measuring luciferase expression in the infected target cells 48 h after infection using the Bright-Glo Luciferase Assay (Promega, Barcelona, Spain). The percentage of inhibition was determined by calculating the difference in relative light units (RLU) between test wells and negative control wells, dividing this result by the difference in RLU.s between positive control wells (without drug) and negative control wells, subtracting from 1 and multiplying by 100. Inhibition curves were defined using a sigmoid dose–response curve with a variable slope. The fold change in drug susceptibility was determined by dividing the 50% inhibitory concentration (IC_{50}) for every sample virus by the IC_{50} for the raltegravir-sensitive virus NL4-3–GFP or NL4-3–RFP. Growth rate assay
Peripheral blood mononuclear cells (PBMCs) from healthy donors were cultured in RPMI 1640 medium supplemented with 20% fetal calf serum (FCS) and stimulated with interleukin-2 (IL-2) and phytohaemagglutinin (PHA) for 3 days before infection. A total of 5×10^6 PHA-stimulated PBMCs were infected with 10,000 TCID_{50} (MOI=0.002) of each viral stock in 1 ml of final volume for 2 h at 37°C. The cells were then washed twice with phosphate-buffered saline and cultured at 10^6 cells/ml in T25 flasks for 10 days. Each day, 250 μl of supernatant was collected and 300 μl of fresh RPMI supplemented with 20% FCS and IL-2 was added. Growth kinetics of the viruses were monitored using a HIV-1 p24 antigen ELISA (Perkin Elmer, Barcelona, Spain) in the supernatant of samples collected from cell cultures. Growth kinetics based on p24 antigen production were analysed by fitting the log-transformed p24 data into a linear model. The growth rate experiments were performed in duplicate.

Competition assay
The fitness of recombinant viruses, which carry the IN from viral isolates from the first and last samples of the same patient, was determined by viral competition after dual infection of MT4 cell cultures. The first and last HIV-1 IN sequences were linked to GFP or RFP reporter genes, respectively. Competition between the viruses generated from each patient was induced with unequal proportions (20:80, 50:50 and 80:20 of the first and last viruses according to titration in TZM-bl cells). A total of 1×10^6 MT4 cells were cultured in RPMI 1640 supplemented with 10% FCS, 50 mg/ml of penicillin and 50 mg/ml of streptomycin before being infected with a total of 20,000 TCID_{50} (MOI=0.02) in a final volume of 1 ml for 2 h at 37°C. Cells were washed twice with phosphate-buffered saline and cultured at 2×10^5 cells/ml in 6-well tissue culture plates (Nunc, Barcelona, Spain). At days 7, 12, 17 and 21,
1x10^6 fresh cells were reinjected by inoculating 100 µl of supernatant from the culture containing the competed viruses. Cells obtained at each passage were centrifuged, genomic DNA was extracted and a multiplex PCR reaction was performed with fluorescently labelled primers specific for the GFP or RFP reporter genes. A total of 0.5 µl of product amplification was mixed with 20 µl of formamide and 1.5 µl of the molecular size marker, labelled with carboxytetramethyl rhodamine (Applied Biosystems). The mixture was denatured at 95°C for 5 min and separated by capillary electrophoresis before being analysed in a 310 Genetic Analyzer (Applied Biosystems). Samples were analysed using GeneScan software (Applied Biosystems). Two peaks (97 bp and 102 bp of the GFP and RFP genes, respectively) were clearly differentiated in the chromatogram. The area of each peak divided by the total area of both peaks gave the relative proportion of virus variants. To ensure that both genes were amplified with the same efficiency by the multiplex PCR reaction, a standard curve was constructed using a gradient of different concentrations of p83-10–GFP and p83-10–RFP plasmids. The correlation coefficient between the percentage of plasmid input and the percentage of gene measured by GeneScan analysis was calculated. The concentrations tested ranged from 0 to 100% of the sample input.

Statistical analysis
Statistical data were estimated with GraphPad Prism (version 4.0) software (San Diego, CA, USA). To compare means between the first and last samples in drug susceptibility and growth rate assays, we applied the Student's t-test. Values of P<0.05 indicated statistical significance.

Results
Patient characteristics
The samples included in this study were taken from HIV-1-infected patients (89% men, median age 45 years) who were longitudinally monitored for up to 13 years. These patients were highly antiretroviral-experienced and had been exposed to a median of 13 antiretroviral drugs with a median of six nucleoside reverse transcriptase inhibitors (NRTIs), two non-NRTIs and five protease inhibitors. Moreover, 40% (18/45) had also been treated with the fusion inhibitor enfuvirtide and none had received IN inhibitors. At the first time point, 4 out of 45 patients (9%) were drug-naive, 22% were taking monotherapy (zidovudine 18% [8/45] and didanosine 4% [2/45]) and 42% were on dual therapy (zidovudine/zalcitabine 29% [13/45], zidovudine/didanosine 9% [4/45], zidovudine/lamivudine 2% [1/45] and zidovudine/stavudine 2% [1/45]). The median plasma viral load in these initial samples was 4.84 log_{10} copies/ml and the median CD4+ T-cell count was 250 cells/mm³. After a median of 10 years of multiple antiretroviral combination regimens, the mean change in HIV-1 RNA was -0.65 log_{10} copies/ml and the CD4+ T-cell count increased by only 15 cells/mm³, reflecting multidrug treatment failure. Subtype determination showed that all patients were infected with HIV-1 subtype B variants.

Natural variability and evolution of the IN gene
The IN gene (867 bases) was amplified and sequenced using 123 plasma samples from 45 patients. Analysis of all the amino acid sequences revealed that the IN of HIV-1 subtype B is a conserved protein with 42% (121/288) of amino acid residues achieving ≥97% conservation and 25% (73/288) completely conserved. The HHCC zinc-binding site, the DDE catalytic triad and the LEDGF/p75 IN-binding domain between residues 128–132 and 161–173 were all ≥97% conserved. No deletions were seen in the IN sequence and only two of the 123 IN sequences (both from the same patient) had an insertion at position 193.

Of the 64 amino acid substitutions previously associated with resistance to IN inhibitors [29], we found 15 in our samples (S17T, V72I, L74I, L101I, K111T, S119R, S119G, T124N, V151I, M154I, K156N, V165I, V201I, L203M and D232N; Table 1). However, no mutations associated with resistance to raltegravir (N155H, Q148H/R/K and G140S/A) [19] or elvitegravir (T66I, E92Q, Q146P and S147G) [20] were detected.

The evolution rate of HIV-1 IN during the study period was 0.06% amino acid substitutions per year. When we analysed codon-specific ratios of dN/dS, we observed that the IN was constrained, with a low dN/dS value (0.15), suggesting that there is no positive selection acting on the IN during antiretroviral treatment targeting PR and RT. Nevertheless, comparison of the first and last sequences of each patient revealed a slight increase in the prevalence of some of the changes associated with resistance to IN inhibitors (Table 1).

Raltegravir susceptibility
The IC_{50} of raltegravir was measured in five selected patients whose HIV-1 IN sequences accumulated between 3 and 14 amino acid substitutions (Tables 2, 3 & 4). None of the IN recombinant viruses derived from patients A1 to A5, either before or after several years of antiretroviral therapy, showed a significant decrease in raltegravir susceptibility with a fold change <3 as compared with the wild type (HIV-1NL4-3) in all cases (Figure 1). In addition, the fold change in raltegravir susceptibility was not different between IN recombinant viruses derived from the first time point samples (mean fold change =1.88, 95% confidence interval [CI] 1.22–2.54) or after several years of antiretroviral therapy (mean fold change =1.75, 95%
CI 1.55–1.94). Conversely, an IN recombinant virus derived from a patient failing a raltegravir-containing regimen (patient B) and harbouring mutations G140S and Q148H (Tables 2, 3 & 4) had a 23.2-fold increase (95% CI 7.3–74.3) in the IC$_{50}$ of raltegravir (Figure 1). However, one HIV-1NL4-3 sample (sample C) containing the T66I substitution, which confers resistance to IN inhibitors from the diketo acid drug family [28], retained full susceptibility to raltegravir (fold change in IC$_{50}$=1.1, 95% CI 0.8–1.5; Figure 1).

Relative replicative fitness in virus mixtures
To measure differences in viral fitness more accurately, dual infections were induced in cell culture. The proportions of IN recombinant viruses derived from the first and last samples were estimated over time by a length polymorphism detection method. Before applying this detection system to growth competition experiments, the method was set up using different mixtures of plasmids containing either the GFP or the RFP reporter genes. The correlation coefficients were 0.999 for both genes (Figure

Table 1. Evolution in frequency of changes at codons previously associated with resistance to different integrase inhibitors

<table>
<thead>
<tr>
<th></th>
<th>N-terminal</th>
<th>Catalytic core domain</th>
<th>C-terminal</th>
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<tbody>
<tr>
<td></td>
<td>17</td>
<td>72</td>
<td>74</td>
</tr>
<tr>
<td>HIV-1 HXB2 strain</td>
<td></td>
<td>S</td>
<td>V</td>
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<tr>
<td>Changes associated with integrase inhibitor resistance</td>
<td>T</td>
<td>I</td>
<td>M/A</td>
</tr>
<tr>
<td>Frequency in first time point (%)</td>
<td>S (78)</td>
<td>I (55)</td>
<td>V (28)</td>
</tr>
<tr>
<td></td>
<td>C (7)</td>
<td>T (2)</td>
<td>I/I (4)</td>
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<tr>
<td></td>
<td>N (12)</td>
<td>I (66)*</td>
<td>I (2)</td>
</tr>
<tr>
<td></td>
<td>C (7)</td>
<td>T (2)</td>
<td>I/I (2)</td>
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<tr>
<td></td>
<td>T (2)</td>
<td>V/I (7)</td>
<td>R (2)</td>
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</table>

*Amino acid substitutions that increase in frequency between the first and the last integrase sequences. HIV-1, HIV type-1.

Table 2. Amino acid substitutions within the HIV-1 integrase (N-terminal domain)

<table>
<thead>
<tr>
<th>Virus</th>
<th>N-terminal domain</th>
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<tbody>
<tr>
<td></td>
<td>3</td>
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<tr>
<td>HIV-1NL4-3</td>
<td>D</td>
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<tr>
<td>Long-term HAART-experienced patients</td>
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</tr>
<tr>
<td>$A^1_{int}$</td>
<td>-</td>
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<tr>
<td>$A^2_{int}$</td>
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<tr>
<td>$A^3_{int}$</td>
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<tr>
<td>$A^4_{int}$</td>
<td>-</td>
</tr>
<tr>
<td>$A^5_{int}$</td>
<td>-</td>
</tr>
<tr>
<td>Raltegravir-experienced patient</td>
<td></td>
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<tr>
<td>$B^1_{int}$</td>
<td>-</td>
</tr>
<tr>
<td>$B^2_{int}$</td>
<td>-</td>
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<tr>
<td>Site-directed mutant</td>
<td>-</td>
</tr>
</tbody>
</table>

$A^1_{int}$, $A^5_{int}$, samples A1–A5 taken at the first time point; $A^1_{int}$, $A^5_{int}$, samples A1–A5 samples taken at the last time point; $B^1_{int}$, sample B taken at the first time point; $B^2_{int}$, sample B taken at the last time point; HAART, highly active antiretroviral therapy; HIV-1, HIV type-1.
The method was sensitive to ≥10% of one of the viral strains (Figure 2). Two recombinant HIV-1\textsubscript{NL4-3} strains, one containing the GFP reporter gene and the other containing the RFP reporter gene, were competed as controls. Results showed that no virus could outgrow the other; therefore, excluding the possibility that fitness differences were affected by the reporter proteins (data not shown).

IN recombinant viruses from the last time point samples (A1\textsubscript{last}, A2\textsubscript{last}, A4\textsubscript{last} and A5\textsubscript{last}) outgrew their counterparts from the first time point samples (A1\textsubscript{first}, A2\textsubscript{first}, A4\textsubscript{first} and A5\textsubscript{first}) when assayed in a drug-free environment (Figure 3A). Only IN recombinant viruses derived from patient A3 showed an identical replication capacity between the first and last samples, which is consistent with the data presented in Table 3.

<table>
<thead>
<tr>
<th>Amino acid substitutions within the HIV-1 integrase (catalytic core domain)</th>
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<tr>
<td>Catalytic core domain</td>
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<tr>
<td>Virus</td>
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<tr>
<td>HIV-1\textsubscript{NL4-3}</td>
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<tr>
<td>Long-term HAART-experienced patients</td>
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<tr>
<td>A1\textsubscript{first}</td>
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<tr>
<td>A5\textsubscript{first}</td>
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<tr>
<td>A5\textsubscript{last}</td>
</tr>
<tr>
<td>Raltegravir-experienced patient</td>
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<tr>
<td>B\textsubscript{first}</td>
</tr>
<tr>
<td>B\textsubscript{last}</td>
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<tr>
<td>Site-directed mutant</td>
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<tr>
<td>T66I</td>
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</table>

A1\textsubscript{last}–A5\textsubscript{last} samples A1–A5 taken at the first time point; A1\textsubscript{first}–A5\textsubscript{first} samples A1–A5 samples taken at the last time point; B\textsubscript{first} sample B taken at the first time point; B\textsubscript{last} sample B taken at the last time point; HAART, highly active antiretroviral therapy; HIV-1, HIV type-1.
with the replication kinetics described below. For each comparison, cultures were set up with three unequal proportions of the competing viruses and all of them provided consistent results over 21 days of culture. However, the IN recombinant virus derived from a patient whose raltegravir-containing regimen failed (patient B) and the HIV-1NL4-3 sample containing the T66I substitution (sample C) were outcompeted by their wild-type IN recombinant counterpart (Figures 3B & 3C), suggesting that selection of IN inhibitor resistance mutations has a fitness cost for the virus in the absence of drug.

Replication kinetics in PBMCs

The replication capacity of these recombinant viruses in the absence of drug was determined by the kinetics of p24Gag production in PBMCs. The slope of the increase in p24Gag for recombinant viruses was compared with the slope of the corresponding HIV-1NL4-3 containing either the GFP or the RFP reporter gene and the percentage of replication capacity was calculated (Figure 3D). Of note, HIV-1NL4-3 containing either the GFP or RFP reporter gene had the same replication capacity. The growth rate of IN recombinant viruses derived from patient samples (A1–A5) ranged from 5 to 117% as compared with the HIV-1NL4-3 replication capacity. All IN recombinant viruses derived from samples taken after several years of antiretroviral therapy (A1last–A5last) had higher replication capacities than their counterpart viruses derived from the first sample (A1first–A5first; Figure 3D). The growth kinetics of the IN recombinant virus derived from the patient failing a raltegravir-containing regimen (patient B) had less replicative capacity as compared with its non-resistant counterpart recombinant virus, but the difference was not statistically significant (Figure 3D). Similarly, the HIV-1 sample containing T66I substitution (sample C) had a 31% lower replication capacity than HIV-1NL4-3 (Figure 3D).

Discussion

A number of studies have identified the presence and frequency of polymorphisms in the HIV-1 IN gene

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**Figure 1. Raltegravir susceptibility**

The fold change (FC) in raltegravir susceptibility was determined by dividing the 50% inhibitory concentration (IC50) for each integrase (IN) recombinant virus by the IC50 for its corresponding HIV type-1 wild type (HIV-1NL4-3). The FC of the IN recombinant viruses from the HIV-1NL4-3 strains, the first A1–A5 samples (cloned into p83-10–green fluorescent protein [GFP]) and the last A1–A5 samples (cloned into p83-10–discosoma red fluorescent protein [RFP]), sample B (with substitutions G140S/Q148H from a patient on a raltegravir-containing regimen before raltegravir treatment and after treatment failure) and sample C with HIV-1 harbouring the substitution T66I (HIV-1NL4-3–GFP and single mutant T66I) are shown. Statistical analyses were performed using the unpaired Student’s t-test.
of treatment-naive patients [30–32]. However, no longitudinal studies of IN evolution in treatment-experienced patients are available, and the effect of these natural changes on phenotypic susceptibility to IN inhibitors and on viral replication has not been fully characterized. Moreover, a stable HAART regimen with inadequate viral suppression has been reported to induce genetic evolution within the PR- and RT-coding regions [33–35].

Our study evaluated the longitudinal evolution of the IN gene over 10 years of infection in heavily antiretroviral-experienced patients and the effects of such changes on phenotypic susceptibility to raltegravir and on replication capacity. Our results show that although the IN gene is highly conserved, a genetic evolution could be seen in the IN of the HIV-1 subtype B obtained from patients exposed to long-term antiretroviral treatment. We found substitutions that have been associated with resistance to different families of IN inhibitors and a slight increase in the prevalence of some of these changes – concentrated in the central domain of the IN – during long-term antiretroviral treatment. These data suggest that IN evolves during long-term treatment and that it potentially modifies IN activity and has an effect on the phenotype and the replicative capacity of the virus. To evaluate this effect, we selected samples with the greatest accumulation of genotypic changes (3–14 non-synonymous substitutions). Our data showed no significant increases in phenotypic susceptibility to raltegravir in recombinant viruses from patients who had accumulated a high number of substitutions in the IN gene during the study period, suggesting that IN changes occurring during several antiretroviral regimens will not diminish the virological response to this inhibitor. This is relevant because the patients selected in this study are one of the main target populations for raltegravir.

Figure 2. Schematic representation of GeneScan detection for competition experiments

(A) Cells obtained at each reinfection were centrifuged, genomic DNA was extracted and a multiplex PCR reaction was performed with fluorescently labelled primers specific for the green fluorescent protein (GFP) or discosoma red fluorescent protein (RFP) reporter genes. (B) Labelled PCR products were denatured, separated by capillary electrophoresis and analysed in a 310 Genetic Analyzer (Applied Biosystems, Barcelona, Spain). Samples were analysed using GeneScan software (Applied Biosystems). Two clearly differentiated peaks appeared in the chromatogram, corresponding to 97 base pairs and 102 base pairs of the GFP and RFP genes, respectively. (C & D) Different proportions of plasmids containing reporter genes GFP or RFP were mixed at different ratios, amplified by the PCR multiplex system and analysed by GeneScan software to test the sensitivity of the GeneScan technique. IN, integrase.
Figure 3. Replication capacity assays in the absence of drugs

(A) Intrapatient competition experiments of integrase (IN) recombinant viruses from long-term highly active antiretroviral therapy-experienced patients (A1–A5).

(B) Competition of IN recombinant viruses from a patient before raltegravir treatment (B first) and after failing raltegravir-containing therapy (B last; raltegravir-resistant virus with substitutions G140S/Q148H).

(C) HIV type-1 wild-type virus (HIV-1 NL4-3) and T66I (HIV-1 harbouring the substitution T66I).

(D) Growth kinetics based on p24Gag production was analysed by fitting a linear model to the log-transformed p24 data by maximum likelihood methods. The slope of recombinant virus p24Gag antigen production after peripheral blood mononuclear cell infection was compared with the slope of a wild-type virus and the percentage of replication capacity was calculated and represented. Error bars represent the so of two independent measurements. Statistical analyses were performed using the unpaired Student’s t-test. GFP, green fluorescent protein; RFP, discosoma red fluorescent protein.
Figure 3. Continued

B

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Days post-infection

C

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Days post-infection

D

Replicative capacity, %

Samples
- HIV-1 NL4-3–GFP (wild type control)
- HIV-1 NL4-3–RFP (wild type control)
- IN recombinant viruses from samples A1–A5 (first time points)
- IN recombinant viruses from samples A1–A5 (last time points)
- IN recombinant viruses from sample B before raltegravir
- IN recombinant viruses from sample B after raltegravir failure
- HIV-1 NL4-3–GFP (wild type) from sample C
- T66I site-directed mutant from sample C
Our data support previous reports that naturally occurring IN polymorphisms had no major effects on susceptibility to some IN inhibitors, such as l-chicoric acids [32] and diketo acids l-731,988 and l-870,810 [36], although the effect of IN polymorphisms on susceptibility has been controversial. Some of the substitutions (such as the V151I mutation), found in some of our patients, have been reported to reduce susceptibility to both l-870,810 and elvitegravir, a strand-transfer IN inhibitor that is currently in clinical development [20]. Furthermore, viruses with IN mutations G140S and Q148H, selected in a patient failing a raltegravir-containing regimen, had a reduced in vitro phenotypic susceptibility to raltegravir. This profile has been reported to induce a seven- to eightfold change in raltegravir resistance due to its location near the catalytic core domain of the enzyme [37]. The single T66I mutant was also shown to retain full susceptibility to raltegravir. This mutation was included because it had been reported to induce cross-resistance to the IN-inhibiting diketo acids l-708,906, l-731,988, GS-9137 and S-1360 [38,39].

Mutations within the IN could have a dramatic effect on both enzyme function and viral replication [40,41]. However, natural variability in viral IN from HIV-infected individuals has been reported to have no effect on growth kinetics, and only minor differences were seen in 3’-end processing, suggesting that in vivo mutability might be restricted by function [32]. This study shows that evolution of HIV-1 IN resulted in an improved in vitro replication capacity and that enhanced fitness was not restricted by the number of amino acid changes accumulated over time. The observation that some viruses had remarkably low replicative capacities is consistent with previous studies on recombinant HIV-1 viral isolates [35]. Although the specific location of certain amino acid substitutions is relevant for HIV IN function [42,43], none of the amino acid changes shown in Tables 2, 3 and 4 are in specific enzyme domains or positions previously reported as relevant for IN activity. These results could reflect the improved fitness of sequential viral isolates from HIV-1-infected patients during disease progression [44]. Furthermore, in the case of the IN gene, accumulation of changes during infection would improve enzyme activity.

Viruses selected in vitro to increasing raltegravir concentrations, containing any of the leading mutations to the raltegravir resistance pathways (Q148H or N155H), have shown severe fitness defects [19]. However, occurrence of the secondary mutation G140S within the Q148H pathway has been reported to increase resistance and moderate replication defects associated with selection of Q148H [19]. This observation might explain the limited fitness impairment shown in IN recombinant viruses from a patient failing a raltegravir-containing regimen with both Q148H and G140S mutations. Furthermore, and in agreement with our data, viruses with the T66I substitution have shown a reduced replication fitness as compared with wild-type viruses [17,28]. This mutation, located in the catalytic core, could impair both 3’ processing and DNA strand-transfer activities of IN [38], thus helping to explain viral fitness impairment. All these data are consistent with the general idea that resistance mutations might impair viral fitness. In particular, they could explain the maintained replication capacity between the first and last samples from patient A3, as this was the only patient in whom three changes previously associated with resistance to IN inhibitors (V72I, L101I and A111T) that did not appear in the first time point were found in the last time point.

The slight increase in the prevalence of changes associated with IN inhibitors seen in our study could support previous reports suggesting some genotypic and phenotypic interactions between the IN and RT enzymes [8,45,46]. Thus, the IN substitution V165I, previously associated with resistance to IN inhibitors, has been shown to be positively associated with substitutions F227L and L210W in RT. Mutations M154I and V165I could be more common in patients whose HAART fails [45].

In conclusion, the presence of natural polymorphisms in HIV-1 IN and the low rate of evolution during long-term combination antiretroviral treatment do not seem to adversely affect in vitro raltegravir susceptibility, and appear to drive the replication capacity of IN recombinant viruses towards improved phenotypes in most patients, suggesting no fitness cost associated with long-term treatment.

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Disclosure statement

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References


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