Impact of the HIV integrase genetic context on the phenotypic expression and in vivo emergence of raltegravir resistance mutations

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Received 23 July 2014; returned 21 August 2014; revised 12 September 2014; accepted 26 September 2014

Objectives: HIV resistance to the integrase inhibitor raltegravir in treated patients is characterized by distinct resistance pathways. We hypothesize that differences in the in vivo dynamics of HIV resistance to raltegravir are due to the genetic context of the integrase present at baseline.

Patients and methods: We studied four patients whose viruses evolved towards different resistance pathways. The integrase baseline sequences were inserted into a reference clone. Primary resistance mutations were then introduced and their impact on viral replication capacity (RC) and resistance was measured.

Results: Patients A and B experienced emergence and persistence of mutation N155H under raltegravir therapy. In the integrase sequence from Patient A, N155H conferred potent resistance coupled with a lower impact on RC than Q148H. In Patient B, instead, selection of N155H could be explained by the dramatic loss of RC induced by the alternative Q148H mutation. In Patient C, N155H initially emerged and was later replaced by Q148H. In this integrase context, N155H resulted in higher RC but lower resistance than Q148H. In Patient D, Q148H rapidly emerged without appearance of N155H. This was the only patient for whom Q148H conferred higher RC and resistance than N155H.

Conclusions: The emergence of different resistance mutations in patients was in full agreement with the impact of mutations in different baseline integrase contexts. Evolution towards different resistance genotypes is thus largely determined by the capacity of different integrase sequences present at baseline to minimize the effect of mutations on virus RC while allowing expression of resistance.

Keywords: integrase strand transfer inhibitors, resistance mechanisms, evolution

Introduction

HIV integrase strand transfer inhibitors (INSTIs) have demonstrated potent antiviral activity in patients infected either by HIV-1 or HIV-2.1,2 INSTIs proved to be valuable components for the therapy of treatment-naive patients3–5 and have dramatically changed the prognosis of HIV infection for patients in whom the virus had developed multiclass resistance.6–10 Like other classes of antiretroviral drugs, however, suboptimal suppression of HIV replication during treatment with INSTIs leads to the emergence of resistance mutations.11 The most complete set of data available today on HIV resistance to INSTIs concerns resistance to raltegravir. Raltegravir resistance has been mainly observed in patients harbouring viruses that were already resistant to other classes of antiretrovirals when raltegravir was introduced into their treatment regimen, resulting in an incomplete suppression of HIV replication and emergence of resistance mutations in the integrase coding sequence.11–18

Evolution of HIV resistance to raltegravir can be divided into three main evolutionary pathways for which the key mutations are N155H, Q148H/K/R and Y143R/H/C, respectively (reviewed in Blanco et al.19). The N155H and Q148H/K/R pathways are more frequently observed than the Y143R/H/C pathway.20 Interestingly, these three evolutionary pathways are distinct and mutually exclusive. Indeed, in patients in whom plasma virus population sequencing indicated the simultaneous presence of N155H and Q148H/K/R, clonal analyses have shown that mutations representative of each pathway were carried by distinct viral
Another remarkable aspect of HIV resistance to raltegravir was observed in patients in whom raltegravir treatment was continued in spite of the presence of major resistance mutations. These studies have revealed that viruses evolved towards increased levels of resistance, which, instead of resulting from accumulation of mutations along one resistance pathway (an evolutionary profile observed for almost all other classes of anti-retroviral agents), was the consequence of a switch from one genotypic pathway to another. In a significant proportion of patients, the first resistant genotypes observed in plasma virus populations belong to the N155H pathway. These genotypes persist in some patients while in others they are replaced by viruses bearing mutations of the Q148H/K/R or, less frequently, Y143R/H/C resistance pathways. Occasionally, genotypes belonging to the Q148H/K/R or Y143R/H/C pathways appear to dominate at the earliest timepoints of the virological failure follow-up. Secondary mutations, specific for each pathway, then accumulate over time.

In this study, we evaluated a potential mechanism explaining these particular raltegravir resistance evolutionary profiles, based on the hypothesis that the selective advantage conferred by key integrase mutations could differ according to the integrase genetic context of the virus present at baseline. Baseline subtype B HIV integrase sequences from four patients whose viruses evolved towards different raltegravir resistance pathways were used to examine the impact of different mutations as a function of the particular integrase genetic background into which they were inserted. Our results show that the integrase genetic context indeed exerts a strong influence on the initial selection of raltegravir resistance pathways.

**Methods**

**Sequencing and cloning of integrase alleles from patients**

This was a non-interventional study with no addition to usual procedures. Biological specimens were obtained for standard viral diagnosis requested by physicians (no specific sampling, no modification of the sampling protocol and no supplementary questions in the national standardized questionnaire). The results were analysed using an anonymized database. French Public Health Law CSP Art. L 1121-1.1 states that such studies on the hypothesis that the selective advantage conferred by key mutations was expressed as a percentage of their reference sequence, and then cloned into pNL4-3 integrase. Then, the larger fragment from AgeI to EcoRI, including the patient-derived integrase sequence, was cloned into plasmids carrying the patient-derived integrase pathways, using oligonucleotides designed to fit each baseline sequence, and then cloned into pNL4-3 as described above. The integrase sequence of each full-length plasmid and the junction sites were verified by standard sequencing (GATC Biotech, Koln, Germany).

**Cell culture and virus production**

HEK293T cells were maintained in DMEM supplemented with 10% heat-inactivated FCS and antibiotics (100 IU/mL penicillin and 100 mg/L streptomycin). The HeLa-derived P4C5 cell line, characterized by constitutive expression of CCR5 and stably transfected with an expression vector for CD4 and CCR5, was maintained in complete DMEM containing hygromycin B (200 mg/L).

Virus production was performed by transfecting HEK293T cells with pNL4-3-based proviral constructs using jetPEI (Polyplus Transfection, Illkirch, France), following the manufacturer’s instructions. An aliquot of supernatant was used to measure virus production by using an HIV p24 ELISA kit (Innogenetics, Ghent, Belgium).

**Virus replication capacity (RC)**

The single-cycle infectivity of the recombinant viruses was determined using indicator P4C5 cells. To this end, P4C5 cells were plated at 8000 cells/well in a 96-well plate 48 h before infection. Cells were infected with serial dilutions of viral supernatant normalized by p24 content (the equivalent of 0.5, 1, 2 and 4 ng/well) in the presence of 20 mg/L DEAE-dextran. Infections were performed in triplicate wells. For each virus input, β-galactosidase activity, resulting from transactivation of the LTR-lacZ reporter cassette in target cells, was measured as OD units using a colorimetric assay based on cleavage of chlorophenol red-β-D-galactopyranoside (CPRG) (Roche, Mannheim, Germany) by β-galactosidase. Briefly, following elimination of the supernatant, the P4C5 cells were lysed in 100 μL of lysis buffer (5 mM MgCl₂ and 0.1% NP-40 in PBS). After incubation for 5 min at room temperature, 100 μL of reaction buffer (6 mM CPRG in lysis buffer) was added to the cell lysates and incubated for 2 h at 37°C. ODs in the reaction wells were read at 570 nm with a reference filter set at 690 nm. After plotting the OD values as a function of the p24 virus input, we calculated the AUC for each virus. For each patient, the RC of the virus carrying the baseline integrase sequence was used as the reference (RC of 100%). The RC of viruses carrying N155H or Q148H integrase mutations was expressed as a percentage of their reference sequence, comparing the AUC values.

**Susceptibility to raltegravir**

P4C5 cells were seeded into 96-well plates in the absence or presence of increasing concentrations of raltegravir (0.32, 1.6, 8, 40, 200, 1000, 5000...
and 25,000 nM). After 24 h, viral supernatants containing the equivalent of 2 ng of p24 antigen were used for infection in triplicate wells in the presence of 20 mg/L DEAE-dextran. Thirty-six hours after infection, the single-cycle titre obtained in the presence of increasing raltegravir concentrations was determined by quantification of the β-galactosidase activity in P4C5 cell lysates using the colorimetric assay described above. The susceptibility of the different viruses was expressed as the concentrations of raltegravir that inhibited 50% of infectious events (IC50), calculated using the dose–response inhibition tool in Prism version 6 (GraphPad Software, La Jolla, CA, USA).

Virus selective advantage
To determine the replicative advantage of mutant viruses as a function of raltegravir concentration, we performed raltegravir susceptibility assays as described above, except that instead of calculating IC50 values, we calculated the ratio of mutant to baseline virus infectivity (in CPRG units) for each drug concentration (selective index). The ratios were then extrapolated as a continuous curve across the range of different drug concentrations tested. Having set the WT infectivity as the reference, the curve representing a mutant virus will be above the WT reference line for drug concentrations in which the mutant displayed a replicative advantage. The height of the peak is proportional to the extent of the replicative advantage.

Results

Raltegravir resistance pathways in treated patients
The treatment history and resistance mutations observed for the four HIV-infected patients studied here were previously described.22 These patients were infected by subtype B HIV and experienced raltegravir therapy failure. The pre-raltegravir baseline majority integrase sequence from each patient is shown in Figure 1. The escape viruses followed three different pathways of resistance to raltegravir. Table 1 summarizes the key data of the patients’ follow-up, including the time at which resistance-associated mutations were detected. In viruses from both Patients A and B, the N155H mutation was initially selected and this mutation remained as the principal resistance mutation throughout their follow-up. Patient A was followed for a relatively short time (8 months overall), with emergence of N155H at month 6 followed by emergence of the accessory mutation V151I at month 8. Patient B was followed for 29 months, a period during which N155H appeared at month 18 (accompanied by the accessory mutation T97A) and later remained as the main resistance mutation, complemented by the accessory mutations T97A, V151I, and G163R. In contrast, Patient C was typical of the frequently described switch in resistance pathway from the N155H pathway to the Q148H/K/R pathway. In this patient, mutation N155H emerged within 2 months of treatment, followed by mutations Q148H and G140S, which were detected as a fraction of genomes by conventional bulk PCR sequencing 4 months after starting raltegravir therapy. Raltegravir failure in Patient D was associated with the selection of Q148H and G140S mutations at month 4, with no trace of N155H at this timepoint.22 The Q148H and G140S mutations then persisted during the nine additional months of follow-up.

Interestingly, while for Patient B one additional change (C43R/C) not described as resistance associated, was detected during follow-up, for the other three patients only resistance-associated mutations distinguished the escape viruses from each respective baseline integrase sequence. This is particularly relevant for Patient C, in whom the two alternative resistance pathways successively emerged in the context of the same integrase sequence.

![Image of baseline integrase sequences](https://academic.oup.com/jac/article-abstract/70/3/731/2911033/)

**Figure 1.** Comparison of the baseline integrase sequences of the four patients using NL4–3 as a reference. Dots indicate sequence identity. The positions corresponding to the main raltegravir resistance mutations of each pathway (Y143, Q148 and N155) are boxed.
Phenotypic characterization of recombinant viruses carrying baseline integrase sequences

To explain the differences in raltegravir resistance evolution profiles in these four patients, the baseline integrase sequences from each patient were cloned into the replication-competent proviral construct pNL4-3. Replacement of the integrase sequence of NL4-3 by the baseline sequences issued from the four patients resulted in very similar IC$_{50}$ values, ranging from 9 nM (baseline sequence of Patient B) to 13 nM (baseline sequence of Patient A), showing that primary integrase sequences were equally susceptible to raltegravir. The RCs of viruses carrying baseline integrase sequences from patients were comparable to or even higher than that of the reference strain NL4-3 (Patient A = 2.4-fold, Patient B = 3.6-fold, Patient C = 1.2-fold and Patient D = 1.6-fold).

Comparison of the effect of mutations N155H and Q148H in patient-derived integrase sequences

We next analysed the consequences of the introduction of resistance mutations N155H and Q148H into the different baseline integrase contexts. Each recombinant virus was phenotypically tested for three parameters: RC in the absence of drug (Figure 2), raltegravir resistance (Figure 3) expressed as IC$_{50}$ and the selective advantage index (Figure 4), which expresses the ratio of infectivity between a mutant and its baseline virus measured as a function of drug concentration.$^{24-26}$ When introduced into the integrase sequence from Patient A, mutation N155H only mildly affected RC to 71% of the baseline sequence from this patient, while Q148H exerted a more deleterious fitness effect, reducing it to 49% (Figure 2). In terms of resistance, N155H promoted a 90-fold increase in IC$_{50}$ (1171 nM) while Q148H only increased IC$_{50}$ by 5-fold (61 nM) (Figure 3). These results show that N155H was the best option for inducing strong resistance

Table 1. Viral and immune characteristics of the four patients

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<th>Patient code</th>
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<th>CD4+ cells/μL</th>
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NA, not applicable.

Figure 2. Impact of raltegravir resistance mutations on virus RC in the absence of raltegravir. Mutant virus RC was measured in a single-cycle assay in the absence of raltegravir and expressed as a percentage of the respective baseline virus RC. The histograms represent the mean and SEM obtained from four independent experiments. Statistical significance was assessed by the Kruskal–Wallis test using GraphPad Prism version 6. Post-test comparisons (performed only if $P < 0.05$) were made using the Mann–Whitney test. *$P < 0.05$. BL, baseline.
to raltegravir with limited impact on virus RC and are fully consistent with the emergence and persistence of this mutation in vivo. By analysing the selective advantage curves (Figure 4), the dominance of mutant N155H becomes even more obvious. Mutant N155H is more infectious than the baseline virus (reference value of 1) for all raltegravir concentrations, while the alternative Q148H mutant is less infectious than the baseline virus even in the presence of raltegravir. Thus, the limited gain in resistance and the higher RC cost of Q148H prevented its emergence in the integrase context of the baseline virus from Patient A.

The situation in Patient B was somewhat different. N155H reduced the RC to 40% of baseline while Q148H was highly deleterious, reducing it to 8% (Figure 2). The level of resistance of these two mutants was comparable, with a 10-fold (111 nM) and 15-fold (131 nM) increase in IC\textsubscript{50} for N155H and Q148H, respectively (Figure 3). The selective advantage curves show a selective index >1 for mutant N155H for a small range of raltegravir concentrations (Figure 4), while the dramatic loss of RC of mutant Q148H does not allow the expression of the resistance potential of the mutation. The RC associated with mutation N155H as compared with Q148H appears to have favoured its initial emergence.

In Patient C, in whom HIV switched from the N155H pathway to the Q148H pathway, introduction of N155H into the baseline integrase sequence reduced RC to 63% and Q148H to 46% of the baseline virus (Figure 2). In terms of resistance, however, the increase in IC\textsubscript{50} for N155H was only 10-fold (104 nM) while Q148H promoted a stronger 17-fold change (168 nM) (Figure 3). It thus appears that the different cost in RC favoured the initial emergence of mutation N155H, but the higher resistance conferred by the Q148H mutation might have overcome the initial fitness disadvantage of this variant. The advantage of mutation Q148H in the context of the baseline sequence from Patient C is apparent also from the selective advantage curves, in particular for raltegravir concentrations >200 nM, for which this mutant shows a higher selective index than mutant N155H (Figure 4).

Virus from Patient D was the only one in which the changes in RC imparted on baseline virus by the raltegravir resistance mutations favoured Q148H (59%) over N155H (46%) (Figure 2). In addition, the 15-fold level of resistance conferred by Q148H (194 nM) was higher than that conferred by N155H (118 nM, 9-fold) (Figure 3). Thus, both phenotypic parameters were in favour of early emergence of Q148H and this mutation conferred a clear selective advantage over a broad range of raltegravir concentrations (Figure 4). Hence, in the particular context of the HIV baseline integrase sequence from Patient D, mutation Q148H was bound to emerge early and persist throughout raltegravir pharmacological pressure, which is what was actually observed in vivo.
Phenotypic expression of resistance mutations in different integrase genetic contexts

In the previous section, for each integrase sequence we compared the effect of mutation N155H with that of Q148H. Further insights can be gained by reanalysing the data comparing the consequences of each mutation as a function of the integrase sequence into which it was inserted (interpatient comparison). Mutation N155H reduced the viral RC to similar levels in different integrase contexts, ranging from 71% to 40% of the respective baseline values. More precisely, N155H reduced the virus RC in the context of integrase from Patient A to 71%, for Patient B to 40%, for Patient C to 63% and for Patient D to 46%. In contrast, the impact of mutation Q148H on the virus RC differed substantially among different integrase sequences, ranging from 59% to as low as 8% of the baseline value (49% for Patient A, 8% for Patient B, 46% for Patient C and 59% for Patient D).

In terms of raltegravir susceptibility, it is interesting to note that mutation N155H alone induced a very high level of resistance in the integrase context of Patient A (IC$_{50}$ of 61 nM, a 5-fold increase), while it was similar for the three other integrase sequences (131 nM, 15-fold increase for Patient B; 168 nM, 17-fold increase for Patient C; and 194 nM, 15-fold increase for Patient D). The resistance phenotypes measured here are similar to those reported in the Stanford database (http://hivdb.stanford.edu/cgi-bin/PositionPhenoSummary.cgi) for viruses carrying the single N155H mutation (17- to 24-fold) or Q148H mutation (18- to 20-fold).

The comparison of the effect of each resistance mutation in different integrase contexts clearly shows that baseline integrase sequences can differently absorb the impact of resistance mutations on the virus RC and allow expression of their potential for resistance to raltegravir.

Discussion

In this study, we found evidence to support the hypothesis that the integrase genetic context present at baseline in HIV-infected patients is a critical factor in determining virus evolution towards different raltegravir resistance pathways. The baseline sequences selected for this study came from four raltegravir-treated patients whose escape viruses had followed different pathways: 155H for Patients A and B, initial emergence of 155H and replacement by...
148H in Patient C and direct emergence of 148H in Patient D. Recombinant viruses carrying the baseline integrase sequences from these four patients did not differ in their susceptibility to raltegravir and displayed adequate virus RC. The agreement between the emergence of specific raltegravir resistance mutations in treated patients and our experimental data on the combined impact of mutations on virus RC and resistance to raltegravir strongly support our hypothesis that the integrase sequence present at baseline favours virus escape towards one or the other resistance pathway.

We showed that the insertion of resistance mutations had different consequences on the virus RC and on resistance to raltegravir, depending on the sequence into which they were inserted. Mutation N155H reduced viral RC to similar levels in different integrase contexts, while the impact of mutation Q148H was more heterogeneous and could dramatically reduce virus infectivity. It thus appears that the capacity to absorb the effect of mutation Q148H on virus RC is a key parameter in determining virus escape towards this resistance pathway. Accordingly, it was in the context of the integrase sequence from Patient D, where the impact was minimal, that the Q148H mutation rapidly emerged. Comparing the consequences of mutations on raltegravir susceptibility, the N155H mutation produced very similar IC50 values in the context of the integrase sequence from Patients B, C and D, but in the integrase context of Patient A the gain in IC50 was 10-fold higher. In striking contrast, insertion of mutation Q148H into the integrase sequence from this patient resulted in the lowest increase in resistance to raltegravir. Thus, baseline sequences may markedly differ also for their capacity to express the potential for resistance. Due to the limited number of patients studied here, however, we cannot draw conclusions regarding possible integrase polymorphism combinations (signatures) that could help predict the nature of the raltegravir evolutionary pathways most likely to emerge in the event of a failure of that antiretroviral drug. Also, stochastic events may play a role in the initial appearance of resistance mutations.

We also considered the alternative possibility that the nucleotide substitution pattern could influence the emergence of alternative resistance mutations. Depending on the number and nature of nucleotide changes involved, some mutations are more likely to be generated, with transitions being largely favoured over transversions.37,28 The baseline codon for N155 was AAT for all patients, and the codon for Q148 was CAA for all patients except Patient D (CAG). Thus, emergence of both resistance mutations would require a transversion: for N155H from AAT to CAT and for Q148H either CAA to CAT or CAG to CAC. These three possible transversions are generated with similar, very low frequency by HIV RT,28 arguing again against the participation of a mutational bias in the emergence of raltegravir resistance mutations, in contrast to the ordered appearance of lamivudine resistance mutations.29

The results described in our study strongly suggest that baseline integrase sequences influence the initial evolutionary choice towards either of the principal resistance pathways that are characteristic of HIV-1 escape and evolution under raltegravir pressure. Whether persistence of any of these mutations and/or pathways remains favoured over time then depends on multiple factors, among which the emergence of secondary mutations, the concentration of raltegravir and the residual virus load are probably relevant parameters. In particular, compensatory mutations may favour the persistence of an escaping virus on an evolutionary pathway or allow the shift towards an alternative solution.

Acknowledgements

Part of the work reported in this manuscript was previously presented at the Frontier in Retrovirology Meeting, Cambridge, UK, 2013 (Poster 56) and at the IAS Conference on HIV Pathogenesis, Treatment and Prevention, Kuala Lumpur, Malaysia, 2013 (Poster WEPE272).

Funding

This work was supported by grants from the French National Agency for Research on AIDS and Viral Hepatitis (ANRS) (grants 12-131 and 13-152) and from Merck Sharp & Dohme-Chibret ( MSD) (grant 1330DLA21). S. R. was a post-doctoral fellow of ANRS.

Transparency declarations

J.-M. M. and C. D. participate in the Advisory Board of Merck, and received a research grant from Merck. All other authors: none to declare.

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