Evolution of a novel pathway leading to dolutegravir resistance in a patient harbouring N155H and multiclass drug resistance

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Objectives: Dolutegravir has been recently approved for treatment-naive and -experienced HIV-infected subjects, including integrase inhibitor (INI)-experienced patients. Dolutegravir is a second-generation INI that can overcome many prior raltegravir and elvitegravir failures. Here, we report the evolution of resistance to dolutegravir in a highly treatment-experienced patient harbouring the major N155H mutation consequent to raltegravir treatment failure.

Methods: Genotypic and phenotypic analyses were done on longitudinal samples to determine viral resistance to INIs. Integrase amino acid sequence interactions with raltegravir and dolutegravir were assessed by molecular modelling and docking simulations.

Results: Five mutations (A49P, L68FL, T97A, E138K and L234V) were implicated in emergent dolutegravir resistance, with a concomitant severe compromise in viral replicative capacity. Molecular modelling and docking simulations revealed that dolutegravir binding to integrase was affected by these acquired dolutegravir mutations.

Conclusions: Our findings identify a novel mutational pathway involving integrase mutations A49P and L234V, leading to dolutegravir resistance in a patient with the N155H raltegravir mutation.

Keywords: HIV-1, integrase inhibitors, mutations, phenotype, genotype

Introduction

Antiretroviral therapy (ART) has revolutionized HIV/AIDS management, reducing viral burden, HIV transmission and the emergence of drug resistance. Nevertheless, selection of salvage therapies for heavily treatment-experienced patients harbouring multiclass drug resistance remains problematic. HIV integrase inhibitors (INIs), including raltegravir, elvitegravir and dolutegravir, are the most recent class of ARV drugs. Overall, raltegravir and elvitegravir share common resistance pathways, with major mutations in the integrase gene at positions Q148H/R/K and N155H for both drugs.1–3 Additionally, the Y143R/C/H pathway has been associated with raltegravir resistance and the T66I/A/K and E92Q mutations preferentially confer elvitegravir resistance.

Dolutegravir is a second-generation INI, showing a higher genetic barrier to resistance. In vitro, dolutegravir is active against viruses with resistance mutations to raltegravir and elvitegravir and has a distinct resistance profile involving mutations such as S153YF, R263K and G118R.4–7 In the VIKING-3 clinical study, dolutegravir remained active in highly treatment-experienced patients with INI-resistant viruses.8 The strongest predictive factor for dolutegravir responsiveness is baseline resistance. Subjects with Q148 plus two or more secondary mutations (G140A/C/S, L74I or E138A/K/T) showed lower likelihood (96%) of achieving HIV RNA ≤ 50 copies/mL than subjects with Y143, N155, T66 or E92 mutations at week 24 on dolutegravir-containing regimens. Overall, dolutegravir can salvage most prior INI-treatment failures depending on the resistance pathway.

Here, we report the evolution of resistance to dolutegravir in a highly treatment-experienced patient harbouring the major N155H mutation consequent to raltegravir treatment failure. Our results show that a dolutegravir-containing regimen was unable to suppress viral replication, leading to rapid development of resistance. Five mutations (A49P, L68FL, T97A, E138K and
L234V) were implicated in emergent dolutegravir resistance with a concomitant severe compromise in viral replicative capacity. Molecular modelling and docking simulations revealed that dolutegravir binding to integrase was affected by these acquired dolutegravir mutations. These results demonstrate that resistance to dolutegravir during salvage therapy may occur via novel pathways. Consequently, genotypic monitoring of dolutegravir clinical resistance in these patients is of great importance.

**Methods**

**HIV viral load measurements and CD4 T cell counts**

HIV RNA plasma viral loads were determined using the Versant HIV-1 RNA 3.0 assay (bDNA, Bayer HealthCare) until June 2010 and the Abbott Real-Time HIV-1 kit thereafter. CD4 T cell counts were evaluated every 3–6 months as suggested by Quebec provincial guidelines (http://publications.msss.gouv.qc.ca/acrobat/f/documentation/2010/10-337-02W.pdf).

**Genotypic resistance analysis**

The full-length integrase (867 bp) and protease/reverse transcriptase (1467 bp) genes were analysed by nested RT–PCR and sequenced using the BigDye Terminator Cycle Sequencing v1.0 Ready Reaction kit (Life Technologies), as previously described. Briefly, sequences were compared with HXB2 by using Seqscape v2.5 (Applied Biosystems) to determine substitutions. Resistance interpretations were subsequently assessed by Stanford HIVdb v7.0 (http://hivdb.stanford.edu/).

**Phenotypic resistance analysis**

The previously genotyped plasmas were sent for analysis of phenotypic susceptibility to INIs, NRTIs, NNRTIs and PIs to Monogram Biosciences, where the highly sensitive phenotypic resistance assays PhenoSense® and PhenoSense® Integrase (Monogram Biosciences) were performed.

**Structural bioinformatics: integrase modelling and dolutegravir docking simulations**

To model interactions of integrase with raltegravir and dolutegravir, integrase amino acid sequences prior to treatment with INIs (29 August 2009) and following raltegravir failure (16 February 2012) and dolutegravir failure (30 October 2013) were submitted to the I-TASSER 3D protein prediction server, as previously described. Apparent binding poses and energies of dolutegravir interactions at the active site of modelled integrase proteins were calculated by in silico docking simulations performed with the program AutoDockVina. The viral DNA mimic, cations and active site water molecules from 3S3M were retained in equivalent positions within the HIV active sites to aid in assessment of INI binding. Prior to docking, ligands and receptor proteins were processed in AutoDockTools. All subsequent image processing and analysis was performed in PyMOL Molecular Graphics System, version 1.3; Schrödinger, LLC.

**Results**

**Patient history**

This patient had been under HIV treatment for 20 years and had received drugs against all available ARV targets, including raltegravir (5 October 2009 to 21 April 2010; 11 May 2011 to 15 February 2012) and dolutegravir (15 February 2012 to 10 March 2014). Evolution of viral load and CD4 T cell counts over time is depicted in Figure 1. Over 20 years of treatment, a slow decline of CD4 T cell count was observed (from 549 cells/mm³ in 1994 to 160 cells/mm³ in 2014). Short treatment interruptions (years 2000, 2008 and 2011) led to episodic bursts in viral load but this did not influence the overall decline (Figure 1). In addition, it
should be noted that viral load since 1994 became undetectable (<40 copies/mL) only once (not shown) as a result of adding dolutegravir to the treatment regimen (1 May 2012). Nevertheless, viral load rebound occurred the following month.

As previously mentioned, the patient was heavily treatment experienced. Genotypic analysis revealed a dominant MDR virus harbouring mutations conferring resistance to NRTIs (M41L, D67N, V118I, M184V, L210W, T215Y) and NNRTIs (K101P, K103N/S, V108I, V179T, V189I), as well as PIs (L10F, I13V, K20M, V32I, L33F, M36I, M46L, I54L, K55R, L63P, A71I, G73A, I84V, L90M). The cumulative effects of this resistance profile on phenotypic susceptibility to NRTIs, NNRTIs and PIs are depicted in Table 1. As expected, the viral population showed multiclass drug resistance to all ARV drugs, with limited residual activity for tenofovir and tipranavir.

**Evolution of resistance to INIs**

From October 2009, INIs were included in ARV regimens. A pre-INI genotype revealed many natural polymorphisms associated with resistance to INIs (R20K, V31I, L41I, L101I, I135V, E157Q, K160Q, V201I, K215N and A265V), none of which conferred reduced phenotypic susceptibility to raltegravir, elvitegravir or dolutegravir (Table 2). Subsequent to raltegravir treatment failure, the patient acquired N155H (October 2010), which persisted thereafter on raltegravir- or dolutegravir-containing regimens. This mutation conferred 14.3-fold and 29.6-fold reductions in raltegravir and elvitegravir susceptibility, respectively (Tables 2 and 3). Although N155H briefly disappeared during a transient treatment interruption (February 2011), re-introduction of raltegravir in May 2011 led to its reappearance, with the subsequent acquisition of S119R, S147V and V151I in February 2012. This resistance profile led to a 34.6-fold and 56.3-fold elevated cross-resistance to raltegravir and elvitegravir, respectively (Tables 2 and 3). The virus remained sensitive to dolutegravir (1.9-fold resistance) and the patient was switched to a dolutegravir-based regimen in February 2012.

The patient developed treatment failure with 63.6-fold resistance to dolutegravir and a maximum of 150-fold cross-resistance to raltegravir and elvitegravir (Table 2). The N155H, S119R, S147V and V151I mutations that were acquired secondary to raltegravir usage persisted throughout dolutegravir treatment. The acquisition of T97A and E138K led to a 37-fold reduction in dolutegravir susceptibility. The sequential acquisition of the A49P, L68FL and L234V mutations after dolutegravir usage led to a further 63-fold decline in phenotypic susceptibility to dolutegravir.

The sequential acquisition of dolutegravir resistance mutations was associated with significant declines in viral replicative capacity (41%) relative to levels observed (101% – 187%) prior to use of dolutegravir in treatment (Table 2). Consistent with this negative effect on replication, we were unable to grow the virus in culture (data not shown), although p24 antigen was detected.

**Integrate modelling**

Insofar as emergent clinical resistance to dolutegravir is rare and largely undefined, structural bioinformatics were used to gain insights regarding the impact of mutations arising following treatment failure on raltegravir- and dolutegravir-based regimens. Three-dimensional models of viral nucleotide sequences of integrate prior to INI exposure (25 August 2009), following raltegravir failure (16 February 2012) and dolutegravir failure (30 October 2013) were obtained (Figure 2). After raltegravir failure via the N155H mutation, the positions of active site catalytic residues (D64, D116, E152) and orientations remained relatively

**Table 1.** Phenotypic profile of NRTIs, NNRTIs and PIs

| Phenotypic susceptibility to NRTIs, NNRTIs and PIs (fold change) 
<table>
<thead>
<tr>
<th>NRTIs</th>
<th>ZDV</th>
<th>d4T</th>
<th>TFV</th>
<th>ABC</th>
<th>ddl</th>
<th>FTC</th>
<th>3TC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13.0</td>
<td>2.64</td>
<td>1.87</td>
<td>6.04</td>
<td>2.19</td>
<td>&gt;</td>
<td>&gt;</td>
</tr>
<tr>
<td>NNRTIs</td>
<td>EFV</td>
<td>ETR</td>
<td>NVP</td>
<td>RPV</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td>&gt;</td>
<td>&gt;</td>
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</tr>
<tr>
<td>PIs</td>
<td>ATV</td>
<td>DRV</td>
<td>AMP</td>
<td>IDV</td>
<td>LPV</td>
<td>NFV</td>
<td>RTV</td>
</tr>
</tbody>
</table>

ZDV, zidovudine; d4T, stavudine; TFV, tenofovir; ABC, abacavir; ddl, didanosine; FTC, emtricitabine; 3TC, lamivudine; EFV, efavirenz; ETR, etravirine; NVP, nevirapine; RPV, ritonavir; DRV, darunavir; AMP, amprenavir; IDV, indinavir; LPV, lopinavir; NFV, nevirapine; RTV, ritonavir; SQV, saquinavir; TPV, tipranavir.

Table 2. Phenotypic profiles for INI-containing regimens

<table>
<thead>
<tr>
<th>Phenotypic susceptibility to INI-containing regimens</th>
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<tbody>
<tr>
<td>VL (copies/mL)</td>
</tr>
<tr>
<td>8170</td>
</tr>
<tr>
<td>RAL FC</td>
</tr>
<tr>
<td>EVG FC</td>
</tr>
<tr>
<td>DTG FC</td>
</tr>
<tr>
<td>RC (%)</td>
</tr>
</tbody>
</table>

VL, viral load; RAL, raltegravir; EVG, elvitegravir; DTG, dolutegravir; Tx-I, treatment interruption on INI regimen; FC, fold change; RC, replicative capacity.
unchanged (Figure 2a), as has been previously reported. The substituted N155H affected the electrochemistry of the active site, potentially forming a salt bridge with E152 and a hydrogen bond with T66, without seriously modifying global integrase structure. Minor steric rearrangements provided by the accessory mutations S119R, I135V and V151I might allow integration in the presence of N155H.

In contrast, the acquisition of the five additional resistance mutations with dolutegravir (A49P, T97A, E138K, S147G and L234V) led to major deviations between pre- and post-dolutegravir integrase structures in the loop region flanked by the C65 and M50 residues (indicated by black arrows in Figure 2b). The mis-positioning of D64 completely outside the active site (Figure 2b and c) suggests a marked impairment in the ability of the virus to integrate. Whereas the A49P and L234V mutations were distal to the active site, T97A, E138K and S147G were in close proximity to the catalytic residues (Figure 2c). More importantly, A49P and L234V were in close proximity to each other, and may have been the catalyst for major structural changes following the use of dolutegravir. Indeed, the disordered loop seen in the structure extending from residue 50 to residue 65 was directly downstream of position 49P (Figure 2c).

**Dolutegravir docking simulations**

To mimic the drug binding of dolutegravir in vivo, dolutegravir was docked to dimeric structures using DNA and Mg$^{2+}$ derived from the prototype foamy virus (PFV) structure 3S3M co-ligands. After docking, the relative position of dolutegravir crystallized into the PFV structure was used as a visual guide for properly docked dolutegravir. As expected, dolutegravir docked easily to both pre-INIs (data not shown) and post-raltegravir integrase structures in the predicted ‘most energetically favourable (1/40)’ poses (Figure 2d). In contrast, dolutegravir did not bind favourably in the post-dolutegravir model (Figure 2e–g). Indeed, docking of dolutegravir to the post-dolutegravir structure shows that binding was favourable (1/40) to the viral DNA mimic (Figure 2e), but less favourable (9/40) to the disordered loop (Figure 2f). Furthermore, it was shown that dolutegravir docked far less favourably (29/40) in a similar position to the co-crystallized pose (Figure 2g) relevant mechanistically for strand-transfer inhibition. Taken together, docking simulations point to compromised dolutegravir binding to integrase as a potential source for emergent resistance. The fact that dolutegravir was still able to bind with the 29th most favourable pose shows that dolutegravir may retain low-level activity despite the presence of some potentially relevant mutations.

**Discussion**

Dolutegravir has been recently approved for treatment of HIV infection. As a second-generation INI, it may be able to overcome many prior raltegravir and elvitegravir failures. This has been demonstrated in both in vitro studies and the VIKING-3 study, where dolutegravir was evaluated in therapy-experienced adults harbouring INI-resistant viruses. The VIKING-3 study showed that viruses with resistance mutations at positions 155, 143 and 155 with T66I or E92Q were more susceptible to dolutegravir than those with mutations at position 148 (if associated with secondary mutations). Importantly, VIKING-3 subjects harbouring viruses with substitutions at codon 66, 149 or 155 achieved viral...
Figure 2. Structural modelling of integrase pre-INIs, post-raltegravir (RAL) and post-dolutegravir (DTG) with simulated binding of DTG to the active site. (a) Overlay of pre-INI (blue) and RAL-exposed (salmon pink) integrase showing 155H forming hydrogen bonds with T66 and E152. (b) Active site catalytic residues (D64, D116, E152) in pre-INI (blue), RAL- (salmon pink) and DTG-exposed (green) integrase. The D64 catalytic residue (green) is outside the active site in the DTG-exposed integrase. Major secondary structure variations are observed along the loop flanked by residue 50 (long black arrows) to residue 65 (short black arrows) (colours: deep blue, INI-naive; brown, RAL-exposed; yellow, DTG-exposed). (c) Overlay of RAL- (salmon pink) and DTG-exposed (green) integrase showing major changes in secondary structure. (d) DTG docking into post-RAL integrase (salmon pink) is in good agreement with the reference crystallized pose (shown in black sticks). (e–g) Docking of DTG to post-DTG integrase (green) shows that it does not bind as the reference position. The favourable binding position is to the viral DNA mimic (1/40) (e); there is less favourable (9/40) binding to the disordered loop (f) and even less favourable (29/40) binding in a similar pose to the reference (g). This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
suppression at week 24 in 100%, 75% and 88% of patients, respectively.\textsuperscript{8,17} However, viral suppression at week 24 was observed in only 59% and 24% of subjects when the viral strains contained a substitution at codon 148 plus one and more than two secondary mutations, respectively. Consequently, dolutegravir has been recommended for salvage therapy in patients resistant to raltegravir or elvitegravir that do not have integrase mutations Q148H/K/R plus two or more secondary mutations.

In the present case, a switch from raltegravir to dolutegravir was performed in February 2012 after the N155H mutation was first detected. To determine whether prior resistance to raltegravir through N155H affected dolutegravir activity, we performed retrospective phenotyping of the virus from the time of switching for raltegravir, elvitegravir and dolutegravir. As expected, this raltegravir-resistant virus was susceptible to dolutegravir. Nevertheless, the dolutegravir activity rapidly diminished, as phenotyping results for December 2012 showed a fold change of 37. This phenotypic resistance was associated with the acquisition of several integrase mutations in addition to the persistence of N155H.

Among the natural polymorphisms identified, V31I, L101I, V201I and A265V are part of 34 highly polymorphic sites in the integrase gene that have been shown to have no effect on viral susceptibility to dolutegravir.\textsuperscript{18} In addition to the N155H mutation, other INI-resistance mutations (T97A, S119R, E138K, S147G and V151I) emerged after re-introduction of raltegravir (Table 3). V151I is a polymorphic accessory INI-resistance mutation selected in raltegravir-treated patients. E138K has been described to confer resistance to dolutegravir and mainly occurs with Q148 mutations.\textsuperscript{19} S147G has been frequently selected in patients receiving elvitegravir, with minimal effects on raltegravir.\textsuperscript{20} T97A alone does not affect raltegravir susceptibility, but has been shown to reduce raltegravir efficacy in combination with Y143C/R.\textsuperscript{21} Interestingly, the addition of the T97A and E138K to the other INI-resistance mutations mentioned above has been observed to be accompanied by a dramatic decrease in viral replicative capacity. Our findings show the acquisition of three hitherto unreported new mutations consequent to dolutegravir treatment failure: A49P, L68F (as a mixture with wild-type) and L234V. More precisely, A49P and L234V were detected after 20 months of dolutegravir therapy, and L68F/L slightly thereafter (23 months). The step-wise acquisition of hitherto unreported integrase resistance mutations suggests a novel dolutegravir resistance pathway rather than selection of rare pre-existent minority species.

With the exception of the N155H substitution, all the accessory amino acid substitutions observed during raltegravir treatment resulted from G→A transitional mutations. In contrast, the previously noted raltegravir-associated mutations T97A, E138K and S147G result from G→A mutations while A49P and L234V represent G→C transversions.\textsuperscript{22} Two major factors may affect the ability of a particular mutation to arise following treatment failure: (i) the ease with which that mutation can occur; and (ii) the selective advantage imparted by the phenotypic expression of that mutation. Even without considering the APOBEC proteins,\textsuperscript{23} transition mutations occur more frequently than transversion mutations. Although the impact of a transition mutation on double-stranded DNA structure is minor, the presence of a transversion mutation usually implies a strong selective pressure [such as for N155H (A→C transversion)], which confers high-level resistance to raltegravir. In our case, two of five dolutegravir-associated substitutions (A49P and L234V) resulted from G→C or C→G transversions, implying strong selective pressure. It is known that major pathways of selectively favourable mutations in HIV either increase or cause resistance and/or recover activity losses caused by a previous resistance substitution. Experiments are currently under way to construct viruses carrying the A49P and/or L234V mutations by site-directed mutagenesis and evaluate their susceptibility to dolutegravir, raltegravir and elvitegravir and their effect on virus replication capacity.

To further characterize these mutations, structural bioinformatics analyses were performed. Two key changes were identified that may have driven the virus to become resistant to dolutegravir: the A49P and L234V mutations (Figure 2). These transversion mutations are within interaction distance of each other (≏4 Å). The A49P substitution is particularly interesting due to the known steric role of proline and the ability of cis–trans isomerization to drastically affect protein structure.\textsuperscript{24} More precisely, this mutation is probably responsible for the mis-positioning of the catalytic D64 residue, as observed in Figure 2(b and c). Indeed, if A49P along with the L234V substitution caused a loss in integration, the impact on dolutegravir binding might have outweighed the need of the virus to integrate; in our binding simulations proper binding of dolutegravir was difficult to achieve (Figure 2e–g). Conversely, post-dolutegravir integrase may be able to fold in a manner that facilitates altered target and viral DNA binding, thereby allowing integration to proceed at a much lower, though uninhibited, rate. To test this hypothesis, more models will need to be tested and molecular dynamics studies performed.

In conclusion, our findings have shown a novel mutational pathway involving A49P and L234V, leading to emergent dolutegravir resistance in a patient on salvage therapy with the N155H mutation. INI salvage therapy with dolutegravir should be used with caution in raltegravir failures even if Q148 resistance mutations are not present.

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Transparency declarations
None to declare.

Author contributions
M. R., I. H. and B. B. designed the experiments, analysed the data and wrote the manuscript. I. H. and D. M. performed the genotyping experiments. C. P. and W. H. performed the phenotyping experiments. P. Q. and M. A.
W. performed the modelling. R. T. was responsible for patient recruitment and provided clinical and laboratory data. All authors edited and approved the final version of the manuscript.

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