G118R and F121Y mutations identified in patients failing raltegravir treatment confer dolutegravir resistance

Soundasse Munir†, Eloise Thierry†, Isabelle Malet2, Frédéric Subra1, Vincent Calvez2, Anne-Geneviève Marcelin2, Eric Deprez† and Olivier Delelis1*†

1Laboratoire de Biologie et Pharmacologie Appliquée, Centre National de la Recherche Scientifique UMR8113, ENS-Cachan, 94235 Cachan, France; 2Laboratoire de Virologie, AP-HP, Groupe Hospitalier Pitie-Salpêtrière, UPMC Université Pierre et Marie Curie, INSERM U943, Paris, France

*Corresponding author. Tel: +33-1-47-40-59-74; Fax: +33-1-47-40-76-71; E-mail: delelis@lbpa.ens-cachan.fr

†These authors equally contributed to the work.

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Objectives: Strand transfer inhibitors (raltegravir, elvitegravir and dolutegravir) are now commonly used to inhibit HIV-1 integration. To date, three main pathways conferring raltegravir/elvitegravir resistance, involving residues Y143, Q148 and N155, have been described. However, no pathway has been clearly described for dolutegravir resistance. The aim of this study was to characterize the susceptibility of two mutations, F121Y and G118R, originally described in patients failing raltegravir-containing regimens, to dolutegravir and raltegravir, and then to compare the resistance of these mutations with that of other well-known mutations involved in raltegravir resistance.

Methods: Both the F121Y and G118R mutations were introduced by site-directed mutagenesis into the pNL4.3 backbone and studied in cell-based and in vitro assays. The effects of the mutations were characterized at the different steps of infection by quantitative PCR.

Results: Results obtained with in vitro and ex vivo assays consistently showed that both mutations impaired the catalytic properties of integrase, especially at the integration step. Moreover, both mutations conferred an intermediate level of resistance to dolutegravir. Interestingly, the F121Y mutation, but not the G118R mutation, displayed differential resistance to raltegravir and dolutegravir. Indeed, the F121Y mutation was more resistant to raltegravir than to dolutegravir.

Conclusions: Mutations at G118 and F121, which have been described in patients failing raltegravir-containing regimens, must be included in drug-resistance-testing algorithms.

Keywords: HIV, integrases, strand transfer inhibitors

Introduction

The integration of HIV-1 DNA into the host genome confers stability on viral DNA and enables the expression of viral genes leading to the subsequent formation of progeny viruses. Therefore, this integration step is a key step in HIV-1 replication. Integration is catalysed by integrase (IN), which is encoded by the pol gene of the virus. IN catalyses two successive reactions during this process (for a review, see Delelis et al.1). The first reaction, named 3′-processing (3′-P), involves the canonical terminal GpT dinucleotides at each end of both long terminal repeats (LTRs). This reaction generates CpA 3′-hydroxyl ends, which participate in the second reaction, named strand transfer.

Due to the central and critical place of integration in HIV-1 replication, IN inhibitors belonging to the class of strand transfer inhibitors (INSTIs) constitute promising compounds that enrich the number of available antiretroviral drugs.2,3 These compounds bind to the complex formed by IN and viral DNA (and not to IN alone) and specifically inhibit the strand transfer reaction by displacing the processed viral DNA end (probably by competition with binding to target DNA mimicking the host cell genome).4,5 Raltegravir and elvitegravir are two potent INSTIs approved by the FDA.6–10 Although these compounds are very effective at inhibiting integration, drug-resistance mutations have been reported that threaten the long-term efficacy of HAART.11 To date, three pathways of resistance have been clearly described in patients receiving raltegravir and in vitro studies have confirmed that resistance involves primary mutations in the IN sequence at positions N155, Q148 and Y143.12–14 Mutations described in patients failing elvitegravir include...
mutations at Q148, E92, N155, T66, S147 and T97. Importantly, there is extensive cross-resistance between raltegravir and elvitegravir. Several second-generation INSTIs have been developed, such as MK-2048 and dolutegravir, which are more potent than first-generation INSTIs, most likely due to the higher stability of IN/DNA/drug ternary complexes. Most strains resistant to raltegravir and elvitegravir do not show cross-resistance to dolutegravir. Indeed, the pathways involving N155 and Y143 residues, which are responsible for raltegravir resistance, are efficiently inhibited by dolutegravir. However, some mutants of the Q148 pathway are resistant to dolutegravir. This mutation is often associated with secondary mutations at position 138 or 140.

In the case of dolutegravir, attempts to select resistant strains using in vitro culture have been unsuccessful. Initially, some mutations, such as those at residues T124, S153 and L101, were identified but the significance of these mutations remains uncertain. More recently, a mutation at position R263, located in the C-terminal region of IN, was identified during in vitro selection; however, this mutation confers only a low level of resistance to dolutegravir. Finally, the selection of viruses resistant to dolutegravir also identified an arginine substitution at position G118, although this mutant was only present among subtype C or CRF02_A/G strains and not subtype B strains. Dolutegravir has been evaluated as first-line therapy in treatment-naive patients in three different Phase III trials, SPRING-2, SINGLE and FLAMINGO, versus third agents currently recommended by the guidelines: raltegravir, efavirenz and boosted darunavir. The primary endpoint was an HIV-1 RNA concentration of <50 copies/mL at week 48. The results of these studies indicated that dolutegravir was non-inferior to raltegravir and was superior to efavirenz and boosted darunavir. Moreover, there was no evidence of treatment-emergent resistance in patients showing virological failure with dolutegravir. Taken together, these studies confirm that dolutegravir possesses a higher genetic barrier for resistance than its first-generation counterparts.

In this study, we characterized mutations at positions F121 and G118 that had recently been described in patients failing raltegravir-containing regimens. Using recombinant IN assays as well as virological experiments, we studied the effect of each mutation on the susceptibility to dolutegravir. We also compared the resistance associated with these mutations with that involving the Q148K residue in combination with the E138K mutation, which is found in strains that are strongly resistant to raltegravir. We show that both G118R and F121Y lead to an intermediate level of resistance to dolutegravir, between the levels associated with E138K/Q148K, which confers the strongest level of resistance. Furthermore, a mutation at position E138 rescued the activity of the Q148K mutant and conferred strong dolutegravir resistance on the E138K/Q148K double mutant, similar to the effect of a mutation at the G140 residue associated with Q148H/R. Although the pathways involved in dolutegravir resistance are not yet known, non-polyomorphic mutations described in patients failing raltegravir display a variable profile of dolutegravir resistance that should be considered before administering dolutegravir.

**Methods**

**Cells and viruses**

HeLa-P4 and 293-T cells were cultured in DMEM supplemented with 10% heat-inactivated FBS. MT4 cells were cultured in RPMI 1640 with 10% FBS. All the cells were maintained at 37°C and 5% CO2. HIV-1 IN mutants were obtained by site-directed mutagenesis using the QuickChange Lightning site-directed mutagenesis kit (Agilent) according to the manufacturer’s instructions. Briefly, the fragment encoding the IN of the replication-competent pHNL4.3 virus was digested with AgeI and EcoRI and inserted into the pGEM-T vector (Promega). After mutagenesis, all the constructs were sequenced and the fragment encoding IN was then inserted into pHNL4.3. HIV-1 stocks were prepared by transfecting 293-T cells with the various HIV-1 molecular clones. Transfection assays were carried out using the calcium phosphate method. Forty-eight hours post-transfection, the viral supernatant was filtered through a filter with a pore size of 0.45 µm and was frozen at −80°C. The concentration of HIV-1 p24 gag antigen in the viral inocula was determined by ELISA (Perkin-Elmer Life Sciences).

**HIV infectivity assay**

Single-cycle titres of the virus were determined in HeLa-P4 cells expressing a β-galactosidase gene under the control of the HIV Tat protein. Briefly, cells were infected in triplicate in 96-well plates with WT or the different IN mutants (the equivalent of 3 ng of p24 gag antigen) with or without increasing concentrations of raltegravir or dolutegravir. Cell survival was estimated with a standard MTT assay. The MTI assay revealed that the highest concentration of dolutegravir used in this study (300 nM) was not cytotoxic [at 48 and 72 h post-infection (p.i.)] (Figure S1, available as Supplementary data at JAC Online). This was also true for raltegravir. The viral titre was then determined by quantifying the β-galactosidase activity in HeLa-P4 lysates with a colorimetric assay based on the cleavage of chlorophenol red-β-D-galactopyranoside (CPRG) by β-galactosidase. The 50% effective concentration (EC50) of each drug was defined as the drug concentration resulting in β-galactosidase activity that was 50% lower than that in infected cells without the drug.

**Viral infections**

Viral infections were performed using MT4 cells (2 × 10⁶/mL with 20 ng of p24 gag antigen per 10⁶ cells) with or without drugs. Four hours p.i., the cells were washed in PBS and new medium was added. At various times p.i., 1 million to 2 million cells were harvested and the dry pellets were frozen at −80°C until use.

**DNA extraction and quantitative PCR**

To digest residual transfection plasmid, the collected cell samples were washed in PBS and incubated with 2 U of DNase I, amplification grade (Invitrogen), for 20 min at room temperature according to the manufacturer’s instructions. As a result of this DNase treatment, the signal from any residual transfection plasmid was negligible for all IN mutant preparations using heat-inactivated viruses. Total DNA from the previously described dry pellets was extracted with the QIAamp blood DNA minikit (Qiagen) according to the manufacturer’s instructions. Viral DNA forms (total viral DNA and integrated viral DNA) were quantified by real-time PCR on a LightCycler instrument using the FastStart DNA master hybridization probe kit (Roche Diagnostics) as previously described. The copy number of total HIV-1 DNA was determined from a standard curve prepared by the amplification of quantities ranging from 10 to 10⁵ copies of
Characterization of IN enzymatic activity in vitro

Recombinant IN from the pNL4.3 strain was produced in Escherichia coli BL21-CodonPlus (DE3)-RIPL (Agilent), and purified under non-denaturing conditions as previously described. An oligonucleotide (ODN) mimicking the unprocessed US LTR end of the viral genome (US5-′-GTG AAA ATC TCT AGT-3′) was radiolabelled with T4 polynucleotide kinase (NEB) and [γ-32P]ATP (Amersham) and were then purified on a Sephadex G-10 column. Double-stranded ODNs were obtained by mixing equimolar amounts of ODNs with the complementary strand USA: 5′-ACT GCT AGA GAT TTT CCA CAC-3′ in the presence of 100 mM NaCl. The 3′-processing assay and strand transfer reactions were carried out at 37 °C with 300 nM of IN in a buffer containing 20 mM HEPES (pH 8.6), 1 mM dithiothreitol, 7.5 mM MgCl2 and 50 mM NaCl in the presence of 6.25 nM USA/USD or 12.5 nM USA/USB-2, respectively. The products were separated in a 16% acrylamide/urea denaturing gel, analysed with a Typhoon TRIO variable mode imager (GE Healthcare) and quantified by assessing IN activity in the presence of various concentrations of INSTIs. The IC50 was determined with Prism 5.0 software.

Steady-state fluorescence anisotropy-based assay

Anisotropy-based assay is a quantitative method to determine the DNA-binding parameters of IN.24 Briefly, IN binding to fluorescein-labelled DNA duplex (composed of a 21-mer 5′-GTG TGG AAA ATC TCT AGT-3′ annealed with its complementary strand) increases the steady-state anisotropy value [measured on a Beacon instrument (Panvera, Madison, WI, USA)], allowing the calculation of the fraction of DNA sites bound to IN as previously described. The DNA-binding step was recorded at 25°C. The formation of IN/DNA complexes was performed by incubating 4 nM fluorescein-labelled ODNs with IN in buffer A (20 mM HEPES (pH 7.5), 1 mM dithiothreitol, 10 mM MgCl2 and 50 mM NaCl). The binding of IN mutants was expressed as a percentage of the binding of the wild-type IN.

Results

Characterization of raltegravir resistance mutations in cell-based assays

Two non-polymorphic mutations (G118R and F121Y) were recently reported in two patients failing raltegravir treatment.28 The G118R mutation was detected in a patient infected with a CRF02_A/G virus whereas the F121Y mutation was detected in a patient infected with a subtype B strain.28 We first investigated the replicative properties of viruses containing G118R or F121Y mutations and their ability to produce viral particles. We compared these strains with the E138K/Q148K double mutant, which is one of the most resistant mutants against both anti-IN drugs.22,23 The E138K and Q148K single mutants were also generated to study the effect of each mutation in the context of the double mutant. First, we transfected 293-T cells with these mutants and examined viral production by measuring the abundance of p24Ag. The production of p24Ag by these mutants was similar to that by the WT virus, showing that none of these mutations substantially impaired the late steps of viral replication, including viral assembly and viral release (Figure 1a).

In order to investigate the effect of these mutations on the early steps of viral replication (viral entry, reverse transcription and integration), we infected Hela-P4 reporter cells expressing β-galactosidase under the control of the viral Tat protein with equivalent amounts of WT and mutant p24Ag antigen and measured the β-galactosidase activity at 40 h.p.i. The mutations affected viral infectivity to different extents (Figure 1b). In cells infected with the G118R or F121Y mutants, β-galactosidase activity was significantly lower than in cells infected with the WT virus (71% and 50% of the WT activity for G118R and F121Y, respectively), reflecting a defect in viral entry, reverse transcription or viral integration. However, this defect was weak compared with that shown by the Q148K mutation, which drastically disrupted viral infectivity (β-galactosidase expression was 10% of the WT activity). Similar findings were reported for the Y143R/C and Q148H mutations conferring raltegravir resistance.29 The single E138K mutation had no effect on viral infectivity because β-galactosidase expression was identical to that in cells infected with the WT virus. Interestingly, the combination of the E138K and the Q148K mutation resulted in the partial recovery (60% of the WT activity) of the low viral infectivity observed in the Q148K mutant. This recovery parallels that of the raltegravir-resistant G140S/Q148H double mutant,29 where the G140S mutation was found to rescue the defect of viral infectivity caused by the Q148H mutation.

Given that some mutations in IN influence the amount of reverse-transcribed viral DNA,23,34 we next studied the potential effect of the mutations on reverse transcription and viral integration by quantifying the total viral DNA and integrated viral DNA as previously described. Quantitative PCR was performed at several timepoints p.i. in MT4 cells infected with viruses harbouring the different mutations G118R, F121Y and the E138K/Q148K double mutant, as well as the E138K and Q148K single mutants. At 10 h.p.i., when reverse transcription was complete, the amount of viral DNA was fairly similar between cells infected with the different mutants, suggesting that none of these mutations drastically impaired reverse transcription activity (Figure 1c). Most of the viral strains, with the notable exception of the Q148K virus, were able to replicate, and cells infected with these strains showed a significant increase in viral DNA load at 72 h.p.i., which followed a transient decrease due to integration (between 24 and 48 h.p.i. in the case of the WT virus). Indeed, at 72 h.p.i., integration may occur leading to new infection cycles and, consequently, an increase in total viral DNA. Furthermore, we noted a strong increase in viral DNA for the E138K mutant as soon as 48 h.p.i., highlighting the strong replicative capability of this mutant. By contrast, the amount of viral DNA in cells infected with the Q148K virus was low at 48 and 72 h.p.i., confirming the severe defect in viral replication as previously observed for the
Taken together, these data show that the E138K mutation improves viral fitness and rescues the defect due to the Q148K mutation, leading to a replicative and resistant strain, similar to that observed for the G140S/Q148H double mutant.

We then quantified the integrated viral DNA at 48 h p.i. because the reverse transcription step was not substantially altered in the different viral strains (Figure 1d). Except for the E138K mutant, all the viral strains showed defects in integration but to different extents. Integration was totally impaired in the Q148K mutant but was partially restored by the E138K mutation in the double mutant. The efficiency of integration was defined as the amount of integrated viral DNA at 48 h p.i. divided by the amount of viral DNA resulting from reverse transcription (at 10 h p.i.). The results are expressed as the percentages of the value obtained for the WT. The results in Figure 1 are the means ± SD (error bars) of three representative independent experiments.

Q148H mutant. Given these findings, the E138K mutation improves viral fitness and rescues the defect due to the Q148K mutation, leading to a replicative and resistant strain, similar to that observed for the G140S/Q148H double mutant.

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Figure 1. Characterization of IN mutants. (a) Quantification of p24 antigen 48 h post-transfection of 293-T cells with 5 µg of WT or mutant viral DNA. (b) Viral infectivity of WT and IN mutants. Viral infectivity was quantified in a single-round replication assay using HeLa-P4 cells. A total of 3 ng of p24 antigen was used for infection. Efavirenz (EFV; 200 nM) was used as a control to ensure that the β-galactosidase activity originated from viral infection. The early steps of infection were assessed by measuring β-galactosidase activity in cell extracts by the CPRG method 48 h p.i. (a and b) p24 antigen and β-galactosidase activity are expressed as percentages of the values obtained for the WT. (c) HIV-1 DNA species monitored by quantitative PCR. MT4 cells were infected with IN mutants (20 ng of p24 antigen/10^6 cells). Total viral DNA was monitored at several timepoints p.i. until 72 h. (d) Integrated viral DNA was quantified at 48 h p.i. The efficiency of integration was defined as the amount of integrated viral DNA at 48 h p.i. divided by the amount of viral DNA resulting from reverse transcription (at 10 h p.i.). The results are expressed as the percentages of the value obtained for the WT. The results in Figure 1 are the means ± SD (error bars) of three representative independent experiments.

Comparative study of resistance profiles against raltegravir and dolutegravir in cell-based assays

After characterizing the replication capacities of the mutants, we investigated the resistance of each strain to raltegravir and
dolutegravir by determining their EC\textsubscript{50} value in infected HeLa-P4 cells (the Q148K mutant was excluded due to its weak infectivity) (Figure 2a). The R263K mutant, which was previously identified by Quashie et al.\textsuperscript{24} by \textit{in vitro} selection, was used as an example of a virus with poor resistance to dolutegravir. The EC\textsubscript{50} value for raltegravir of the WT virus was 10 nM, in accordance with previous studies.\textsuperscript{29} The mutants displayed three types of response in terms of raltegravir resistance (the FCs are reported in Table 1): (i) the E138K mutant showed no resistance (EC\textsubscript{50} of 10 nM; FC = 1); (ii) the F121Y and G118R single mutants both showed an intermediate level of resistance (EC\textsubscript{50} of 100 and 200 nM, respectively; FC = 10 and 20, respectively); and (iii) the E138K/Q148K and G140S/Q148H double mutants were highly resistant to raltegravir (EC\textsubscript{50} >300 nM; FC >30).

Regarding resistance to dolutegravir, the EC\textsubscript{50} of dolutegravir for the WT virus (2.5 nM) confirmed that this compound was a more potent IN inhibitor than raltegravir (Figure 2a). The calculated EC\textsubscript{50} values for dolutegravir revealed that the different mutations could be classified into three levels of resistance, as described for raltegravir resistance (Figure 2a). Dolutegravir inhibited E138K and R263K with an EC\textsubscript{50} of 2.7 nM (FC = 1.1) and 5 nM (FC = 2), respectively, confirming that E138K does not confer dolutegravir resistance and that R263K displays poor resistance to dolutegravir, as described by Quashie et al.\textsuperscript{24} The F121Y and G118R mutations conferred a stronger resistance to dolutegravir (8 and 50 nM, and FC = 3 and 20, respectively), although this was weaker than the resistance of the E138K/Q148K mutant, which showed the strongest resistance to dolutegravir (EC\textsubscript{50} of 300 nM, FC = 120), in

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<td>WT</td>
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<td>G118R</td>
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<td>F121Y</td>
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Figure 2. Resistance of IN mutants to dolutegravir (DTG) and raltegravir (RAL). (a) HeLa-P4 cells were infected, in triplicate, with WT or mutant virus (3 ng of p24\textsubscript{gag}) in the presence of increasing concentrations of DTG or RAL. The CPRG assay was used to quantify \(\beta\)-galactosidase activity in cell extracts 48 h p.i. The EC\textsubscript{50} corresponds to the concentration of IN inhibitor required to inhibit \(\beta\)-galactosidase activity by 50% of its value in untreated infected cells. DTG was used for the left and middle panels. The symbols corresponding to each protein are shown in the figures. DTG (white symbols) and RAL (black symbols) were used for the experiment in the right panel. The data shown are the means ± SD (error bars) of three independent experiments. The EC\textsubscript{50} (nM) values for DTG and RAL are reported in the table below the panels. (b) The same experiment described in Figure 1(d) was conducted without (grey bars) or with (black bars) 50 nM DTG. Integrated viral DNA was quantified 48 h p.i. The ratio of integrated viral DNA was defined as the amount of integrated viral DNA at 48 h p.i. divided by the amount of viral DNA resulting from the reverse transcription. For each virus, the amount of integrated viral DNA in the condition without DTG was defined as 100% integration. The results in Figure 2 are the means ± SD (error bars) of three representative independent experiments.
Raltegravir IC50 10

Dolutegravir EC50 20

EC50 values were obtained from cell culture assays and IC50 values were obtained from assays using recombinant IN proteins.

ND, not determined.

a low strand transfer reaction efficiency (Figure 3b, right panel).

recombinant IN. Although the 3′-processing activity of the F121Y and G118R mutants was similar, it was significantly higher than that of the F121Y mutant (Figure 3b, right panel). Thus, the G118R mutation impairs the 3′-processing activity but not the strand transfer reaction. By contrast, the F121Y mutation, which inhibited the 3′-processing reaction to a similar extent to the G118R mutation, also showed a low strand transfer reaction efficiency (Figure 3b, right panel).

An impairment of 3′-processing activity may be due to a defect in the catalytic activity or DNA binding of IN. To distinguish between these two possibilities, we performed fluorescence anisotropy experiments to study the DNA-binding properties of IN. Only G118R IN showed an impairment in DNA binding (44% lower than WT IN; Figure S3), suggesting that the defect in 3′-processing observed for this mutant was due to a defect in its ability to bind DNA. F121Y and WT IN bound DNA with a similar affinity, suggesting that the low 3′-processing and strand transfer activities of the F121Y mutant are the result of a defect in its catalytic properties.

We also performed strand transfer experiments with a 3′-processed DNA substrate to overcome the effects of mutation on the 3′-processing reaction.35 As described above, the strand transfer efficiency of the G118R mutant was similar to that of the WT and higher than that of the F121Y mutant (Figure 3c, compare left panel, lanes 1, 11 and 21). The weak strand transfer reaction efficiency found in vitro for the F121Y mutant is consistent with the poor integration of this mutant into the genome of MT4 cells (see Figure 1d).

The G140S/Q148H mutant was previously reported to be highly resistant to raltegravir.39,36 The Q148H mutation was responsible for raltegravir resistance and the detrimental effect of this mutation on the overall IN activity was restored by the G140S mutation. We also analysed the effect of single E138K and Q148K mutations as well as that of the E138K/Q148K double mutation on IN activity. The 3′-processing activity of the E138K mutant was similar to that of the WT (around 80% of WT activity) after 3 h of incubation at 37°C (Figure 4a, left panel, grey bars). Under the same conditions, the 3′-processing activities of the Q148K and the E138K/Q148K mutants were severely impaired (3% and 11% of the WT activity for Q148K and E138K/Q148K, respectively). Interestingly, increasing the incubation time at 37°C up to 24 h led to a partial recovery of the 3′-processing activity for the E138K/Q148K mutant (37% of the WT activity) but not for the Q148K mutant (Figure 4a, right panel). Furthermore, when the strand transfer was normalized by the 3′-processing activity and expressed as a percentage of the WT, the strand transfer activity of the E138K and E138K/Q148K mutants was significantly lower than that of the WT virus, whereas the strand transfer activity of the Q148K mutant was similar to that of the WT virus (Figure 4a, right panel). It has previously been suggested that the inactive-to-active transition, which characterizes IN binding to DNA, accounts for the slow single turnover rate constant of the 3′-processing reaction.37

**Table 1.** FCs in IC50 and EC50 of dolutegravir and raltegravir for each IN mutant

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<td>Dolutegravir IC50</td>
<td>18 ± 3.1</td>
<td>5 ± 1.2</td>
<td>0.9 ± 0.1</td>
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<td>Dolutegravir EC50</td>
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<td>3.2 ± 1</td>
<td>1.1 ± 0.4</td>
<td>ND</td>
<td>120 ± 9.3</td>
<td>2 ± 0.1</td>
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<td>Raltegravir IC50</td>
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<td>20 ± 2</td>
<td>1 ± 0.3</td>
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<td>ND</td>
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ND, not determined.

EC50 values were obtained from cell culture assays and IC50 values were obtained from assays using recombinant IN proteins. Each value represents the mean ± SD for three independent experiments.

agreement with other studies.32,33 Notably, dolutegravir inhibited the replication of the G140S/Q148H mutant with higher efficiency (EC50 = 10 nM, FC = 4) and then displayed an intermediate profile of resistance to dolutegravir whereas it was highly resistant to raltegravir (see above). Thus, in contrast to E138K/Q148K, G140S/Q148H displays differential susceptibility to dolutegravir/raltegravir, meaning that dolutegravir but not raltegravir can discriminate between E138K/Q148H and G140S/Q148H mutants. We observed a similar tendency for F121Y.

The resistance of the E138K/Q148K double mutant to dolutegravir was stronger than that of the G140S/Q148H virus. Moreover, both the G118R and F121Y mutants, which were identified in clinical trials using raltegravir, are resistant to dolutegravir, albeit to different extents.

We then assayed the resistance of all the mutants to dolutegravir by measuring the efficiency of integration in the presence of 50 nM dolutegravir (which fully inhibits the integration of the WT virus in HeLa-cell-based assays) (Figure 2b). No integrated viral DNA was detected for WT and E138K, confirming that E138K is not involved in dolutegravir resistance. Furthermore, for the three other mutants (G118R, F121Y and E138K/Q148K), the amount of integrated DNA correlated with the resistance factor measured in the HeLa-cell-based assay (see Figure 2).

**Characterization of the 3′-processing and strand transfer activities of recombinant mutants**

The above-mentioned mutations could affect the 3′-processing reaction and/or the strand transfer reaction mediated by IN. We used a 21-mer DNA substrate mimicking the blunt viral DNA end to investigate the effect of the mutations on both activities of recombinant IN. Although the 3′-processing efficiency of both the F121Y and G118R mutants was similar, it was significantly lower than that of the WT (43% of the WT activity) (Figure 3a, compare lanes 1, 11 and 21; and Figure 3b, left panel). After normalization in terms of the 3′-processing activity, we found that the strand transfer efficiency of the G118R mutant was similar to that of the WT and significantly higher than that of the F121Y mutant (Figure 3b, right panel). Thus, the G118R mutation impairs the 3′-processing activity but not the strand transfer reaction. By contrast, the F121Y mutation, which inhibited the 3′-processing reaction to a similar extent to the G118R mutation, also showed a low strand transfer reaction efficiency (Figure 3b, right panel).

An impairment of 3′-processing activity may be due to a defect in the catalytic activity or DNA binding of IN. To distinguish between these two possibilities, we performed fluorescence anisotropy experiments to study the DNA-binding properties of IN. Only G118R IN showed an impairment in DNA binding (44% lower than WT IN; Figure S3), suggesting that the defect in 3′-processing observed for this mutant was due to a defect in its ability to bind DNA. F121Y and WT IN bound DNA with a similar affinity, suggesting that the low 3′-processing and strand transfer activities of the F121Y mutant are the result of a defect in its catalytic properties.

We also performed strand transfer experiments with a 3′-processed DNA substrate to overcome the effects of mutation on the 3′-processing reaction.35 As described above, the strand transfer efficiency of the G118R mutant was similar to that of the WT and higher than that of the F121Y mutant (Figure 3c, compare left panel, lanes 1, 11 and 21). The weak strand transfer reaction efficiency found in vitro for the F121Y mutant is consistent with the poor integration of this mutant into the genome of MT4 cells (see Figure 1d).

The G140S/Q148H mutant was previously reported to be highly resistant to raltegravir.39,36 The Q148H mutation was responsible for raltegravir resistance and the detrimental effect of this mutation on the overall IN activity was restored by the G140S mutation. We also analysed the effect of single E138K and Q148K mutations as well as that of the E138K/Q148K double mutation on IN activity. The 3′-processing activity of the E138K mutant was similar to that of the WT (around 80% of WT activity) after 3 h of incubation at 37°C (Figure 4a, left panel, grey bars). Under the same conditions, the 3′-processing activities of the Q148K and the E138K/Q148K mutants were severely impaired (3% and 11% of the WT activity for Q148K and E138K/Q148K, respectively). Interestingly, increasing the incubation time at 37°C up to 24 h led to a partial recovery of the 3′-processing activity for the E138K/Q148K mutant (37% of the WT activity) but not for the Q148K mutant (Figure 4a, right panel). Furthermore, when the strand transfer was normalized by the 3′-processing activity and expressed as a percentage of the WT, the strand transfer activity of the E138K and E138K/Q148K mutants was slightly higher than that of the WT virus, whereas the strand transfer activity of the Q148K mutant was similar to that of the WT virus (Figure 4a, right panel). It has previously been suggested that the inactive-to-active transition, which characterizes IN binding to DNA, accounts for the slow single turnover rate constant of the 3′-processing reaction.37

**Comparative in vitro study of mutants resistant to raltegravir and dolutegravir**

After showing that the mutants exhibit different levels of resistance to raltegravir and dolutegravir in a cellular model, we next...
were resistant to raltegravir and dolutegravir (IC\textsubscript{50}, raltegravir 160 nM, and dolutegravir 5 nM) (Figure 3c and Figure 4b). Thus, both the F121Y and G118R mutants showed moderate resistance to raltegravir and dolutegravir (FC\textsubscript{raltegravir} 10 and 10, FC\textsubscript{dolutegravir} 5 and 18, for F121Y and G118R, respectively (Table 1)). These data are in accordance with results obtained from the HeLa-cell-based assay (see Figure 2 and Table 1). We also confirmed that E138K was not associated with raltegravir or dolutegravir resistance, in contrast to the Q148K and E138K/Q148K mutants (IC\textsubscript{50}, raltegravir 800 nM for E138K/Q148K in comparison with WT IC\textsubscript{50} 5 nM, and IC\textsubscript{50}, dolutegravir 27 and 92 nM for Q148K and E138K/Q148K, respectively, in comparison with WT IC\textsubscript{50} 5 nM) (Figure 4b and Table 1). These values are consistent with those found in the HeLa-cell based assay. These findings thus confirm that: (i) the resistance of E138K/Q148K to raltegravir and dolutegravir is higher than that of F121Y and G118R, and (ii) G140S/Q148H and F121Y appear to display differential susceptibility to raltegravir and dolutegravir.

These results are in accordance with previous reports demonstrating that the activities of N155H/S/T, Y143C/H/R and Q148H INs and to a lesser extent that of the Q148K/R mutants, which are involved in raltegravir resistance, are inhibited by dolutegravir. Interestingly, the G118R and F121Y INs, which were previously described in patients failing raltegravir treatment, showed resistance to both raltegravir and dolutegravir \textit{in vitro}. Moreover,
the level of resistance of the G118R mutant to dolutegravir was higher than that of the F121Y mutant, which suggests that G118R is involved in dolutegravir resistance.

Discussion

The need for the integration step for HIV-1 replication has led to the development of anti-IN compounds. Although raltegravir and elvitegravir have shown potency in clinical practice, many reports have described the emergence of raltegravir and elvitegravir resistance involving three main pathways with the residues Y143, Q148 and N155.14,29,42,43 Dolutegravir was recently approved by the US FDA and belongs to the second generation of inhibitors targeting IN. The VIKING trial demonstrated that dolutegravir is efficient against most of the viruses resistant to raltegravir or elvitegravir but is ineffective against viruses harbouring Q148 pathway mutations with a secondary mutation such as G140, L74 or E138.40

In this study, we characterized two novel mutations described in patients failing raltegravir treatment: G118R and F121Y. We investigated the resistance of these mutants to raltegravir and dolutegravir in relation to other well-known mutations involved in raltegravir-resistance pathways. Mutant strains could be divided into three different groups based on their level of resistance to dolutegravir: the first group included only the E138K mutation, which was not resistant; the second one encompassed the G118R and F121Y mutations, which showed an intermediate level of resistance; and the third one comprised mutants such as the E138K/Q148K double mutant with strong resistance. Interestingly, the level of resistance of some mutants depended on the INSTI studied. This was exemplified in our study by the G140S/Q148H and the F121Y mutants, which showed differential resistance to raltegravir and dolutegravir.

This is also the case for the well-established raltegravir-resistant Y143 and N155 pathways that were poorly resistant to dolutegravir (FC <1.9).23 A structural study performed by Hare...
et al. 44 on primate foamy virus (PFV) IN has to some extent revealed the relationship between the intasome structure and differential resistance to INSTIs. Only raltegravir establishes a π–π stacking interaction between its oxadiazole ring and the Y143 residue, which is required for its stability in the intasome. By contrast, dolutegravir does not establish such a stable π–π interaction, explaining why dolutegravir, but not raltegravir, remains active against the Y143H mutant. In the case of the N155H mutant, the His residue at position 155 forms an interaction with the phosphoamidate group of the 3′-adenosine of the processed DNA. The resistance of the N155H mutant to raltegravir is explained by the incapability of raltegravir to disrupt this interaction. By contrast, dolutegravir disrupts the His–phosphate interaction, which renders this mutant susceptible to dolutegravir. 44

The Q148 pathway belongs to the third group of resistance, i.e. highly resistant to dolutegravir. Importantly, the Q148H/K mutation is often associated with a secondary mutation such as E138K or G140S. Indeed, the inactive-to-active transition is strongly impaired by the Q148K mutation and this defect can be partially reversed by the E138K mutation. This situation is similar to that previously described for the G140S/Q148H mutant.29 Thus, the Q148K mutation dramatically impairs the 3′-processing activity but not the strand transfer reaction, suggesting that direct binding of IN to the 3′-processed DNA substrate overcomes this limiting transition step. The Q148 residue plays a key role in this transition that occurs on the blunt DNA substrate and allows 3′-processing. These secondary mutations rescue the impaired activity of the Q148H/K single mutant but they also enhance resistance to some extent; although these mutants alone are neutral in terms of resistance, the resistance of double mutants to INSTIs is consistently higher than that of the Q148H/K mutant alone. Remarkably, the G140S/Q148H double mutant showed differential resistance to raltegravir and dolutegravir whereas this difference was less pronounced for the E138K/Q148H double mutant: the G140S/Q148K mutant was strongly resistant to raltegravir but displayed lower resistance to dolutegravir, whereas the E138K/Q148K mutant showed strong resistance to both compounds. We wondered whether this difference was due to the nature of the mutation at position 148 (H versus K) or to the position of the secondary mutation (position 138 versus 140). However, no structural data are available regarding the differential effect of mutations at position Q148 (only the structure of the Q148H mutation is available). Moreover, structural studies have been performed with the PFV-1 intasome, where the equivalent of the residue G140 in HIV-1 IN is S209 (the PFV S217H single mutant is thus equivalent to the HIV-1 G140S/Q148H double mutant) and the effect of mutation at position 138 has not yet been explored. Consequently, it is not possible to use this structure to study Q148H/K mutants and the influence of any mutation at the 138 or 140 position. However, functional studies have investigated the role of amino acid substitutions involved in the Q148 pathway in a systematic manner.23 Given that E138K/Q148H and G140S/Q148K are either poorly or not resistant to dolutegravir, it appears that both the nature of the mutation at position 148 (K>Q) and the position of the secondary mutation itself (position 138) are important for determining resistance against dolutegravir. Therefore, the unique combination of the two mutations, E138K and Q148K, confers strong resistance to dolutegravir.

The G118R and F121Y mutations belong to the second group, which is associated with an intermediate level of resistance. Here, we used biochemical and virological assays to show that these two mutations, which occurred in patients failing raltegravir treatment, are associated with dolutegravir resistance (20-fold and 3.2-fold for G118R and F121Y, respectively). The G118R mutation, which was first selected in vitro against another second-generation inhibitor, MK-2048, 17 was suggested to be specific for second-generation INSTIs. However, our study shows that this mutation, which is found in raltegravir-treated patients, displays a similar resistance profile against raltegravir and dolutegravir and therefore does not confer differential susceptibility to the two compounds. By contrast, our data show that F121Y displays differential susceptibility to raltegravir and dolutegravir, similar to our findings with the G140S/Q148H mutant. Both residues, G118 and F121, are located in the vicinity of S119, which is essential for the interaction with the target DNA. Although the 3′-processing efficiency of both the F121Y and G118R mutants is lower than that of WT IN, only the F121Y mutant is characterized by a low strand transfer reaction efficiency. This finding, based on in vitro assays involving recombinant proteins, is consistent with results obtained in cell-based assays (integration assays), which showed that the integration efficiency of the F121Y mutant was lower than that of the G118R mutant. This suggests that the residue at position 121 is more important for the target DNA-binding site than the residue at position 118, because mutation at position 121 severely impairs the strand transfer reaction and differentially modulates INSTI resistance.

Overall, our data show that G118R and F121Y represent new raltegravir-resistance pathways that may also be involved in dolutegravir resistance (G118R→F121Y). Furthermore, the Q148 pathway, which was previously described as being involved in raltegravir and elvitegravir resistance, is involved in dolutegravir resistance when associated with E138K, especially when the Q residue is replaced with a K. Thus, these profiles need to be monitored in the future in patients treated with dolutegravir.

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Transparency declarations
None to declare.

Supplementary data
Figures S1 to S3 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


