The combination of the R263K and T66I resistance substitutions in HIV-1 integrase is incompatible with high level viral replication and the development of high-level drug resistance.

Jiaming LIANG1,2, Thibault MESPLÈDE1, Maureen OLIVEIRA1, Kaitlin ANSTETT1,3, and Mark A. WAINBERG1,2,3.

1McGill University AIDS Centre, Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, Quebec, Canada.

2Division of Experimental Medicine, Faculty of Medicine, McGill University, Montréal, Québec, Canada.

3Department of Microbiology and Immunology, Faculty of Medicine, McGill University, Montréal, Québec, Canada.

Correspondence to: Mark A. Wainberg, McGill AIDS Centre, 3755 Ch. Côte-Ste-Catherine, Montréal, QC H3T1E2, Canada. E-mail: mark.wainberg@mcgill.ca

Running head: Incompatibility of R263K and T66I

Abstract: 235

Importance: 131

Text: 2531
Key Words

HIV-1, integrase, drug resistance, integrase inhibitors, R263K, dolutegravir, raltegravir, elvitegravir
ABSTRACT

Background: The R263K substitution in integrase has been selected in tissue culture with dolutegravir (DTG) and been reported in several treatment-experienced individuals receiving DTG as part of salvage therapy. R263K seems to be incompatible with the presence of common resistance mutations associated with raltegravir (RAL), a different integrase strand transfer inhibitor (INSTI). T66I is a substitution that is common in individuals who have developed resistance against a different INSTI termed elvitegravir (EVG), but it is not known whether these two mutations might be compatible in the context of resistance against DTG or what impact the combination of these substitutions might have on resistance against INSTIs. E138K is a common secondary substitution observed with various primary resistance substitutions in RAL- and EVG- treated individuals.

Methods: Viral infectivity, replicative capacity, and resistance against INSTIs were measured in cell-based assays. Strand-transfer and 3’processing activities were measured biochemically.

Results: The combination of R263K and T66I decreased HIV-1 infectivity, replicative capacity, and strand-transfer activity. The addition of the E138K substitution partially compensated for these deficits and resulted in high levels of resistance against EVG but not against DTG or RAL.

Conclusions: These findings suggest that the presence of T66I will not compromise the activity of DTG and may also help to prevent the additional generation of R263K. Our
observations support the use of DTG in second-line therapy for individuals who experience treatment failure with EVG due to the T66I substitution.

**Importance**

The integrase strand transfer inhibitors (INSTIs) elvitegravir and dolutegravir are newly developed inhibitors against human immunodeficiency virus-1 (HIV-1). HIV drug-resistant mutations in integrase that can arise in individuals treated with elvitegravir commonly include the T66I substitution whereas R263K is a signature resistant substitution against dolutegravir. In order to determine how different combinations of resistance integrase mutations can influence the outcome of therapy, we report here the effects of the T66I, E138K, and R263K substitutions, alone and in combination, on viral replicative capacity and resistance to integrase inhibitors. Our results show that the addition of R263K to the T66I substitution diminishes viral replicative capacity and strand-transfer activity while not compromising susceptibility to dolutegravir. This supports the use of dolutegravir in second-line therapy for patients failing elvitegravir who harbor the T66I resistance substitution.
Introduction

Recent strategies to treat HIV-1 infection involve the use of integrase strand-transfer inhibitors (INSTIs) that are the most potent antiretroviral drugs (ARVs) to date and include raltegravir (RAL), elvitegravir (EVG), and dolutegravir (DTG) (1). Despite this, the emergence of drug-resistance mutations in integrase (IN) represents a concern for the future use of these drugs, and various resistance mutations against RAL and EVG, that are associated with treatment failure, have been characterized (2). A high degree of cross-resistance also exists between RAL and EVG, since the major resistance substitutions for RAL are located at positions G140, Y143, Q148, and N155, while those for EVG are at positions T66, E92, G140, S147, Q148, and N155 (1, 3). Although resistance in initial therapy has not yet been reported for DTG, patients can fail DTG if they were previously treated with RAL or EVG and possess relevant mutations for those drugs (4-6).

In contrast, a R263K substitution was selected in tissue culture with DTG and this substitution has been reported in several treatment-experienced, INSTI-naïve individuals who were not fully suppressed when receiving DTG-based therapy (7). We showed that R263K alone or in combination with other secondary mutations confers low-level resistance to DTG and that viruses containing R263K possess significantly reduced viral replication capacity (8-10).

It is also notable that R263K has been shown to emerge secondary to the T66I substitution during tissue culture selections with EVG (11). Here, we have examined the effect of combining the T66I and R263K substitutions on HIV-1 viral replicative capacity and levels of resistance against various INSTIs and have also studied this in the context
of the secondary E138K mutation that commonly arises during the emergence of clinically relevant resistance for both RAL and EVG.

Material and methods

Cells and reagents

TZM-bl and 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM). PM-1 cells were cultured in Roswell Park Memorial Institute medium (RPMI). Both DMEM and RPMI were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin. Cord-blood was obtained from the Department of Obstetrics, Jewish General Hospital, Montréal, Canada. Primary human cord-blood mononuclear cells (CBMCs) were isolated from cord-blood using Ficoll Hypaque (GE Health Care Life Sciences), and the CBMCs were stimulated with phytohemagglutinin. CBMCs were grown in RPMI. Cells were maintained at 37ºC under 5% CO2. RAL, EVG, and DTG were provided by Merck & Co., Inc., Gilead Sciences, and ViiV Healthcare Inc respectively.

Generation of Replication-Competent Genetically Homogenous Virus

pNL4-3IN(T66I), pNL4-3IN(R263K), pNL4-3IN(T66I/R263K), and pNL4-3IN(T66I/E138K/R263K) were produced using site-directed mutagenesis. The production of the pNL4-3IN(R263K) and pNL4-3IN(E138K/R263K) plasmids has been reported previously (10). The primers used for T66I mutagenesis were: sense: 5’-CCAGGAATATGGCAGCTAGATTGTATACATTTAGAAGGAAAAGTT-3’ and antisense: 5’-AACCTTTTCTTCTAAATGTATACAATCTAGCTGCCATATTCTGG-3’. All plasmids were verified by sequencing. To produce replication-competent
genetically homogenous viruses, 12.5 µg of pNL4-3IN(WT), pNL4-3IN(T66I), pNL4-3IN(R263K), pNL4-3IN(T66I/R263K), or pNL4-3IN(T66I/E138K/R263K) plasmid were used to transfect 293T cells using Lipofectamine 2000 (Invitrogen). Fresh medium was added at 4 h post transfection. After 48 h, culture fluids were harvested and passed through a 0.45-µm filter. Quantification of viruses was performed using p24 and RT assays as described previously (12).

**Tissue culture selections with RAL or EVG**

CBMCs were infected with NL4.3IN(WT), NL4.3IN(T66I), or NL4.3IN(E138K/R263K) viruses and then grown in the presence of increasing concentration of RAL or EVG. Viral replication in culture was monitored by RT assay and aliquots of culture fluids were collected weekly. Viral RNA was extracted from the aliquots using a RNA extraction kit (Qiagen) and amplified by RT-PCR (Life Technologies) as previously described (13). The PCR products were then sequenced to detect emergence of drug-resistance mutations.

**HIV-1 infectivity and replicative capacity**

HIV-1 infectivity was measured by short-term TZM-bl assay. Briefly, 30,000 TZM-bl cells/well were infected with serially diluted viruses in a 96-well flat-bottom plate. Cells were lysed at 48 h after infection and luciferase levels were measured to directly monitor short-term infectivity. Fold decreases in infectivity were represented as the relative EC$_{50}$, which is the amount of virus (previously quantified using RT assay) needed for TZM-bl cells to produce half of the maximal level of luciferase in an infection. HIV-1 replicative capacity was measured as counts per minute (cpm) in PM-1 cells following HIV-1 infection over 21 days. Both assays have previously been described (14).
Susceptibility to antiretroviral compounds

Susceptibilities of virus to ARVs were measured by addition of serially diluted DTG, RAL, or EVG to TZM-bl cells prior to infection using the viruses described above. Luciferase levels were measured after 48 h of incubation, similar to the protocol for the infectivity assay described above, and IC$_{50}$ values were determined.

Generation of plasmids for integrase protein expression and purification

Expression plasmids pET-15b coding for soluble integrases that were either wild-type (WT) or containing the R263K or E138K/R263K substitutions were generated using site-directed mutagenesis as previously described (10). The T66I, T66I/R263K, and T66I/E138K/R263K combinations of mutations were produced using the primers described above. The pET-15b plasmids were then used to express recombinant proteins in BL21 (DE3) bacterial cells. The protocol for protein expression and purification of his-tagged integrase has been described (9).

Cell-free strand-transfer assay

Integrase strand-transfer activities of WT integrase enzyme and integrase proteins containing the T66I, R263K, T66I/R263K, E138K/R263K, or T66I/E138K/R263K substitutions were measured as previously described (15). Briefly, 300 nM of processed LTR-DNA duplexes were coated onto Costar 96-well DNA-binding plates (Corning) by overnight incubation at 4°C. The plates were washed once with blocking buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.25% BSA) and then incubated with the same buffer for 30 min at 37°C or overnight at 4°C. Immediately before the strand-transfer assay, plates
were washed once with PBS pH 7.4 and Assay Buffer (50 mM MOPS pH 6.8, 0.15% CHAPS, 50 mM NaCl, 30 mM MnCl₂, 50 µg/mL BSA). 400 nM of purified integrase proteins were resuspended in assay buffer with 5 mM DTT and added to the microplates for a 30 min incubation at room temperature. Serially-diluted biotinylated-target DNA (0 - 60 nM) was then added to each well for 1 h at 37°C. The plates were then washed twice with wash buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20, 2 mg/mL BSA). Streptavidin-Eu solution (50 µM DTPA, 0.025 µg/mL Eu-labeled streptavidin) diluted in wash buffer was added for 30 min at room temperature. Finally, the plates were washed twice with wash buffer and 80 µl Wallac enhancement solution (PerkinElmer) was added. Time-resolved fluorescence was read using a FLUOstar Optima multilabel plate reader (BMC Labtech).

**3’ processing assay**

The 3’ processing activities of WT integrase enzyme or enzymes containing the T66I, R263K, T66I/R263K, E138K/R263K, T66I/E138K/R263K substitutions were measured as described (16). The 3’ processing assay was similar to the strand-transfer assay. Serial dilutions of unprocessed-LTR DNA duplex with 3’-biotinylation were used to coat the plates at concentrations between 0 to 40 nM. After addition of purified integrase proteins, the plates were incubated for 2 h to allow 3’ processing to occur.

**Data analysis**

Each experiment was performed at least twice using three or four replicate samples. Data analysis was performed using GraphPad Prism 5.0.
Results

Emerging substitutions in NL4.3\textsubscript{IN(WT)}, NL4.3\textsubscript{IN(T66I)}, and NL4.3\textsubscript{IN(E138K/R263K)} under RAL or EVG drug pressure

To confirm previous findings and verify the possibility of T66I/E138K/R263K triple substitutions, selection studies were performed using CBMCs infected with NL4.3\textsubscript{IN(WT)}, NL4.3\textsubscript{IN(R263K)}, and NL4.3\textsubscript{IN(E138K/R263K)} under increasing concentrations of RAL or EVG (Table 1). Together with several substitutions, the T66I substitution emerged from the NL4.3\textsubscript{IN(WT)} or NL4.3\textsubscript{IN(R263K)} under RAL or EVG pressure, respectively, and from NL4.3\textsubscript{IN(E138K/R263K)} with both drugs. In contrast, the T66I substitution was not detected when RAL selection experiments were initiated with a virus containing the R263K substitution nor did emerged from the WT virus under EVG pressure.

Combining the T66I and R263K substitutions impairs viral infectivity

To determine the effects of the T66I, E138K and R263K substitutions on viral infectivity, TZM-bl cells were infected with NL4.3\textsubscript{IN(WT)}, NL4.3\textsubscript{IN(T66I)}, NL4.3\textsubscript{IN(R263K)}, NL4.3\textsubscript{IN(T66I/R263K)}, or NL4.3\textsubscript{IN(T66I/E138K/R263K)} virus (Figure 1). The NL4.3\textsubscript{IN(T66I)}-, NL4.3\textsubscript{IN(R263K)}, and NL4.3\textsubscript{IN(T66I/E138K/R263K)} viruses showed only slight impairments in infectivity relative to WT (Figure 1a and c), whereas NL4.3\textsubscript{IN(T66I/R263K)} displayed a significant defect in infectivity (Figure 1b). Relative infectivity was decreased by 8-fold by the T66I/R263K combination of substitutions. The addition of E138K to T66I/R263K partially restored infectiousness (1.45-fold decrease in infectivity relative to WT) (Figure 1d).
The T66I/R263K combination of substitutions impairs viral replicative capacity

A single cycle of infection does not always capture replicative defects. Therefore, we also assessed the long term replicative capacity of the different viruses that contained T66I using PM-1 cells. Infections were carried out with NL4.3\textsubscript{IN(WT)}, NL4.3\textsubscript{IN(T66I)}, NL4.3\textsubscript{IN(R263K)}, NL4.3\textsubscript{IN(T66I/R263K)}, or NL4.3\textsubscript{IN(T66I/E138K/R263K)} over 21 days (Figure 2). RT activity was measured in culture fluids at days 3, 7, 14, and 21. Similar to the results of the TZM-bl infectivity assay, we found that the T66I substitution alone had little effect on viral replicative capacity (Figure 2a) and R263K decreased viral replication to a similar extent as previously reported (12). In contrast, the NL4.3\textsubscript{IN(T66I/R263K)} virus showed a major defect in replicative capacity (Figure 2c). Although the T66I/R263K containing-virus yielded similar levels of RT activity at day 3 in comparison to the other viruses tested, replication gradually decreased over the subsequent 18 days while the other viruses attained higher levels of replication at or after day 7. In particular, the NL4.3\textsubscript{IN(T66I/E138K/R263K)} virus showed partially restored replicative capacity in comparison to the NL4.3\textsubscript{IN(T66I/R263K)} virus.

Strand-transfer activities of recombinant integrase containing the T66I, E138K, and/or R263K substitutions

To determine whether the deficits in replicative capacity observed with mutated viruses in PM1 cells were caused by changes in integrase activity, cell-free biochemical strand-transfer assays were performed using purified recombinant integrases containing the T66I, E138K, and/or R263K substitutions. Maximal enzyme activity ($V_{max}$) and the amount of
target LTR-DNA used to reach half $V_{\text{max}}$ ($1/2\text{MaxDNA}$) were calculated for each of the recombinant integrase enzymes. The relative $V_{\text{max}}$ was measured for each recombinant integrase and maximal strand-transfer activity of WT integrase was arbitrarily set at 100% (Table 2). The results show that the presence of the T66I substitution increased $1/2\text{MaxDNA}$ by 2.4-fold while decreasing $V_{\text{max}}$ to 67% of WT. Similarly, the R263K substitution increased $1/2\text{MaxDNA}$ by 2-fold, and the E138K/R263K substitutions in tandem decreased $V_{\text{max}}$ to 38.7% of WT. For T66I/R263K substitutions, $1/2\text{MaxDNA}$ was increased by 2.3-fold and $V_{\text{max}}$ decreased to 13% of WT. The three T66I/E138K/R263K substitutions together resulted in a slightly decreased $1/2\text{MaxDNA}$ (1.2-fold) but $V_{\text{max}}$ was only 17% of WT.

3’ processing activity of recombinant integrase enzymes containing the T66I, E13K8, and/or R263K substitutions

3’ processing is a rate-limiting step in HIV-1 integration and a 3’ processing assay was performed to determine the effects of the T66I, E138K, and R263K substitutions on enzyme activity. The results show that no significant differences were observed among the various recombinant integrase enzymes that were tested (Table 3).

The T66I/R263K combination of substitutions confers significant resistance to EVG but remains susceptible to DTG

The T66I substitution in integrase has been previously reported to confer major resistance to EVG while increasing HIV-1 susceptibility to DTG. Previously, we showed that R263K, alone or in combination with several secondary mutations, conferred moderate-
level resistance to EVG and low-level resistance against DTG (8-10). Now, we conducted resistance assays using TZM-bl cells to determine the effects of the T66I substitution when combined with R263K and/or with the E138K secondary substitution on resistance to each of DTG, RAL, and EVG (Table 4). Compared to the IC₅₀ of WT, and in agreement with previous studies (17), the T66I substitution alone increased susceptibility to DTG by 1,000-fold, conferred low-level resistance against RAL (2.4-fold), and higher level resistance against EVG (10-fold). Viruses containing the T66I/R263K combination of substitutions were susceptible to DTG (0.089-fold), slightly resistant to RAL (1.6-fold), and more resistant to EVG (22-fold). The T66I/E138K/R263K virus was susceptible to DTG (0.027-fold), slightly resistant to RAL (2.5-fold), and highly resistant to EVG (164-fold).

Discussion

T66I was originally described as a change in integrase selected in tissue culture under EVG pressure. It was later shown to be common in the genomes of viruses isolated from individuals failing EVG treatment (18). Other substitutions that are associated with treatment failure under EVG-based therapy include E92Q, G140S/A, S147G, Q148H/R/K, and N155H (18), of which the latter also emerged from the WT virus under EVG pressure in the current study (Table 1). T66I can also be found in viruses from individuals who have experienced treatment failure with RAL, though more rarely (19). This may be due to the high versus low levels of resistance conferred by this substitution against EVG and RAL, respectively (17), an observation that we have confirmed here (Table 4). In addition, we have confirmed that T66I does not confer resistance against
DTG but significantly increases HIV-1 susceptibility to this drug. Structural models derived from the crystal structure of the prototype foamy virus integrase protein suggest that the T66I substitution might increase susceptibility to DTG by disrupting an electrostatic interaction between T66 and N155 (15). No single integrase substitution besides R263K has ever been shown to confer significant levels of resistance against DTG (17, 20), helping to explain the prevalence of R263K in some treatment-experienced, INSTI-naïve individuals who experienced DTG-based treatment failure (7).

We have shown previously that the R263K substitution is also associated with decreases in viral DNA integration and viral replication capacity (8), suggesting that the development of resistance both in tissue culture and in vivo involves a balance between levels of resistance and replicative capacity. The emergence of the T66I/R263K combination of substitutions in tissue culture selection with EVG has been documented (11, 21) and we show here that this combination severely impaired both integrase strand-transfer activity and HIV-1 replicative capacity (Table 2 and Figure 2, respectively). In contrast, the T66I/R263K combination of substitutions has not been observed in the presence of RAL (19, 22), suggesting that the low levels of resistance against RAL that are associated with this combination are not sufficient to compensate for deficits in replication capacity, that are related to decreased strand-transfer integrase activity but not 3’-processing activity (Tables 2 and 3).

The positive effect of E138K on strand-transfer activity seems to be due to an improvement in DNA binding activity, as shown by decreases in $1/2M_{1/2}$DNA values when this substitution was present (Table 2). In contrast, E138K had little effect on maximal strand-transfer activity. These findings correlate with the E138K-associated partial
compensation of defects in infectivity and replicative capacity that were observed with the T66I/R263K combination of mutations (Figures 1 and 2). The addition of the E138K substitution to the T66I/R263K combination also increased levels of resistance against RAL and EVG by 1.5- and 7.5-fold, respectively (Table 4).

Importantly, the addition of R263K to T66I did not confer resistance against DTG, although it may have moderated the increase in susceptibility to this drug that was associated with T66I alone (Table 4). Furthermore, the T66I/R263K combination of substitutions severely impaired viral replicative capacity (Figure 2). This suggests that patients who experience EVG-based treatment failure with an emergent T66I substitution can be successfully treated with DTG and may not be able to develop the R263K substitution in combination with T66I. Given the high prevalence of the latter substitution in individuals who have failed EVG (18), our results provide additional justification for the use of DTG in second-line therapy after development of T66I.

In the current study, we also tested the ability of E138K, a secondary substitution that has been observed together with R263K in tissue culture, to act together with the T66I/R263K combination of mutations to modulate strand-transfer activity and replicative capacity (Table 2 and Figures 1 and 2). However, viruses that contain the T66I/E138K/R263K combination of substitutions remained highly susceptible to DTG (Table 4).

Funding

This work was supported by the Canadian Institutes for Health Research (CIHR).
Acknowledgments

JL performed experiments, analysed the data and wrote the initial manuscript. TM designed and performed experiments, analyzed the data and corrected the manuscript. MO and KA performed experiments. MAW supervised the project and revised the manuscript. All authors read and approved the final version of the paper.

Conflicts of interest

The authors declare they have no conflict of interest.

Legends for illustrations

**Figure 1.** Viral infectivity in TZM-bl cells. TZM-bl cells were infected with (a) NL4.3\textsubscript{IN(WT)}, NL4.3\textsubscript{IN(T66I)}, or NL4.3\textsubscript{IN(R263K)}; (b) NL4.3\textsubscript{IN(WT)} or NL4.3\textsubscript{IN(T66I/R263K)}; (c) NL4.3\textsubscript{IN(WT)} or NL4.3\textsubscript{IN(T66I/E138K/R263K)} virus over 48h. Infectivity of NL4.3\textsubscript{IN(WT)} virus is represented in (a-c) for comparison. Luciferase levels were measured and (d) fold decrease in infectivity was calculated. Error bars indicate means ± standard deviation.

**Figure 2.** Viral replicative capacity in PM-1 cells. PM-1 cells were infected with NL4.3\textsubscript{IN(WT)} or (a) NL4.3\textsubscript{IN(T66I)}, (b) NL4.3\textsubscript{IN(R263K)}, (c) NL4.3\textsubscript{IN(T66I/R263K)}, and (d) NL4.3\textsubscript{IN(T66I/E138K/R263K)} viruses over 21 days. Replicative capacity of the above-mentioned viruses was normalized to reverse transcriptase (RT) levels of the NL4.3\textsubscript{IN(WT)} virus at day 7. Supernatants were collected at days 3, 7, 14, and 21 at which time RT levels were measured as counts per minute (cpm). Error bars indicate means ± standard deviation.
Table 1. New substitutions emerging from NL4.3<sub>IN(WT)</sub>, NL4.3<sub>IN(R263K)</sub>, and NL4.3<sub>IN(E138K/R263K)</sub> infections of CBMCs under raltegravir (RAL) or elvitegravir (EVG) drug pressure at week 30.

Table 2. Strand-transfer activity of recombinant subtype B integrase containing the T66I, E138K, and/or R263K substitutions.

Table 3. 3’ processing activity of recombinant subtype B integrase containing the T66I, E138K, and/or R263K substitutions.

Table 4. Susceptibilities of NL4.3<sub>IN(WT)</sub>, NL4.3<sub>IN(T66I)</sub>, NL4.3<sub>IN(R263K)</sub>, NL4.3<sub>IN(T66I/R263K)</sub>, and NL4.3<sub>IN(T66I/E138K/R263K)</sub> viruses to dolutegravir (DTG), raltegravir (RAL), and elvitegravir (EVG) as represented by IC<sub>50</sub> and fold-change (FC) relative to NL4.3<sub>IN(WT)</sub> virus.

References


Table 1. New substitutions emerging from NL4.3<sup>WT</sup>, NL4.3<sup>R263K</sup>, and NL4.3<sup>E138K/R263K</sup> infections of CBMCs under raltegravir (RAL) or elvitegravir (EVG) drug pressure at week 30.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (μM)</th>
<th>WT</th>
<th>R263K</th>
<th>E138K/R263K</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAL</td>
<td>0.05-2.5</td>
<td>T66I, T97A, G163R</td>
<td>-</td>
<td>H51N, T66I, T97A, S119R, Y143H</td>
</tr>
<tr>
<td>EVG</td>
<td>1</td>
<td>N155H, R263K</td>
<td>M50I, T66I</td>
<td>M50I, T66I, S119R, S147G</td>
</tr>
</tbody>
</table>
Table 2. Strand-transfer activity of recombinant subtype B integrase enzymes containing the T66I, E138K, and/or R263K substitutions.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Relative V\text{max} (%)</th>
<th>95% CI of Relative V\text{max} (%)</th>
<th>1/2MaxDNA (nM)</th>
<th>95% CI (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100</td>
<td>91.6 to 108.5</td>
<td>10.15</td>
<td>7.5 to 12.8</td>
</tr>
<tr>
<td>T66I</td>
<td>67.3</td>
<td>56.4 to 78.2</td>
<td>24.4</td>
<td>15.5 to 33.2</td>
</tr>
<tr>
<td>R263K</td>
<td>89.1</td>
<td>74 to 104.3</td>
<td>20.1</td>
<td>12.1 to 28.2</td>
</tr>
<tr>
<td>T66I/R263K</td>
<td>13.0</td>
<td>10.4 to 15.6</td>
<td>23.2</td>
<td>12.9 to 33.6</td>
</tr>
<tr>
<td>E138K/R263K</td>
<td>38.7</td>
<td>32 to 45.5</td>
<td>5.15</td>
<td>2.0 to 8.3</td>
</tr>
<tr>
<td>T66I/E138K/R263K</td>
<td>17.1</td>
<td>13.9 to 20.2</td>
<td>8.4</td>
<td>3.5 to 13.3</td>
</tr>
</tbody>
</table>
Table 3. 3’ processing activity of recombinant subtype B integrase containing the T66I, E138K, and/or R263K substitutions.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Relative V_{max} (%)</th>
<th>95% CI of Relative V_{max} (%)</th>
<th>1/2MaxDNA (nM)</th>
<th>95% CI (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100</td>
<td>81.9 to 118.1</td>
<td>1.3</td>
<td>0.62 to 1.9</td>
</tr>
<tr>
<td>T66I</td>
<td>70.85</td>
<td>55.8 to 85.9</td>
<td>0.95</td>
<td>0.33 to 1.58</td>
</tr>
<tr>
<td>R263K</td>
<td>69.2</td>
<td>55.7 to 82.6</td>
<td>0.99</td>
<td>0.41 to 1.6</td>
</tr>
<tr>
<td>T66I/R263K</td>
<td>106.7</td>
<td>77 to 135.7</td>
<td>1.7</td>
<td>0.53 to 2.9</td>
</tr>
<tr>
<td>E138K/R263K</td>
<td>120.2</td>
<td>98.1 to 154.4</td>
<td>1.83</td>
<td>0.85 to 2.8</td>
</tr>
<tr>
<td>T66I/E138K/R263K</td>
<td>97.6</td>
<td>76.2 to 114.9</td>
<td>1.26</td>
<td>0.55 to 1.97</td>
</tr>
</tbody>
</table>
Table 4. Susceptibility of NL4.3<sub>WT</sub>, NL4.3<sub>T66I</sub>, NL4.3<sub>R263K</sub>, NL4.3<sub>T66I/R263K</sub>, and NL4.3<sub>T66I/E138K/R263K</sub> viruses to dolutegravir (DTG), raltegravir (RAL), and elvitegravir (EVG) represented by IC<sub>50</sub> and fold change (FC) relative to NL4.3<sub>WT</sub> virus.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DTG</th>
<th>RAL</th>
<th>EVG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</td>
<td>95% CI (nM)</td>
<td>FC</td>
</tr>
<tr>
<td>WT</td>
<td>0.3</td>
<td>0.2 to 0.5</td>
<td>1</td>
</tr>
<tr>
<td>T66I</td>
<td>0.0004</td>
<td>0.0002 to 0.0007</td>
<td>0.001</td>
</tr>
<tr>
<td>R263K</td>
<td>1.5</td>
<td>1.2 to 1.7</td>
<td>4.8</td>
</tr>
<tr>
<td>T66I/R263K</td>
<td>0.03</td>
<td>0.02 to 0.04</td>
<td>0.09</td>
</tr>
<tr>
<td>T66I/E138K/R263K</td>
<td>0.008</td>
<td>0.004 to 0.02</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Genotype IC<sub>50</sub> (nM) 95% CI (nM) FC IC<sub>50</sub> (nM) 95% CI (nM) FC IC<sub>50</sub> (nM) 95% CI (nM) FC
WT 0.3 0.2 to 0.5 1 1.3 0.3 to 5.3 1 6.4 2.5 to 16.6 1 T66I 0.0004 0.0002 to 0.0007 0.001 3.2 1.3 to 7.8 2.4 61 42.6 to 87.6 10 R263K 1.5 1.2 to 1.7 4.8 1.8 0.8 to 4.2 1.3 28 16.3 to 47 4 T66I/R263K 0.03 0.02 to 0.04 0.09 2.2 0.5 to 8.9 1.6 141 95 to 209.3 22 T66I/E138K/R263K 0.008 0.004 to 0.02 0.03 3.4 0.9 to 12.6 2.5 1,054 881.6 to 1261 164
Figure 1. Viral infectivity in TZM-bl cells. TZM-bl cells were infected with (a) NL4.3<sub>IN(WT)</sub>, NL4.3<sub>IN(T66I)</sub>, or NL4.3<sub>IN(R263K)</sub>; (b) NL4.3<sub>IN(WT)</sub> or NL4.3<sub>IN(T66I/R263K)</sub>; (c) NL4.3<sub>IN(WT)</sub> or NL4.3<sub>IN(T66I/E138K/R263K)</sub> virus over 48h. Infectivity of NL4.3<sub>IN(WT)</sub> virus is represented in (a-c) for comparison. Luciferase levels were measured and (d) fold decrease in infectivity was calculated. Error bars indicate means ± standard deviation.
Figure 2. Viral replicative capacity in PM-1 cells. PM-1 cells were infected with NL4.3<sub>IN(WT)</sub>, or (a) NL4.3<sub>IN(T66I)</sub>, (b) NL4.3<sub>IN(R263K)</sub>, (c) NL4.3<sub>IN(T66I/R263K)</sub>, and (d) NL4.3<sub>IN(T66I/E138K/R263K)</sub> viruses over 21 days. Replicative capacity of the above-mentioned viruses was normalized to reverse transcriptase (RT) levels of the NL4.3<sub>IN(WT)</sub> virus at day 7. Supernatants were collected at days 3, 7, 14, and 21 at which time RT levels were measured as counts per minute (cpm). Error bars indicate means ± standard deviation.