Resistance mutations against dolutegravir in HIV integrase impair the emergence of resistance against reverse transcriptase inhibitors

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Objective: Among 1222 antiretroviral-naive patients who received dolutegravir (DTG) as part of first-line therapy, none has developed resistance against this compound after 48–96 weeks of follow-up. Moreover, only four occurrences of virological failure with resistance mutations have been documented in previously drug-experienced patients who received DTG as a first time integrase inhibitor as a component of a second-line regimen. The R263K integrase resistance mutation was observed in two of these individuals who received suboptimal background regimens. We have previously selected mutations at position R263K, G118R, H51Y, and E138K as being associated with low-level resistance to DTG. Now, we sought to investigate the facility with which resistance on the part of R263K-containing viruses might develop.

Design and methods: We tested the ability of DTG-resistant viruses containing either the R263K or G118R and/or H51Y mutations to develop further resistance against several reverse transcriptase inhibitors during in-vitro selection experiments.

Results: Our results show that DTG-resistant viruses are impaired in their ability to acquire further resistance to each of nevirapine and lamivudine as a consequence of their relative inability to develop resistance mutations associated with these two compounds.

Conclusion: Our findings provide an explanation for the fact that no individual has yet progressed to virological failure with resistance mutations associated with dolutegravir in clinical trials in which patients received dolutegravir together with an optimized background regimen.   © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins

Introduction

Since the discovery of the first inhibitors of HIV replication, drug resistance has been a major problem in regard to HIV therapy and eradication [1,2]. Although combined antiretroviral therapy (cART) has helped to reduce the emergence and spread of drug–resistant strains, drug resistance has emerged in the aftermath of treatment failure for every antiretroviral drug studied to date. This includes each of compounds that block the reverse transcriptase, protease, and integrase enzymes of HIV.

Integrase inhibitors (INIs) are the most recent category of antiretroviral drug to be developed and include raltegravir (RAL), elvitegravir (EVG), and dolutegravir (DTG). However, the latter seems to be unique in that no mutations associated with drug resistance have yet occurred in previously treatment–naive patients [3]. It

has been hypothesized that this may be due to greatly reduced viral replication capacity that is associated with resistance to this drug [4]. However, tissue culture selection studies have shown that R263K and G118R mutations can arise under DTG pressure [5] and other in-vitro studies with DTG have led to the selection of a variety of mutations that confer very low-level resistance [6]. We demonstrated that both R263K and G118R impart a low level of resistance against DTG that is associated with a decrease in replication capacity [5,7]. R263K was later detected in two highly treatment-experienced patients who failed DTG in a trial termed SAILING (A Study of GSK1349572 Versus Raltegravir (RAL) With Investigator Selected Background Regimen in Antiretroviral-Experienced, Integrase Inhibitor-Naive Adults) [8], in which participants had never previously received an INI, but had preexisting resistance to two or more classes of antiretroviral drugs at baseline. Thus, these individuals had limited options in regard to background regimens.

We also detected several secondary mutations in association with R263K and G118R in our in-vitro selection studies, but these substitutions (e.g. H51Y, E138K) were unable to compensate for the negative effect that R263K has on HIV replication capacity and integrase strand transfer activity and, in fact, resulted in a further diminution in both viral and integrase fitness [4,7]. Nevertheless, viruses containing R263K are replication competent and are able to propagate in cell culture for at least 2 years. Given the recent approval of DTG by the U.S. Food and Drug Administration, there is now a need to better understand how DTG resistance mutations will affect the ability of HIV to develop resistance to other classes of antiretroviral drugs, including the reverse transcriptase inhibitors that constitute the backbone of almost all HIV treatment regimens. Here, we report the results of tissue culture selection studies that have employed viruses containing the DTG resistance mutations G118R and R263K, alone and in combination with secondary mutations that were grown in the presence of the nucleoside reverse transcriptase inhibitor (NRTI) lamivudine (3TC) and the nonnucleoside reverse transcriptase inhibitor (NNRTI) nevirapine (NVP). Our results show that viruses that were resistant to DTG were impaired in their ability to develop resistance against each of 3TC and NVP. These results help to explain why DTG resistance mutations that have the potential to lead to virological failure were only observed during the SAILING study [9] and not in clinical trials [10] that employed DTG in first-line therapy.

Methods

Cells and antiviral compounds
Primary human cord blood mononuclear cells (CBMCs) were obtained through the Department of Obstetrics, Jewish General Hospital, Montreal, Canada. ViiV Healthcare Ltd. (Research Triangle Park, North Carolina, USA) kindly provided DTG. 3TC and NVP were obtained from the NIH AIDS Research and Reference Reagent Program (https://www.aidsreagent.org/).

Site-directed mutagenesis and virus production
The H51Y and R263K mutations in integrase have been described previously [5,7]. Briefly, these substitutions were introduced into the pNL-4.3 plasmid by site-directed mutagenesis (SDM) using a QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, California, USA) and the presence of mutations was confirmed by sequencing. The primers employed have been published elsewhere [5,7]. Genetically homogeneous replication-competent wild-type and mutated NL4.3 viruses were produced by transfecting the corresponding sequenced plasmid into 293T cells using Lipofectamine 2000 (Invitrogen, Burlington, Ontario, Canada). At 48 h posttransfection, cell culture fluids were harvested, filtered through 0.45 μm, and treated with benzonase (EMD, Mississauga, Ontario, Canada) to degrade the plasmids. Reverse transcriptase activity and p24 antigen levels were used to quantify virus production.

Selection of resistant viruses in cord blood mononuclear cells
CBMCs were stimulated with phytohemagglutinin and infected with relevant viruses at a multiplicity of 0.1 for 2 h at 37°C and subsequently grown in the presence of RPMI1640 media (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Drug concentrations were gradually increased over 25 weeks, as described previously [11]. Reverse transcriptase activity in culture fluids was measured weekly to monitor HIV-1 replication. Every week, the infected cells were re-cocultured with fresh donor cells and the drug concentrations were adjusted appropriately to permit the emergence of resistant variants. Increments of 2.5-fold in DTG concentration were attempted at each passage, unless the pressure on viral survival was too great, in which case, the previous concentration was again used or in some cases, drug pressure was eliminated to allow recovery of viral growth. At each passage, an aliquot of the culture fluid was stored at −70°C.

Nucleic acid extraction, amplification, and sequencing analysis
Viral RNA was extracted from culture fluids using the QIAamp viral extraction kit (Qiagen, Toronto, Ontario, Canada) and reverse transcribed into DNA using the SuperScript III one step RTPCR with a Platinum Taq kit (Invitrogen). The reverse transcriptase region was amplified by nested PCR using a standard protocol (Virco BVBA, Beerse, Belgium), purified using the QIAquick PCR purification kit (Qiagen), and genotyped by a standard protocol (Virco BVBA), as described previously [12]. In the case of the integrase gene, the primers and

protocols used for amplification and sequencing have been published previously [13]. Both reverse transcriptase and integrase were sequenced using the BigDye1 Terminator v1.1 Cycle Sequencing Kit and an ABI 3130xl Genetic Analyzer. Sequencing results were analyzed using SeqScape software version 2.5.

Results

Dolutegravir resistance mutations delay dose escalation during HIV-1 tissue culture selection with reverse transcriptase inhibitors

To understand the possible effects of the G118R and R263K DTG mutations on the occurrence of resistance to other drugs, we performed selections with HIV-1 NL4.3 bearing the above DTG mutations, alone or in combination with the secondary H51Y substitution, in the presence of 3TC or NVP. The results of Fig. 1 (a and b) show that the wild-type virus developed resistance against 3TC and NVP, respectively, within about 8 weeks, but that the R263K and G118R viruses were somewhat delayed in this regard. The G118R and H51Y/G118R-containing viruses were unable to generate resistance to 3TC and were only able to grow in 10 μmol/l NVP after 20–25 weeks (Fig. 1a and b). Viruses containing the R263K/H51Y substitutions were virtually unable to generate any resistance to 3TC over 24 weeks (Fig. 1a).

For periods after 25 weeks, wild-type and H51Y viruses became 1000-fold resistant against 3TC, whereas the level of resistance to this drug was only 100-fold for the H51Y/R263K virus and only 25-fold for both the G118R and H51Y/G118R viruses. All viruses except G118R/H51Y were able to develop 1000-fold resistance against NVP after 25 weeks, whereas the resistance level was 250-fold for the G118R/H51Y viruses. In contrast, the latter viruses attained five-fold resistance against DTG under further selection, whereas all other viruses including the wild-type viruses were only able to generate 2.5-fold resistance against this drug following extended selection. Thus, the R263K, G118R, H51Y/R263K, and H51Y/

G118R mutations can inhibit the development of resistance against reverse transcriptase inhibitors. In contrast, the H51Y mutation alone had little or no influence on the generation of drug resistance, consistent with the fact that this mutation does not on its own confer significant resistance to DTG nor does it impact on viral replication capacity [7]. As a control, we also studied whether viruses resistant to RAL and/or EVG could develop resistance to 3TC and NVP and found that no delays occurred in appearance of either resistance or relevant resistance mutations. In brief, viruses containing mutations at positions E92Q or N155H developed the V106A and Y181C mutations within 7 weeks of selection with NVP and developed the M184I/V mutations within 7 weeks of selection with 3TC. In addition, viruses containing both the E92Q and N155H mutations developed both the K103T and Y188C mutations after 7 weeks of selection with NVP and mixtures of mutations at position M184I after 7 weeks of selection with 3TC.

Mutations in the R263K and G118R resistance pathways delay the emergence of resistance mutations against reverse transcriptase inhibitors

To characterize resistance mutations against reverse transcriptase inhibitors that may have arisen in the presence of 3TC and NVP, the reverse transcriptase region of the HIV-1 *pol* gene was genotyped (Table 1). During the 3TC selections, both the wild-type and H51Y-containing viruses acquired the M184V/I resistance mutations as early as week 6. However, the appearance of M184I was delayed when the R263K virus was employed and no 3TC mutation could be detected for the H51Y/R263K or H51Y/G118R viruses at times up to 11 weeks. The M184I substitution was finally detected at week 25 in the case of the H51Y/R263K viruses.

In the presence of NVP, a mixture of V106A/V was detected after 6 weeks of selection for both the wild-type and H51Y viruses, whereas only the mutated V106A was detected at week 8. The same V106A substitution only emerged between weeks 11 and 14 when selection studies
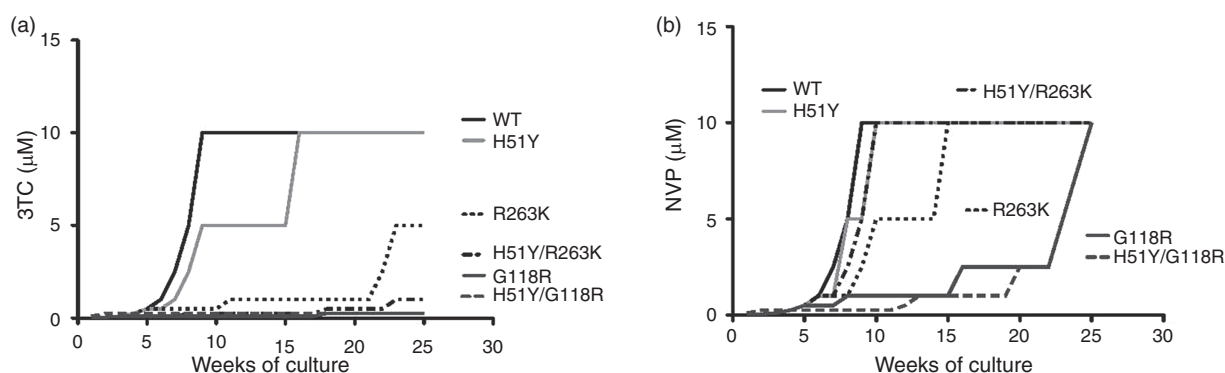

Fig. 1. Dolutegravir resistance mutations delay reverse transcriptase inhibitor dose-escalation during in-vitro selection studies in the presence of **(a)** lamivudine (3TC) or **(b)** nevirapine (NVP). WT, wild-type.

Table 1. Emergent mutations in reverse transcriptase detected by genotyping viruses containing dolutegravir resistance-associated mutations grown under drug pressure.

Virus	Week 6	Week 8	Week 11	Week 15	Week 25
Selections with 3TC					
WT	**M184V**	**M184V**	**M184V**	ND	ND
H51Y	**M184I**	**M184I**	**M184I**	ND	R83K/R, **M184I**
R263K	None	**M184I**	**M184I**	ND	**M184I**
H51Y/R263K	ND	None	None	ND	**M184I**
G118R	ND	ND	None	None	None
H51Y/G118R	ND	None	None	None	None
Virus	**Week 6**	**Week 8**	**Week 11**	**Week 15**	**Week 25**
Selections with nevirapine					
WT	**K103K/T,V106A/V**	**V106A, P225H/P**	**V106A**	ND	V75L/V, **V106A, P225H**
H51Y	**V106A/V**	**V106A**	**V106A, P225H/P**	ND	**V106A, P225H**
R263K	ND	None	**V106A/V**	**V106A**	**V106A**
H51Y/R263K	**M230L/M**	**V106A/V, M230M/L**	**V106A**	ND	**V106A**
G118R	ND	None	None	None	D123D/**N,Y181C**, T369I
H51Y/G118R	ND	None	None	None	**V108I**, I142I/T, T369I/T, E399E/G

Dolutegravir-resistance mutations delay the emergence of resistance mutations against reverse transcriptase inhibitor (RTI) in reverse transcriptase. The resistance mutations characterized are presented in bold. ND, not done; WT, wild-type.

were initiated with the R263K–containing virus. The H51Y/R263K virus first generated the M230L/M polymorphism before acquiring the V106A resistance mutation at week 11. At week 25, both the wild–type and H51Y viruses carried the V106A/P225H mutations. The G118R and H51Y/G118R viruses did not develop any relevant resistance mutations, except for the Y181C NNRTI mutation, which emerged between weeks 15 and 25.

Presence of the H51Y and R263K mutations following selection with reverse transcriptase inhibitors

In order to identify potential reversions of the DTG–related mutations, the integrase regions of the *pol* genes of the DTG–resistant viruses were genotyped during selections with the various reverse transcriptase inhibitors (Table 2). This analysis was also performed in the absence of drug in order to assess the possibility of spontaneous reversion. The results show that each of the R263K and G118R mutations, alone or in combination with H51Y, was conserved throughout the 25 weeks of selection, both in the presence of RTI and with no drug added. In the absence of treatment, likely polymorphisms at positions H51N, D6D/N, and H171H/Y were detected with the H51Y virus at week 25.

Previous results from our laboratory have shown that the H51Y/R263K and H51Y/G118R combinations severely decreased HIV-1 replication capacity [7]. Consistent with this result, genotyping showed that these combinations of mutations were unstable in the absence of pressure with DTG or the other drugs (Table 2). In addition, a H51N substitution was observed at week 6, both in the presence of NVP and in the absence of drug. This change prevailed up to week 25 in the presence of

NVP. In the absence of drug, the H51N/R263K combination was associated with an E138E/K mixture at week 6 and E138E/K was also detected later at week 12 in the H51Y/R263K viruses. However, only H51N and R263K were detected after 25 weeks of NVP pressure. The E138K substitution was also detected together with H51Y/R263K between weeks 6 and 12 in the presence of 3TC. After 25 weeks of cell culture with 3TC, genotyping revealed the presence of a H51S/E138K/R263K combination.

Stability of the H51Y and R263K mutations in the presence of dolutegravir

We also performed tissue culture selection experiments in the presence of DTG using the wild-type, H51Y, R263K, G118R, H51Y/R263K, and H51Y/G118R viruses (Fig. 2 and Table 3). DTG concentrations were raised to a maximum of 0.025 μmol/l for both the wild-type and mutated viruses after 6 and 2 weeks of selection, respectively (Fig. 2). Integrase genotyping shows that the wild-type virus developed the R263K mutation after about 12 weeks (Table 3), confirming that this substitution can emerge under DTG pressure, as reported previously [5]. Viruses containing the R263K or G118R substitutions did not generate additional mutations over the 25 weeks of selection in this study. The R263K and G118R substitutions each conferred approximately two–fold to three–fold resistance to DTG as previously shown [5]. When selections were initiated with H51Y virus, a R262K mutation emerged between weeks 12 and 25. Consistent with our results with reverse transcriptase inhibitors, neither the H51Y/R263K nor H51Y/G118R combinations of mutations were negatively selected when grown in the presence of DTG. However, an additional E138K mutation emerged in the R263K virus at week 12 and was conserved over the 25 weeks of selection. None

Table 2. Changes in integrase sequences identified by genotyping at the indicated times following replication of wild-type, H51Y, R263K, H51Y/R263K, G118R, and H51Y/G118R viruses in cord blood mononuclear cells in the presence of nevirapine and lamivudine as well as in the absence of drug.

Virus	Week 6	Week 12	Week 25
3TC			
WT	ND	ND	None
H51Y	H51Y	ND	H51Y
R263K	R263K	ND	R263K
H51Y/R263K	H51Y, E138K, R263K	H51Y, E138K, R263K	H51S, E138K, R263K
G118R	ND	G118R	G118R
H51Y/G118R	H51Y/G118R	ND	ND
Nevirapine			
Virus	Week 6	Week 12	Week 25
WT	ND	ND	None
H51Y	H51Y	ND	H51Y
R263K	R263K	ND	R263K
H51Y/R263K	H51N, R263K	H51N, R263K	H51N, R263K
G118R	ND	G118R	G118C
H51Y/G118R	ND	H51Y, G118R	ND
No drug			
Virus	Week 6	Week 12	Week 25
WT	ND	ND	None
H51Y	H51Y	ND	D6D/N, H51Y, H171H/Y
R263K	R263K	ND	R263K
H51Y/R263K	H51N, E138E/K, R263K	H51Y, E138K/E, R263K	H51N, R263K
G118R	ND	G118R	G118R
H51Y/G118R	ND	H51Y, G118C	H51Y, G118C

Dolutegravir-resistance mutations are stable in the presence of reverse transcriptase inhibitor (RTI) as well as in the absence of drugs. 3TC, lamivudine; ND, not done.
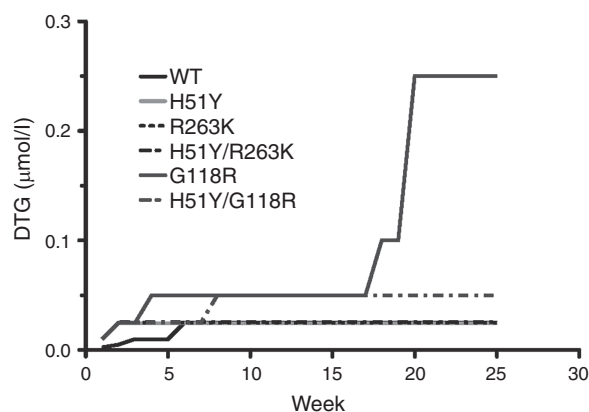
of these viruses attained high levels of resistance against DTG.

Discussion

To better understand the possible generation of resistance against reverse transcriptase inhibitors in patients treated with DTG, we conducted selection studies using each of 3TC and NVP using NL4.3 viruses harboring the H51Y, R263K, G118R, H51Y/R263K, and H51Y/G118R integrase mutations. We have previously shown that

Fig. 2. Development of low-level resistance during dolutegravir selections with dolutegravir-resistant viruses. DTG, dolutegravir; WT, wild-type.

H51Y has little or no effect on HIV–1 replication capacity [7] and our results here show that this mutation alone was innocuous in regard to the acquisition of resistance against the reverse transcriptase inhibitors employed. However, the H51Y substitution permitted the emergence of a previously unreported substitution, R262K, after 25 weeks of culture in the presence of DTG. This suggests that the 50-51 region of integrase functionally interacts with the C–terminal region of the protein, in agreement with our previously published in-silico homology model of HIV-1 integrase [5].

Our results also show that the presence of R263K or G118R, alone or in combination with H51Y, can delay the emergence of resistance mutations associated with each of NVP and 3TC. For example, the emergence of the M184I mutation from the R263K virus was delayed by at least 2 weeks compared with the presence before week 6 of the M184V and M184I mutations in the wild-type and H51Y viruses, respectively. In view of the fact that 3TC is a NRTI, whereas NVP is an NNRTI, it seems unlikely that these delays could have resulted from direct interactions between the reverse transcriptase and integrase enzymes during the viral replication cycle [14,15]. In contrast, these delays may have been caused by the negative impact that DTG-resistance mutations have on viral replication. Indeed, we have shown that both the R263K mutation [5] and the G118R mutation [16] decrease both the viral replication capacity and the integrase enzyme activity and that the addition of H51Y to either of these mutations failed to restore these activities [7,17]. In fact, the addition of H15Y to R263K

Table 3. Changes in integrase sequences identified by genotyping at the indicated times following replication of wild-type, H51Y, R263K, H51Y/R263K, G118R, and H51Y/G118R viruses in cord blood mononuclear cells in the presence of dolutegravir.

Virus	Week 6	Week 12	Week 25
Dolutegravir			
WT	ND	R263K	R263K
H51Y	ND	H51Y	H51Y, R262K
R263K	ND	R263K	R263K
H51Y/R263K	H51Y, R263K	H51Y, E138K, R263K	H51Y, E138K, R263K
G118R	ND	ND	T66I, G118R, E138K
H51Y/G118R	ND	ND	H51Y, G118R

Changes in integrase sequences in the presence of DTG. ND, not done.

further decreased viral fitness and enzyme activity compared with R263K only [7]. These observations help to explain the relative inability of DTG-resistant viruses to develop resistance mutations against NVP and 3TC.

We have previously shown that the H51Y/R263K or H51Y/G118R combination has a profoundly negative effect on HIV replication capacity. Accordingly, it is not surprising that this combination of mutations was unstable in the absence of DTG pressure (Table 2). H51Y was the more fragile substitution and was lost after 25 weeks of selection in the presence of NVP or 3TC, as well as in the absence of drug. Likely polymorphisms at positions Y51N and Y51S were observed; however, Y51N was detected after only 6 weeks of culture, both in the absence of drug and under NVP pressure, whereas Y51S was detected after 25 weeks of selection with 3TC. The H51Y/R263K virus also acquired the integrase E138K mutation in the presence of DTG (Table 3). We previously identified E138K as a secondary mutation that is associated with G118R during in-vitro selection studies with another INI, MK2048, and we have shown that the addition of E138K to G118R can partially restore the viral replication capacity of viruses containing G118R, while increasing levels of resistance to MK2048 [16]. In addition, E138K has been previously reported to be a secondary mutation in association with Q148R and several other primary substitutions that confer resistance to both RAL and EVG [18–30]. However, E138K does not seem to be able to compensate for the diminished fitness that is associated with the R263K mutation [31]. We are currently investigating E138K in regard to its effects on resistance to DTG as well as on in-vitro strand transfer activity.

In clinical trials performed to date (reviewed in [10]), only four treatment-experienced INI-naive participants receiving DTG failed therapy with resistance mutations in integrase. All these individuals were enrolled in the SAILING trial and, in two cases, the R263K mutation was detected [8,9]. A third participant developed a T97A/E138T/A combination of mutations consecutive to a preexisting Q148 mutation at baseline, whereas a fourth individual developed the polymorphic V151I/V substitution that has been previously shown to have little or no effect on INSTI susceptibility [9,32]. Individuals participating in the SAILING study were highly antiretroviral therapy-experienced and had been treated with suboptimal regimens due to the presence of multiple resistance mutations in each of the reverse transcriptase and protease regions of the viral genome. In contrast, no virological failure with resistance mutations has been reported for DTG in studies performed to date on treatment-naive individuals [10]. Yet, patients who have failed RAL-containing or EVG-containing regimens and who have developed mutations in integrase associated with these two drugs may not always respond well to DTG and may, in fact, develop further RAL-associated mutations in the event that treatment with DTG is not successful [8].

We believe that the results reported in the present study might in part explain the absence of development of resistance in treatment-naive individuals treated with DTG together with an optimized background therapy and suggest that the presence of mutations associated with resistance to DTG can impair the ability of HIV to acquire further resistance against NVP and 3TC, a NNRTI and NRTI, respectively. Additionally, our results show that the presence of DTG can prevent the reversion of integrase mutations that are associated with DTG resistance. Our data are consistent with the notion that R263K may not be a deleterious mutation for DTG in contrast to the mutations in the RAL/EVG pathways that have the ability to severely compromise DTG activity [8]. We conclude that the presence of DTG when used together with other active drugs may not lead to virological failure even if the R263K substitution is present. This hypothesis could be tested by ultrasensitive sequencing of integrase from residual plasma viral RNA in individuals who have been successfully treated with DTG or from the DNA of lymphocytes of such individuals.

Acknowledgements

Conflicts of interest
There are no conflicts of interest.

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