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HIV-1 resistance dynamics in patients with virological failure to dolutegravir maintenance monotherapy.

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ABSTRACT

Introduction

A high genetic barrier to resistance to the integrase strand transfer inhibitor (INSTI) dolute-gravir has been reported *in vitro* and *in vivo*. We describe the dynamics of INSTI resistance associated mutations (INSTI-RAMs) and mutations in the 3'-polypurine tract (3'-PPT) in relation to virological failure (VF) observed in the randomized dolutegravir as maintenance monotherapy study (DOMONO, NCT02401828).

Methods

From ten patients with VF, plasma samples were collected before the start of cART and during VF, and were used to generate Sanger sequences of integrase, the 5' terminal bases of the 3' long terminal repeat (LTR), and the 3'-PPT.

Results

Median HIV-RNA at VF was 3,490 (interquartile range 1.440-4.990) c/mL. INSTI-RAMs were detected in 4 patients (S230R, R263K, N155H, and E92Q+N155H), no INSTI-RAMs were detected in 4 patients, and sequencing of the integrase gene was unsuccessful in 2 patients. The time to VF ranged from 4 to 72 weeks. In 1 patient, mutations developed in the highly conserved 3'-PPT. No changes in the terminal bases of the 3'-LTR were observed.

Conclusions

The genetic barrier to resistance is too low to justify dolutegravir maintenance monotherapy, because single INSTI-RAMs are sufficient to cause VF. The large variation in time to VF suggests that stochastic reactivation of a pre-existing provirus containing a single INSTI-RAM is the mechanism for failure. Changes in the 3'-PPT point to a new dolutegravir resistance mechanism *in vivo*.

INTRODUCTION

The second-generation integrase strand transfer inhibitor (INSTI) dolutegravir (DTG) seems to have a high genetic barrier to resistance *in vitro*, with significant loss of replication capacity in viruses with INSTI resistance associated mutations (RAMs) compared with wildtype viruses.[1,2] The high genetic barrier to DTG resistance when administered as DTG-containing cART is already demonstrated *in vivo*: no RAMs associated with decreased DTG susceptibility were observed in any of the 1067 treatment-naive patients who initiated DTG-containing cART in pivotal phase 3 studies.[3–5] In treatment-experienced but INSTI-naive patients taking a DTG-containing cART-regimen, virological failure (VF) with development of INSTI-RAMs is rare.[6,7] Furthermore, mutations selected by the first generation INSTI raltegravir (RAL) and elvitegravir (EVG) might have limited impact on DTG susceptibility, enabling successful treatment with DTG-containing cART in a large proportion of patients who previously developed VF during use of RAL- or EVG-containing cART, although dosing DTG twice daily is necessary for certain INSTI-RAMs.[7–11]

Given the high genetic barrier to DTG resistance and other favorable properties of DTG, such as few drug-drug-interactions, few adverse events, and a low pill-burden, we hypothesized that DTG could be used as monotherapy to maintain viral suppression in HIV-1 infected adults with long-term virological suppression during cART. More recently, DTG maintenance monotherapy has been investigated in small, retrospective, observational studies and case series, which showed high rates of virological suppression without emergence of INSTI-RAMs in INSTI-naive patients. These studies included a total of 118 patients, in five of whom VF led to development of INSTI-RAMs, but all five were INSTI-experienced or had treatment-compliance issues. Also, these studies were done in routine clinical care and without standardized monitoring and ethics committee approval.[12–16] As these results lacked a control arm, we conducted the randomized, controlled Dolutegravir as Maintenance Monotherapy for HIV-1 study (DOMONO, NCT02401828) and showed that DTG maintenance monotherapy in HIV-1 infected patients led to a higher rate of VF (8/95, 8%) compared with patients who continued cART (3/152, 2%).[17]

Since INSTI-RAMs in the integrase gene are rarely detected in patients with VF to DTG-containing cART,[5,6] novel DTG resistance pathways outside of the integrase gene may exist. Experiments with purified integrase enzyme and long terminal repeat (LTR) duplexes showed that mutations in the four terminal bases of the LTR could confer INSTI resistance.[18] Furthermore, *in vitro* DTG resistance selection experiments showed that mutations in the 3'-polypurine tract (3'PPT) confer high-level resistance to RAL, EVG, and DTG.[19] Both resistance pathways have not been reported yet in HIV-infected individuals. Here, we describe the dynamics of resistance-associated mutations in the integrase gene and the 3'-PPT in HIV-1 infected individuals with VF during DTG maintenance monotherapy.

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METHODS

Study population

Patients with VF during DTG maintenance monotherapy were included from two studies performed in the outpatient clinic of the Erasmus University Medical Center (Rotterdam, The Netherlands) and the University Medical Center Groningen (Groningen, the Netherlands). The first group of patients consisted of participants in the DOMONO main study. The most important inclusion criteria were a CD4 T-lymphocyte nadir *above* 200 cells/mm³, an HIV-RNA zenith below 100.000 c/mL (except when measured during the acute phase of infection), and no previous VF and/or any documented RAMs, according to The Stanford HIV Drug Resistance Database.[20] The second group of patients consisted of participants in the DOMONO pilot study. This pilot study had the same inclusion and exclusion criteria as the main study, with the exception that patients with a CD4 T-lymphocyte nadir *below* 200 cells/mm³ were included.

Study procedures

HIV-1 RNA in plasma was quantified with the COBAS® ampliprep/COBAS®Taqman® HIV-1 v2 test (Roche diagnostics, Almere, The Netherlands). VF was defined as a confirmed plasma HIV-RNA level above 200 c/mL. Adherence was defined as the percentage self-reported adherence. DTG plasma-concentrations at the moment of VF were determined and interpreted according to the Therapeutic Drug Monitoring protocol of Radboud University Medical Center (Nijmegen, the Netherlands).[21] In all patients, Sanger sequences covering the integrase gene, and the 3'-PPT and the four 5' terminal bases of the 3'-LTR in the nef gene were determined using stored plasma samples with detectable HIV-RNA before patients started on cART (baseline samples) and in plasma samples at time of VF during treatment with DTG monotherapy. For nef amplification, RNA was isolated with the High Pure Viral RNA kit (Roche, #11858882001), according to the manufacturer's instructions. Of 60 μL of eluted RNA, 12.5 μL was used for complementary DNA synthesis, using 50 pmol oligo dT primer (20T+TRAAG, Eurogentec) and superscript IV (ThermoFisher) with the following polymerase chain reaction (PCR) conditions: 5 minutes at 30°C, 2 hours at 50°C, and 10 minutes 80°C. After RNase H inactivation (by incubation for 20 minutes at 37°C and 15 minutes at 70°C), 5 μL of complementary DNA was used for PCR analysis. Primers were as follows: HXB2-8343for//T (or C): AGAGTTAGGCAGGGATAT(C)TCACC and HXB2-9632rev: GCACTCAAGGCAAGCTTTATTGAGGCT. PCR conditions were as follows: 2 minutes at 94°C and 40 cycles of 10 seconds 94°C, 30 seconds at 60°C, 1 minute at 68°C, and 10 minutes at 68°C (extension). PCR samples were separated on 1% agarose, and bands were cut and extracted with QIAEX II gel extraction kit (Qiagen) before sequencing. For integrase sequencing, nucleic acids were extracted using MPLC (Roche) after ultracentrifugation of 1.5 mL ethylenediaminetetraacid-plasma at 26.000xg for 1 hour at 4°C. Subsequently, the HIV-1

integrase gene was amplified with the OneStep RT-PCR kit (Qiagen, Venlo, The Netherlands) and 20 pmol of the primers described in supplementary Table 1, according to manufacturer's protocol, using the following thermal profile: 30 minutes at 50°C, 15 minutes at 95°C, and 25 cycles of 1 minute at 95°C, 1 minute at 55°C, 2 minutes at 72°C, and 10 minutes at 72°C. Nnested PCR was then performed with the HotStar HiFidelity Polymerase kit according to the manufacturer's protocol and the following thermal profile: 5 minutes at 95°C and 30 cycles of 15 seconds at 94°C, 1 minute at 50°C, 2 minutes at 72°C and 10 minutes at 72°C. One microliter of the amplicon was sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and 5 pmol of sequencing primers described in supplementary Table 1. The sequenced PCR products were purified using Performa DTR V3 purification plate (Edgebio, Sopachem, Ochten, The Netherlands) and analyzed on an ABI 3130XL sequencer (Applied Biosystems). The sequence data were analyzed using a Sequence Navigator software sequencer (Applied Biosystems) and SeqMan v10.1.2 (DNASTAR, Madison,WI). Resulting HIV-1 integrase sequences were further analyzed using the Stanford database to check for known drug RAMs, and baseline sequences were compared to rebound sequences using BioEdit, v7.2.0.[20]

Ethical considerations

The DOMONO main study and its pilot study were approved by the Dutch Medical Ethics Committee and were performed in accordance with good clinical practice and the Helsinki Declaration. All participating subjects provided verbal and written informed consent before study procedures.

RESULTS

Patient population and baseline characteristics

Eight of 95 participants of the DOMONO main study had VF. Of the four participants of the DOMONO pilot study, two had VF. In total, 10 patients experienced VF during DTG maintenance monotherapy. Baseline characteristics at entry of DTG maintenance monotherapy study are listed in Table 1. All participants were male with a median age of 46 years and were infected with HIV-1 subtype B. The median HIV-RNA zenith was 29.750 c/mL and the median CD4 T-lymphocyte nadir was 235 cells/mm^3. In three patients (patient 5, 9, 10) cART was initiated within a year after the HIV-diagnosis. Nine of the participants with VF were INSTI-naive, whereas one patient (patient 3) was previously treated successfully with RAL-containing cART and DTG-containing cART. The median duration of plasma HIV-RNA suppression (defined as a plasma HIV-RNA lower than 50 c/mL) during cART (ie. before initiation of DTG maintenance monotherapy) was 61 months.

	Patients (N=10)
Male sex, N (%)	10 (100)
Age, years, median (Q1,Q3)	46 (39,52)
Mode of transmission, N (%)	
MSM	7 (70)
HSX	2 (20)
Other	1 (10)
Ethnicity, N (%)	
Caucasian	7 (70)
Caribbean/Surinam	3 (30)
Time on cART, median (Q1, Q3), months	71 (47, 104)
Time suppressed on cART, median (Q1, Q3), months	61 (41, 101)
INSTI-naive, N (%)	9 (90)
HIV-1 subtype B, N (%)	10 (100)
HIV-RNA zenith, median (Q1, Q3), c/ml	29.750 (18.250, 66.625)
CD4 T-lymphocyte nadir, median (Q1, Q3), cells/mm^3	235 (183, 300)

Table 1. Baseline characteristics of patients with virological failure on dolutegravir maintenance monotherapy. DTG=dolutegravir, MSM=men having sex with men, HSX=heterosexual, cART=combination antiretroviral therapy, INSTI=integrase strand transfer inhibitor.

Characteristics of virological failure during DTG maintenance monotherapy

Clinical and virological characteristics at the moment of VF are shown in Table 2 and 3. Median HIV-RNA (IQR) at VF was 3.490 (1.440-4.990) c/mL. Interestingly, the time to VF after the start of DTG maintenance monotherapy varied considerably, with patient 4 already experiencing VF at week 4 and patient 7 experiencing VF at week 72. The median time to VF was 33 weeks. Self-reported adherence was more than 95%, and DTG plasma-concentrations during DTG maintenance monotherapy were therapeutic (i.e. above 0.50 mg/L) in all patients at time of VF. Two patients received co-medication (patient 3: oxazepam, buprenorphine/naloxone, fluticasone, quetiapine, levomepromazine, and prednisolone; patient 5: simvastatin, acetylsalicylic acid, tolbutamid, perindopril, and metoprolol), but no drug-drug-interactions were detected. No INSTI-RAMs were detected in the samples obtained from patients before they started therapy. At the moment of VF detection, INSTI-RAMs were detected in patients 2, 5, 7, and 9, whereas no known INSTI-RAMs were detected in patients 1, 4, 8, and 10; integrase gene sequencing was unsuccessful in patients 3 and 6 owing to low plasma HIV-RNA levels of 1.440 and 678 c/mL respectively. Single INSTI-RAMs were detected in three patients: S230R (in patient 2), R263K (in patient 5), and N155H (in patient 7). In patient 9, the combination of E92Q and N155H was detected. Figure 1 shows the courses of plasma HIV-RNA levels and the INSTI-RAMs detected during treatment with DTG maintenance monotherapy.

Patient	Study	Relevant medical history	HIV-RNA zenith (c/ml)	CD4-T-lymphocyte nadir (cells/mm³)	cART before start DTG*	INSTI naive*	Interval between HIV-diagnosis and start cART (months)	Time suppressed on cART before DTG* (months)	HIV-RNA at VF (c/mL)	DTG-plasma level at VF in mg/L$	Adherence (according to clinician)
4	Main	-	18.500	290	TDF/FTC/RPV	Yes	27	29	71.600	1.29 (+14h)	>95%
6	Main	-	7.420	220	TDF/FTC/EFV	Yes	28	103	678	2.00 (+19h)	>95%
10	Pilot	-	66.500	100	TDF/FTC/RPV	Yes	1	59	798	5.31 (+19h)	>95%
1	Main	-	17.500	280	TDF/FTC/RPV	Yes	66	47	3.510	2.59 (+16h)	>95%
2	Main	-	99.270	330	TDF/FTC/RPV	Yes	51	22	1.570	2.96 (+22h)	>95%
3	Main	Psychiatric problems, alcohol-abuse, ablatio retinae with operation.	56.300	210	TDF/FTC/DTG	No (RAL, DTG)	105	48	1.440	1.00 (+24h)	>95%
9	Pilot	-	24.900	70	TDF/FTC/EFV	Yes	0	100	54.200	0.86 (+16)	>95%
8	Main	-	67.000	230	TDF/FTC/RPV	Yes	47	64	4.990	1.44 (+24h)	>95%
5	Main	POAD, coronary artery disease, DM2	34.600	240	TDF/FTC/NVP	Yes	7	84	3.470	0.70 (+13h)	>95%
7	Main	-	20.100	380	TDF/FTC/NVP	Yes	58	14	4.180	2.15 (+9h)	>95%

Table 2. Overview of clinical characteristics of patients failing dolutegravir maintenance monotherapy. VF=virological failure, INSTI=integrase strand transfer inhibitor, DTG=dolutegravir, TDF=tenofovir disoproxil fumarate, FTC=emtricitabine, RPV=rilpivirine, EFV=efavirenz, RAL=raltegravir, NVP=nevirapine, cART=combination antiretroviral therapy, POAD=peripheral occlusive arterial disease, DM2=Diabetes Mellitus type 2. DTG*=DTG maintenance monotherapy. $ Timing of sample in hours after last DTG intake.

Patient	Study	Time to VF*	INSTI-RAMs detected at VF	INSTI-RAMs detected at baseline	Additional changes in integrase at VF compared to baseline
4	Main	4	No	No	VI32I, LS45L, T112A
6	Main	12	below limit of detection	No	-
10	Pilot	24	No	No	IV72V, LI101I
1	Main	30	No	No	A10D, D11E, N17S, L45Q, I50M, T111K, A112T, T124S, I135V, KR211K, I220L
2	Main	30	S230R	No	EV13E, LV45L
3	Main	36	below limit of detection	No	-
9	Pilot	42	E92Q, N155H	No	T112I
8	Main	48	No	No	G24GNDS, R111RK, A124AT, A229D, S283G
5	Main	60	R263K	No	ED10E, ED11E, L45I, F121FV, RK231K, LF234L
7	Main	72	N155H	No	D41G, T111A, S119R, N155H, M208I

Table 3. Virological characteristics and mutations in the integrase gene of HIV from patients with VF on dolutegravir maintenance monotherapy. VF=Virological Failure, DTG=dolutegravir, INSTI-RAMs=Integrase Strand Transfer Inhibitor – Resistance Associated Mutations. *In weeks after start DTG maintenance monotherapy.
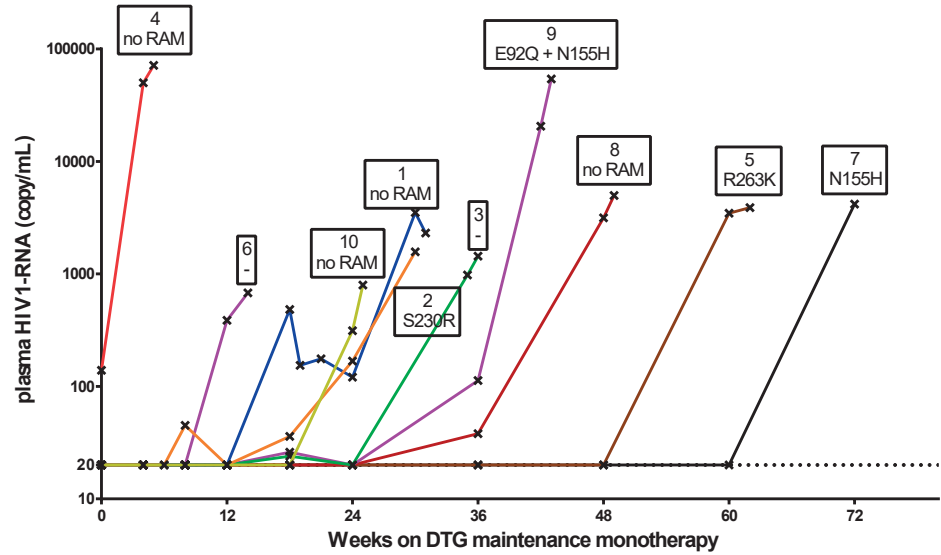

Figure 1. Overview of the course of the plasma HIV-RNA and INSTI-RAMs in all patients with VF. The dotted line represents the limit of quantification of plasma HIV-RNA (20 copy/mL). -=sequencing of integrase gene unsuccessful due to low plasma HIV-RNA level.

To investigate the role of an alternative resistance mechanism, we sequenced the nef gene, including the 3'-PPT and four 5' terminal bases of the 3'-LTR, because mutations in these sites can confer resistance to INSTIs.[18,19] No mutations were observed in the four 5' terminal bases of the 3'-LTR at baseline or at VF in any of the patients. In 9 of 10 patients, we did not detect changes in the 3'-PPT located in the nef gene. However, in patient 10, who did not develop INSTI-RAMs in the integrase gene, the wildtype G-stretch of the 3'-PPT was observed at baseline (i.e. GGGGGG), but at VF two changes were detected, i.e. GGG**A**G**C** (Figure 2).
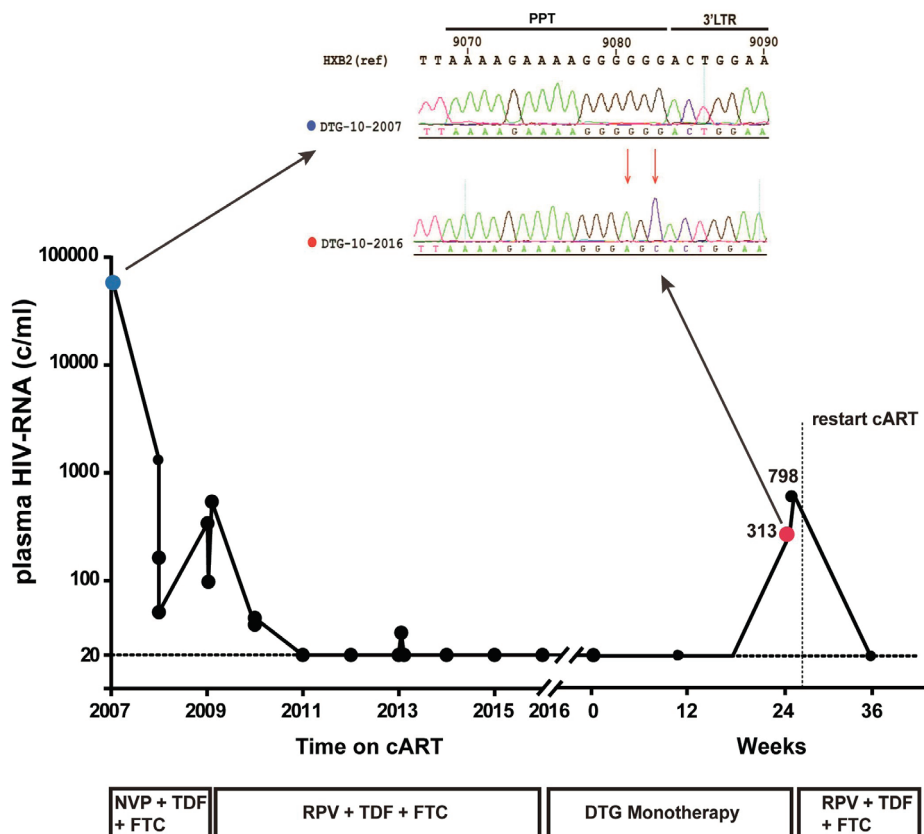

Figure 2. Changes in the G-stretch of the 3'-PPT in patient 10 at VF and course of plasma HIV-RNA. NVP=nevirapine, TDF=tenofovir disoproxil fumarate, FTC=emtricitabine, RPV=rilpivirine, DTG=dolutegravir. DTG-10-2007 is the sequence generated from HIV-RNA in plasma prior to cART initiation. DTG-10-2016 is the sequence generated from HIV-RNA in plasma at VF during DTG maintenance monotherapy. HXB2 is the sequence from the HXB2 reference strain.

Virological response after restart of cART

At the moment of confirmed VF, DTG maintenance monotherapy was stopped, and all patients restarted their previous cART regimen. The patients responded well to cART reinitiation, including the patient who restarted with dolutegravir, emtricitabine, and tenofovir. In all patients, plasma HIV-RNA levels declined to lower than 20 c/mL and remained lower than 20 c/mL for a mean of 39.7 weeks of follow-up.

DISCUSSION

In the DOMONO study, we recently showed that a higher proportion of patients treated with DTG maintenance monotherapy experienced VF, compared with patients who continued to receive cART.[17] In addition, 50% (2/4) of participants in the subsequent DOMONO pilot study, in which patients had a CD4 T-lymphocyte nadir of lower than 200 cells/mm^3, had VF. The same observation was previously made during protease inhibitor maintenance monotherapy.[22] Moreover, of the ten patients with VF in the main study and pilot study combined, we observed the emergence of INSTI-RAMs in four INSTI-naive patients: N155H (in patient 7), E92Q + N155H (in patient 9), R263K (in patient 5), and S230R (in patient 2). These results are in contrast with findings of observational cohorts, in which VF during DTG maintenance monotherapy often occurred in INSTI-experienced patients, in patients with previous VF during INSTI-containing therapy, and/or in patients with suboptimal adherence.[12–16] Only Blanco et al are reporting VF with emergence of INSTI-RAMs in INSTI-naive patients.[23] Emergence of the R263K mutation has previously been observed in the SAILING study in treatment-experienced, INSTI-naive patients who experienced VF on DTG-containing cART[6,24], and in the VIKING study, the N155H mutation emerged in patients with HIV resistant to RAL and/or EVG, who were treated with DTG-containing cART.[11,25] Furthermore, emergence of the N155H and E92Q+N155H was noted in the retrospective, observational Redomo-study in patients with VF on DTG maintenance monotherapy.[23] To our best knowledge, the S230R mutation has been described previously in one patient experiencing VF during DTG-containing cART.[26] Interestingly, development of the S230R mutation was observed in 1 of 5 HIV-1 infected humanized mice treated with DTG monotherapy. However, in that study, the S230R mutation was detected in combination with E138K, G140S, Q148H and N155H.[27] *In vitro*, the N155H, N155H + E92Q and the R263K mutation caused 1-2-, 2.5-, and 2-4-fold changes in the IC$_{50}$ of DTG and conferred only low-level resistance to DTG.[9,28,29] Pham et al. characterized the S230R substitution and showed that it resulted in a 4-fold change in IC$_{50}$ of DTG in tissue culture resulting in low level resistance to DTG.[30] When combining these results with our observations *in vivo*, it appears that single INSTI-RAMs that result in a relatively small fold increase in the IC$_{50}$ of DTG are sufficient to cause VF in patients receiving DTG maintenance monotherapy. In our study, plasma HIV-RNA levels were relatively

low in eight of the ten patients who experienced VF (lower than 1000 c/mL) compared with their pre-cART HIV-RNA zenith. This suggests that DTG still exhibited partial antiretroviral activity. It should be noted that our patients received DTG 50 mg once daily and that treatment with higher doses of DTG may be sufficient to overcome this partial resistance and fully suppress replication of these viruses.[11]

In four patients with VF, no known INSTI-RAMs were detected (patient 1, 4, 8, and 10). For these patients, we compared the pre-cART integrase sequence with the integrase sequence during VF and found amino acid changes at the following positions: E10, E11, S17, S24, V32, L45, M50, I72, L101, K111, T112, T124, I135, K211, I220, D229, and S283. Mutations at these positions occur at frequencies of more than 1% in INSTI-naive patients.[31] These mutations have not been shown to increase the IC_{50} of DTG *in vitro*,[31-33] although we cannot exclude that certain combinations of these mutations result in a decreased susceptibility to DTG. VF could also be explained by therapy non-adherence, but this seems unlikely because only therapy-adherent patients with no history of VF were included in our study, and DTG plasma-concentrations were therapeutic at the time of VF.

To find an explanation for VF, we searched for mutations outside of the integrase gene that may confer INSTI resistance in all ten patients. Therefore, we sequenced the 5' terminal bases of the U3 region of the LTR (i.e. ACTG), which are the substrate for integrase in the integration process.[18] Dicker et al showed with purified integrase enzymes and LTR duplexes that certain mutations in the four terminal bases of the LTR led to decreased binding of INSTI to integrase, while the strand-transfer activity of integrase remained relatively intact.[18] These mutations have not been reported in HIV culture experiments or *in vivo*. In this study, we did not detect changes in the ACTG motif of the 3'-LTR in patients with VF.

Simultaneously, we sequenced the 3'-PPT region adjacent to the 3'-LTR, because mutations in this region have been linked to INSTI resistance.[19] Malet et al recently showed in *in vitro* HIV culture experiments involving high doses of DTG that mutations located in the nef gene led to INSTI resistance.[19] The AàC mutation located 6 nucleotides upstream of the 3'-PPT motif in combination with four changes in the G-stretch of the 3'-PPT motif (GGGGGG à **GCAGTdel**), conferred high level resistance to DTG, RAL, and EVG. In our study, we detected mutations in the G-stretch of the 3'-PPT in the virus from patient 10 that were not present at baseline (GGGGGG à GGG**AGC**). Interestingly, a high DTG plasma-concentration was detected during VF in this patient, and the mutations in 3'-PPT described by Malet et al were also selected using high DTG concentrations *in vitro*. In addition, no known INSTI-RAMs in the integrase gene were detected in this patient.

The 3'-PPT is more than 99.9% conserved among HIV-1 sequences in the Los Alamos database, as it serves as hybridization site for the RNA primer for plus-strand DNA synthesis during reverse transcription. Removal of these RNA primers is pivotal because it defines the end of the linear proviral DNA for integration.[34] The GGGGGG à GGGAGC mutations in the 3'-PPT observed in patient 10 result in a glycine to alanine change in the Nef protein, and the mutations in the 3'-PPT described by Malet et al result in a truncated Nef protein. It seems unlikely, however, that the Nef protein plays a role in INSTI resistance, since there is hardly data that the Nef protein plays any role in the proviral integration process and because a previous study reported full susceptibility to RAL of Nef-deficient HIV.[35] The terminal 200–250 base pairs of each proviral DNA end are the primary protein binding sites of the HIV intasome.[36,37] Since the 3'-PPT in the unintegrated linear proviral DNA intermediate is located approximately 9000 base pairs from the 5'-LTR and approximately 800 base pairs from the end of the 3'-LTR, it is unlikely that the 3'-PPT interacts with the HIV intasome.

Notwithstanding the findings above, the 3'-PPT plays a major role in defining the proviral DNA end of the 5'-LTR. Previous studies on the reverse transcription process showed that mutations in the G-stretch of the 3'-PPT can lead to incomplete removal of the 3'-PPT RNA primer by RNAse H or to an alternative starting point for plus-strand DNA synthesis.[34,38-40] Both scenarios would lead to an altered end of the unintegrated linear proviral DNA intermediate at the 5'-LTR site. In addition, production of viral particles from unintegrated proviral DNA has been proposed. In patient 10, we observed the emergence of different mutations in the 3'-PPT, compared with the virus described by Malet et al. This indicates that other mutations in 3'-PPT may also cause INSTI resistance. However, experiments with site-directed mutants are needed to prove that the GGGGGG à GGGAGC mutations in 3'-PPT indeed cause INSTI resistance. In addition, further studies are needed to unravel the mechanism of INSTI resistance caused by mutations in 3'-PPT.

Still, for three patients we could not find a cause for virological failure. Of note is patient 4 who had adequate plasma DTG levels and already failed four weeks after start of DTG maintenance monotherapy with a plasma HIV-RNA level of 71.600 c/mL. Ultra-deep sequencing of plasma HIV-1 RNA or proviral DNA in peripheral blood mononuclear cells during VF might show minority variants that harbor RAMs in integrase or 3'-PPT, which could partially explain VF. Unfortunately, samples needed for these analyses were not available. VF in these patients might also be explained by yet another novel resistance pathway, which would require whole-virus genome sequencing at baseline and during VF, and phenotyping of the emerged mutations with site-directed mutants. In addition, VF in these patients could be explained by replication of HIV in sanctuary sites in which DTG does not penetrate with subsequent spillover of HIV to peripheral blood.

The origin of the viruses detected during VF in this study is currently unknown. These viruses might originate from ongoing low level replication in sanctuary sites during cART that are not detectable in plasma. However, Joos et al showed that after patients with long-term virological suppression (plasma HIV-1 RNA lower than 50 c/mL) stopping cART, the rebound viruses detected in plasma originate from reactivation of latently infected cells instead of viral lineages that continuously replicate at low levels.[41] Reactivation of HIV from latently infected cells is a stochastic process which occurs on average every 5–8 days, and depends on the size of the replication competent viral reservoir. This may also explain why two of the four patients who had a CD4 T-lymphocyte nadir lower than 200 cells/mm^3 and were included in the DOMONO pilot study, went on to have VF. Indeed, a low CD4 T-lymphocyte nadir is correlated with a larger viral reservoir.[42,43] Owing to the error-prone nature of HIV replication, minority populations with drug resistant mutants harboring single resistance mutations are most likely generated before therapy is initiated and persist in the proviral archive.[44,45] We hypothesize that the viruses detected during VF in this study originate from stochastic reactivation of a single cell harboring a provirus with a preexisting INSTI-RAMs. Reactivation of wild type virus from latently infected cells will not result in further rounds of replication, because these viruses are inhibited by DTG. However, reactivation of a virus carrying a single INSTI-RAM is not fully inhibited by DTG and further rounds of replication will lead to detectable HIV-RNA levels in plasma. This hypothesis is supported by the large variation observed in the timing to failure due to stochastic nature of reactivation of proviruses carrying a single INSTI-RAM.

In conclusion, the genetic barrier to DTG resistance is too low to justify DTG maintenance monotherapy, even in treatment-adherent patients with a relatively low HIV-RNA zenith and high CD4 T-lymphocyte nadir. The exact mechanism of VF in patients with long-term virological suppression who switch to DTG maintenance monotherapy is currently unclear, but we propose stochastic reactivation of a single cell harboring a provirus with a pre-existing INSTI-RAM as mechanism for VF. Mutations in the G-stretch of the 3-PPT region might confer an alternative DTG resistance pathway.

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SUPPLEMENTARY DATA CHAPTER 3

Primer	Sequence (5'-3')	Position according to HXB2*
Primers outer PCR		
5'INoutF1	ggAATCATTCAAgCACAACCAgA	4059-4081
3'INoutR2	TGTATGCAGACCCCAATATGTT	5262-5241
Primers nested PCR		
5'INinF1	TATCTggCATgggTACCAgCAC	4143-4164
3'INinR1	TAgTgggATgTgTACTTCTgAAC	5217-5195
Sequence primers		
KVL076	GCACAYAAAGGRATTGGAGGAAATGAAC	4161-4188
KVL082	GGVATTCCCTACAATCCCCAAAG	4647-4669
INT3	TTCGGGTTTATTACAG	4897-4912
INTSeq5	CTGGCTACATGAACTGCTAC	4470-4452
INT4	CTTGTATTACTACTGC	4986-4971
3'INinR1	TAgTgggATgTgTACTTCTgAAC	5217-5195

Table S1. Primers for integrase sequencing. * Genbank accession no. K034550.