Mutation Q95K enhances N155H-mediated integrase inhibitor resistance and improves viral replication capacity

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Received 21 April 2010; returned 10 June 2010; revised 6 July 2010; accepted 23 July 2010

Objectives: The genetic barrier to development of raltegravir resistance is considered to be low, requiring at least one primary integrase mutation: Y143C, Q148H/K/R or N155H to confer raltegravir therapy failure. However, during continued raltegravir treatment failure, additional mutations may be selected. In a patient failing raltegravir therapy, we investigated the impact of multiple integrase mutations on resistance and viral replication. Furthermore, in vivo fitness was investigated during failure of raltegravir-containing highly active antiretroviral therapy and after raltegravir was discontinued from the regimen.

Methods: Patient-derived viral integrase genes were cloned into a reference strain. These recombinant viruses were used to determine the contribution of individual integrase mutations to raltegravir resistance and replication capacity in vitro. To determine in vivo fitness, the relative proportion of specific integrase mutations was monitored over time by in-depth clonal analysis of the viral integrase at baseline, during and after raltegravir treatment.

Results: Raltegravir therapy failure was associated with the initial selection of primary resistance mutation N155H. This mutation conferred a 3.8-fold reduction in raltegravir susceptibility and a severe reduction in viral replication. Acquisition of integrase mutation Q95K increased resistance (6.2-fold) and partly restored viral replication. Selection of a third mutation, V151I, further increased raltegravir resistance (20-fold), but decreased viral replication. After prolonged raltegravir interruption, raltegravir resistance mutations were lost, demonstrating the reduced replication capacity of the resistant virus.

Conclusions: We describe selection of Q95K as a secondary resistance mutation during raltegravir therapy failure. In the background of N155H, Q95K enhances raltegravir and elvitegravir resistance and improves the impaired replication of the virus.

Keywords: HIV, secondary resistance mutation, treatment interruption, fitness

Introduction

Raltegravir, the first integrase inhibitor (INI) in clinical practice, is well tolerated and has potent antiviral activity.1,2 Virological failure in patients on raltegravir is often related to selection of mutations in the target gene. The genetic barrier towards the development of raltegravir resistance is considered to be low: a single mutation at codon 143, 148 or 155 of the viral integrase is sufficient to induce virological failure.1 However, during continued raltegravir therapy failure, additional substitutions in the viral integrase are selected.1,3 These secondary mutations may increase the level of raltegravir resistance and/or improve viral replication.3

In a patient who experienced continuous virological failure during raltegravir-containing therapy we performed an in-depth analysis. We describe the impact of selected integrase mutations on the evolution of INI resistance during raltegravir-based salvage therapy and after partial treatment interruption (PTI).

Materials and methods

The patient was a participant in the AIDS Therapy Evaluation Project Netherlands (ATHENA) observational cohort, which has been approved by the local and national institutional review boards.

Amplification, sequencing and cloning

Viral RNA was isolated from longitudinally obtained plasma samples. From viral RNA, amplicons containing the reverse transcriptase-integrase

Drug susceptibility analysis

MT4 cells, stably carrying the enhanced green fluorescent protein (eGFP) coding sequence under control of the HIV-1 LTR promoter (MT4-LTR-eGFP cells), were inoculated with virus at a multiplicity of infection (moi) of 0.001 in the presence of 3-fold dilutions of the INIs raltegravir and elvitegravir. Four replicate determinations were performed for each drug concentration. After 3 days, infection was quantified by UV microscopy measuring HIV Tat-induced eGFP expression. Using HIV-1, wild-type virus as reference, fold change values were calculated by dividing the mean 50% inhibitory concentration (IC50) for a recombinant virus by that for the IIIB reference strain. Repeat testing of RT-IN amplicons showed low inter-assay variability and high reproducibility.

Drug susceptibility and replication capacity of viral clones

Viral clones harbouring the mutations of interest were generated by site-directed mutagenesis on the patient-derived baseline sample. Mutations were introduced by amplifying the RT-IN fragment in the presence of a third primer harbouring the mutation of interest. Patient-derived baseline RT-IN genes with the desired mutation were then cloned into the Hxb2 backbone. Individual clones were tested for drug susceptibility and viral replication. Drug susceptibility was determined as described above. To determine viral replication, the amount of p24 of each viral clone was measured by ELISA (Aalto Bio Reagents, Dublin, Ireland). SupT1 cells (2.0 \times 10^6) were infected with an equivalent of 75 ng of p24 of each virus in the absence of raltegravir. All infections were performed in duplicate. Cultures were maintained for 14 days and once daily 300 \muL of cell-free virus supernatant was harvested for p24 analysis.

Quantification of viral populations

Amplified integrase genes (nucleotides 4143–5217) from viral isolates were cloned into a cloning vector using the pGEM®-T Easy Vector System (Promega, Leiden, The Netherlands). Minimum input in the PCR was 750 copies of RNA. Individual colonies were cultured and DNA was isolated using the Miniprep system. Integrase sequences were obtained using the method and primers as described.

Results

We studied a patient from our outpatient clinic experiencing virological failure during a raltegravir-containing antiretroviral regimen. This patient was heavily therapy experienced and harboured multiple protease inhibitor (PI), nucleoside reverse transcriptase inhibitor (NRTI) and non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance mutations in the viral genome [international AIDS society (IAS) defined resistance mutations, protease: L10I, V11I, K20I, M36I, K43T, M46I, ISV4, I62V, L63P, A71V, G73S, V82C, ISV4, L90M; reverse transcriptase: M41L, L74I/V, A98G, K103KN, M184V, L210W, T215Y and K219E].

In 2007 therapy was modified to a maintenance regimen containing zidovudine + lamivudine, tenofovir and raltegravir (through early access in the named patient programme from Merck). The genotypic sensitivity score (GSS) of this regimen was 1.5 based on the Stanford algorithm.

After brief virological suppression (plasma HIV-1 RNA <50 copies/mL) a viral rebound was observed. In an attempt to re-suppress viral load, tipranavir was added to the therapy regimen. Unfortunately, the HIV-1 RNA load further increased. Therapy failure was not associated with selection of additional resistance mutations in the viral protease (PR) or RT gene. We investigated whether the observed rise in HIV-1 RNA could be attributed to resistance to raltegravir by analysing longitudinal samples from the patient drawn before raltegravir treatment was initiated (sample #1, Figure 1a) and during therapy failure (samples #2–5, Figure 1a).

Raltegravir therapy failure was associated with the gradual accumulation of mutations in the viral integrase: initially the N155H mutation was detected in the quasispecies, followed by selection of Q95K and later V151I (Figure 1a). Viral populations from longitudinal samples were tested for raltegravir (and elvitegravir) susceptibility. In the sample in which key raltegravir resistance mutation N155H was first detected (sample #2 in Figure 1), the overall virus population was still susceptible to raltegravir. Two weeks later, viruses with the N155H substitution dominated the viral population. In addition, mutation Q95K appeared. The viral population with Q95K/N155H showed a 6-fold reduction in raltegravir susceptibility. Five weeks later the frequently reported secondary mutation V151I appeared. Ultimately, virus harbouring all three mutations (Q95K/V151I/N155H) dominated the viral population, which was associated with a further decrease in raltegravir (20-fold) and elvitegravir susceptibility (Figure 1b).

Drug susceptibility and replication capacity of site-directed mutants

Because of the presence of quasispecies we wanted to investigate the contribution of each individual mutation to resistance and replication. Therefore we generated site-directed mutants from patient-derived viruses. Raltegravir and elvitegravir susceptibilities of viral clones were concordant with the population data (Figure 2a). Mutations Q95K and V151I, individually or concurrently, did not result in a reduction in raltegravir susceptibility. The single mutant Q95K had no effect on viral replication, while V151I slightly lowered replication (data not shown). Primary raltegravir resistance mutation N155H resulted in a 3.8-fold reduction in raltegravir susceptibility and severely impaired viral replication (Figure 2b). Subsequent selection of Q95K (Q95K/N155H) resulted in a small but statistically significant (Student's t-test P=0.03) increase in raltegravir resistance to 6.2-fold and partially restored the decreased replication caused by N155H. Further addition of V151I reduced viral replication and resistance was increased to >20-fold, resulting in an overall higher fitness of the triple mutant (Q95K/V151I/N155H) in the presence of raltegravir.

Partial treatment interruption

To assess added value of raltegravir in a future salvage regimen, raltegravir therapy was interrupted while tipranavir and the NRTI
backbone were maintained. Although HIV-1 RNA had been stable for several months, interruption of raltegravir treatment resulted in a 0.8 log10 copies/mL increase in HIV-1 RNA concentrations (Figure 1a). Clonal analysis showed no changes in the viral integrase during the first 2 weeks of PTI (Figure 1a and c: samples #5 and #6, 18/18 and 18/18 clones analysed). One month after PTI, clonal analysis revealed a more heterogeneous population: 14/18 (77.8%) clones harboured all three resistance mutations, 1/18 (5.6%) was of intermediate nature (V151I/N155H) and 3/18 (16.7%) had baseline genotype without integrase resistance mutations (sample #7, Figure 1a and c). After 7 weeks of PTI (sample #8, Figure 1a and c), the viral population had completely returned to the baseline integrase genotype displaying full susceptibility to raltegravir. In an ultimate attempt to control viral replication it was decided to initiate mega highly active antiretroviral therapy (megaHAART) including raltegravir, maraviroc, tenofovir, emtricitabine, etravirine and fosamprenavir/ritonavir, resulting in complete viral suppression (<50 copies/mL).

Discussion
In the majority of cases raltegravir resistance develops via one of three pathways represented by the initial selection of Y143R, Q148H/K/R or N155H. These mutations result in raltegravir resistance but also impair viral replication. It has been shown that additional mutations can enhance resistance and/or restore viral replication.3,6 We identified Q95K as a secondary mutation that contributes to virological failure during raltegravir therapy via the N155H pathway. Q95K has been observed during elvitegravir in vitro...
selection, and during raltegravir failure in one patient of the BENCHMRK studies. It was demonstrated to confer only a 2.6-fold increase in IC50 of elvitegravir, whereas its contribution to raltegravir resistance was unknown.7,8

We show that Q95K alone has no effect on INI resistance or viral replication but enhances N155H-mediated resistance and partially restores the reduced replication caused by N155H. The clinical relevance of this mutation needs to be assessed in clinical cohorts, such as the European integrase resistance study group (CORONET).

The other secondary mutation, V151I, has been observed frequently in combination with N155H and has been reported to increase resistance.3 In our patient, selection of V151I also leads to an increase in resistance. Despite the fact that V151I slightly lowers viral replication, the overall in vivo fitness of the triple variant (Q95K/V151I/N155H) in the presence of raltegravir is improved since it rapidly dominates the viral population. In the absence of raltegravir, during PTI, the resistance mutations disappear suggesting a negative effect of these mutations on viral replication. This notion is in line with our in vitro experiments, demonstrating reduced viral replication of the resistant virus in the absence of raltegravir and is supported by previous observations during withdrawal of integrase inhibitors.9,10

In conclusion, selection of the integrase Q95K mutation contributes to viral escape during raltegravir therapy. It partly rescues the impaired viral replication caused by N155H and enhances raltegravir and elvitegravir resistance.

Acknowledgements
Part of this work was presented at the European HIV Drug Resistance Workshop in Sorrento, Italy, 2010 (Abstract no. 34).

Funding
The work was supported by the Dutch AIDS Fund (project number 2006028) and the European Union (grant number LSHP-CT-2007-037693).

Figure 2. Drug resistance and replication capacity of viral clones. All clones are identical (complete RT-IN inserts) to the patient-derived baseline clone except for the indicated integrase mutations. (a) Raltegravir and elvitegravir susceptibilities of individual clones. Values are averages of three separate experiments. *Due to poor infectious virus titre, experiments with this clone could not be repeated. (b) Replication capacities of the different site-directed mutants were determined by monitoring p24 production in the absence of raltegravir. All viruses were tested in duplicate. Error bars indicate the SEM.
Transparency declarations
Both A. M. J. W. and M. N. have received a research grant from Merck and M. N. has served as a consultant to Merck. All other authors have nothing to declare.

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