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Development of a phenotypic susceptibility assay for HIV-1 integrase inhibitors

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A B S T R A C T

Phenotypic resistance analysis is an indispensable method for determination of HIV-1 resistance and cross-resistance to novel drug compounds. Since integrase inhibitors are essential components of recent antiretroviral combination therapies, phenotypic resistance data, in conjunction with the corresponding genotypes, are needed for improving rules-based and data-driven tools for resistance prediction, such as HIV-Grade and geno2pheno$_{[integrase]}$. For generation of phenotypic resistance data to recent integrase inhibitors, a recombinant phenotypic integrase susceptibility assay was established. For validation purposes, the phenotypic resistance to raltegravir, elvitegravir and dolutegravir of nine subtype-B virus strains, isolated from integrase inhibitor-naïve and raltegravir-treated patients was determined. Genotypic resistance analysis identified four virus strains harbouring RAL resistance-associated mutations. Phenotypic resistance analysis was performed as follows. The HIV-1 integrase genes were cloned into a modified pNL4-3 vector and transfected into 293T cells for the generation of recombinant virus. The integrase-inhibitor susceptibility of the recombinant viruses was determined via an indicator cell line. While raltegravir resistance profiles presented a high cross-resistance to elvitegravir, dolutegravir maintained *in-vitro* activity in spite of the Y143R and N155H mutations, confirming the strong activity of dolutegravir against raltegravir-resistant viruses. Solely a Q148H + G140S variant presented reduced susceptibility to dolutegravir. In conclusion, our phenotypic susceptibility assay permits resistance analysis of the integrase gene of patient-derived viruses for integrase inhibitors by replication-competent recombinants. Thus, this assay can be used to analyze phenotypic drug resistance of integrase inhibitors *in vitro*. It provides the possibility to determine the impact of newly appearing mutational patterns to drug resistance of recent integrase inhibitors.

1. Introduction

Since HIV-1 requires lifelong treatment, it is highly desirable that antiretroviral drugs have the following characteristics: (1) High potency against viral replication, (2) good long-term tolerability, and (3) a high barrier to resistance. These characteristics are displayed by HIV-1 integrase inhibitors (INIs), the most recent class of antiretroviral drugs. Antiretroviral activity of INIs arises from their ability to inhibit the strand-transfer activity of HIV-1 integrase (IN), thus preventing the integration of proviral DNA into the host genome, which is an indispensable step in viral replication (Hazuda et al., 2000). Raltegravir (RAL), elvitegravir (EVG), and dolutegravir (DTG) are the currently available INIs.

In 2007, the first INI, RAL, was approved for the treatment of patients infected with HIV-1, followed by EVG in 2012. These first-generation INIs are highly efficacious in the treatment of HIV-1-infected subjects, but suffer from a low barrier to resistance, resulting in rapid emergence of resistance mutations (Geretti

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et al., 2012; Grobler and Hazuda, 2014; Quashie et al., 2013). Resistance to RAL is induced by three clearly identified major resistance pathways, involving primary/major mutations at the IN residues Y143, Q148, and N155, respectively. Since primary/major resistance mutations for EVG include T66I, E92Q, Q148H/R/K, and N155H, RAL and EVG present broad cross resistance (Blanco et al., 2011; Garrido et al., 2012; Geretti et al., 2012; McColl et al., 2007; Sichtig et al., 2009). In contrast, pathways leading to primary resistance to DTG have not yet been identified, but likely involve the accumulation of multiple mutations (Kobayashi et al., 2011).

DTG is a second-generation INI that was approved in 2014 for use in treatment-naïve and treatment-experienced patients, including INI-experienced patients. Compared to RAL and EVG, DTG has a higher resistance barrier since resistance is only slowly selected *in vitro* and has not emerged in studies of therapy-naïve patients, up to date (Llibre et al., 2015; Raffi et al., 2013; Wainberg et al., 2013; White et al., 2014). Antiviral activity of DTG in spite of resistance to RAL and EVG has been shown *in vitro*, with only limited cross resistance to RAL and EVG resistant viruses (Seki et al., 2015; Underwood et al., 2012; Van Wesenbeeck et al., 2011). This has been confirmed in clinical studies with patients harbouring RAL-resistant HIV-1 strains (Arribas et al., 2010; Eron et al., 2010). Nevertheless, decreased susceptibility to DTG is shown by isolates with a major INI-resistance mutation at residue Q148 and additional minor INI-resistance mutations (Abram et al., 2013; Underwood et al., 2012). DTG is highly efficacious in treatment-naïve and treatment-experienced patients, regardless of previous therapies. In the clinic, this has led to an increase in INI use in first and second-line therapies. Some mutations selected *in vitro* and *in vivo* are potentially involved in resistance to DTG, e.g. mutations at residues G118, F121, E138, S153, and R263 (Kobayashi et al., 2011; Quashie et al., 2012). However, their impact on DTG resistance and cross-resistance to other INIs remains unclear.

Resistance development is characterized by the appearance of a major resistance mutation followed by the accumulation of resistance mutations that further increase drug resistance or restore viral replication capacity. Thus, it is important to investigate the complete mutation patterns of HIV-1 IN and not only single resistance mutations. We therefore developed a phenotypic IN susceptibility assay that allows for assessment of resistance and cross-resistance to INIs in patient-derived viruses.

We will use our newly developed phenotypic resistance assay for generating genotype-phenotype pairs, which will in turn be used for analysing INI resistance in different HIV-1 strains. Specifically, we want to produce genotype-phenotype pairs that are independent of controlled clinical studies and use them for characterization of INI-resistance mutations that are associated with therapy failure. Furthermore, this data will be used for improving the interpretation of HIV-1 genotypes with respect to resistance to INIs. Genotype-phenotype pairs can be used for identifying complex resistance patterns and for the improvement of the bioinformatics tool geno2pheno$_{[integrase]}$, which offers freely available phenotype-based INI-resistance predictions. geno2pheno$_{[integrase]}$ version 2.0 was trained on 285 RAL- and 228 EVG genotype-phenotype pairs from the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu). These genotype-phenotype pairs originate from controlled clinical studies and were predominantly generated with commercial phenotypic assays.

2. Materials and methods

2.1. Materials

2.1.1. Viral samples

Subtype-B viral samples were obtained from patients of the RESINA cohort (BMG: IIA5-2013-2514AUK375). Analysis of INI resistance was performed on nine plasma samples harbouring viruses with different genotypic integrase profiles. Five samples were obtained of patients without INI treatment history harbouring viruses presenting genotypically none of the known INI resistance mutations (#1-5) as negative control. For phenotypic determination of INI resistance, four plasma samples of patients after RAL-therapy failure harbouring viruses with described RAL resistance mutations (#6-9) were analysed. Subtyping was performed with the COMET HIV-1 subtyping tool, version 0.2 (http://comet.retrovirology.lu/).

2.1.2. Integrase inhibitors

RAL was kindly provided by Merck. EVG and DTG were purchased from Selleckchem (pure substances).

2.2. Methods

We performed genotypic and phenotypic INI-resistance analyses on the IN gene region of nine patient-derived HIV-1 subtype-B virus strains. For this purpose, we considered RAL, EVG, and DTG. The samples were selected according to their genotypic resistance profile.

2.2.1. Genotypic INI-resistance analysis

For the selection of convenient plasma samples for the validation of the phenotypic susceptibility assay for INIs, genotypic resistance analysis of the integrase gene region was performed as previously described (Sichtig et al., 2009). Mutations in each IN sequence were determined by comparison to *HXB2*, an HIV-1 subtype-B reference strain. The prediction of genotypic resistance of the viruses against RAL, and cross-resistance against EVG and DTG and the scored INI resistance-associated mutations were defined according to published data (reviewed in (Blanco et al., 2011; Quashie et al., 2013)) and the Stanford Drug Resistance Database (http://hivdb.stanford.edu).

2.2.2. Vector design

We cloned the HIV-1 IN region of each viral isolate into the HIV-1 complete-genome vector pNL4-3 (GenBank accession no. AF324493 constructed by (Adachi et al., 1986)). For this purpose, the vector had to be modified to pNL4-3ΔIN. The EcoRI restriction site at nucleotide (nt) positions 5743–5748 and the NcoI site at nt positions 10,565–10,570 were deleted. Furthermore, we inserted an EcoRI site at nt positions 4171–4176 (*pol* gene) and an NcoI site at nt position 5098–5103 (Fig. 1a). We used site-directed mutagenesis for inserting the EcoRI and the NcoI sites and for deleting the EcoRI at nt positions 5743–5748 (see primers in Table 1), in accordance with the manufacturer's protocol (QuickChangeTM Site Directed Mutagenesis Kit, Stratagene). For deleting the NcoI site at nt postion 10565 in pNL4-3, we digested the vector with the NcoI enzyme, subsequently filling up the 5′-overlapping single-stranded DNA via Klenow fragment and religating the vector. In addition to deleting the NcoI site, this resulted in a 4 nt insertion (CATG).

2.2.3. IN amplification and purification

Viral RNA from 1000 μl plasma was isolated automatically using the MagNA Pure Compact Nucleic Acid Isolation Kit I and the MagNA Pure^{TM} Compact System (Roche Diagnostics), according to the manufacturer's instructions. cDNA of the RNA was generated using the SuperScript III RT Kit (Invitrogen) with the primer 5220as (Table 1). Amplification of the integrase gene was performed via nested PCR with the HotStarTaq-Polymerase (Qiagen), according to the company's protocol. The first PCR was performed with the outer primers 4140 s and 5220as and the second PCR was performed with the inner primers 4166MfeI and 5077PciI (Table 1). These primers are

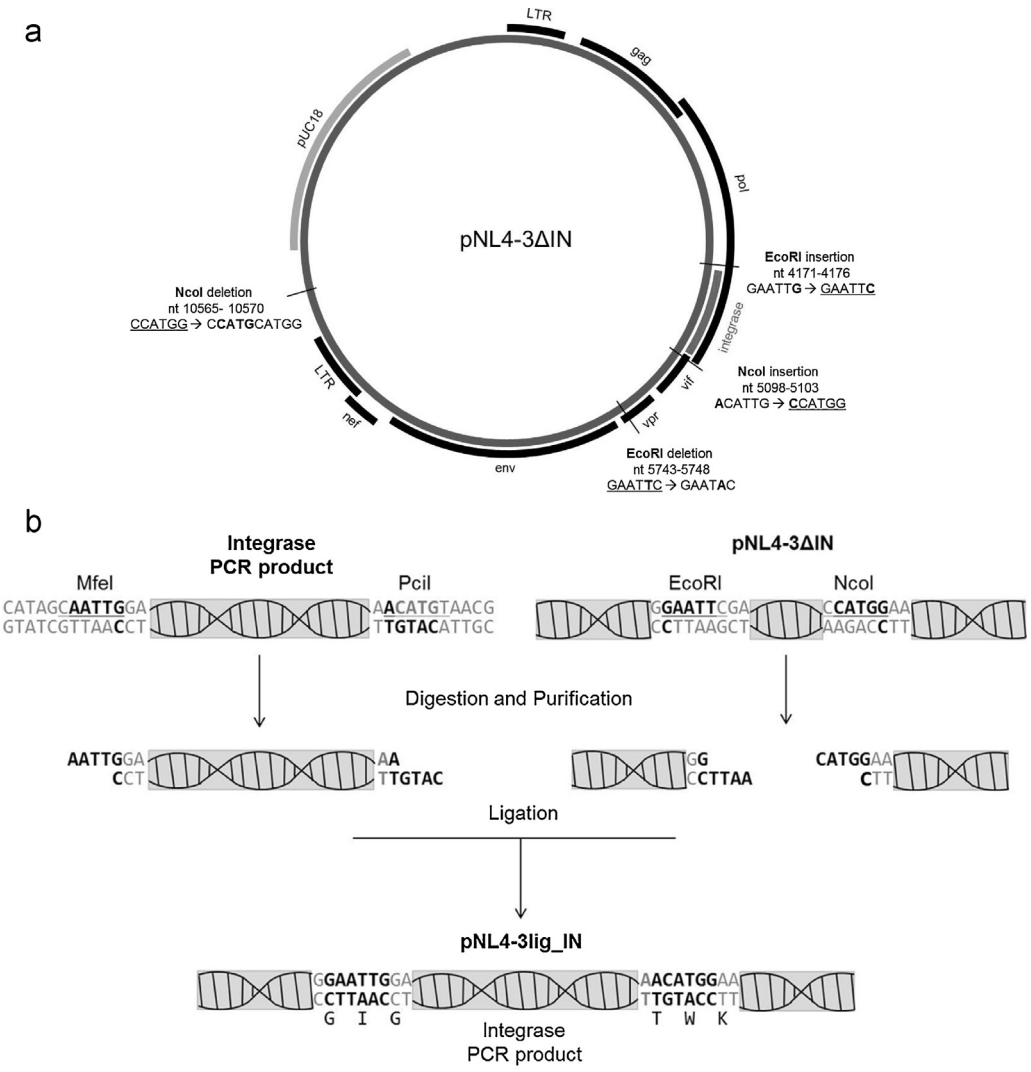

Fig. 1. (a) Schematic representation of the pNL4-3ΔIN construct.
The EcoRI restriction site at position 5743–5748 and the NcoI restriction site at position 10565–10570 were deleted, while an EcoRI (4171–4175) and an NcoI (5098–5103) restriction site flanking the integrase gene were introduced. The nucleotide positions highlighted in bold were exchanged by site-directed mutagenesis. (b) Schematic representation of the cloning of the amplified IN gene into pNL4-3ΔIN to NL4-3lig_IN. The IN PCR amplicons were flanked with the recognition sites for MfeI and PciI, which overhangs are compatible cohesive to the EcoRI and NcoI sites of pNL4-3ΔIN allowing a ligation without produced amino acid exchanges. The nucleotides highlighted in bold present the restriction overhangs generated by the different enzymes.

Table 1
Oligonucleotides used for vector preparation, IN cloning and IN sequencing.

Primer	Sequence 5′ → 3′	Position (NL4-3)	Application
t5747a-s	AAGCCATAATAAGAATACTGCAACAACTGCTGTTTATCCATTTC	5731 ⟶ 5774	EcoRI-Deletion
t5747a-as	GATAAACAGCAGTTGTTGCAGTATTCTTATTATGGCTTCCACTC	5725 ⟵ 5768	EcoRI-Deletion
g4176c-s	GTACCAGCACACAAAGGAATTCGAGGAAATGAACAAG	4155 ⟶ 4191	EcoRI-Insertion
g4176c-as	CTTGTTCATTTCCTCGAATTCCTTTGTGTGCTGGTAC	4155 ⟵ 4191	EcoRI-Insertion
a5098c-s	CAAGTAGACAGGATGAGGATTAACCCATGGAAAAGATTAGTAAAACA	5074 ⟶ 5120	NcoI-Insertion
a5098c-as	TGTTTTACTAATCTTTTCCATGGGTTAATCCTCATCCTGTCTACTTG	5120 ⟵ 5174	NcoI-Insertion
5′-INT	ATTGGAGGAAATGAACAAGT	4173 ⟶ 4192	PCR
3p31	ATCCTGTCTACYTGCCACACAA	5066 ⟵ 5087	PCR
4140s	GTCTACCTGGCATGGGTACCAGCAC	4140 ⟶ 4164	PCR
5220as	CCCTAGTGGGATGTGTACTTCTGA	5197 ⟵ 5220	PCR
4166MfeI	CAAAGCAATTGGAGGAAATGAACAAGT	4166 ⟶ 4192	PCR
5077PciI	CGTTACATGTTCTAATCCTCATCCTGTCTAC	5077 ⟶ 5107	PCR

flanked with restriction sites for MfeI and PciI which have compatible cohesive ends to the restriction sites EcoRI and NcoI, allowing a successful cloning of the integrase gene into pNL4-3ΔIN (Fig. 1b). Thermal cycling of both PCRs consisted of an activation step at 95 °C for 15 min. The first PCR consisted of 35 cycles. Each cycle spent 30s at 95 °C, 30s at 58 °C and 2 min at 72 °C. The second PCR was a touchdown PCR comprising 45 cycles of 30s at 95 °C, 45s at 65° down to 58 °C in 4 cycles and 3 min at 72 °C. Both PCRs had a final elongation step for 10 min at 72 °C.

The 911 bp PCR amplicons were purified (QIAquick PCR Purification Kit, Qiagen) and quantified.

2.2.4. Cloning strategy

In order to produce pNL4-3ΔIN vectors for ligation with IN PCR-products, the restriction sites of the modified pNL4-3 vector were digested with the endonucleases EcoRI and NcoI for 6 h at 37 °C. Subsequently, the NL4-3ΔIN-fragment (13902 bp) was purified by gel extraction.

In order to enhance the ligation efficiency of IN PCR-products (IN-MfeI/PciI) and the pNL4-3ΔIN vector, we used the pDrive cloning vector (Qiagen) in an intermediate step. Specifically, 100 ng of the purified IN PCR product and 1 μl of the pDrive cloning vector were ligated and transformed into competent JM109 cells. After cultivation and plasmid preparation, the DNA clones were verified by restriction analysis and sequencing. The integrase genes of the respective clones were cleaved using the framed endonucleases MfeI and PciI and extracted with a gel. 20 ng of the extracted fragments and 60 ng of pNL4-3ΔIN (ratio 1:5) were used for the ligation reaction (15 h at 15 °C). After transformation into JM109 cells, plasmid DNA was isolated and controlled by restriction analysis with MfeI with expected fragment sizes of 1747, 3930, and 9151 bp. All positive clones were analysed by sequencing in order to verify the mutations. For the generation of a wildtype reference strain pNL4-3lig_IN, the integrase gene of pNL4-3 was amplified and ligated in into pNL4-3ΔIN, using exactly the same procedure applied to the patients' virus variants. Note that neither the insertion nor the deletion restriction sites changed the amino acid sequence of the pNL4-3lig_IN after cloning, since compatible restriction sites were used for ligating the vector and the insert, thus restoring the original amino acid sequence of the NL4-3 backbone (Fig. 1b).

2.2.5. Cultivation of recombinant virus

For the generation of recombinant virus, the plasmid DNA (10 μg), which contained the recombinant virus genome, was transfected into 293T cells (1.5 million cells per plate), using the SuperFect transfection reagent (Qiagen), in accordance with the manufacturer's protocol. Recombinant viral particles were generated by the transfected 293T cells and were harvested from the supernatant two days after transfection. For this purpose, the supernatant was centrifuged at 1500 rpm for 5 min, aliquoted and stored at −80 °C. In order to generate high-titer infectious-virus supernatants, 2 ml of the 293T supernatants were used to infect 10 ml CEMx174 cells (100.000 cells/mL) and cultured for 3–5 days until a cytopathic effect (CPE) was visible. Supernatant aliquots were stored at −80 °C. The IN genotype of the cultivated viruses was verified by sequencing analysis. Due to the differential replication capacity of each recombinant strain, virus titers varied between samples. For this reason, phenotypic susceptibility was tested with sample-specific supernatant volumes. These volumes were determined with a virus titration assay. Specifically, the indicator cell line CEMx174-SIV-SEAP (Means et al., 1997) was infected with 1, 5, 10, or 20 μl of the virus stock as, described by Walter et al. (Walter et al., 1999). In CEMx174-SIV-SEAP cells, HIV-1 Tat protein transactivates the stably integrated SEAP reporter construct, which leads to a strong increase in SEAP activity. This activity can be measured in the supernatant using the Phospha-Light™ SEAP (Secreted Placental Alkaline Phosphatase) Reporter Gene Assay System (Applied Biosystems, Part Number T1015) three days after infection. Equivalent supernatant volumes were determined by normalization with respect to 20.000 relative light units (RLU). The equivalent supernatant volume for each sample was used as the input for the susceptibility assay.

2.2.6. Susceptibility assay

Walter et al., 1999; describes a procedure for phenotypic resistance analysis (Fig. 2). (Walter et al., 1999). Since this procedure had only been established for NRTIs, NNRTIs, and PIs, it was adapted in order to perform INI resistance and cross resistance analysis of the recombinant clones, as follows. The INIs were used in a serial dilution with a dilution factor of 3. The concentrations of RAL, EVG, and DTG ranged between 0 and 10 μM, 0–3 μM, and 0–0.1 μM, respectively. Equivalent supernatant volumes, as calculated by the virus titration assay, were used to determine the viral susceptibility to INIs in a 96-well format. For this purpose, CEMx174-SIV-SEAP cells were infected in triplicate with the calculated 20.000 RLU dose of the recombinant virus clones in the presence of the respective drug concentrations. For standardisation, the susceptibility of the reference strain NL4-3lig_IN to the different INIs was determined on every 96-well plate, additionally.

The replicative capacity of the recombinant viruses was determined three days after infection by measuring the SEAP activity of the virus cultures and of the reference virus. Using the decline of RLUs in the serial drug dilutions, the mean 50% inhibitory drug concentration (IC_{50}) was determined. The fold change (FC) values were calculated by dividing the determined mean IC_{50} value of a recombinant virus clone by that for the reference strain NL4-3lig_IN.

2.2.7. Analysis

The correlation of the determined FC values measured in the established phenotypic susceptibility assay and the predicted FC values by geno2pheno$_{[integrase]}$ was calculated with Pearson's correlation coefficient (R) (http://www.socscistatistics.com/tests/pearson/Default2.aspx).

3. Results

3.1. Genotypic resistance analysis

For evaluating the phenotypic resistance test for INIs, nine different patient-derived subtype-B virus strains with and without RAL resistance-associated mutations were analysed. The selection of the viruses was based on the integrase genotypes which were analysed in the context of routine diagnostics of resistance testing.

Three of the viruses isolated from RAL-experienced patients harboured mutations conferring resistance to RAL *in vivo*, including the primary mutation N155H combined with the secondary mutation G163R (#7), the primary mutation Y143R associated with the secondary mutations T97A and E138K (#8), and the primary mutation Q148H with the secondary mutation G140S (#9). The virus strain #6 presented only the secondary RAL-resistance mutation V151I (Table 2). The genotyped viruses #1-5 presented none of the described RAL-resistance mutations.

3.2. Phenotypic resistance analysis

After characterization of the IN genotypes, the integrase region of the selected viruses was amplified and cloned into the modified pNL4-3 vector (pNL4-3ΔIN) for generating recombinant viruses in cell culture. Sample-specific volumes of virus supernatants (20.000 RLU dose determined via virus titration assay) were used for the measurement of virus replication under drug exposure by means of the susceptibility assay. A schematic overview of the IN susceptibility assay is pictured in Fig. 2.

The determined IC_{50} values of the five HIV-1 clones without genotypic resistance to all INIs (#1-5) were predominately comparable to those of the reference strain NL4-3lig_IN, with the exception of clone #2 (Table 3, Fig. 3). This wildtype strain (#2) presented unexpected susceptibility results to EVG and DTG. The mean IC_{50}-value for EVG was 23.78 nM (range 3.32–4.40) and for DTG 2.89 nM (range 1.41–1.94), with a mean calculated fold change (FC) of 3.78 and 1.59, respectively. As these increased fold changes are most probably a result of a reduced replication capacity of the recombinant virus and not of an initial reduced susceptibility to the drugs, clone #2 was excluded of the analysis. The mean IC_{50}-values

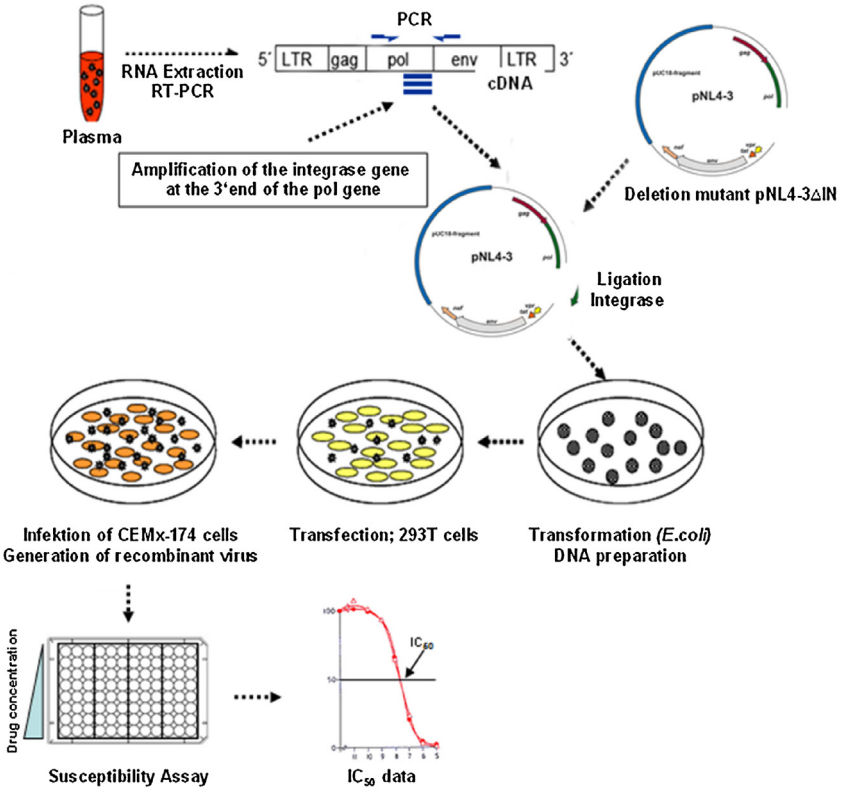

Fig. 2. Schematic representation of the phenotypic IN resistance assay.
IN RT-PCR products were cloned into a modified IN-deleted pNL4-3 backbone. Recombinant plasmid DNA was transfected and viruses were cultivated. Finally, viral replication was measured with a reporter gene assay.

Table 2
Substitutions of RAL-treated patients analysed for phenotypic INI-susceptibility.

Sample	Substitutions (relative to the reference strain HXB2)
#1–4773	D10E, K14R, R20 K, A23 V, V31I, V72I, G123S, A124T, R127 K, T206S, N232D, L234G, N254Q
#2–4867	D3G, D10E, E11D, R20 K, V31I, M50I, L101I, T112I, G123S, A124T, R127 K, K136Q, G163E, G193E,S230N, N232D
#3–7459	D10E, K14R, R20 K, V31I, P90S, S119G, T122I, G123S, A124T, T125A, R127 K, K156N, V201I, T206S, D229N, S230N, N232E, S283G
#4–7480	D10E, K14R, V72I, A91E, L101I, T112A, S119R, G123S, A124N, R127 K, K156N, S195C, N232D, M275L
#5–307	D10E, E11D, V31I, V72I, L101I, K111T, S119P, G123S, A124T, R127 K, F181Y, K219N, N222 K, N232D
#6–4939	D10E, I113 V, G123S, A124T, R127 K, V151I, N232D, L234V
#7–4833	D10E, S17N, V31I, V72I, V75A, T112 V, G123S, A124N, T125A, V126L, R127 K, **N155H**, G163R, S230N, N232D, D256E, D278G
#8–5150	D10E, S17N, M22I, A23 V, L28I, L45 V, L68 V, V72I, T97A, L101I, S119T,G123S, A124T, R127 K, E138 K, **Y143R**, N232D, D256E
#9–6834	D10E, S17N, L28I, S39C, L45 V, V72I, I113L, G123S, A124N, T125A, R127 K, G140S, **Q148H**, G163Q, V201I, N232D, V259I, K266R, S283G

Mutations highlighted bold: Primary RAL resistance-associated mutations.
Mutations underlined: Secondary RAL resistance-associated mutations.

for the remaining four strains without defined RAL resistance mutations was 9.83 nM for RAL (range 8.23–13.53 nM), compared with 6.31 nM for EVG (range 4.00–9.55 nM), and only 1.97 nM for DTG (range 1.67–2.14 nM). Their calculated mean fold changes (FC) for RAL ranged from 1.38 to 2.12, for EVG from 0.64 to 1.52, and for DTG from 0.92–1.18.

The IC_{50} values and FCs of clone #6, harbouring only the minor RAL resistance mutation V151I, were comparable to the wild-type values for all INIs with a mean FCs of 1.97 for RAL (range 1.20–2.86), 0.95 for EVG (range 0.92–0.98) and 1.31 for DTG (range 1.23–1.35) (Table 3).

The clones harbouring RAL resistance mutations, #7, #8, and #9, revealed higher IC_{50} and FC values for RAL and EVG compared to the wildtype strains, as expected (Table 3; Fig. 4). Regarding DTG, the RAL-resistant clones #7 and #8 presented no reduction in susceptibility (mean FCs of 1.3 and 0.6, respectively). Overall, clone #9 (Q148H + G140S) presented the highest resistance against RAL and EVG (FC > 200), and also reduced sensitivity to DTG (mean FC 3.44; range 3.13–3.73).

For the determination of the IC_{50} values and the calculation of the FC values, the phenotypic analyses were performed in triplicate, independently (see standard deviation). For proving our data, the calculated FC values were compared to predicted FC values obtained with geno2pheno$_{[integrase]}$ (Table 3). There is a strong positive correlation between measured and predicted FC values. The Pearson's correlation coefficients (R) were 0.98 for RAL and 1.00 for EVG. As this phenotype-based interpretation tool only offers predictions for RAL and EVG, virtual determination of FC values for DTG was not possible.

4. Discussion

HIV integrase inhibitors are potent antiretroviral drugs that efficiently decrease viral load in HIV-1 infected patients. Since 2007,

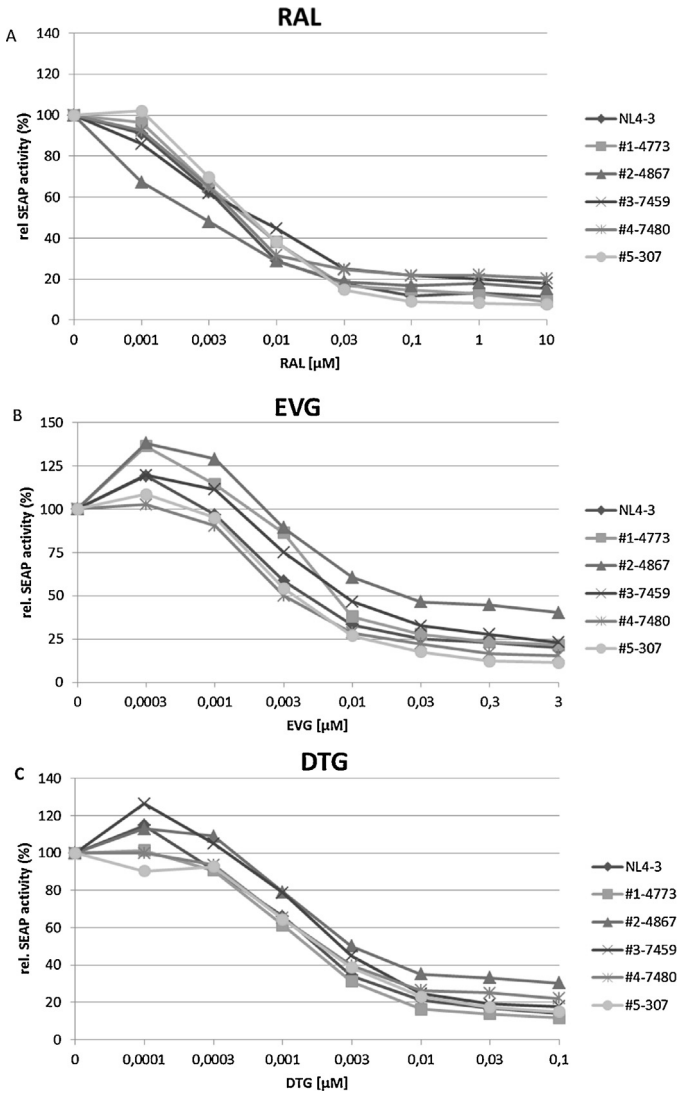

Fig. 3. Relative SEAP activity of the HIV-1 clones #1-5 without described RAL-associated mutations in the presence of the integrase inhibitors RAL, EVG and DTG. Relative SEAP (Secreted Placental Alkaline Phosphatase-gene) activity of the HIV-1 subtype B clones #1-5 in the presence of increasing concentrations of A: raltegravir (RAL), B: elvitegravir (EVG) and C: dolutegravir (DTG).

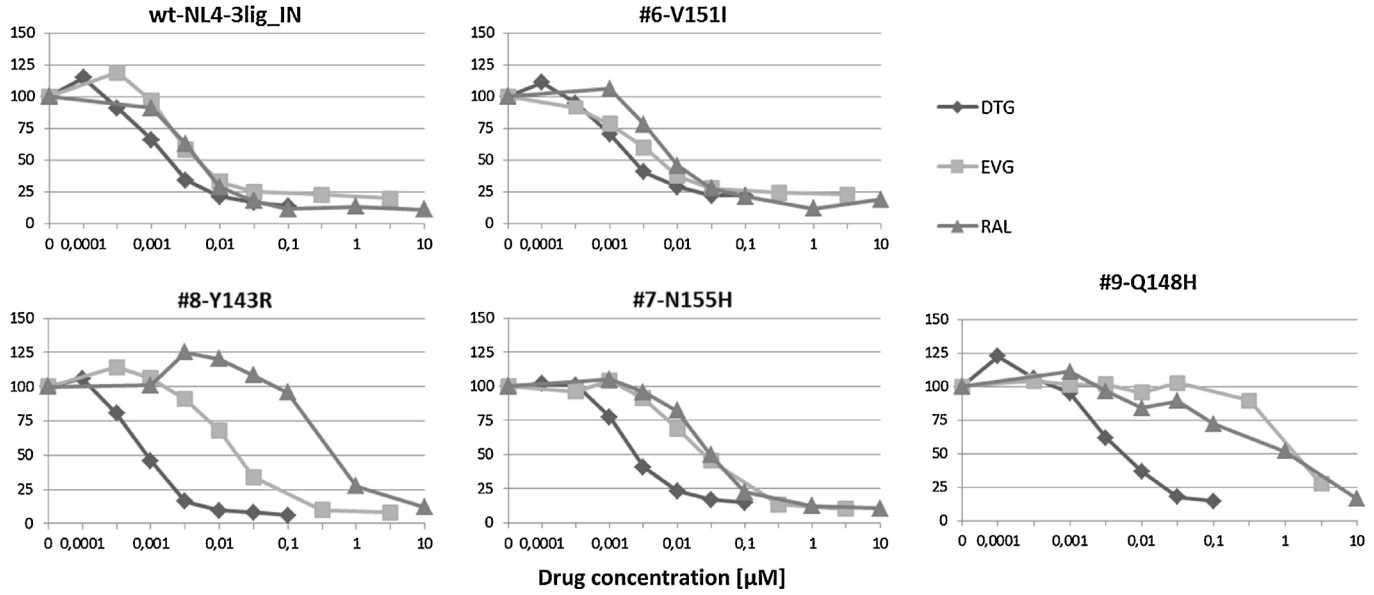

Fig. 4. Relative SEAP activity of the HIV-1 clones #6-9 harbouring described RAL-associated mutations in the presence of the integrase inhibitors RAL, EVG and DTG.

Table 3
Determined IC_{50}-values and calculated fold changes against INIs.

Sample	RAL					EVG				DTG				
	RAMs		IC_{50}	FC			IC_{50}	FC				IC_{50}	FC	
	primary	**secondary**	**[nM]**	**mean**	**± SD**	**Range**	**g2p**	**[nM]**	**mean**	**± SD**	**Range**	**g2p**	**[nM]**	**mean**	**± SD**	**Range**
NL4-3lig_IN	Ø	Ø	5.6	1.0	–	–	1.7	6.3	1.0	–	–	1.9	1.8	1.0	–	–
#1–4773	Ø	Ø	8.23	1.38	0.88	0.48–2.24	0.8	7.27	1.15	0.34	0.90–1.55	1.3	1.67	0.92	0.14	0.78–1.06
#2–4867	Ø	Ø	4.16	0.77	0.68	0.06–1.41	0.8	23.78	3.78	0.56	3.32–4.40	0.8	2.89	1.59	0.30	1.41–1.94
#3–7459	Ø	Ø	13.53	2.12	1.48	0.70–3.66	3.4	9.55	1.52	0.69	0.80–2.18	0.9	2.06	1.14	0.16	0.97–1.29
#4–7480	Ø	Ø	9.29	1.63	0.94	0.86–2.67	1.9	4.00	0.64	0.40	0.36–1.09	1.5	2.02	1.12	0.29	0.88–1.44
#5–307	Ø	Ø	8.25	1.53	0.94	0.45–2.21	1	4.42	0.70	0.24	0.44–0.93	1.8	2.14	1.18	0.17	1.00–1.35
#6–4393	Ø	V151I	10.30	1.97	0.84	1.20–2.86	1.7	5.97	0.95	0.04	0.92–0.98	1.9	2.37	1.31	0.06	1.23–1.35
#7–4833	N155H	G163R	37.31	7.00	3.01	3.52–8.81	30.9	28.69	4.56	1.90	2.98–6.66	41.6	2.40	1.32	0.30	0.99–1.56
#8–5150	Y143R	T97A. E138 K	695.56	145.75	72.91	96.71–229.54	562	20.23	3.21	0.59	2.54–3.63	2.1	1.03	0.57	0.35	0.32–0.96
#9–6834	Q148H	G140S	1499.96	231.50	132.15	133.27–381.74	622.5	2005.30	318.61	80.07	265.37–410.69	2784.5	6.24	3.44	0.30	3.13–3.73

RAM: resistance-associated mutation; RAL: Raltegravir; EVG: Elvitegravir; DTG: Dolutegravir; FC: fold change; SD: standard deviation; g2p: geno2pheno$_{[integrase]}$; light grey: excluded from the analysis due to insufficient amount of RLUs for reliable phenotypic resistance data.

the INI RAL is a successful component of antiretroviral therapies. However, single mutations in the integrase are sufficient to confer high-level resistance (reviewed in (Metifiot et al., 2010)), characterizing RAL as a drug with a low genetic barrier to resistance. This characteristic led to a large increase of INI-resistant viruses in HIV-1 infected patients, due to the emergence of INI-resistant viruses upon therapy failure, but also due to the transmission of INI resistant viruses (Boyd et al., 2011; Hurt, 2011; Young et al., 2011). With the approval of the INIs EVG and DTG in the recent years, two further potent INIs are available. However, the sequential use of RAL and EVG is not recommended, due to the high level of cross-resistance to these compounds (Garrido et al., 2012). Thus, currently only DTG can be used in a combination therapy after RAL or EVG have failed, since DTG is characterized to be widely active against HIV-1 variants isolated from RAL-treated patients (Underwood et al., 2012).

We devised a recombinant susceptibility assay, which facilitates the investigation of the complete integrase gene region. This will permit the analysis of genotypic and phenotypic resistance and cross-resistance to the different INIs and the identification of currently insufficiently characterized resistance profiles of the recent INIs, available or under development.

After successful cloning and cultivation of recombinant virus with representative mutations for RAL-naive and RAL-experienced viruses, the different clones were analysed for their susceptibility against RAL, EVG, and DTG. The virus strains without detected resistance mutations demonstrated FCs predominantly comparable to the wild-type reference strain NL4-3lig_IN for RAL, EVG and DTG. The viruses harbouring described RAL-resistance mutations presented reduced susceptibility to the different INIs according to their mutation pattern. The primary RAL resistance mutation N155 and Q148 resulted in high resistance to RAL and EVG, while the primary RAL mutation Y143 just influenced the RAL susceptibility, proving the low impact of the Y143R on EVG and DTG resistance (Metifiot et al., 2011; Underwood et al., 2012). The determined FCs of the analysed virus strains harbouring different RAL-specific mutation patterns in our phenotypic susceptibility assay are comparable to other *in-vitro* data (Canducci et al., 2011; Van Wesenbeeck et al., 2011). Beside the Y143R mutation, DTG also maintained activity to the isolate with the N155H mutations. Thus, the activity of DTG was only reduced for the virus strain carrying the Q148H mutation in combination with a secondary RAL-resistance mutation, as also reported by other *in vitro* studies (Underwood et al., 2012).

The phenomenon of the determination of high IC_{50} values of recombinant clones for specific drugs despite of genotypic fully susceptibility, as determined for the recombinant wildtype clone #2, can most probably be attributed to a reduced replication capacity. Generally, reduced replication capacity can be observed in resistant variants but was also in susceptible isolates down to 65% compared to the reference strain depending on the analysed drug (De Luca et al., 2007; Walter et al., 2002). Replication reduced virus variants need higher amounts of input virus and show unexpected high IC_{50} values in our assay system. The virus titration assay calculates the virus input inducing 20.000 RLUs 3 days after infection. As replication competent viruses finalize at least two replication cycles in 3 days, replication reduced variants achieve less. Due to the reduced replication capacity a higher amount of virus supernatant is required to gain a reporter gene expression comparable to the reference strain. It contains more infectious virus particles and also more natural secreted tat protein, the inducer of the SEAP reporter gene, which lead to an incomplete inhibition of the virus replication (Xiao et al., 2000). The variation of the IC_{50} values between the different antiretroviral drugs is drug dependent (De Luca et al., 2007). These data illustrate the limitations of phenotypic susceptibility testing by the use of recombinant viruses, as other regions outside the integrase gene region play a role in determining viral fitness.

There is a significant influence of *pol* fragments on the replication capacity of HIV-1; the conditions for the phenotypic resistance analysis of chimeric viruses are complex (Brumme et al., 2011; Iordanskiy et al., 2010). This limitation was also observed during our first attempt to generate chimeric viruses of non-B integrase genes in the pNL4-3 subtype-B backbone, as these recombinants were not able to generate a sufficient number of recombinant particles as required by the susceptibility assay.

Overall, the determined IC_{50} values of the wild type strains were 5-fold and 3.2-fold higher for RAL and EVG compared to DTG, confirming the low DTG drug concentrations needed for the inhibition of viral replication (Seki et al., 2015). The intrinsic variability of our phenotypic IN susceptibility assay is comparable to the reverse-transcriptase and protease inhibitor assay described by Walter et al. (Walter et al., 1999).

Mean FC values for RAL and EVG measured by our phenotypic susceptibility assay are strongly positively correlated to FC values predicted by geno2pheno$_{[integrase]}$. Similar results were observed for a phenotypic drug susceptibility assay for HIV protease and reverse transcriptase which was compared to predicted values produced with a genotype-based phenotyping algorithm (Pattery et al., 2012; Van Houtte et al., 2009).

Phenotypic resistance testing is time consuming and fails to produce (accurate) results in a variety of settings, e.g. low viral load of the plasma samples and reduced fitness of the generated recombinants. However, it is an invaluable tool for resistance determination in patients who harbour viruses with complex genetic patterns or for drugs with insufficiently characterized mutational resistance profiles.

In order to improve the INI-resistance predictions by geno2pheno$_{[integrase]}$, more genotype-phenotype pairs are required. In this context, it is especially important that independent data describing phenotypic resistance after therapy failure in routine clinical practice be generated. Furthermore, the generation of resistance data for DTG and other, investigational INIs will allow geno2pheno$_{[integrase]}$ to offer predictions for these drugs. Last but not least, the generation of a sufficient amount of phenotypic resistance data for INIs will allow for the robust determination of clinically-relevant cutoffs for the fold-change in drug resistance.

In conclusion, the established phenotypic INI resistance assay permits resistance analysis of the complete IN gene of patient-derived viruses for INIs. Thus, the assay can be used for the analysis of newly appearing mutational resistance patterns of recent INIs providing resistance data for interpretation system geno2pheno$_{[integrase]}$ in future.

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