Primary mutations selected \textit{in vitro} with raltegravir confer large fold changes in susceptibility to first-generation integrase inhibitors, but minor fold changes to inhibitors with second-generation resistance profiles

Olivia Goethals\textsuperscript{a}, Ann Vos\textsuperscript{a}, Marcia Van Ginderen\textsuperscript{a}, Peggy Geluykens\textsuperscript{a}, Veerle Smits\textsuperscript{a}, Dominique Schols\textsuperscript{b}, Kurt Hertogs\textsuperscript{a}, Reginald Clayton\textsuperscript{a,*}

\textsuperscript{a} Tibotec Virco Virology BVBA, Gen De Wittelaan L 118 3, 2800 Mechelen, Belgium
\textsuperscript{b} Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroederstraat 10, 3000 Leuven, Belgium

**A B S T R A C T**

Emergence of resistance to raltegravir reduces its treatment efficacy in HIV-1-infected patients. To delineate the effect of resistance mutations on viral susceptibility to integrase inhibitors, in vitro resistance selections with raltegravir and with MK-2048, an integrase inhibitor with a second-generation-like resistance profile, were performed. Mutation Q148R arose in four out of six raltegravir-selected resistant viruses. In addition, mutations Q148K and N155H were selected. In the same time frame, no mutations were selected with MK-2048. Q148H/K/R and N155H conferred resistance to raltegravir, but only minor changes in susceptibility to MK-2048. V54I, a previously unreported mutation, selected with raltegravir, was identified as a possible compensation mutation. Mechanisms by which N155H, Q148H/K/R, Y143R and E92Q confer resistance are proposed based on a structural model of integrase. These data improve the understanding of resistance against raltegravir and cross-resistance to MK-2048 and other integrase inhibitors, which will aid in the discovery of second-generation integrase inhibitors.

© 2010 Elsevier Inc. All rights reserved.

**Introduction**

The human immunodeficiency virus (HIV) causes acquired immunodeficiency syndrome (AIDS). Infection with HIV remains a significant burden on global human health, with an estimated 33.4 million people currently living with HIV and 2.7 million new infections reported in 2008 (WHO. Aids epidemic update. 2009).

The viral enzymes reverse transcriptase, protease and integrase (IN) are essential for the HIV replication cycle and are encoded by the pol gene. HIV-1 IN, a 32 kDa enzyme (288 amino acids), comprises three distinct functional domains. The N-terminal domain (amino acids 1–50) is believed to be involved in protein multimerization and contains an H-H-C-C zinc finger-like motif, which coordinates zinc. The catalytic core domain (amino acids 51–212) contains the catalytic triad of acidic residues D64, D116 and E152 comprising a highly conserved DD-35-E motif that likely coordinates two divalent metal ions, probably magnesium, and is required for catalytic activity (Asante-Appiah and Skalka, 1997; Esposito and Craigie, 1999). The C-terminal domain (amino acids 213–288) has DNA-binding activity and is therefore thought to play a role in binding to viral and host DNA.

During the integration process, IN recognizes specific sequences in the long terminal repeats (LTRs) of the viral genome and binds to the viral DNA in preparation for catalytic activity. When DNA-bound, the IN enzyme removes a GT dinucleotide, adjacent to a conserved 3′ CA sequence, from each viral cDNA 3′-end in a process termed 3′-processing. The 3′-processed DNA product, as part of the preintegration-complex, is then trafficked to the nucleus and imported through the nuclear envelope for subsequent IN-facilitated strand transfer into the cellular chromosomal DNA. Following strand transfer, the gaps in the DNA are likely annealed by host DNA repair enzymes, and the proviral DNA is established within the genomic DNA of the infected cell (Asante-Appiah and Skalka, 1997; Engelman et al., 1991; Esposito and Craigie, 1999).

With the possible exception of the V(d)J polynucleotide transferase RAG1, there is no likely mammalian homologue for IN (Meleka et al., 2002). Also, IN is essential for viral infectivity (LaFemina et al., 1992; Sakai et al., 1993) and the IN sequence in the pol gene is highly conserved among HIV-1 clinical isolates (Cannon et al., 1994; Ceccherini-Silberstein et al., 2009; Reinke et al., 2001). Therefore, IN has remained an attractive target for antiretroviral therapy over the last decade (Pommier et al., 2005; Witvrouw et al., 2004). Recent progress has resulted in the IN inhibitors (INIs) elvitegravir (EVG) and raltegravir (RAL), with EVG reaching late stage clinical trials (Sato et al., 2006) and RAL approved for use in treatment-experienced patients (Cahn and Sued, 2007; Grinsztejn et al., 2007) and very
Recently in treatment-naive patients (Klein and Struble, 2009). Also, in an effort to design novel second-generation INIs, a series of compounds was optimized resulting in two INIs, MK-2048 and compound G (Cmpd G), with improved resistance profiles compared with the first-generation INIs (Vacca et al., 2007).

The use of combinations of antiretroviral drugs in highly active antiretroviral therapy (HAART) has proven remarkably effective in controlling the progression of HIV disease and prolonging survival (Palella et al., 1998; Richman, 2001), but the efficacy of regimens can be compromised by the development of resistance (DeGruttola et al., 2000; Ledergerber et al., 1999; Richman, 2006). All approved antiretroviral drugs have elicited resistance mutations, and the search for next-generation inhibitors with increased efficacy, superior resistance profiles, higher genetic barriers to resistance development and improved safety profiles in all classes of antiretrovirals, is a current focus of the pharmaceutical industry and other research institutes. Following clinical validation of IN as a target for antiretroviral therapeutic intervention (DeJesus et al., 2006; Grinsztejn et al., 2007), INIs are expected to become frequently used in HAART, and the corresponding resistance mutations will result in a clear requirement for second generation INIs to maintain the efficacy of regimens containing an INI component. Currently, emerging data from clinical studies of RAL and EVG elucidate relationships between certain mutations and the loss of efficacy of INIs (Cooper et al., 2008; McColl et al., 2007). In order to establish the requirements of a second generation INI, many factors, including resistance profile, genetic barrier, safety profile, dosing, and necessity for boosting must be considered. A comprehensive understanding of the resistance profile of first-generation INIs, the pathways employed by the virus to circumvent inhibition, and the degree of cross-resistance to other INIs will therefore enable evaluation of investigational compounds as potential second-generation INIs.

RAL showed excellent therapeutic efficacy in patients infected with HIV-1, including treatment-naive HIV-1-infected patients (Marlowitz et al., 2006) and patients with multidrug-resistant HIV-1 and a history of treatment failure (Grinsztejn et al., 2007; Steigbigel et al., 2008). Resistance to RAL has been investigated in vitro (Kobayashi et al., 2008; Wai et al., 2007) and in vivo (Charpentier et al., 2008; Cooper et al., 2008; Malet et al., 2008). A resistant virus with genotype E138A/G140A/Q148K was selected in vitro by Wai et al. (2007). N155H and Q148K/R pathways with additional mutations were reported by Kobayashi et al. (2008). However, virologic failure in patients on RAL treatment was generally associated with a mutation at one of the three residues Y143, Q148 or N155 (Cooper et al., 2008). Other clinical studies highlighted the selection of additional minor resistance mutations including E92Q, E92A/T66A and E157Q (Charpentier et al., 2008; Malet et al., 2008). Secondary mutations described in the Q148H/K/R pathway include L74M+ E138A, E138K, or G140S. Mutations associated with the N155H pathway include L74M, E92Q, T97A, E92Q + T97A, Y143H, G163K/R, V151I, or D232N. The Y143R/H/C mutation seems uncommon (Miller et al., 2008).

Here, we report the results from a series of in-vitro resistance selection (IVRS) experiments with RAL in parallel with MK-2048 and Cmpd G, and the profiling of the selected and recombinant viruses containing mutants from this and other resistance studies (Goethals et al., 2008; Kobayashi et al., 2008) to delineate the contribution of each mutation to the susceptibility to RAL, MK-2048, Cmpd G and a panel of diverse INIs.

Results

Selection of HIV-1 strains resistant to RAL

A parallel IVRS methodology in 96-well plates (Goethals et al., 2008) was used to select six strains with reduced susceptibility to RAL.

Table 1

<table>
<thead>
<tr>
<th>Passage Selected mutations</th>
<th>Mutations post-reculture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus 1 43 V54I E138K G140A Q148R</td>
<td>V54I E138K G140A Q148R</td>
</tr>
<tr>
<td>Virus 2 38 E138K G140A Q148R</td>
<td>E138K G140A Q148R</td>
</tr>
<tr>
<td>Virus 3 19 E138K G140A Q148K</td>
<td>E138K G140A Q148K</td>
</tr>
<tr>
<td>Virus 4 51 L63I L74M A128T E138K Q148R V151I</td>
<td>L63I L74M A128T E138K Q148R V151I</td>
</tr>
<tr>
<td>Virus 5 33 L74M E92Q V151I N155H</td>
<td>L74M E92Q V151I N155H</td>
</tr>
<tr>
<td>Virus 6 19 E138E/K Q148R</td>
<td>E138E/K Q148R</td>
</tr>
</tbody>
</table>

* Sequencing results are reported as amino acid changes compared with HIV-1 (HXB2D) wild type reference sequence. Mutations present in more than 25% of the global virus population were detected as a mixture with the wild-type virus.

The drug-resistant strains were selected by serial passage of HIV-1 IIIB in the presence of increasing concentrations of RAL. When the viruses were propagated at a final concentration of 6 µM, a concentration 600 times higher than the EC50 of RAL, viruses were genotyped to identify mutations. One selected strain required 19 passages to accumulate mutations enabling replication in the presence of 6 µM RAL, where other strains required 33, 38, 43 or 51 passages.

Six parallel IVRS experiments elucidating different resistance pathways (Table 1) were performed. Viruses with primary mutations Q148R, Q148K or N155H were selected. Four out of six viruses selected the Q148R mutation. A new mutation V54I was selected as an additional mutation in a virus containing the Q148R mutation.

In parallel with RAL, viruses were propagated in the presence of increasing concentrations of MK-2048 or Cmpd G (Table 2). After 41 passages, one virus was selected with 1.6 µM of MK-2048 and one with 1 µM of Cmpd G. Although both viruses were propagated at a concentration 500 times higher than the EC50 of their respective

Table 2

<table>
<thead>
<tr>
<th>Selection with Passage Selected mutations</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus 7 MK-2048</td>
<td>G140E/G</td>
</tr>
<tr>
<td>Virus 8 Cmpd G</td>
<td>no mutations selected</td>
</tr>
</tbody>
</table>

* Sequencing results are reported as amino acid changes compared with HIV-1 (HXB2D) wild type reference sequence. Mutations present in more than 25% of the global virus population were detected as a mixture with the wild-type virus.

Fig. 1. Cross-resistance of six different RAL-selected HIV-1 strains. The viruses found in the IVRS experiments with RAL were tested for susceptibility to RAL and other INIs. Relative changes in susceptibility of the viruses compared with wild-type IIIB virus are displayed on the graph.
selecting compound, no mutations could be selected. Only a mixture of a mutant and wildtype, i.e. G140E/G in virus 7, was observed which was selected with 1.5 µM MK-2048.

**Phenotypic (cross)-resistance of the RAL-selected HIV-1 strains to a panel of INIs**

All six RAL-selected HIV-1 strains were re-cultured and the antiviral activity of RAL was determined. In parallel, the susceptibility of the selected HIV strains to a panel of INIs, namely EVG, PACA, L-870,810, PICA and two INIs with a second-generation resistance profile, MK-2048 and Cmpd G, was determined to establish the degree of cross-resistance (Fig. 1). The re-cultured viruses were genotyped to confirm that mutations had been preserved throughout the reculturing process (Table 1). All primary mutations were maintained in the six selected viruses after reculture, except in virus 6, were the Q148R mutation reverted to a mixture Q148Q/R.

In comparison with wild-type virus, viruses 1 to 5 showed large reductions in susceptibility to RAL, EVG, PACA and L-870,810 (>240-fold) (Fig. 1). Virus 6 (E138E/K Q148Q/R) was more susceptible
to RAL, EVG, PACA and L-870,810 (susceptibility reduction between 11- and 65-fold) than viruses 1 to 5. Viruses 1 and 2, two viruses harboring the primary mutation Q148R and additional mutations G140A, E138K or V54I, showed greatly reduced susceptibility to MK-2048 (370- and 140-fold, respectively), with more moderate reductions in susceptibility to Cmpd G (85- and 25-fold, respectively). Virus 1 differs from virus 2 by only one extra mutation V54I, but confers a higher reduction in susceptibility to all INIs than virus 2, except to PACA, were a similar reduction was observed. Virus 3 (E138K G140A Q148K S230R), virus 4 (L63I L74M A128T E138K Q148R V151I), and virus 5 (L74M E92Q V151I N155H E157Q) were 50 to 70-fold less susceptible to MK-2048 and 5- to 15-fold to Cmpd G. Virus 6 (E138E/K Q148Q/R) displayed wild-type susceptibility to MK-2048 and Cmpd G. Viruses 1 to 5 conferred a 30-fold reduction in susceptibility to PICA, where virus 6 showed only 6-fold reduction in susceptibility to PICA (Fig. 1).

Effect of the site-directed mutants on the susceptibility of HIV-1 (HXB2D) to different INIs

Mutations selected in this study and reported in previous studies from this lab and others, including clinical data (Cooper et al., 2008; McColl et al., 2007), were incorporated in a wild-type HIV-1 backbone (HXB2D) by site-directed mutagenesis, and susceptibility of the resulting viruses to each INI was determined (Fig. 2). Specifically, the Q148R, the Q148K and the N155H mutation, all with their additional mutations selected during IVRS experiments, and the clinically observed mutations N155S, T66I, Q148H, G140S, and Y143C were included. Twenty-three combinations of single, double, triple or quadruple mutations were tested for susceptibility to RAL and to other INIs (Fig. 2). AZT, which was used as a control antiviral, showed EC50 values for the IN mutants that was consistent with the EC50 for wild-type virus, indicating full susceptibility to the reverse transcriptase inhibitor.

Small reductions (~6-fold) in virus susceptibility to EVG, RAL, or any other INI used in this study was observed when the impact of a singular V54I, L74M, A128T, E138K, G140A/S, V151I, E157Q or S230R mutation was tested (Fig. 2A). The mutations G140A/S and E138K have been previously observed accompanying one of the primary resistance mutations Q148K/H/R and are considered as secondary mutations (Nakahara et al., 2009).

The T66I mutation, while conferring 25-fold reduced susceptibility to EVG, caused only 1- to 6-fold reduction in susceptibility to the other INIs (Fig. 2A). Y143C, a resistance associated mutation (RAM) of RAL (Cooper et al., 2008), conferred a 4-fold reduction in susceptibility to RAL, but did not reduce susceptibility to the other tested INIs (~3-fold). The single mutations E92Q, N155H/S and Q148K/R, which are considered as RAMs of RAL or EVG (Cooper et al., 2008; McColl et al., 2007), conferred low to moderately reduced susceptibility to RAL and PICA (~14-fold) but moderately to highly reduced susceptibility to EVG, PACA and L-870,810 (~6- to 90-fold). Q148H confers a 6-fold reduction in susceptibility to RAL and a 5-fold reduction to L-870,810 and PACA, but wildtype susceptibility to the other INIs. The mutations Q148R and E92Q showed wild-type susceptibility to MK-2048 and Cmpd G, while the mutation N155H conferred 6- and 4-fold reduced susceptibility to respectively MK-2048 and Cmpd G (Fig. 2A).

Compared with the effect of Q148R and G140A on their own, combining both mutations in a common backbone resulted in a large decrease in susceptibility (7- to 564-fold) to the INI panel (Fig. 2B). The triple mutant Q148R/G140A/E138K conferred similar reductions in susceptibility as the double mutant Q148R/G140A to most of the members of the INI panel, but led to a decreased susceptibility to Cmpd G. The mutation V54I had no additional effect on virus susceptibility to the tested INIs, when added to the triple mutant Q148R/G140A/E138K.

The mutation Q148H on its own conferred between 1- and 6-fold decreases in susceptibility to the panel of INIs, while addition of the G140S mutation led to a further decrease in susceptibility between 3-

Fig. 3. Replication kinetics of recombinant viruses. MT4 cells were infected with recombinant viruses with mutations at the 148 locus, at an MOI of 0.0001. Cells were determined daily in cell-free supernatant. Experiments were performed in duplicate.

Fig. 4. Three-dimensional docking model of HIV-1 IN with RAL

RAL was docked into the model of Chen et al. (2008) and it adopted a binding mode, common to other potent inhibitors, sharing four key elements (Fig. 4):

1) The inhibitor-binding site exists only upon 3′-processing of viral DNA, removing a GT dinucleotide;
2) The hydrophobic tail (benzyl moiety) binds in a putative hydrophobic pocket formed mostly by the flexible active-site loop (site 1);
3) The polar moiety chelates only the Mg2+ coordinated by D116 and E92 (site 3).
4) A polar interaction between the inhibitor and a triad motif consisting of G65, H67 and E92 (site 3).
suggesting a potentially higher genetic barrier to resistance development than selected with MK-2048 or Cmpd G during the same time frame, different mutations were selected with RAL, no mutations were selected. This might explain why Y143R was not selected. Although the substitution Q148R (CAA/G to CGA/G) requires only one nucleotide change, the Q148 and N155 were highlighted in the model. All residues clustered proximal to the catalytic triad represented by the DDE-motif of IN. Q148 and Y143 are located in the flexible loop (site 1), where the benzylo moiety of RAL is situated. The glutamic acid at position 92 is located at site 3.

Discussion

Here we report the results from IVRS studies with RAL and the effects of selected mutations on the virus susceptibility to RAL and on cross-resistance to other first-generation INIs and to the second-generation INIs MK-2048 and Cmpd G. In parallel with the RAL IVS studies, resistance selection was performed with MK-2048 and Cmpd G.

RAL failure is associated with IN mutations in at least three distinct genetic pathways defined by two or more mutations including a signature mutation at Q148H/K/R, N155H, or Y143R/H/C, and one or more additional minor mutations (Cooper et al., 2008; Johnson et al., 2008). The existence of several INI resistance pathways is similar to what has been described for other antiretroviral classes, such as nucleoside-, and non-nucleoside reverse transcriptase inhibitors or protease inhibitors (Clavel and Hance, 2004). However, the determinants of the evolution toward these different profiles are unknown. They could be related to different factors such as naturally occurring polymorphisms in IN (Lataille et al., 2007), pharmacokinetic factors such as RAL trough levels (Winslow et al., 1996), or sequences of other proteins like reverse transcriptase, which may interact with IN (Wu et al., 1999; Zhu et al., 2004).

Here, distinct resistance pathways were selected with RAL, i.e. the Q148R, the Q148K and the N155H pathways. Some mutations may develop less readily than others, for example, the substitution Y143R requires at least two nucleotide changes (TAC to CGC), while the substitution Q148R (CAA/G to CGA/G) requires only one nucleotide change. This might explain why Y143R was not selected. Although different mutations were selected with RAL, no mutations were selected with MK-2048 or Cmpd G during the same time frame, suggesting a potentially higher genetic barrier to resistance development and possible second-generation resistance profiles of the two investigational compounds MK-2048 and Cmpd G.

When testing the RAL-selected viruses for susceptibility to a panel of INIs, high-level cross-resistance between RAL, EVG, PACA and L-870,810 was observed. The similarity between the pharmacophores of these compounds suggests a similar binding mode, which might explain the similar changes in virus susceptibility to those compounds. Less cross-resistance was observed between RAL and MK-2048, Cmpd G, or PICA, where PICA has a different pharmacophore compared with RAL. The pharmacophore of PICA is a pyrido hydroxamic acid moiety, while the pharmacophore of RAL is a diketo amide. It is expected that both pharmacophores share a similar binding mode at the catalytic triad, albeit with small differences in their geometry. This variability in both pharmacophores may explain the difference in susceptibility and the difference in potency of the compounds. But although the change in susceptibility compared with wild type of the most heavily mutated viruses was several times lower for PICA than for RAL, the lower potency and higher toxicity of PICA likely precluded its further development. Less cross-resistance was also seen between RAL and MK-2048 or Cmpd G in line with previous observations (Vacca et al., 2007; Wai et al., 2007), highlighting their potential as second-generation INIs.

For the first time, V54I is reported as an emergence selected by RAL in vitro. Comparison of the two RAL-selected viruses, virus 1 (Q148R G140A E138K V54I) and virus 2 (Q148R G140A E138K), differing from each other only by the mutation V54I, showed that virus 1 conferred a slightly higher reduction in susceptibility to all INIs, except PACA, compared with virus 2, which does not harbor V54I.

To test the individual or the combined effect of the selected mutations on viral susceptibility to the INI panel, single, double, triple and quadruple mutations were constructed in a wild-type HIV-1 backbone via site-directed mutagenesis. In addition, the mutations N155S, T66I, Q148H, G140S, Y143C selected by RAL (Cooper et al., 2008) or EVG (McColl et al., 2007) in the clinic, were included in the analysis.

The IN mutation T66I, previously identified as a resistance-associated mutation of EVG (Goethals et al., 2008), has also been selected in vitro with S-1360 (Fikkert et al., 2004), L-708,906 (Fikkert et al., 2003) and L-731,988 (Hazuda et al., 2000), but is not considered as a resistance mutation of RAL, as confirmed in our study.

E92Q has been previously selected with EVG (Goethals et al., 2008), and is considered as a RAM for EVG. Hombrouck et al. (2008) selected E92Q during IVRS studies with the naphthyridine L-870,810, and Kobayashi et al. (2008) selected E92Q with EVG, S-1360 and L-870,810. E92Q has been associated occasionally with failure in patients on RAL treatment as an additional minor mutation of RAL accompanying the primary N155H mutation (Johnson et al., 2008; Malet et al., 2008). Therefore, it will be preferably for a second generation INI to effectively inhibit the N155H/E92Q virus.

Mutation Y143C, although associated with virologic failure in patients treated with RAL, was not selected by our IVRS studies with RAL. Y143C seems to be exclusively associated with low level of resistance to RAL, but remains uncommon.

Mutation N155S was previously selected in vitro by a diketo acid analog (Hazuda et al., 2004) and by S-1360 (Kobayashi et al., 2008). Only a 4-fold reduction in susceptibility to RAL was conferred by N155S, where in comparison the N155H mutant showed a 12-fold reduction in susceptibility to RAL. Thus, the change of the amino acid at position 155 from a serine to a histidine would increase resistance to RAL. The N155H mutation is one of the major pathways selected with RAL or with EVG in vivo (Cooper et al., 2008; McColl et al., 2007). Here we observe N155H as the only primary mutation that confers, as a single mutation, a reduction in susceptibility to Cmpd G and MK-2048, although the reduction is minor (4- and 6-fold respectively).

Another major pathway selected with RAL or EVG involves the mutation of the glutamine at position 148 to a lysine, a histidine or an arginine. Because Q148H is the most common mutation selected with
RAL in the clinic (Johnson et al., 2008), it will be essential for the next-generation compounds to inhibit the viruses with this mutation. We observed that Q148H alone retained wild-type susceptibility to MK-2048 and Cmpd G, highlighting their potential as second generation inhibitors.

In our study, the additional effect of G140A in combination with Q148R caused an additional decrease in susceptibility to the INIs and a reduction in replication capacity compared with the recombinant virus with the Q148R mutation alone.

Delelis et al. (2009) demonstrated that in the Q148H/G140S mutant virus, the G140S rescued the replication deficiency of the Q148H mutant, while the mutation Q148H is responsible for resistance to RAL. Fransen et al. (2008) found that Q148H and G140S in combination reduced susceptibility to RAL much more than either mutation alone. We also observed a large decrease in susceptibility to all INIs due to the addition of G140S to Q148H and we speculate that Q148H/G140S would evolve from the Q148H virus, due to both a recovery of replicative capacity and a decrease in susceptibility. G140S in combination with the Q148K mutation had only a limited additional effect on the susceptibility to most INIs. Fransen et al. (2008) even observed a suppressed resistance conferred by Q148K and G140S together compared with Q148K alone. Nakahara et al. (2009) have reported an additional 2-fold increase in susceptibility to 5–1360 when adding the G140S mutation to Q148K. Moreover, a reduced viral fitness of the Q148K virus compared with wild-type virus was reported, where the fitness was partially recovered by addition of the secondary mutation G140S to Q148K. The addition of E138K to the mutant viruses Q148K/G140S, Q148H/G140S or Q148R/G140A did not confer increased resistance against the INIs, but E138K did partly compensate the reduced replication of the mutant virus Q148R/G140A.

The addition of V54I to the Q148R/G140A/E138K recombinant virus did not confer further resistance to RAL or to the other INIs, but slightly improved the replication capacity of the triple mutant virus. To our knowledge, V54I has not been identified as a polymorphism (Lataillade et al., 2007) and due to the better replication capacity of the recombinant virus with V54I, we suggest it is a compensatory mutation resulting from IVRS with RAL.

To better understand the influence of the found mutations on inhibitory activity, RAL was docked into the model of Chen et al. (2008). The mutations, associated with resistance to RAL, i.e. E92Q Y143R/C, Q148R/H/C and N155S/H are highlighted in the model. All primary mutations clustered proximal to the catalytic triad represented by the DDE motif of IN (Fig. 4). Various mechanisms by which resistance is conferred by these mutations are likely complex and can be proposed. A first mechanism of resistance is based on a change in an amino acid resulting in loss of the group/moiety to which the drug is bound. In the model of Chen, Q148 and Y143 are located in the hydrophobic pocket and are key residues making direct contact with the benzyl moiety of RAL; mutation of these two residues can result in loss of direct drug–enzyme contact, hence leading to resistance. E92 is located at site 3 in the model and makes a polar interaction with RAL; substitution of E92 to a glutamine disturbs this polar interaction, leading to a reduced drug–enzyme contact and consequently to resistance to RAL. Another mechanism of resistance is based on the possible influence of IN mutations on the positions of the Mg2+ ions, thereby interfering with the chelating function of the inhibitor. In the model, the residue at position 155 makes contact with one of the Mg2+ ions. Therefore, mutation N155H may affect the coordination of the Mg2+ ions within the architecture of the catalytic site and can subsequently interfere with the chelating activity of RAL. Another possibility is that N155H, which is in close proximity of the viral DNA, may indirectly interrupt the binding of the inhibitor by altering formation of the complex between IN and viral DNA. A high resolution X-ray diffraction analysis of the crystal structure of the inhibitor-bound IN complex with DNA, which has remained elusive to date, would help to test these hypotheses.

Here we examined the association of selected mutations with the activity of a panel of INIs, including second-generation INIs, and the profiles of drug-resistant mutants in vitro. This research enabled us to identify several mutational pathways leading to resistance against first-generation INIs like RAL, and illustrates the higher genetic barrier to resistance development of second-generation INIs. A better understanding of the mechanisms leading to INI resistance is needed to optimize their use in HIV-infected patients and, enables the design of new second-generation INIs.

Materials and methods

Abbreviations

EC50, inhibitor concentration required for 50% inhibition of HIV-1 replication in cell culture; CC50, cytotoxicity, inhibitor concentration required for 50% inhibition of cell growth; HIV-1, human immunodeficiency virus type 1; HIV-1 (IIIB), wild-type HIV-1; IN, integrase; INI, integrase inhibitor; LTR, long terminal repeat; HAART, highly active antiretroviral therapy; SDM, site-directed mutant; EGFP, enhanced green fluorescent protein; MOI, multiplicity of infection; CCID50, infectious dose required for infection of 50% of cell culture;
Antiviral compounds

Elvagtevirag (EVG; GS-9137; Sato et al., 2006); Raltegravir (RAL; MK-0518; Anker and Corales, 2008); L-870,810 (Hudson et al., 2004); PACA (a 3-Hydroxy-4-oxo-4,6,7,8,9,10-hexahydropyrido-[1,2,3]-azepine-2-carboxamide; Belyk et al.;) PICA (a 9H-Pyrido[3,4-b]indole-3-carboxamide; Kuki et al., 2005); Compound G (6H)-2′-[(3-chloro-4-fluorophenyl)methyl]-8-ethyl-1,2,6,7,8,9-hexahydro-10-hydroxy-N,6-dimethyl-1,9-dioxo-yrazino[1′.2′.1.5]pyrrolo[2,3-d]pyridazine-4-carboxamide (Cmpd G; Wai et al., 2007) and MK-2048 (Vacc et al., 2007); and the nucleoside reverse transcriptase inhibitor Zidovudine (AZT; Horwitz et al., 1964) were obtained from commercial suppliers or were synthesized in-house. Chemical structures are shown in Fig. 5 and resulting activity and toxicity in Table 3.

Cells and viruses

The human T-lymphoblastoid cell line MT4 was kindly provided by Dr. Naoki Yamamoto (National Institute of Infectious Diseases, AIDS Research Center, Tokyo, Japan). The cell line was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 0.1% NaHCO3, antibiotics (0.02% gentamicin, 0.8% G418), and stored in a humidified incubator with a 5% CO2 atmosphere at 37°C.

MT4–LTR–EGFP cells were obtained by transfecting MT4 cells with a selectable construct encompassing the coding sequences for the HIV-LTR as a promoter for the expression of enhanced green fluorescent protein (EGFP) and subsequent selection of permanently transfected cells. MT4-CMV-EGFP cells were obtained by selection for permanently transformed MT4 cells with an EGFP reporter gene under control of the consecutive CMV promoter.

HIV-1 (IIIIB) was provided by Dr. Guido van der Groen (Institute of Tropical Medicine, Antwerp, Belgium). HIV-1 molecular clone pHXB2D was provided by Centre for AIDS Reagents (ARP026; Brussels, Belgium).

Site-directed mutants (SDMs) of IN coding sequences were constructed in the pUC19-5′HXB2D vector (XbaI–SalI fragment of pHXB2D), containing the HIV-1 clone HXB2D IN coding sequence, by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and high-performance liquid chromatography-purified primers (Genset Oligos, La Jolla, CA). Plasmid sequences were confirmed by dideoxy sequencing.

Generation of the SDM virus stocks

MT4 cells were subcultured at a density of 250,000 cells/ml on the day before transfection. Cells were pelleted and resuspended in phosphate-buffered saline at a concentration of 3.1×106 cells/ml. A 0.8 ml portion (2.5×106 cells/ml) was used for each transfection. Transfections were performed with the Bio-Rad Gene pulser (Bio-Rad, Hercules, CA) with 0.4 cm electrode cuvettes (Bio-Rad). Cells were electroporated with 10 μg of Sall-linearized pUC19-3′/HXB2D (Sall–XbaI fragment of pHXB2D) and 5 μg Sall-digested SDM at 250 μF and 300 V, followed by a 30 min incubation at room temperature. Next, 10 ml of fresh culture medium was added to the suspension of transfected cells, which was then incubated at 37°C in a humidified atmosphere with 5% CO2. Cell cultures were monitored for the appearance of cytopathic effect (CPE). At virus breakthrough (full CPE), culture supernatant was typically harvested by centrifugation at 8 to 10 days after transfection and was stored at −80°C for subsequent drug susceptibility determination.

Antiviral assays

The antiviral activity of inhibitors was determined in a cell-based HIV-1 replication assay in triplicate. Briefly, MT4–LTR–EGFP cells (400,000 cells/ml) were infected with HIV-1 (IIIIB, HXB2D, selected viruses, or SDM strains; multiplicity of infection MOI = 0.0025) in the presence or absence of inhibitor. After 3 days of incubation, the inhibition of HIV replication was quantified by measuring EGFP fluorescence, and expressed as EC50 values. The cytotoxicity of inhibitors was determined in parallel on mock-infected MT4-CMV-EGFP cells (150,000 cells/ml) cultured in the presence or absence of test compound concentrations (data not shown). After three days of incubation, inhibition of cell proliferation was quantified by measuring the EGFP fluorescence, and expressed as CC50 values (cytotoxicity, 50% inhibitory concentration of cell growth).

Genotyping

Viral RNA was extracted from culture supernatant or virus stock using a NucliSens easyMAG apparatus (bioMérieux, Marcy l’Etoile, France), a high-throughput automated nucleic acid extraction system, cDNA encoding reverse transcriptase and IN was generated with Expand Reverse Transcriptase (Roche Diagnostics, Basel, Switzerland), followed by amplification of the IN region by nested PCR (1456 bp). PCR products were genotyped by automated population-based full-sequence analysis (ABI PRISM BigDye Terminator cycle sequencing; Applied Biosystems, Foster City, CA). Sequencing results were reported as amino acid changes compared with the HIV-1 (IIIIB) or (HXB2D) wild-type reference sequence. Mutations present in more than 25% of the total virus population were detected as a mixture with the wild type virus.

Automated IVRS experiments in 96-well plates (Goethals et al., 2008)

In vitro selection was performed in 96-well plates, with each row representing a separate IVRS experiment. In contrast with the classical IVRS experiment, where the compound concentration is only increased at viral breakthrough, this method uses a protocol where the virus is challenged with an increased compound concentration at each passage. HIV-1 IIIIB was used as starting strain for the IVRS with RAL, whereas HIV-1 HXB2D was used as starting strain for IVRS with MK-2048 and Cmpd G.

Replication kinetics of recombinant viruses

MT4 cells (120,000 cells) were infected with HIV-1 (HXB2D or recombinant viruses; MOI of 0.0001) in the absence of inhibitor (Fig. 3). Every day, cells were examined for the appearance of HIV-1-induced CPE. In addition, aliquots of cell-free supernatants were taken for determination of viral p24 levels (HIV-1 p24 enzyme-linked immunosorbent assay, Perkin Elmer, Waltham, MA).

Molecular modeling

Compounds were docked, using glide (ref: Glide, version 5.0, Schrödinger, LLC, New York, NY, 2008.), in a model provided by Chen et al. (2008), who developed a model of HIV-1 IN bound to the viral DNA, thereby mimicking the strand transfer complex. In this model an
X-ray structure of the IN CCD is combined with viral DNA, which is based on the crystal structure of DNA-bound Tn5, a structural homologue of HIV-1 IN. The flexible loop (residues 140–152) was computationally constructed in the presence of viral DNA, since the biologically relevant conformation of this loop may be dependent on the presence of bound DNA.

Acknowledgments

The authors gratefully acknowledge Dr. James M. Chen, Dr. Manuel Tsang and Dr. Xiaowu Chen of Gilead for providing the coordinates, upon which the model shown in Fig. 4 was based, and Dr. Anneleen Hombrouck, Dr. Dirk Jochmans and Luc Geeraert for discussion and critical reading of the manuscript.

References


