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Primary mutations selected *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

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ABSTRACT

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.

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 pre-integration-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 (Melek 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

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recently in treatment-naïve 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-naïve HIV-1-infected patients (Markowitz 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
Overview of genotypes of the viruses found with the automated IVRS experiments in 96-well plates at a final concentration of 6 μM RAL.

	Passage	Selected mutations[a]	Mutations post-reculture[a]
Virus 1	43	V54I E138K G140A Q148R	V54I E138K G140A Q148R
Virus 2	38	E138K G140A Q148R	E138K G140A Q148R
Virus 3	19	E138K G140A Q148K S230R	E138K G140A Q148K S230R/S D232D/N
Virus 4	51	L63I L74M A128T E138K Q148R V151I	L63I L74M A128T E138K Q148R V151I
Virus 5	33	L74M E92Q V151I N155H E157Q	L74M E92Q V151I N155H E157Q D232D/N V265A/V
Virus 6	19	E138E/K Q148R	E138E/K Q148Q/R

[a] Sequencing results are reported as amino acid changes compared with HIV-1 (IIIB) 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.

Table 2
Overview genotypes of the viruses selected with MK-2048 or Cmpd G.

	Selection with	Passage	Selected mutations[a]
Virus 7	MK-2048	41	G140E/G
Virus 8	Cmpd G	41	no mutations selected

[a] 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 EC_{50} 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 EC_{50} of their respective

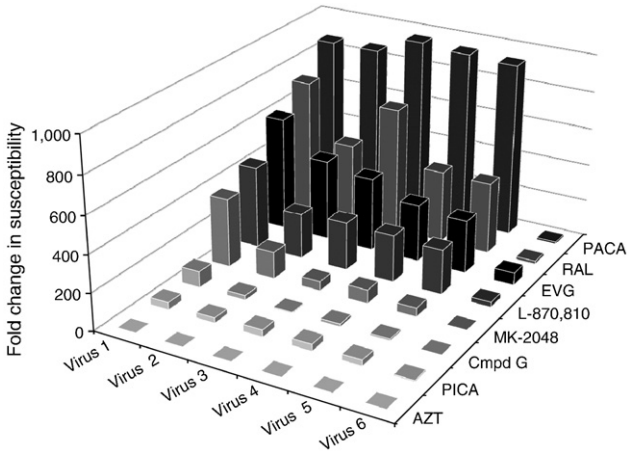

Fig. 1. Cross-resistance of six different RAL-selected HIV-1 strains. The viruses found in the IVRS experiments with RAL were tested for susceptibility to RAL and other INIs. Relative changes in susceptibility of the viruses compared with wild-type IIIB virus are displayed on the graph.