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Insights into the genetic basis for natural phenotypic resistance of human rhinoviruses to pleconaril

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Abstract

Recent phylogenetic analyses of the deduced amino acid sequence of the major viral capsid protein (VP1) of all human rhinovirus (HRV) serotypes revealed two distinct species within the genus: species A (75 serotypes) and species B (25 serotypes). Pleconaril is a novel capsid inhibitor of HRVs. All 75 species A serotypes and 18 of the 25 species B serotypes are susceptible to inhibition by pleconaril in cell culture. The seven resistant serotypes are HRV-4, -5, -42, -84, -93, -97 and -99. We were interested in understanding the genetic basis for phenotypic resistance to pleconaril among these naturally occurring viruses. We compared the 25 amino acids of VP1 that comprise the drug-binding pocket of susceptible and resistant species B viruses. A consistent difference was observed at two positions: the vast majority of susceptible viruses had tyrosine and valine at VP1 residues 152 and 191, respectively (Y_{152} and V_{191}); all resistant viruses had phenylalanine and leucine at these positions (F_{152} and L_{191}). HRV-14, a pleconaril susceptible virus, has a drug-binding pocket amino acid composition that differs from the naturally resistant HRV-5 and HRV-42 only at these two positions. To gain further insight into the role of these specific residues in natural resistance to pleconaril, we substituted the amino acids at these two positions individually and in combination in an infectious clone of HRV-14 and tested the rescued virus for susceptibility to pleconaril and virion stability. The results indicate that substitution of V_{191} to Leu in HRV-14 has a profound negative impact on drug susceptibility but that full resistance to pleconaril is only seen when combined with Phe at position 152 in a HRV-14 double variant (F_{152}, L_{191}). These data identify L_{191} in species B HRV as a potentially key residue in conferring significantly reduced susceptibility to pleconaril. These results may be useful in distinguishing naturally occurring viral resistance to pleconaril from treatment-emergent resistance.

Keywords: Pleconaril; Picornavirus; Common cold; Antiviral; Natural phenotypic resistance

Human rhinoviruses (HRVs), the major etiologic agents of common colds, are the most prevalent viral infectious agents of humans. They comprise a distinct genus with about 100 members or serotypes within the family Picornaviridae. Others and we have recently published phylogenetic analyses that distinguish two species within the HRV genus that include all but one of the currently recognized HRV serotypes (Ledford et al., 2004; Savolainen et al., 2002). Species A consists of 75 virus serotypes, while species B is populated with 25 serotypes. The lone outlier, HRV-87, is phylogenetically related to species D members of the enterovirus genus of the Picornaviridae (Blomqvist et al., 2002; Savolainen et al., 2002).

Atomic structures of three species A (HRV-1A, HRV-2 and HRV-16) and two species B (HRV-3 and HRV-14) viruses have been reported (Arnold and Rossmann, 1990; Kim et al., 1989; Oliveira et al., 1993; Rossmann et al., 1985; Verdaguer et al., 2000; Zhao et al., 1996). These viruses share a common architecture in which a single strand of positive sense RNA is protected by a protein coat or capsid composed of 60 copies of each of four structural proteins, designated VP1–VP4. The core structure of VP1, VP2 and VP3 is an eight-stranded anti-parallel β-barrel, while VP4 exists as an extended polypeptide chain on the inner surface of the virus capsid in association with the viral RNA. Located within the β-barrel of VP1 is a surface-accessible hydrophobic pocket into which bind several chemical classes of low molecular weight compounds called capsid inhibitors (Andries et al., 1992; Kim et al., 1993; Ledford et al., 2004; Smith et al., 1986; Zhang et al., 2004). Upon binding, capsid

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inhibitors affect the functions associated with the virus capsid, such as virus attachment to cellular receptors and uncoating of viral RNA (Pevear et al., 1989; Zeichhardt et al., 1987).

Pleconaril is a potent capsid inhibitor of HRVs (and human enteroviruses) (Groarke and Pevear, 1999; Pevear et al., 1999). Pleconaril inhibits the replication in cell culture of all 75 HRV species A serotypes and 18 of the 25 species B viruses (50% effective concentration (EC_{50}) range of 0.02–6.68 μM) at non-toxic drug concentrations. The seven naturally occurring species B viruses that are resistant to pleconaril inhibition (HRV-4, -5, -42, -84, -93, -97 and -99: $EC_{50} > 12.5$ μM) form a phylogenetic cluster (Ledford et al., 2004). Nevertheless, these seven viruses are quite closely related to the remaining species B HRV, and in particular, in the 25 amino acid residues of VP1 that comprise the drug-binding pocket (Kim et al., 1993; Ledford et al., 2004). Of these 25 amino acid residues, 17 are completely conserved across all species B viruses, and another seven positions are occupied by one of only two different residues (Ledford et al., 2004). However, all seven naturally resistant HRVs differ from the majority sequence of HRV species B members at two amino acid positions in the drug-binding pocket: VP1 amino acid positions 152 and 191 (Tyr_{152} and Val_{191} in the majority sequence; Phe_{152} and Leu_{191} in the resistant viruses; Table 1). The resistant viruses HRV-5 and -42 differ from the pleconaril-susceptible HRV-14 (EC_{50} value of 0.19 μM) only at these two positions. In addition, HRV-26, which is about 10-fold less susceptible to pleconaril inhibition than HRV-14, differs from HRV-14 at only position 191 (Val_{191} in HRV-14, Leu_{191} in HRV-26; Table 1). None of the species B HRV serotypes differed from HRV-14 only at position 152. These comparisons implicate position 191 as an important determinant of drug susceptibility.

We have previously reported that the 25 amino acid residues that comprise the drug-binding pocket of HRVs determine susceptibility to inhibition by pleconaril (Ledford et al., 2004). For example, group A and group B HRVs with identical drug-binding pocket residues had a mean difference in the EC_{50} value of only 2.1-fold (95% confidence interval = 1.7–2.5-fold) and 2.8-fold (95% confidence interval = 2.3–3.4-fold), respectively. In other words, two viruses with identical amino acid compositions of their drug-binding pockets were inhibited by pleconaril in an essentially identical manner. With this knowledge in hand, we sought to establish more definitively the role of VP1 residues 152 and 191 in natural phenotypic resistance to pleconaril, by engineering the amino acids at these two positions in various combinations in an infectious clone of HRV-14, and testing the rescued virus for susceptibility to pleconaril. The HRV-14 cDNA plasmid contains the entire 7226 bp HRV-14 genomic sequence under control of a T7 RNA polymerase promoter. Viral sequence terminates with a 42-residue polyadenosine tail, followed by a unique Mlu1 restriction enzyme site for plasmid linearization to generate runoff T7 transcripts.

The HRV-14 cDNA (wild-type (wt) sequence of Y_{152}, V_{191}; Table 1) was utilized to generate plasmids encoding the single F_{152} or L_{191} changes, as well as the double variant, F_{152}/L_{191} in the drug-binding pocket. Single nucleotide changes in the codons encoding VP1 amino acids at positions 152 and 191 were introduced into the DNA plasmid (see legend to Table 2).

Table 1
Alignment of VP1 amino acids in drug-binding pocket of select HRV species B viruses[a]

HRV serotype	EC_{50} (μM)[c]	Amino acid at VP1 position																							
		104	105	106	107	124	126	128	130	150	151	152	174	175	176	186	188	191	197	199	215	219	221	224	245	267
Majority Sequence[b]		I	N	L	S	F	S	Y	I	A	M	Y	P	S	V	F	V	V	Y	C	I	N	M	M	H	G
14	0.19	I	N	L	S	F	S	Y	I	A	M	Y	P	S	V	F	V	V	Y	C	I	N	M	M	H	G
26	1.60	I	N	L	S	F	S	Y	I	A	M	Y	P	S	V	F	V	L	Y	C	I	N	M	M	H	G
5	>10	I	N	L	S	F	S	Y	I	A	M	F	P	S	V	F	V	L	Y	C	I	N	M	M	H	G
42	>10	I	N	L	S	F	S	Y	I	A	M	F	P	S	V	F	V	L	Y	C	I	N	M	M	H	G

[a] Amino acids in the drug-binding pocket are defined as those for which any atom is within 4 Å of compounds bound in HRV-1A or HRV-14 (Kim et al., 1993).
[b] The majority sequence is defined as the amino acid residue that appears most frequently at that position in the 25 HRV species B viruses (Ledford et al., 2004).
[c] EC_{50} is defined as the concentration of pleconaril that protects 50% of a HeLa cell monolayer from virus-induced cytopathology as described previously (Ledford et al., 2004).

Table 2
Susceptibilities to pleconaril inhibition of wild type HRVs and engineered variants

Virus	EC_{50} (μM) ± S.D.[a]
HRV-5, wt (F_{152}/L_{191})	>10.0 ± 0
HRV-42, wt (F_{152}/L_{191})	>10.0 ± 0
HRV-26, wt (Y_{152}/L_{191})	1.6 ± 0.1
HRV-14, wt (Y_{152}/V_{191})	0.19 ± 0.09
HRV-14, F_{152}	0.23 ± 0.03
HRV-14, L_{191}	5.9 ± 3.5
HRV-14, F_{152}/L_{191}	>10.0 ± 0

All wild-type HRV serotypes were purchased from the ATCC (Rockville, MD, USA) and passaged in HeLa-OH-I cells, which were a gift from Dr. Frederick Hayden, University of Virginia. Virus susceptibility to inhibition by pleconaril was determined in a 96-well format assay that measured protection of a HeLa-OH-I cell monolayer from virus-induced CPE. An infectious cDNA clone of HRV-14 in a pUC19 plasmid derivative was a gift from Dr. Wai-Ming Lee, University of Wisconsin (Lee et al., 1993). The cDNA was transformed into "Max Efficiency" DH5α competent cells (Invitrogen Corp., Carlsbad, CA, USA), extracted using QIAprep spin mini prep kit (Qiagen Inc., Valencia, CA, USA) and double restriction digested with Nde I (New England Biolabs Inc., Beverly, MA, USA) and Mlu I (Promega Corp., Madison, WI, USA) to confirm the presence of the 7.226 kbp insert. A positive clone was scaled up and plasmid DNA was extracted using Qiagen plasmid maxi prep kit (Qiagen Inc.). Single nucleotide changes were engineered into the HRV-14 infectious cDNA using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) following the manufacturer's protocol. The site-directed altered plasmids were transformed into XL10-Gold Ultracompetent cells (Stratagene) and plasmid DNA extracted as described above. The resulting purified DNA was sequenced through the entire capsid region to confirm the presence of the desired nucleotide changes (Biotech Core Inc., Mountain View, CA, USA or ACGT Inc., Wheeling, IL, USA). The double mutant (F_{152}/L_{191}) was engineered using the F_{152} variant as the basis for the mutagenesis.

[a] Each EC_{50} value represents the mean of at least two individual determinations.

RNA transcribed from sequence-confirmed, linearized plasmids was transfected into HeLa-OH-I cells. Virus-induced cytopathic effects (CPE) were monitored daily. The virus was harvested when CPE was complete. The entire capsid region of the recovered viruses was RT-PCR amplified and resequenced to confirm the nucleotide sequence after virus amplification in cell culture.

Transfection of RNA transcripts from all the three engineered plasmids resulted in the recovery of infectious virus with only intended nucleotide changes in the capsid-coding region. These recovered viruses, as well as several wild-type viruses, were then evaluated for susceptibility to inhibition by pleconaril in cell culture (Table 2). The results demonstrate that the amino acid in position 191 plays a dominant role in determining susceptibility to pleconaril. Replacement of V_{191} with leucine in drug-susceptible HRV-14 resulted in a ∼30-fold reduction in drug susceptibility of the variant virus (EC_{50} value of 5.9 μM; Table 2). Replacement of Y_{152} with phenylalanine did not affect drug susceptibility (EC_{50} values of 0.19 μM versus 0.23 μM, respectively; Table 2). However, the double variant containing both Y_{152} to phenylalanine and V_{191} to leucine changes was fully resistant to pleconaril inhibition in the cell culture (EC_{50} > 10 μM). Taken together, these results indicate that L_{191} is the major driver of reduced drug susceptibility in species B HRVs, but that complete resistance to drug inhibition requires the presence of changes at both the 152 and 191 positions in the drug-binding pocket relative to HRV-14. These observations are consistent with data showing that HRV-26, which has a Y_{152}/L_{191} amino acid pattern in the drug-binding pocket, retains limited susceptibility to pleconaril inhibition (Table 2).

During the course of clinical studies of pleconaril in subjects with community-acquired common colds, variant viruses with reduced susceptibility to pleconaril inhibition were recovered from about 10% of subjects who could be evaluated (Hayden et al., 2003). Sequencing of pre- and post-treatment-initiation virus isolates revealed that approximately one-third of the subjects from whom a reduced susceptibility variant was recovered had been infected with a species B virus (manuscript in preparation). However, in no instance was an amino acid change at position 191 observed in pleconaril treatment emergent viruses. Such a change would have required a single nucleotide transversion, from guanosine to cytosine or uracyl at the second position of the codon. Thus, viruses with reduced drug susceptibility that emerge as a consequence of antiviral treatment differed from naturally occurring drug-resistant viruses.

This lack of selection of variant viruses with an amino acid change at position 191 suggests that such a virus has a fitness liability that affects its viability in pleconaril-treated patients. One correlate of HRV fitness examines the stability of virions. All naturally occurring (wild-type) HRVs show a loss of infectivity upon exposure to reduced pH (Hughes et al., 1973, 1974). The inactivation profile is characteristic for each individual serotype. This profile likely reflects a balance between virion stability in the environment and the virus' ability to disassemble (uncoat) during infection. Significant changes in the virion stability upset this balance, resulting in a fitness cost. For example, viruses engineered or selected in cell culture for resistance to capsid inhibitors (Groarke and Pevear, 1999), or isolated from drug-treated subjects in clinical studies (Yasin et al., 1990), show significant changes in their physical stability profile relative to their wild-type parent. When examined, such virus variants showed substantially attenuated growth in vivo (Groarke and Pevear, 1999; Yasin et al., 1990).

The pH-inactivation profiles for wild-type HRV serotypes 5, 14, 26 and 42 are shown in Fig. 1A. As expected, all showed a loss of infectivity of at least 10-fold by 60 min exposure to pH 5.4. The pH-stability profile of the engineered virus variants was compared to that of their parental wild-type HRV-14. The infectivity of HRV-14 L_{191} was completely stable to treatment at a pH as low as 4.8 (Fig. 1B). The variant virus HRV-14 F_{152} was as susceptible as wild-type HRV-14 to low pH inactivation. Finally, the double variant carrying both the F_{152} and L_{191} amino acid changes regained a pH-inactivation profile characteristic of wild-type viruses.

The dramatically altered pH-stability profile of the L_{191} HRV-14 variant suggests an overly stable virion, which would be expected to carry a fitness liability. This may in turn explain why this variant has not been observed to date in patients, in response to pleconaril treatment (D. Pevear, unpublished data). Future studies will be required to further examine the fitness of these isolates.

More significantly, the results suggest that natural phenotypic resistance to pleconaril is readily distinguishable from

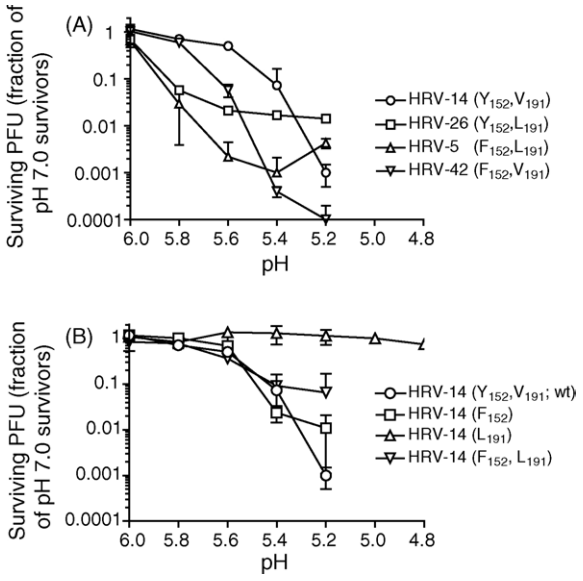

Fig. 1. pH-inactivation profiles of wild-type HRVs (panel A) and engineered HRV-14 variants (panel B). Viruses were incubated at room temperature for 1 h in 500 μL of 0.1 M sodium acetate buffered to the appropriate pH with glacial acetic acid. The solution was then neutralized by addition of 1.5 mL of 1 M Tris buffer, pH 8 and frozen at −80 °C. Virus titers were determined by standard plaque assay on HeLa-OH-I cells.

treatment-emergent resistance, in that changes at the 152 and 191 positions in VP1 have not been observed to date in clinical isolates of HRV species B viruses in response to pleconaril treatment.

In summary, the results reported here identify the molecular basis for drug resistance to pleconaril among naturally occurring HRV serotypes. The ability to distinguish these naturally resistant viruses from those that arise in patients in response to pleconaril treatment suggests that genetic analyses may be useful in the development of a resistance-monitoring program should pleconaril be approved for human use.

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