

Conservation of Amino Acids in Human Rhinovirus 3C Protease Correlates with Broad-Spectrum Antiviral Activity of Rupintrivir, a Novel Human Rhinovirus 3C Protease Inhibitor

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The picornavirus 3C protease is required for the majority of proteolytic cleavages that occur during the viral life cycle. Comparisons of published amino acid sequences from 6 human rhinoviruses (HRV) and 20 human enteroviruses (HEV) show considerable variability in the 3C protease-coding region but strict conservation of the catalytic triad residues. Rupintrivir (formerly AG7088) is an irreversible inhibitor of HRV 3C protease with potent *in vitro* activity against all HRV serotypes (48 of 48), HEV strains (4 of 4), and untyped HRV field isolates (46 of 46) tested. To better understand the relationship between *in vitro* antiviral activity and 3C protease-rupintrivir binding interactions, we performed nucleotide sequence analyses on an additional 21 HRV serotypes and 11 HRV clinical isolates. Antiviral activity was also determined for 23 HRV clinical isolates and four additional HEV strains. Sequence comparison of 3C proteases ($n = 58$) show that 13 and 11 of the 14 amino acids that are involved in side chain interactions with rupintrivir are strictly conserved among HRV and HEV, respectively. These sequence analyses are consistent with the comparable *in vitro* antiviral potencies of rupintrivir against all HRV serotypes, HRV isolates, and HEV strains tested (50% effective concentration range, 3 to 183 nM; $n = 125$). In summary, the conservation of critical amino acid residues in 3C protease and the observation of potent, broad-spectrum antipicornavirus activity of rupintrivir highlight the advantages of 3C protease as an antiviral target.

The picornavirus family consists of over 200 medically important viruses, including human rhinoviruses (HRV) and human enteroviruses (HEV). HRV, comprising over 100 different serotypes, are a major cause of mild upper respiratory infections (reviewed in references 4, 8, and 40). Although HRV infections are usually mild and self-limiting, they can also be associated with exacerbation of disease in individuals with underlying respiratory disorders (4, 48). The HEV include over 70 viruses that are associated with diverse clinical syndromes ranging from mild, self-limiting infections to fulminant and potentially fatal disease (3, 52, 53). Earlier clinical studies with agents that inhibit virus attachment and/or uncoating (e.g., tremacamra, a soluble intracellular adhesion molecule-1, pirodavir, and pleconaril) and nonspecific antiviral agents such as α -2 β interferon have demonstrated that prevention and early treatment of HRV colds could provide clinical benefit (3, 18–23, 53). Recently, a retrospective analysis of two multicenter clinical trials demonstrated that pleconaril, a capsid-function inhibitor (17), significantly reduced the duration and severity of picornavirus-induced colds (24). To date, however, no antiviral agents have been approved for the prevention or treatment of HRV infection.

We have focused our antiviral strategy on the inhibition of 3C protease, a viral enzyme that is absolutely required for the proteolytic cleavage of viral precursor polyproteins to functional pro-

teins (10–14, 42, 46, 47) and, thus, is essential for viral replication. Initial sequence comparisons of the 3C protease-coding regions from six HRV serotypes (15, 28, 38, 57, 58, 63), together with the experimentally derived three-dimensional structure of 3C protease (41), indicate significant homology in the substrate-inhibitor binding site, including strict conservation of the three amino acid residues that comprise the catalytic triad (His 40, Glu 71, and Cys 147). Consistent with this finding, rupintrivir (formerly AG7088), a novel, irreversible inhibitor of 3C protease (10–13, 61) has demonstrated broad-spectrum, potent *in vitro* antiviral activity against all picornaviruses tested, including 48 HRV serotypes, 4 HEV strains, and 46 untyped field isolates of HRV (34, 46, 64). Furthermore, recent data demonstrating the ability of rupintrivir to moderate illness severity and reduce viral load in human subjects following experimental HRV infection provide proof of concept for the mechanism of 3C protease inhibition (25). To better understand the relationship between the broad-spectrum *in vitro* antiviral activity of rupintrivir and 3C protease-rupintrivir binding interactions, we performed nucleotide sequence analyses on an additional number of laboratory strains and clinical isolates of HRV and antiviral assays to include HRV clinical isolates and additional HEV strains. Our results indicate that among the HRV and HEV strains evaluated, 13 and 11, respectively, of the 14 amino acid residues previously shown to be critical for 3C protease inhibitor binding (41) are absolutely conserved. This conservation is consistent with the comparable *in vitro* antiviral activity of rupintrivir against numbered HRV serotypes, HRV isolates, and HEV strains evaluated (50% effective concentration range, 3 to 183 nM).

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MATERIALS AND METHODS

Antiviral compound. Rupintrivir (molecular weight, 598.7; aqueous solubility of 15 $\mu\text{g/ml}$) was synthesized at Pfizer Global Research and Development (formerly Agouron Pharmaceuticals, Inc.), San Diego, Calif. (11).

Cells and virus strains. H1-HeLa cells, MRC-5 cells, all numbered HRV serotypes, and HEV were purchased from the American Type Culture Collection (Manassas, Va.). Nasal lavage samples from subjects experimentally infected with challenge strains of HRV 39, HRV Hanks, HRV 23, or HRV 16 were kindly provided by Frederick G. Hayden (Department of Internal Medicine, University of Virginia Health Sciences Center, Charlottesville, Va.) and Ronald B. Turner (Department of Pediatrics, University of Virginia Health Sciences Center). Field isolates from symptomatic individuals were identified as HRV by the optical immunoassay OIA (45) and were generously provided by Rachel Ostroff (Thermo BioStar, Inc., Boulder, Colo.). Clinical viral isolates were obtained by inoculating H1-HeLa cells with nasal lavage samples. All HRV clinical isolates were propagated and antiviral assays were performed in H1-HeLa incubated at 34°C. Cells were grown in minimal essential medium (Invitrogen, Carlsbad, Calif.) with 10% fetal bovine serum (HyClone Lab. Inc., Logan, Utah). Coxsackieviruses B2 and B5 (CVB 2 and CVB 5) were propagated and antiviral assays were performed in H1-HeLa cells incubated at 37°C. Echovirus serotypes 6 and 9 (EV 6 and EV 9) were propagated and antiviral assays were performed in MRC-5 cells incubated at 37°C. Cell-free HRV lysates were either extracted immediately or stored at -80°C for subsequent RNA isolation.

DNA sequence analysis. RNA was purified from virus by silica-based extraction by using the RNeasy method (QIAGEN, Valencia, Calif.), and then cDNA was synthesized from viral RNA by using a First Strand synthesis kit (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.) and random nanomers. For each strain, the target region (corresponding to bases 5116 to 5827 in HRV 89) was amplified by PCR with *Taq* 2000 polymerase (Stratagene, San Diego, Calif.). The 3C protease nucleotide sequences from HRV serotypes 3, 9, 10, 11, 13, 15, 17, 19, 23, 24, 31, 36, 39, 52, 56, 59, 62, 78, 81, 87, and HRV Hanks were determined, when possible, by sequencing of PCR products. Otherwise, the PCR amplicons were subcloned into pGEM-T Easy vectors (ProMega Corp., Madison, Wis.) and sequenced by using T7 and SP6 primers. The difficulty in amplifying the 3C protease regions from very diverse HRV serotypes was addressed by using multiple degenerate PCR primers. The sense PCR primers used were 5'-CARGGNCCWTAATCNGGNWANC-3', 5'-CARGGNCCATAYTCNGGNRANCC-NAAR-3', and 5'-CAGGGACCATACTCAGG-3'. The antisense PCR primers used were 5'-ATCYTYAANACACTWGGNTGNAYTWNNGTTT T-3' and 5'-ATCYTYAANACACTWGG-NTGNAYT-3'. Since previous studies identified the sequence flanking the 3C protease of HRV 39, species-specific primers consisting of the sense primer 5'-AAAACCATGGAGCGGAGGGTA G-TTGC-3' and antisense primer 5'-CACAGGATCCTTATTACATTCCTG GTTTTCTTTGA-3' were used for HRV 39. The DNA sequences were analyzed, relevant regions were translated, and phylogenetic relationships based on similarities of 3C protease amino acid sequences were determined by DNASTAR analysis software (DNASTAR Inc., Madison, Wis.). For illustration purposes, a phylogenetic tree was generated by Clustal W analysis in MegAlign. The peptide sequences for 3C protease from HRV serotypes 1A (63), 1B (28), 2 (57), 14 (58), 16 (38), and 89 (15) and from all HEV (6, 7, 9, 29-33, 36, 39, 44, 49, 50, 54, 59, 65-69) were obtained from the GenBank database (Bethesda, Md.).

Antiviral assays. The ability of rupintrivir to protect cells against infection with HRV clinical isolates and HEV was measured in 96-well plates by a modified and standard cytopathic effect (CPE) inhibition assay, respectively, by using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) dye reduction method (HEV) (34, 56, 62, 64). In the standard CPE assay, H1-HeLa and MRC-5 cells were initially resuspended at a final concentration of 2×10^5 or 5×10^4 cells per ml, respectively, and infected with virus (50 μl) at a constant multiplicity of infection (MOI) determined to yield ≥ 65 to $\leq 95\%$ cell death (CPE) for each individual virus (MOI range, 0.004 to 1) or mock infected with medium only. At 1 to 6 days later, cultures were evaluated for CPE microscopically, XTT and phenazine methosulfate were added to the test plates, and the amount of formazan produced was quantified spectrophotometrically at 450 nm with adjustment for background at 650 nm. Data were expressed as the percentage of formazan produced in compound-treated cells compared to formazan produced in wells of uninfected, compound-free cells. The 50 and 90% effective concentrations (EC_{50} and EC_{90}) were calculated as the concentrations of compound that increased the percent of formazan production in infected, compound-treated cells to 50% and 90% of that produced by uninfected, compound-free cells, respectively. The modified CPE assay, which allows for the simultaneous determination of antiviral activity

TABLE 1. In vitro antiviral activity of rupintrivir against HRV clinical isolates and HEV^a

Virus	Antiviral activity	
	EC_{50} (nM)	EC_{90} (nM)
HRV clinical isolate ^b		
100	22 \pm 20	32 \pm 8
103	58, 24	316, 746
108	43, 19	182, 42
113	46, 26	464
119	29 \pm 11	145 \pm 130
123	23	ND
126	23	794
129	19	39
142	19	38
O	54 \pm 51	275 \pm 258
G	14, 20	66, 1078
I	104, 16	604, 29
W	10, 3	44, 7
J	18 \pm 6	69 \pm 90
R	14 \pm 8	204 \pm 291
Q	17	33
B	12	40
C	12	28
T	42 \pm 28	123 \pm 24
E	23	562
15	26 \pm 7	262
28	8	18
32	7	19
Enterovirus		
CVB 2	22, 137	88, 254
CVB 5	7, 47	147, 83
EV 6	51, 34	94, 59
EV 9	11, 18	65, 34

^a Antiviral activity was determined by measuring XTT dye reduction following 2 to 7 days of infection by using the standard or modified CPE inhibition assays as described in Materials and Methods. Results represent the mean \pm standard deviation (three to five experiments) or individual values (one or two experiments). ND, not determined.

^b Clinical isolates were derived from nasal lavage samples collected from subjects infected with HRV serotype 39 (designated 100, 103, 108, 113, 119, 123, 126, 129, 142, O, G, I, W), Hanks (designated J, R, Q, and B), 16 (designated C, T, and E), or 23 (designated 15, 28, and 32).

and optimal CPE, was utilized with HRV clinical isolates. Briefly, each clinical HRV isolate was inoculated at six serial 10-fold dilutions in quadruplicate for a 50% tissue culture infectious dose determination (back titration) and at three 10-fold virus dilutions for compound titration. The EC_{50} values were determined by utilizing the inoculum dilution for which the calculated 50% tissue culture infectious dose per milliliter was between 32 and 320. XTT and phenazine methosulfate were added to the test plates and processed as above.

RESULTS

In vitro antiviral activity of rupintrivir against HRV clinical isolates and HEV strains. Previous studies have demonstrated potent, broad-spectrum antiviral activity of rupintrivir against 48 HRV numbered serotypes and 4 related picornaviruses as well as 46 untyped HRV field isolates (34, 46). To extend these findings, we evaluated the antiviral activity of rupintrivir against 23 clinical isolates of HRV derived from subjects during experimental challenge studies (Table 1). Four additional laboratory strains of HEV, selected for their diversity and association with human disease (CVB 2 and 5 and EV 6 and 9), were likewise evaluated (Table 1). Rupintrivir demonstrated potent in vitro antiviral activity against 23 of 23 HRV clinical isolates tested in H1-HeLa cell protection assays, with a mean

EC₅₀ of 24 nM (range, 3 to 104 nM) and a mean EC₉₀ of 214 nM (range, 7 to 1078 nM). Potent in vitro activity was also demonstrated against four HEV strains with a mean EC₅₀ of 41 nM (range, 7 to 137 nM) and a mean EC₉₀ of 103 nM (range, 34 to 254 nM). Overall, rupintrivir demonstrated comparable antiviral potencies (61-fold range [3 to 183 nM] in EC₅₀ values) against all the HRV and HEV isolates tested ($n = 125$; Table 1) (34, 46).

Amino acid sequence analyses of HRV and HEV 3C protease. High-resolution crystallographic studies have identified 23 different amino acid residues located in the substrate (inhibitor) binding site of HRV 2 3C protease that interact with rupintrivir (41). Inhibitor binding interactions have been identified, 17 of which occur with side chains of 14 amino acids (including Phe 25, His 40, Glu 71, Ile 125, Leu 127, Ser 128, Asn 130, Thr 142, Cys 147, His 161, Gly 163, Gly 164, Asn 165, and Phe 170), while rupintrivir interactions with nine other amino acids involve polypeptide main chain atoms only (Fig. 1). To understand the extent of conservation of the 14 amino acid residues that are involved in side chain interactions with rupintrivir and to correlate this with the observed spectrum of rupintrivir in vitro antiviral activity, we performed sequence analysis on the 3C protease-coding regions from 21 HRV serotypes and 11 HRV clinical isolates. The amino acid sequences from the combined 32 HRV isolates were aligned together with the published sequences from 6 HRV serotypes and 20 HEV strains ($n = 58$) (Fig. 2), and the extent of homology was evaluated. Of the 14 amino acids involved in side chain interactions with rupintrivir, 13 (93%) and 11 (79%) were absolutely conserved (identical) in HRV and HEV strains, respectively. Among the HRV strains, only Asn 130 was not conserved. Among the HEV strains, Asn 130 was not conserved, and Ile 125 and Ser 128 were replaced with conservative (similar in charge and polarity) substitutions of Leu 125 in all HEV analyzed and Gly 128 in 17 of 20 HEV analyzed (3 of 20 were Ser), respectively. Rupintrivir demonstrated comparable in vitro antiviral activity against the HEV (mean EC₅₀ of 65 nM) and HRV (mean EC₅₀ of 22 nM) strains in which matching sequence data were available ($n = 41$; Table 1) (46). This suggests that the differences in level of conservation (11 and 13 of 14 amino acids conserved for HEV and HRV, respectively) did not have an effect on rupintrivir antiviral activity.

Despite the strict conservation of amino acids involved in critical binding interactions with rupintrivir, the overall level of diversity among the complete 3C proteases from the picornaviruses evaluated ($n = 58$) is reflected in the low overall homology at the amino acid level (42 of 183 amino acids, or <23% identity and approximately 80% dissimilarity) (Fig. 3). Furthermore, the levels of variability between the HRV and HEV strains evaluated were comparable to the levels of variability observed among all HRV isolates evaluated ($\leq 41\%$ amino acid identity). Consistent with the two genetic subgroups A and B that have been previously identified (26, 35, 55), HRV isolates also appear to cluster into two groups based on the level of homology of their 3C proteases. Specifically, one cluster contained HRV serotypes 14, 3, 17, and 52 (HRV group B viruses) and, with the exception of HRV 87, all other HRV analyzed belong to a second cluster, identified as HRV group A viruses. Although both subgroups share less than 42% amino acid sequence identity, within each subgroup they share comparable homology (greater than 73 and 77% for groups A

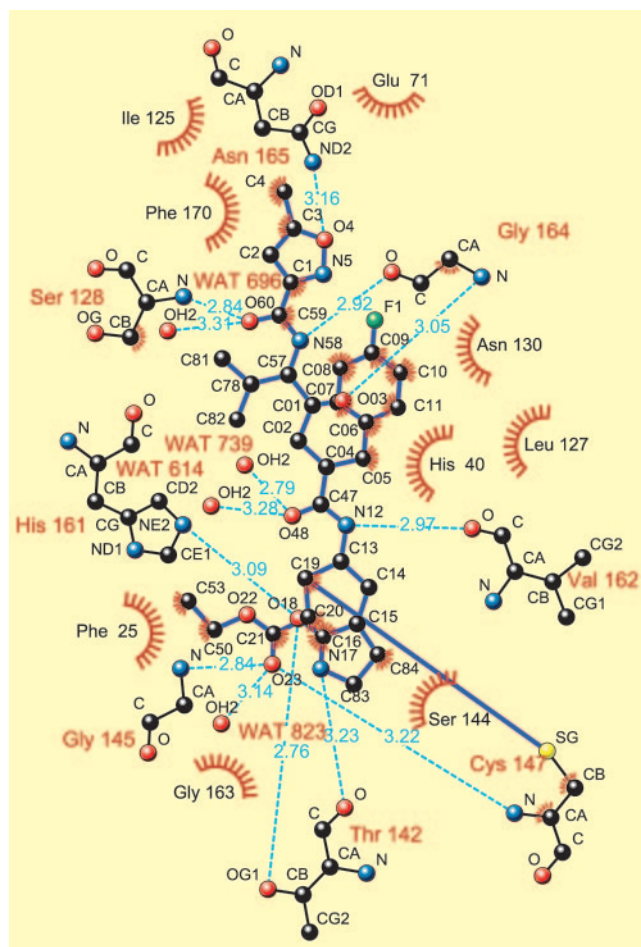


FIG. 1. Interactions between HRV 2 3C protease and rupintrivir. All amino acid residues having side chains interacting directly with the bound inhibitor are included (Phe 25, His 40, Glu 71, Ile 125, Leu 127, Ser 128, Asn 130, Thr 142, Cys 147, His 161, Gly 163, Gly 164, Asn 165, and Phe 170). Amino acid residues (Ser 144, Gly 145, and Val 162) having only polypeptide main chain interactions are shown. Other amino acid residues (Asn 22, Gly 23, Lys 24, Leu 126, Lys 143, and Tyr 146) having only polypeptide main chain interactions are not shown. Hydrogen bonds are indicated by dashed lines. The covalent bond between the sulfur γ atom (SG) of Cys 147 and carbon 19 atom (C19) of the inhibitor is shown by a solid line. The drawing was prepared by using the program LIGPLOT (60).

and B, respectively). In addition, sequence analysis of 3C proteases from the HRV clinical isolates derived following inoculation of subjects in experimental human clinical studies were identical to the specific challenge virus strain utilized, e.g., HRV Hanks and isolates J and Q; HRV 39 and isolates 119 and 138. In a similar manner, the three field isolates (M24, M36, and MR) demonstrated greater than 95% homology with other known HRV numbered serotypes.

HRV serotype 87 is currently considered an unclassified rhinovirus falling into neither group A nor B. The comparison of the 3C protease amino acid sequences for HRV 87 demonstrated less than 40% amino acid homology with other HRV strains but a greater similarity (53 to 83% identity) with HEV strains. In this analysis, HRV 87 related most closely to HEV 70, sharing 83% identity. To confirm this finding, we per-

Majority	GPEEEFGRSLI KHNSCVVTTQNGKFTGLGI YDRVAI LPTHADPGKEI QI DGI ETKVLDSYDL YNKDGVKLEI TVLKLDRNEKFRDI RKYI PE																																																																																									
	10	20	30	40	50	60	70	80	90																																																																																	
Rupintrivir	NT	AL	L	LRK	I	LT	EK	E	S	H	I	CV	Q	DNV	VN	QKI	QI	K	K	K	VDP	NTN	L	I	I	E	GF	S																																																														
HRV 3	NT	AL	L	LRK	I	LT	EK	E	S	H	I	CV	Q	DNV	VN	QKI	QI	K	K	K	VDP	NTN	L	I	I	E	GF	S																																																														
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HRV 11																																																																																										
HRV 13																																																																																										
HRV 15																																																																																										
HRV 17	NT	AL	L	LRK	I	VTI	TK	E	A	VH	CVV	SQ	DD	VL	VN	QP	V	LK	K	K	LDP	PENTN	L	I	I	AF	AD																																																															
HRV 19																																																																																										
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HRV 31																																																																																										
HRV 36																																																																																										
HRV 39																																																																																										
HRV 52	NT	AM	L	LRK	I	VT	TK	E	A	H	CVV	SQ	DD	VL	VN	QP	V	LK	K	K	MGP	PENTN	L	I	I	AF	A																																																															
HRV 56																																																																																										
HRV 59																																																																																										
HRV 62	D																																																																																									
HRV 78	V																																																																																									
HRV 81																																																																																										
HRVHanks																																																																																										
HRV 1a																																																																																										
HRV 2																																																																																										
HRV 14	NT	AL	L	LRK	I	MTI	SK	E	H	CVI	Q	DD	VL	VN	QKI	R	K	K	K	VDP	PENI	N	L	I	I	GF	S																																																															
HRV 16																																																																																										
HRV 89																																																																																										
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M12																																																																																										
CVB 2	AF	AV	AMM	R	ST	K	EY	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																																
CVB 3	AF	AV	AMM	R	ST	K	EY	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																																
CVB 5	AF	AV	AMM	R	ST	K	EY	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																																
EV 6	AF	AV	AMM	R	ST	K	EY	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																																
EV 9	AF	AV	AMM	R	ST	K	EY	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																																
EV 11	AF	AV	AMM	R	ST	K	EY	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																																
CAV 21	GFDY	AV	AMM	R	ST	K	EY	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																																
EV 70	SLD	AQ	AI	MRK	T	VI	AR	SK	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																															
HRV 87	GFD	AQ	AI	M	K	T	VI	AR	SK	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																														
CVB 1	VF	AV	AMM	R	ST	K	EY	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																																
CVB 4	AF	AV	AMM	R	ST	K	EY	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																																
CVB 6	AF	AV	AMM	R	ST	K	EY	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																																
CAV 9	AF	AV	AMM	R	ST	K	EY	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																																
EV 12	AF	AV	AMM	R	ST	K	EY	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																																
EV 30	AF	AV	AMM	R	ST	K	EY	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																																
CAV 16	SLD	AL	L	RR	I	RQ	Q	DQ	H	M	VR	L	V	R	SQ	T	WE	H	K	L	I	N	I	AVE	V	DEQ	N	L	L	V	T	T	F																																																									
CAV 24	GFDY	AV	AMM	R	ST	K	EY	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																																
EV 71	SLD	AL	L	RR	I	RQ	Q	DQ	H	M	VR	L	V	R	SQ	T	WE	H	K	L	I	N	I	AVE	V	DEQ	N	L	L	V	T	T	F																																																									
PV 1	GFDY	AV	AMM	R	ST	K	EY	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																																
PV 2	GFDY	AV	AMM	R	ST	K	EY	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																																
PV 3	GFDY	AV	AMM	R	ST	K	EY	E	M	W	V	R	K	PT	LM	N	DQ	VG	AKE	VD	TN	L	L	N	GG	FLAK																																																																

FIG. 2. Amino acid sequences of 3C proteases from HRV and HEV ($n = 58$) including PV (poliovirus), CVB, coxsackievirus A (CAV), EV (echovirus serotypes 6, 9, 11, 12, or 30 or enterovirus serotypes 70 or 71), HRV clinical isolates (designated 100, 103, 108, 113, 119, 138, J, and Q), and HRV field isolates (M24, M36, and MR). Dashes represent homologous sequences. Fourteen amino acids of 3C protease that have side chain interactions with rupintrivir, including the catalytic triad (His 40, Cys 147, and Glu 71), are boxed in gray.

formed sequence analyses in the 3C protease-coding region of a second independent lot of HRV 87 as well as the 5' untranslated region of the genome. Results confirmed our observation, demonstrating that HRV serotype 87 is more closely related to HEV than to HRV (data not shown).

DISCUSSION

Prevention and treatment of picornavirus infection remain a significant unmet medical need. Given the remarkable heterogeneity of HRV (composed of over 200 different serotypes)

Majority	NEDDYPECNLAL SANQPEN TI I PVGDVVS YGNI LL SGTQTARMLKYNYP T KSGYCGGVLYKI GQVLGI HVGGNGRDGFSAML LRSYFTETQ																									
	100	110	120	130	140	150	160	170	180																	
Rupintrivir																										
HRV 3	DL	EGLDAT	VVHS	GFT	LD	PI	TMA	L	N	N	P	T	I	R	D	T	Q	CTT	KI	F	Q	Q	KKQ	V	K	
HRV 9	R		V	V	P	S	I													SI	I				N	
HRV 10		D			T	N																	A		S	
HRV 11	R				T	N																	A		S	
HRV 13	R		D		V	P	N	I																	NQ	
HRV 15	S		D		V	V	P	S	I																S	
HRV 17	DP	EGM	GT	VI	NS	NFKD	LE	QLTFA	L	N	SVP	N	I	R	E	RT	Q	CST	KI	Y	Q	Q	RKQ	V	K	
HRV 19	K		D		V	P	LS																S		S	
HRV 23		D		L		P	N																		AD	
HRV 24		D	S			T	N	I															A		S	
HRV 31		D				T	N																A		S	
HRV 36	R		A		L	D	P	N	AE															K	G	
HRV 39						T	LN																		D	
HRV 52	DP	EGM	GT	VI	NS	NFKD	LE	QLTFA	L	N	SVP	N	I	R	E	RT	Q	CST	KI	Y	Q	Q	KKQ	V	K	
HRV 56		D				T	N																A		S	
HRV 59	S		D	S			T	N	G																S	
HRV 62	S		D			T	N																A		S	
HRV 78	H		M			V	EQP	E	C															I	N	
HRV 81	T				V	P	K	I															S		Q	
HRV/Hanks	D				N		T	N	I														A		S	
HRV 1a	T				D	P	K	I																	GQI	
HRV 2			N		L		P	N																	DV	
HRV 14	DL	EGVDAT	VVHS	NFT	LE	P	TMA	L	N	S	P	N	I	R	D	A	T	Q	CAT	KI	F	Q	Q	KKQ	V	K
HRV 16	S				V		T	P	K															S		Q
HRV 89	R		A		L	D	P	S	A																K	G
HRV 1b	T				V	P	K	I																		D
100					T	LS																				D
103					T	LS																				D
108					T	LS																				D
113					T	LS																				D
119					T	LS																				D
138					T	LS																				D
J	D				N		T	N	I														A		S	
Q	D				N		T	N	I														A		S	
M24	S				D	S			I	N															S	
M36	T				V	P	K	I																	D	
M12	T				V	P	K	I																	D	
CVB 2	E	VEVN	AV	I	NTSKFP	MY	Q	TD	FLN	G	P	K	M	F	RA	Q	MST	K	HQ	A	KH	NDE				
CVB 3	E	VEVN	AV	I	NTSKFP	MY	Q	TE	FLN	G	P	K	M	F	RA	Q	MST	K	HQ	A	KH	NDE				
CVB 5	E	VEVN	AV	I	NTSKFP	MY	Q	TD	FLN	G	P	K	M	F	RA	Q	MST	K	HQ	A	KH	NDE				
EV 6	E	VEVN	AV	I	NTSKFP	MY	Q	TD	FLN	G	P	K	M	F	RA	Q	MST	K	HQ	A	KH	NDE				
EV 9	E	VEVN	AV	I	NTSKFP	MY	Q	TD	FLN	G	P	K	M	F	RA	Q	MST	K	HQ	A	H	N	E			
EV 11	E	VEVN	AV	I	NTSKFP	MY	Q	TD	FLN	G	P	K	M	F	RA	Q	MST	K	HQ	A	KH	NDE				
CAV 21	QI	TETNDGV	I	VNTSKYP	MYV	A	TEQ	YLN	G	R	T	M	F	RA	Q	I	TCT	K	I	M	SH	A	A	K	QS	
EV 70	Y		NDAI	SVNTSKFP	MY	Q	TLN	FLN	G	P	H	I	M	F	RA	Q	VTTT	K	I	AQ	A		QN		K	
HRV 87	C		NDAV	SVHTSKFP	MY	Q	TN	FLN	G	P	H	I	M	F	RA	Q	VTTT	K	I	AQ	A		H		D	
CVB 1	E	VEVN	AV	I	NTSKFP	MY	Q	TE	FLN	G	P	K	M	F	RA	Q	MST	K	HQ	A	KH	NDE				
CVB 4	E	VEVN	AV	I	NTSKFP	MY	R	TD	FLN	G	P	K	M	F	RA	Q	MST	K	HQ	A	KH	NDE				
CVB 6	E	VEVN	AV	I	NTSKFP	MY	Q	TD	FLN	G	P	K	M	F	RA	Q	MST	K	HQ	A	KH	NDE				
CAV 9	E	MEVN	AV	I	NTSKFP	MY	Q	TD	FLN	G	P	K	M	F	RA	Q	MST	K	HQ	A	KH	NDE				
EV 12	E	AEVN	AV	I	NTSKFP	MY	Q	TD	FLN	G	P	K	M	F	RA	Q	MST	K	HQ	A	H	N	E			
EV 30	E	AEVN	AV	I	NTSKFP	MY	Q	TD	FLN	G	P	K	M	F	RA	Q	MST	K	V	HQ	A	H	N	E		
CAV 16	I	SAASDAT	VI	NTEHMPSMFV		Q	FLN	G	P	H	TMM	F	A	Q	VTSV	K	I	I	Q	C	G	K	ASE			
CAV 24	QI	TETNDGV	I	VNTSKYP	MYV	A	TEQ	YLN	G	R	T	M	F	RA	Q	I	TCT	K	I	M	SH	A	A	K	QS	
EV 71	TI	SGASDAT	VI	NTEHMPSMFV		Q	FLN	G	P	H	TMM	F	A	Q	VTSV	K	I	I	Q	C	G	K	ASE			
PV 1	QI	TETNDGV	I	VNTSKYP	MYV	A	TEQ	YLN	G	R	T	M	F	RA	Q	I	TCT	K	I	M	SH	A	A	K	QS	
PV 2	QI	TETNDGV	I	VNTSKYP	MYV	A	TEQ	YLN	G	R	T	M	F	RA	Q	I	TCT	K	I	M	SH	A	A	K	QS	
PV 3	QI	TETNDGV	I	VNTSKYP	MYV	A	TEQ	YLN	G	R	T	M	F	RA	Q	I	TCT	K	I	M	SH	A	A	K	QS	

FIG. 2—Continued.

and HEV (including poliovirus, coxsackievirus, and echovirus), an antiviral agent should at minimum demonstrate significant antiviral potency against the majority of virus serotypes and strains. Although small molecule inhibitors targeting viral attachment and uncoating have demonstrated antiviral activity, these inhibitors are often limited in their spectrum of activity (2, 34, 46). The clinical relevance of this limitation was recently

shown for pleconaril; in two pivotal human clinical trials 13% of baseline isolates were not susceptible to pleconaril (16).

We have focused our research efforts on the HRV 3C protease, an enzyme that mediates viral proteolytic processing and is absolutely required for virus replication. Nucleotide sequence comparisons of a limited number of HRV serotypes had revealed considerable variability in the 3C protease-coding

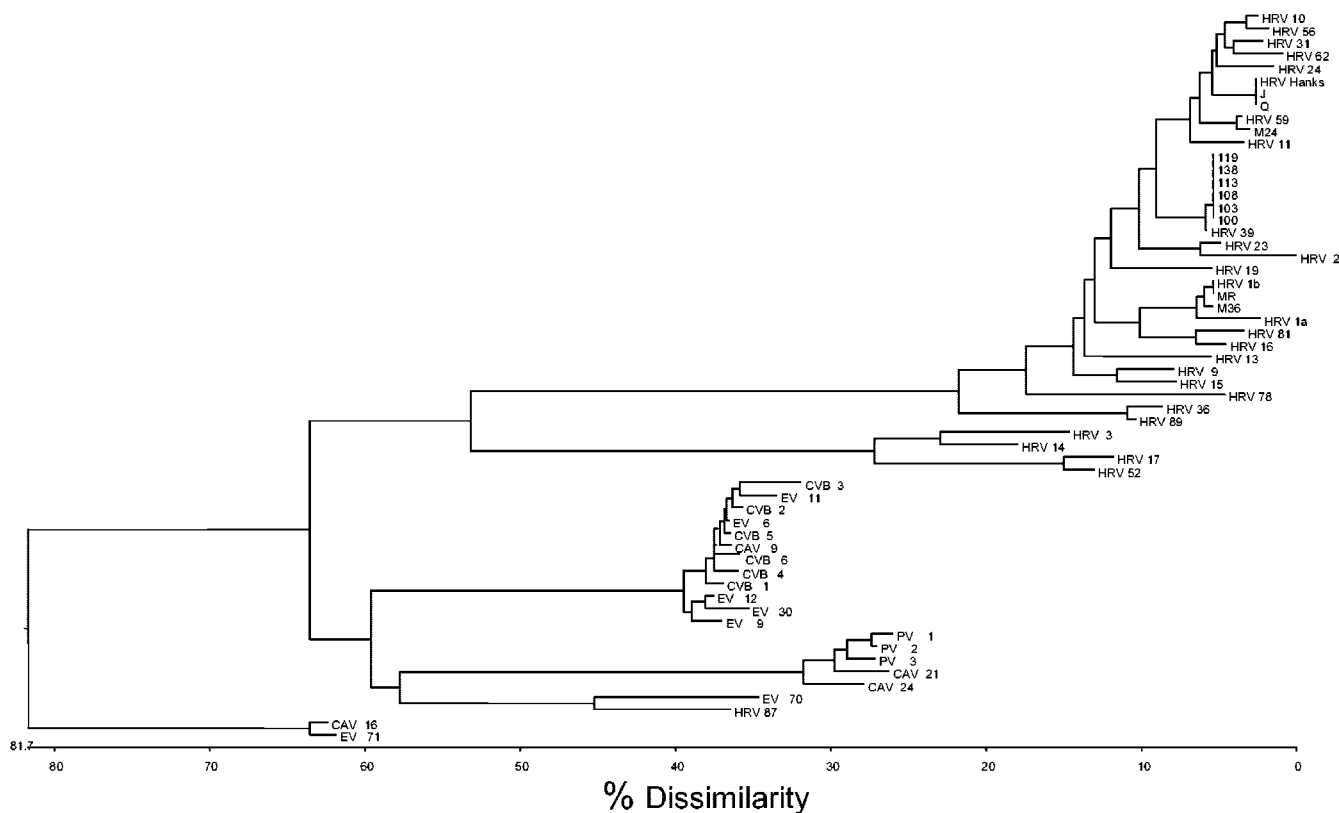


FIG. 3. Phylogenetic tree of HRV and HEV based on homology of 3C protease amino acid residues was generated by MegAlign by using Clustal W analysis as described in Materials and Methods. The length of each pair of branches represents the distance between the sequences, and the horizontal axis indicates the approximate percent dissimilarities of the 3C protease amino acid sequences.

regions but strict conservation of critical amino acids located in the enzyme active sites, suggesting that inhibitors against 3C should have broad-spectrum activity. This was demonstrated in studies with rupintrivir, a novel inhibitor of 3C protease. In vitro antiviral assays, rupintrivir was active against all numbered HRV serotypes, untyped field isolates, and related picornaviruses tested (34, 46). Moreover, rupintrivir demonstrated comparable antiviral potencies against all HRV serotypes tested, exhibiting only a 27-fold range in EC_{50} values (46). This was in contrast to results observed with pirodavir and pleconaril, inhibitors of capsid function. These compounds demonstrated a wider range in antiviral potencies (1,590- to 2,707-fold range in EC_{50} values) and were not active against all HRV serotypes tested. In this study, we have further evaluated the in vitro antiviral activity of rupintrivir against HRV clinical isolates that were derived from nasal lavage specimens obtained from human volunteers after experimental challenge as well as four additional HEV strains. Consistent with results from previous studies, rupintrivir inhibited the replication of all isolates tested. Importantly, rupintrivir demonstrated comparable antiviral potencies across all picornaviruses tested ($n = 125$; 61-fold range in EC_{50} values) (34, 46).

To better understand the relationship between the broad-spectrum antiviral activity of rupintrivir and the level of homology among different 3C protease-coding regions, we performed nucleotide sequence analyses on 33 HRV numbered serotypes and clinical isolates. Previous studies by Matthews et

al. (41) show that extensive interactions occur between rupintrivir and the substrate (inhibitor) binding pocket residues of 3C protease. Specifically, examination of the high-resolution X-ray structure of rupintrivir bound to type 2 rhinovirus 3C protease (41) indicates extensive protein-inhibitor interactions involving 23 different amino acid residues. Individual amino side chains are involved in protein-inhibitor contacts for 14 of these residues, while for the remaining nine interacting residues, only polypeptide main chain atoms directly contact rupintrivir. We anticipate that side chain variation (nonconservation) for residues making only main chain contact with rupintrivir will have a minor effect on inhibitor binding. On the other hand, serotype-dependent substitutions for amino acids having side chain interactions with rupintrivir could be expected to directly affect inhibitor binding. In this regard, it is remarkable that of the 14 residues involved in such side chain-mediated interactions with rupintrivir, 13 are identically conserved in all known HRV 3C protease sequences. The single exception is Asn 130 (type 2) located at the back of the S2 specificity pocket. It is known that other 3C protease inhibitors with bulky substituents that penetrate deeply into the S2 pocket lose potency against certain HRV serotypes (41). It is likely that the high conservation of these extended binding site residues is directly related to stringent requirements for recognition, binding, and cleavage of viral precursor polyprotein sequences. Among HEV strains, Asn 130 was also not conserved, and Ile 125 and Ser 128 were replaced with conserva-

tive substitutions. The small variations in the EC₅₀ values are well below the level at which they could be rationalized by structure. Since the MOIs utilized in these experiments did not have a significant effect on virus susceptibility to rupintrivir (different MOIs yield comparable EC₅₀ values), it is likely that these variations are due to assay variability. Overall, these observations provide a compelling molecular explanation for the broad-spectrum potency of rupintrivir across a range of different picornavirus strains.

With the exception of HRV 87, phylogenetic analyses based on 3C protease-deduced amino acid sequences also confirm the segregation of numbered HRV prototype strains into two known genetic groups (5, 26, 43, 55). Specifically, HRV serotypes 3, 14, 17, and 52 appeared to form one cluster (group B), and all others appeared to cluster in the other group (group A). In terms of picornavirus evolution, it is noteworthy that relationships that have been defined for other regions of the genome (e.g., 5' untranslated region and VP1 and VP4/2 capsid proteins in the P1 region) are also exemplified in 3C protease, which occupies the terminal third or P3 region of the genome. The preservation of these relationships suggests that significant intertypic recombination has not occurred (51) and implies that recombination is not a primary mechanism for diversification in HRV. Consistent with analyses based on other regions of the picornavirus gene regions (1, 27, 37, 55), our phylogenetic analysis of 3C protease amino acid sequences also suggests that HRV 87 is more closely related to other HEV than to other HRV. Although HRV 87 also differs from other HRV in its receptor specificity, HRV 87 exhibits acid lability, a property typical for HRV and not other HEV. Despite these differences and consistent with the level of 3C protease homology, HRV 87 demonstrates susceptibility to rupintrivir comparable to other HRV and HEV.

In this study, we utilized nucleotide sequence and structural analyses to better understand the molecular basis for the broad-spectrum antiviral activity of rupintrivir. In summary, the absolute conservation of the amino acids involved in side chain interactions with rupintrivir explains rupintrivir's broad-spectrum activity and comparable antiviral potencies across diverse picornavirus strains and further highlights 3C protease as a novel antiviral target.

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