Structural Basis for Antiviral Inhibition of the Main Protease, 3C, from Human Enterovirus 93

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Members of the Enterovirus genus of the Picornaviridae family are abundant, with common human pathogens that belong to the rhinovirus (HRV) and enterovirus (EV) species, including diverse echo-, coxsackie- and polioviruses. They cause a wide spectrum of clinical manifestations ranging from asymptomatic to severe diseases with neurological and/or cardiac manifestations. Pandemic outbreaks of EVs may be accompanied by meningitis and/or paralysis and can be fatal. However, no effective prophylaxis or antiviral treatment against most EVs is available. The EV RNA genome directs the synthesis of a single polyprotein that is autocatalytically processed into mature proteins at Gln \( \downarrow \) Gly cleavage sites by the 3C protease (3C\(^{\text{pro}}\)), which has narrow, conserved substrate specificity. These cleavages are essential for virus replication, making 3C\(^{\text{pro}}\) an excellent target for antivirus drug development. In this study, we report the first determination of the crystal structure of 3C\(^{\text{pro}}\) from an enterovirus B, EV-93, a recently identified pathogen, alone and in complex with the anti-HRV molecules compound 1 (AG7404) and rupintrivir (AG7088) at resolutions of 1.9, 1.3, and 1.5 \( \AA \), respectively. The EV-93 3C\(^{\text{pro}}\) adopts a chymotrypsin-like fold with a canonically configured oxyanion hole and a substrate binding pocket similar to that of rhino-, coxsackie- and poliovirus 3C proteases. We show that compound 1 and rupintrivir are both active against EV-93 in infected cells and inhibit the proteolytic activity of EV-93 3C\(^{\text{pro}}\) in vitro. These results provide a framework for further structure-guided optimization of the tested compounds to produce antiviral drugs against a broad range of EV species.

Enteroviruses (EVs) are small, nonenveloped, icosahedral, positive-sense, single-stranded RNA viruses classified into a genus of the Picornaviridae family, one of the largest and most important families of viral pathogens of vertebrates, including humans (36). The Enterovirus genus encompasses 234 human pathogens that form 7 species spread worldwide: human enteroviruses A through D (HEV-A, HEV-B, HEV-C, and HEV-D) and human rhinoviruses A through C (HRV-A, HRV-B, and HRV-C) (23). Echoviruses and coxsackievirus B (CV-B) are classified within the HEV-B species, and polioviruses (PVs) are classified within HEV-C. There are also EVs that infect nonhuman primates, cattle, and swine that may play roles in zoonotic spread and the emergence of new human pathogens. In humans, infections range from asymptomatic to more severe illnesses that are manifested as aseptic meningitis, encephalitis, gastroenteritis, myocarditis, paralysis, and poliomyelitis, with high mortality rates in infected newborn infants. Outbreaks of different EVs are frequently reported. For example, EV-71 of HEV-A caused serious complications—encephalitis and myocarditis—and death during epidemics of hand, foot, and mouth disease in Asia in 1997, 1998, 2000, and 2008 (44). The disease manifestation of acute flaccid paralysis is also associated with nonpoliovirus EVs within the HEV-B species, including newly discovered viruses like EV-93, which is the subject of this study (20). Despite the enormous health care impact of EV infections, no antiviral drugs have been approved to control these infections (for a recent review, see reference 11). The EV genome is a positive-sense, single-stranded RNA of between 7.4 and 7.5 kb with a single open reading frame translated into a large polyprotein of approximately 2,200 amino acids (~250 kDa) (13, 38). This polyprotein is rapidly processed by co- and posttranslational cleavages into three precursor molecules, P1, P2, and P3, and then into mature viral proteins: the structural proteins VP4 to VP1 from P1 and the nonstructural proteins associated with replication, 2A to 2C and 3A to 3D from P2 and P3, respectively, from the N to the C terminus (22). Most of these cleavages are mediated by the 3C protease (3C\(^{\text{pro}}\)) either alone or as a domain of 3CD\(^{\text{pro}}\). In addition to its key role in processing the polyprotein, 3C\(^{\text{pro}}\) cleaves a number of host proteins to remodel the cellular environment for virus reproduction (12, 43). Due to its central role in the control of genome expression, 3C\(^{\text{pro}}\) could be considered the “main” protease (49). Crystal structures have been determined for EV 3C\(^{\text{pro}}\) from CV-B3, HRV-2, HRV-14, PV-1, and EV-71 (7, 10, 27, 30, 31, 34). The protease adopts a...
chymotrypsin-like fold with the Cys-His-Glu catalytic triad and its orally bioavailable analogue compound 1 (AG7404). Asterisks indicate carbon atoms that make an irreversible covalent bond with the active-site cysteine residue of 3Cpro.

FIG. 1. Chemical structure of rupintrivir (AG7088) and its orally bioavailable analogue compound 1 (AG7404).
antiviral method and using the semiempirical quantum-mechanical optimization method AM1 (33). Geometry restraint information for rupintrivir was calculated with SKEWTER within CCP4i (9). The lyophilized peptide and antiviral agents were dissolved in 100% dimethyl sulfoxide (DMSO) at a concentration of 0.5 mM. Cleavage reaction inhibition by two antiviral agents, rupintrivir (AG7088) and compound 1, was tested in infected cells.

**Protoactivity in infected cells.** The antiviral activities of rupintrivir (AG7088) and compound 1 (AG7404) were determined by incubating monolayers of human rhabdomyosarcoma cells (RD) with 100 or 1,000 50% cell culture infective doses (CCID50) of EV-93 in two 96-well plates. After an adsorption period of 2 h at 37°C, the virus was removed and serial dilutions of one compound per plate were added. The cultures were further incubated for 7 days, until the complete cytopathogenic effect (CPE) was observed in the wells with infected cells. The cultures were then further incubated for 7 days, until the complete cytopathogenic effect (CPE) was observed in the wells with infected cells. The cultures were then further incubated for 7 days, until the complete cytopathogenic effect (CPE) was observed in the wells with infected cells.

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**RESULTS AND DISCUSSION**

Structure determination and model quality. EV-93 3Cpro was expressed in E. coli cells and purified at a high yield (~25 mg per liter of cell culture). Needle-like crystals, up to approximately 10 by 10 by 170 μm, were obtained in a few weeks from a solution containing PEG 8000, magnesium acetate, and cacodylate. Further optimizations of the crystallization conditions, including additive screen and macro- or microseeding, did not yield larger crystals of the native protein. Taking advantage of the highly focused beam of the ID23-2 line at the European Synchrotron Radiation Facility (ESRF; Grenoble, France), we collected two data sets from a single crystal, irradiating each time a fresh crystal portion along its long dimension, with a shifted but overlapping 2θ-angle range. Merging the two data sets at high resolution (Table 1). The stability of the protein was confirmed by tandem mass spectrometry (MS-MS) fragmentation and SDS-PAGE.

**Table 1. Crystallographic data and refinement statistics**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>EV-93 3Cpro</th>
<th>EV-93 3Cpro-compound 1</th>
<th>EV-93 3Cpro-rupintrivir</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution range (Å)</td>
<td>66.4–1.9 (2.0–1.90)</td>
<td>39.0–1.32 (1.39–1.32)</td>
<td>30.0–1.50 (1.55–1.50)</td>
</tr>
<tr>
<td>Space group</td>
<td>P21</td>
<td>P21</td>
<td>P21</td>
</tr>
<tr>
<td>Cell dimensions (Å, degrees)</td>
<td>a = 39.07, b = 65.22, c = 66.36, β = 90.67</td>
<td>a = 39.04, b = 64.45, c = 68.74, β = 90.81</td>
<td>a = 39.00, b = 63.91, c = 66.36, β = 90.43</td>
</tr>
<tr>
<td>No. of observed reflections</td>
<td>26,356 (3,828)</td>
<td>79,978 (11,666)</td>
<td>52,513 (5,196)</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>4,4 (3.3)</td>
<td>3.4 (3.3)</td>
<td>3.7 (3.6)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.9 (100)</td>
<td>100 (100)</td>
<td>100 (100)</td>
</tr>
<tr>
<td>Mean multiplicity</td>
<td>5.1 (1.0)</td>
<td>5.2 (1.5)</td>
<td>17.7 (2.5)</td>
</tr>
<tr>
<td>Rmerge</td>
<td>0.127 (0.412)</td>
<td>0.085 (0.508)</td>
<td>0.063 (0.488)</td>
</tr>
<tr>
<td>Rwork</td>
<td>0.148</td>
<td>0.125</td>
<td>0.166</td>
</tr>
<tr>
<td>Rfree</td>
<td>0.210</td>
<td>0.170</td>
<td>0.198</td>
</tr>
<tr>
<td>No. of protein atoms (non-H)</td>
<td>3,037</td>
<td>3,322</td>
<td>3,011</td>
</tr>
<tr>
<td>No. of water molecules</td>
<td>339</td>
<td>543</td>
<td>375</td>
</tr>
<tr>
<td>No. of hetero compounds</td>
<td>2 Mg2+ ions, 6 glycerol molecules</td>
<td>2 compound 1 molecules, 8 ethylene glycol molecules, 3 HPO4 2− ions, 2 NH4+ ions</td>
<td>2 rupintrivir molecules, 2 Mg2+ ions, Cl− ions</td>
</tr>
<tr>
<td>RMSD for bond length (Å); RMSD for bond angles (°)</td>
<td>0.012; 1.40</td>
<td>0.012; 1.60</td>
<td>0.008; 1.41</td>
</tr>
<tr>
<td>Mean B value (Å2)</td>
<td>11.89</td>
<td>9.29</td>
<td>12.21</td>
</tr>
<tr>
<td>Mean B value for inhibitor molecules (Å2)</td>
<td>20.2, 19.7</td>
<td>16.8, 22.0</td>
<td></td>
</tr>
<tr>
<td>Ramachandran analysisa,d</td>
<td>88.8/10.2/1.0</td>
<td>89.1/9.6/1.3</td>
<td>97.26/2.74/0.0</td>
</tr>
<tr>
<td>Values for indicated protease or compounda</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**

1. Values in parentheses refer to the high-resolution shell.
2. Rmerge = Σh,θ |Ihθ|−<Ihθ>/Σh,θ Ihθ, where Ihθ is the ith-intensity measurement of reflection h and <Ihθ> is the average intensity for multiple measurements.
3. Rwork = Σh|Fh|−|Fobs|/Σh|Fobs|, Rfree was calculated for 5% of the reflections not used for refinement.
4. Ramachandran analysis was done with PROCHECK.

**In vitro proteolytic activity assay.** A peptide representing the predicted 3Cpro cleavage site of EV-93 (Ac-RHSGATLEALFQ | GPPVYREIKIS-NH2 | Gencepep) was used to test EV-93 3Cpro proteolytic activity in vitro and its inhibition by two antiviral agents, rupintrivir (AG7088) and compound 1 (AG7404). The hydrolyzed peptide and antiviral agents were dissolved in 100% dimethyl sulfoxide (DMSO) at a concentration of 0.5 mM. Cleavage reaction mixtures containing 30 μM peptide, 3 μM EV-93 3Cpro, 50 mM HEPES (pH 7.2), and 150 mM NaCl in a total volume of 100 μl were incubated at 30°C. The reactions were stopped after 2 h by the addition of 0.5% (final concentration) trifluoroacetic acid (TFA) or by freezing at −20°C. Samples were analyzed by reverse-phase high-performance liquid chromatography (HPLC) on a Source SRPC ST 4.6/150 column (GE Healthcare) using a 2% to 90% linear gradient of acetonitrile in 0.1% TFA. To keep them independent of the initial amount of the substrate, trans cleavage efficiencies (E) are reported as the fraction of the substrate converted to products, based on the integrated peak areas at 215 nm corresponding to the remaining substrate (r) and the products (p), as follows: E = −Σp/Σr + Σp), where the substrate and products were analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) and comparison with the reference samples (the synthesized full peptides, Ac-RHS VGATLEALFQ-OH and H-GPPVYREIKIS-NH2). The presence of the protein was confirmed by tandem mass spectrometry (MS-MS) fragmentation and SDS-PAGE.

**Protein structure accession numbers.** Atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 3Q3X, 3Q3Y, and 3RUO for EV-93 3Cpro, EV-93 3Cpro-compound 1, and EV-93 3Cpro-rupintrivir, respectively.
during irradiation was most likely due to its low solvent content, 37.5%, corresponding to dense crystal packing with two molecules in the asymmetric unit.

We solved the structure of EV-93 3C\textsuperscript{pro} by molecular replacement, using the structure of the homologous PV-1 3C\textsuperscript{pro} as a search model, the two proteins sharing 61% of identical amino acids. The structure of EV-93 3C\textsuperscript{pro} was refined at 1.9 Å to a final $R_{\text{work}}$ of 14.8% ($R_{\text{free}} = 21\%$; see Table 1 for refinement statistics). We modeled all residues except the His\textsubscript{6} tag and the last two and three C-terminal residues for chains A and B, respectively, which we did not model because of poor electron density. Figure 2 shows the electron density in a representative region of the structure. The Ramachandran plot shows 88.4% of the residues to be in the most favored regions, 10.3% and 1.3% in the additionally and generously allowed regions, respectively (26). The two molecules of the asymmetric unit are related by a noncrystallographic 2-fold axis. The refined structure contains 339 water molecules, 2 magnesium ions, and 6 glycerol molecules.

The structure of EV-93 3C\textsuperscript{pro} in complex with compound 1 was solved by molecular replacement using the native structure and was refined at 1.32 Å to a final $R_{\text{work}}$ of 12.5% ($R_{\text{free}} = 17\%$; Table 1). The final model encompasses all EV-93 3C\textsuperscript{pro} amino acids except the first N-terminal and the last three C-terminal (and His\textsubscript{6} tag) residues. One molecule of compound 1 per protein chain, including molecules from the 1-fluorobenzene-4-yl group, which is different from the corresponding compound 1 group (Fig. 1, P2 position), was clearly defined in both 2Fo–Fc and Fo–Fc electron density maps. Given the higher resolution of the EV-93 3C\textsuperscript{pro}–compound 1 complex structure, the molecular replacement calculation was repeated with protein molecule A of the complex structure (without ligand and solvent atoms) as the initial model. The electron density maps showed a position for the rupintrivir molecule identical to that shown when using the native structure as the starting model, so the molecule was added. The structure was refined at 1.50 Å to a final $R_{\text{work}}$ of 16.6% ($R_{\text{free}} = 19.8\%$; Table 1). The structure also contains 375 water molecules, 2 Mg\textsuperscript{2+} ions, and 1 Cl\textsuperscript{−} ion.

Overall structure and active site of EV-93 3C\textsuperscript{pro}. EV-93 3C\textsuperscript{pro} folds into two antiparallel \(\beta\) barrels (residues 15 to 77 and 97 to 173, respectively) that are oriented 90° apart, linked by a 20-amino-acid loop with a short \(\alpha\)-helix in its middle, and flanked by two other \(\alpha\)-helices at the N and C termini, 14 and 6 amino acids long, respectively (Fig. 3a). The two barrels are topologically equivalent and are formed by six antiparallel \(\beta\) strands with the first four (A to D) organized into a Greek key motif. Our structure confirms that EV-93 3C\textsuperscript{pro} adopts a chymotrypsin-like fold similar to that of other picornavirus 3C\textsuperscript{pro}s. The RMSDs between 3C\textsuperscript{pro}s from EV-93 and other EVs are 0.28 Å (160 C\textsuperscript{a} atoms) for CV-B3, 0.70 Å (152 C\textsuperscript{a} atoms) for PV-1, 0.77 Å (150 C\textsuperscript{a} atoms) for HRV-2, 1.02 Å (150 C\textsuperscript{a} atoms) for HRV-14, and 1.16 Å (170 C\textsuperscript{a} atoms) for EV-71 (7, 10, 27, 30, 34). These figures underline the high conservation of the 3C\textsuperscript{pro} structure in viruses of the Enterovirus genus. Analysis of EV-93 3C\textsuperscript{pro} interfaces with the PISA server (24) suggests that the homodimer formed by the two chains of the asymmetric unit (Fig. 3a) might be stable.
in solution with an interface area of 1,710 Å² mainly involving parts of the H9251 1-helix and B2 strand (residues 1, 5, 107, 113, and 143 from both chains). However, a gel filtration chromatogram showed only one peak corresponding to a monomer of EV-93 3Cpro, and there is no further evidence that dimerization is required for the proteolytic activity of EV-93 3Cpro.

The active-site residues are located in the cleft between the two barrels with the nucleophilic Cys147 from the C-terminal barrel and the general acid base pair His40–Glu71 from the N-terminal barrel (Fig. 3). Early in the refinement of the native structure of EV-93 3Cpro, residual-difference density close to the sulfur of the active-site cysteine in both copies indicated that Cys147 residues were oxidized at least to the stage of sulfenic acid, SO₂⁻, and partially to that of sulfonic acid, SO₃⁻. We therefore modeled both residues as the latter case, with partial occupations for the O atom (Fig. 3b). Since the purified protein is active (see below), such oxidation must have occurred during crystallization or X-ray data collection.

Oxidized active-site Cys residues were also observed in other picornaviral 3Cpros (2, 3). As a result, the side chains of His40 adopt two conformations, one corresponding to the canonical orientation, as seen in other EV 3Cpros, and making hydrogen bonds with the two other active-site residues, and the second corresponding to the oxidized-cysteine state, being rotated about 120° out of the active site.

Substrate hydrolysis by cysteine proteases occurs through a covalent tetrahedral intermediate between the active-site nucleophile and the carbonyl carbon of the scissile bond. The resulting oxyanion is stabilized by strong hydrogen bonds with amide groups of the protease, which are collectively called the oxyanion hole. In the EV-93 3Cpro structure (Fig. 3b), the amide groups of Cys147, Gln146, and Gly145 form this oxyanion hole with a conformation similar to that of other EV 3Cpros, which is adequate to stabilize the tetrahedral intermediate.

3C proteases were shown to recognize amino acid residues around the cleavage site, mostly at the P4…P1 positions that fit into corresponding specific binding subsites (S4…S1, S1') of the protease. Based on comparison of the structure of HRV-14 3Cpro covalently bound to a peptide (acetyl-LEALFQ-ethyl propionate) inhibitor, including P6 to P1 substrate residues (7), with the EV-93 3Cpro structure, we propose that the substrate binding pocket of EV-93 3Cpro is formed by residues belonging to the β strands B2, E2, F2 and to the C2 to D2 loop head of the oxyanion hole (Fig. 3b). In particular, mutations in the B2 to C2 loop were shown to have a significant impact on the proteolytic activity of 3Cpro from foot-and-mouth disease virus, which belongs to another genus of the Picornaviridae family (47).

Rupintrivir and compound 1 inhibit EV-93 replication in infected cells. Since compound 1 and rupintrivir, developed as irreversible inhibitors of HRV 3Cpro, inhibit 3Cpros encoded by viruses of HEV species (40, 41), we reasoned that they could also be active against EV-93. Indeed, rupintrivir inhibited EV-93 replication in infected RD cells, a prototype cell line for enterovirus growth (48), with a mean EC₅₀ of 33 nM (range, 17 to 50 nM). The EC₅₀ for compound 1 was 93 nM (range, 37 to 112 nM). These results are consistent with mean EC₅₀ obtained for related HEVs: 88 nM (range, 7 to 183 nM) and 75 nM (range, 7 to 249 nM) for rupintrivir and compound 1, respectively (40, 41). We were unable to isolate a drug-resistant virus after 15 successive passages of the virus in the presence of either of the compounds at concentrations that allowed observation of the full cytopathogenic effect caused by the virus. For HRVs, most mutations conferring resistance to

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**FIG. 3.** Crystal structure of EV-93 3Cpro. (a) Ribbon representation of the two molecules present in the asymmetric unit with the noncrystallographic 2-fold axis (●) perpendicular to the plane. The protease folds into two antiparallel β barrels (in green and orange tones from the N to the C terminus), forming the chymotrypsin-like fold. The catalytic triad is highlighted as stick representations. (b) Active site of EV-93 3Cpro. Key residues are highlighted as ball-and-stick representations (stereo view). Main-chain amides forming the oxyanion hole are indicated by “n.”
rupintrivir were obtained after only a few serial passages, 3 for HRV-14, 4 for HRV-2 and HRV-39, and 6 for HRV-Hanks (6), and thus 15 passages were considered sufficient. As mutating residues in HRVs are mostly conserved in sequence and structure compared to those in EV-93, we cannot rule out that the difference between EV-93 and HRVs in obtaining drug-resistant viruses is due to different passaging procedures.

**Rupintrivir and compound 1 inhibit EV-93 3C\textsuperscript{pro} protease in vitro.** In order to verify that compound 1 and rupintrivir could also target the EV-93 3C protease, we tested the effect of each antiviral agent on the *in vitro* proteolytic activity of EV-93 3C\textsuperscript{pro} on a cognate peptide substrate (P13 to P11) that mimics the 2C \textsubscript{1} \textsubscript{3}A cleavage site. As illustrated in Fig. 4A, reverse-phase HPLC analyses of overnight incubations of the peptide with EV-93 3C\textsuperscript{pro} provide clear evidence of the expected proteolytic cleavage. The identities of the substrate and its cleavage products were verified by MALDI-TOF and comparison with the reference samples, while the presence of the protein was confirmed by MS-MS fragmentation and SDS-PAGE. The proteolytic assay performed with the C147A mutant of EV-93 3C\textsuperscript{pro} showed no effect on the substrate and did not render detectable cleavage products (Fig. 4D), thereby confirming that Cys\textsuperscript{147} is the active nucleophilic residue. Proteolytic assays performed in the presence of DTT showed similar results (Fig. 4B and Table 2).

**FIG. 4.** Inhibition of the *in vitro* proteolytic activity of EV-93 3C\textsuperscript{pro} by rupintrivir. Reverse-phase chromatograms show in red the products of the digestion of the EV-93 2C \textsubscript{1} \textsubscript{3}A peptide (Ac-RHSVGATLEFQ-GPPVYREIKIS-NH\textsubscript{2}) by EV-93 3C\textsuperscript{pro} (p) without rupintrivir (r) (A) or with rupintrivir at two inhibitor-to-protein molar ratios, 1:3 (B) and 30:3 (C). (D) C147A mutant protein. Chromatograms of the substrate peptide alone are shown in blue in all panels.

**TABLE 2.** *trans*-cleavage efficiencies of 2C \textsubscript{1} \textsubscript{3}A peptide by EV-93 3C\textsuperscript{pro}.

<table>
<thead>
<tr>
<th>Protein Inhibitor</th>
<th>Inhibitor-to-protein molar ratio ((\mu)M)</th>
<th>Cleavage efficiency (E^a)</th>
</tr>
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<tbody>
<tr>
<td>EV-93 3C\textsuperscript{pro}</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>EV-93 3C\textsuperscript{pro} purified</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>EV-93 3C\textsuperscript{pro} C147A mutant</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>EV-93 3C\textsuperscript{pro} Rupintrivir</td>
<td>1:3</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>3:3</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>30:3</td>
<td>0</td>
</tr>
<tr>
<td>EV-93 3C\textsuperscript{pro} Compound 1</td>
<td>1:3</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3:3</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>30:3</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) ++++, efficiency of >90%; ++, efficiency of <20%; +, efficiency of <10%; 0, no visible product peaks.
ence of an inhibitor with three different inhibitor-to-protease molar ratios indicated that both rupintrivir and compound 1 efficiently inhibit native EV-93 3Cpro in vitro (Fig. 4B and C and Table 2). No cleavage product was detected with a 10-fold excess of inhibitors over the enzyme, and cleavage efficiencies of less than 10% were observed with equimolar amounts of the two antivirals relative to the 3Cpro (Table 2). These results are consistent with prior results for potency and irreversible inhi-

![Stereo views of compound 1 and rupintrivir bound to the EV-93 3Cpro active-site pocket.](image-url)

FIG. 5. Stereo views of compound 1 and rupintrivir bound to the EV-93 3Cpro active-site pocket. (a and b) Refined structures of EV-93 3Cpro in complex with compound 1 (a) and rupintrivir (b). Both compounds are represented as sticks, with their 2Fo−Fc-weighted electron density contoured at 1.5 σ and represented as a blue mesh and their Fo−Fc-weighted-difference electron densities contoured at −3 σ and +3 σ and shown in red and green, respectively. EV-93 3Cpro residues interacting with the compounds are shown as sticks and are labeled. (c) Conservation of the compound 1 and rupintrivir binding pocket. The rupintrivir structure (yellow carbon atoms) is overlaid on the EV-93 3Cpro-compound 1 structure (green carbon atoms). The EV-93 3Cpro molecular surface is colored from cyan to magenta for variable to conserved residues, respectively, based on the multiple-sequence alignment presented in Fig. 6. EV-93 3Cpro residues interacting with the antiviral compounds are shown as sticks and are labeled. Positions P4 to P1' are labeled in all figures.
bition by these compounds of 3C pro from HRVs and EVs (40, 41) and corroborate their strong antiviral effect against a broad spectrum of picornaviruses.

Rupintrivir and compound 1 as potential antivirals against all EVs. To characterize the molecular interactions of compound 1 and rupintrivir with EV-93 3C pro, we cocrystallized their complexes and solved their crystallographic structures at very high resolutions. The structure of EV-93 3Cpro-compound 1 is the first known structure of a protease in complex with this antiviral agent. The electron density allowed us to unequivocally and precisely build one molecule of compound 1 or rupintrivir per protein (Fig. 5). In both cases, the inhibitor electrophilic carbon (Fig. 1, asterisk) is covalently bound to the active-site Cys 147 after its Michael addition, forming a stable tetrahedral adduct and resulting in the irreversible inactivation of the protease. Compound 1 binds to EV-93 3Cpro in a partially extended conformation with its peptidomimetic backbone making antiparallel -sheet-type hydrogen bonds with part of the solvent-exposed β strand, E2, of the protein (residues 162 to 164). The inhibitor's P4 part (Fig. 5a) lies in the deep groove formed by the β strands E2, F2, and B2 and interacts with protein residues 125 to 128, 164 to 165, 168, and 170. The P3 part makes two main-chain hydrogen bonds with Gly163–164, and the hetero ring is mainly solvent exposed, interacting with Gly 128 only on one side. P2 2-propynyl stacks against His 40, and residues 71 and 127 further constrain its conformation. The P1 part is deeply inserted between strands E2 (residues 162 to 164) and loop 142 to 144, making this pocket wider by about 1 Å than that of the native structure. A P1 glutamine-like side chain makes hydrogen bonds with Thr142 and His161, most probably mimicking the recognition of the natural substrate P1 Gln, which is highly conserved in 3C cleavage sequences. The P1 carbonyl oxygen of the ethyl ester is positioned above the oxyanion hole formed by the amide groups of Cys147, Gln 146, and Gly 145 but makes a hydrogen bond only with the latter. The ethoxycarbonyl group is more mobile and is either solvent exposed or interacts with residues 23 to 25.
The binding mode of rupintrivir 1 to EV-93 3Cpro is very similar to that of compound 1. The differences between the two antivirals lie in the least-conserved P3 and P2 positions (Fig. 1 and 5b), where a 2-pyridon-1,3-diy group that cycles with the following amine is replaced by a valine amino acid (4-methyl-pentan-2-one-1,3-diy) and the ethynyl group is replaced by a 1-fluorobenzen-4-yl group in rupintrivir. Variations at the P3 position imply the loss of van der Waals contacts with Gly128 in the case of rupintrivir, since its valine side chain is exposed to the solvent and does not interact with the protein. In contrast, compared with that in compound 1 the P2 substitution in rupintrivir results in additional stacking interactions of the 1-fluorobenzen-4-yl ring with His60 and Glu71 side chains and new interactions of the fluorine atom with Thr130 and Arg39. The stacking interactions of the inhibitor ring with His60 and Glu71 were also observed in the HRV-2 3Cpro-rupintrivir complex (30), as was the H bond of the fluorine atom with Thr130, which is an Asn in HRV-2 3Cpro. The contact with Arg39 is not present in the HRV-2 3Cpro-rupintrivir complex, since this residue is a Thr in the HRV-2 protein and has a much shorter side chain. The enhanced interactions of rupintrivir with the proteases at site S2 could explain why EC50s for this antiviral are almost systematically lower than those obtained for compound 1 against EV-93 and HRVs (see above and references 40 and 41). Interactions of rupintrivir with EV-93 and HRV-2 3Cpro’s also changed for the following residues: Leu125 and G128, which correspond to Ile and Ser in the HRV-2 protein, respectively, and Asn165 and Thr142, which have different side chain conformations in the two complexes.

A noticeable feature of the binding pocket is the conservation of EV-93 3Cpro residues interacting with compound 1 within all EVs. For 11 EV species representing the entire genetic diversity of this genus, most of the 3Cpro residues making side chain interactions (71%; in red in Fig. 6) or only main-chain interactions (62%; in blue) with compound 1 are identical or physico-chemically similar. The four not strictly conserved residues involved in side chain interactions (Leu125, Gly128, Thr130, and Phe170) make steric interactions with compound 1 or rupintrivir that are compatible with the amino acid diversity observed. Thr130 interacts only with the fluorine end of the 1-fluorobenzen-4-yl group of rupintrivir (Fig. 1, P2), as was discussed above. A higher level of conservation of the 3C inhibitor binding pocket is observed in HRV serotypes (5).

In studies to control natural rhinovirus infection by 3Cpro inhibitors, compound 1 or rupintrivir showed unsatisfactory performance and was therefore excluded from further clinical development (39). Our results with EV-93 indicate that these compounds could be valuable antivirals against other EV species. The high level of conservation among EVs of the residues forming the 3Cpro binding pockets for compound 1 and rupintrivir and the broad-spectrum antiviral activity of these compounds in vitro reinforce their potential as excellent candidates for developing potent antivirals against all EVs (28). These results also suggest that the level of conservation of the residues forming the substrate binding pocket could be useful in the process of designing antiviral compounds against new, emerging enteroviruses.

Conclusion. In summary, we report the first determination of the crystallographic structure of the main protease from a human enterovirus B (EV-93 3Cpro) alone and in complex with the HRV antiviral molecules compound 1 and rupintrivir at resolutions of 1.9, 1.3, and 1.5 Å, respectively. The chymotrypsin-like fold of the protease presents the catalytic triad Cys-His-Glu in the cleft between the two six-stranded β barrels, adjacent to a canonically configured oxyanion hole. We showed that compound 1 and rupintrivir inhibit the proteolytic activity of EV-93 3Cpro in vitro and are active against EV-93 in infected cells. The primary and tertiary structures of the 3Cpro binding pockets for these two compounds are highly conserved among EVs, which explains their broad-spectrum antiviral capacity. These results reinforce the structural framework for designing antiviral drugs against the 3Cpro to control enterovirus infections.

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REFERENCES

Crystallographic analysis reveals a trypsin-like polypeptide fold, RNA-binding site, and means for irreversible inhibitors of human rhinovirus 3C protease with potent antiviral activity.


