3C Protease of Enterovirus 68: Structure-Based Design of Michael Acceptor Inhibitors and Their Broad-Spectrum Antiviral Effects against Picornaviruses

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We have determined the cleavage specificity and the crystal structure of the 3C protease of enterovirus 68 (EV68 3Cpro). The protease exhibits a typical chymotrypsin fold with a Cys...His...Glu catalytic triad; its three-dimensional structure is closely related to that of the 3Cpro of rhinovirus 2, as well as to that of poliovirus. The phylogenetic position of the EV68 3Cpro between the corresponding enzymes of rhinoviruses on the one hand and classical enteroviruses on the other prompted us to use the crystal structure for the design of irreversible inhibitors, with the goal of discovering broad-spectrum antiviral compounds. We synthesized a series of peptidic α,β-unsaturated ethyl esters of increasing length and for each inhibitor candidate, we determined a crystal structure of its complex with the EV68 3Cpro, which served as the basis for the next design round. To exhibit inhibitory activity, compounds must span at least P3 to P1; the most potent inhibitors comprise P4 to P1. Inhibitory activities were found against the purified 3C protease of EV68, as well as with replicons for poliovirus and EV71 (50% effective concentration [EC50] = 0.5 μM for the best compound). Antiviral activities were determined using cell cultures infected with EV71, poliovirus, echovirus 11, and various rhinovirus serotypes. The most potent inhibitor, SG85, exhibited activity with EC50 of ~180 nM against EV71 and ~60 nM against human rhinovirus 14 in a live virus–cell-based assay. Even the shorter SG75, spanning only P3 to P1, displayed significant activity (EC50 = 2 to 5 μM) against various rhinoviruses.

Enteroviruses comprise several pathogens that are implicated in a large variety of clinical manifestations ranging from mild illnesses to more serious or even life-threatening diseases, such as meningitis, encephalitis, myocarditis, pancreatitis, acute paralysis, or neonatal sepsis. In recent years, China and several countries in South East Asia have been hit by outbreaks of hand, foot, and mouth disease caused by enterovirus (EV) 71 or coxsackievirus A16 (more than 488,000 cases in the 2008 epidemic in China alone [3]).

To date, no approved specific antiviral therapy for diseases caused by enteroviruses is available. There is an urgent need for safe and broad-spectrum drugs against the existing pathogenic EVs. The same holds true for other members of the picornavirus family, in particular rhinoviruses, since it is now clear that some of the latter cause exacerbations of asthma and chronic obstructive pulmonary disease (1). Also, drugs against poliovirus are needed to aid in the completion of polio eradication (4).

The enteroviral genome consists of a single-stranded, positive-sense RNA of approximately 7,500 bases in length. The coding region of the viral genome is divided into three parts (P1, P2, and P3) encoding the four structural (derived from P1) and seven nonstructural viral proteins (derived from P2 and P3). The genome gives rise to the viral polyprotein, which is processed co- and posttranslationally through a series of primary and secondary proteolytic cleavages by the virus-encoded proteases 2Apro and 3Cpro/3CDpro. 2Apro cleaves the bond between the P1 and P2 segments of the viral polyprotein, whereas the 3Cpro and its precursor, 3CDpro, are responsible for generating the majority of precursor and mature proteins (see, for example, reference 1).

Here, we describe the substrate cleavage specificity and the crystal structure (at 2.4-Å resolution) of the 3C protease of enterovirus 68 (EV68, also called EV-D68 to indicate its membership in the human enterovirus D family). This is the first crystal structure of a protein from a group D enterovirus. Like the other members of the enterovirus family, the EV68 3Cpro is a cysteine protease containing a Cys...His...Glu catalytic triad and exhibiting a two-domain fold similar to that of the serine proteases of the chymotrypsin family. A structural comparison of the 3C proteases of known crystal structure revealed an intermediate position of the EV68 3Cpro between the corresponding enzymes from human rhinovirus 2 (HRV2) and those from enteroviruses and prompted us to use the structure for the design of broad-spectrum antivirals directed against picornaviruses in general, even though EV68 itself...
does not play any important role as a pathogen. Because of their importance in the viral replication cycle and their unique specificity for glutamine in the P1 position of the substrate (which is not found in any known host-cell protease), 3C proteases are attractive targets for antiviral drug discovery (5, 6). However, only one such drug candidate has entered clinical trials thus far. Rupintrivir (AG7088) was developed as an inhibitor of the 3Cpro of human rhinoviruses, but development was stopped after phase II because of limited efficacy in the treatment of the common cold (7). Carrying an \( /H9251, /H9252\)-unsaturated alkyl ester moiety, rupintrivir acts as a Michael acceptor for the nucleophilic Cys residue in the catalytic center of the 3Cpro (8). A series of studies found \( /H9251, /H9252\)-unsaturated alkyl esters to be the most active rhinovirus 3C protease inhibitors among various electrophilic compounds (9). We and others have occasionally described \( /H9251, /H9252\)-unsaturated esters as inhibitors of coronavirus or enterovirus proteases (10–14), but the only systematic study involving X-ray crystallography and structure-based drug design, as well as \textit{in vivo} evaluation, deals with the coronavirus main protease as a target (14).

We synthesized a series of peptides of increasing lengths that carry an \( /H9251, /H9252\)-unsaturated ester as a Michael acceptor for the nucleophilic cysteine residue of the enzyme. A crystal structure of each compound in complex with the EV68 3Cpro revealed details of the interactions occurring in the specificity subsites of the enzyme and suggested modifications to be applied in the next round of the design process. We found that the potency of the compounds increased with length. SG85, which occupied 3Cpro subsites S4 to S1, was the most potent among these inhibitors, with \( k_{\text{obs}}/[I] = 202,200 \text{ M}^{-1} \text{s}^{-1} \). Most members of this series of compounds were not toxic to Vero A, HeLa Rh, BGM, RD, and Huh-T7 cells. They proved also active on the replication of subgenomic replicons and in cells infected with other picornaviruses such as enterovirus 71 (also called EV-A71 to indicate its classification as a human enterovirus A [HEV-A] species), poliovirus, echovirus 11, and rhinoviruses. Thus, our structure-based drug design approach underlines the suitability of the picornaviral 3C protease as a target for broad-spectrum antivirals.

**MATERIALS AND METHODS**

**Synthesis of \( /H9251, /H9252\)-unsaturated ester compounds.** SG74 was synthesized according to a published procedure (15) (see Fig. 1 and the supplemental material). To a solution of SG74 (for SG75 and SG81) or SG81 (for SG82, SG83, SG84, SG85, and SG98) (1 mmol, 1 eq) in 1 ml of CH2Cl2, 0.5 ml of trifluoroacetic acid (TFA) was added at 0°C. The dark brown solution was stirred at room temperature for 2 h and then concentrated in vacuo. Next, 0.5 ml of dimethylformamide (DMF) was added to neutralize the residual in situ, and the resulting solution was used for the next step without further purification. Protected amino acid (1 mmol, 1 eq), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI)-HCl, and then NaBH4, 25°C, 18 h, 62%; (g) (i) TFA:CH2Cl2 (1:2), 2 h; (h) EDCI, HOBr, Boc-Phe-OH, DMF, 0 to 25°C, 18 h, 74%. (B) Reagents and conditions are presented as follows: (a) (i) TFA:CH2Cl2 (1:2), 2 h; (ii) EDCI, HOBr, protected amino acid, DMF, 0 to 25°C, 18 h, 46 to 70%.
resulting solution was stirred at room temperature for 18 h. The reaction mixture was then diluted with ethyl acetate, and the organic phase was washed with 10% citric acid, saturated aqueous NaHCO₃, and brine, dried over MgSO₄, filtered, and concentrated in vacuo. The resulting white solid was further purified through silica gel chromatography.

**Recombinant protein production.** cDNA corresponding to the 3Cpro domain of EV68 (strain 3799) was amplified prior to cloning into the pOPINE plasmid in order to produce the EV68 3Cpro in fusion with a lysine residue and a His₆ tag at the C terminus (16). The coding region was verified by DNA sequencing, and the construct was transformed into *Escherichia coli* Tuner (DE3) placI cells for expression.

The protein was purified using immobilized-metal-affinity chromatography (HisTrap FF column; GE Healthcare) and eluted with 25 mM Tris–HCl (pH 8.0), 300 mM NaCl, and 250 mM imidazole. Further purification was achieved by size-exclusion chromatography using 25 mM Tris–HCl (pH 8.0), 200 mM NaCl, 5 mM diithiothreitol (DTT), and a HiLoad 16/60 Superdex 75 Prep-Grade column. Protein concentration was determined using the Bio-Rad protein assay, and the sample was stored at −70°C.

**Crystallization and data collection.** The protein was concentrated to 10 mg/ml for crystallization. Initial crystallization screening was performed using the sitting-drop vapor diffusion method in 96-well Intelli-Plates (Dunn Laboratories). Several commercial kits (Sigma, Jena Bioscience, and Hampton Research) were used for screening. Using a Phoenix robotic system (Art Robbins), drops were made of 260 nl of protein and 260 nl of precipitant solution. The optimized crystallization condition identified was 0.07 M soya bean trypsin inhibitor (Rayonics). The data were processed with MOSFLM (17) and reduced using the SCALA (18) program from the CCP4 suite (19).

**Crystallization using the sitting-drop vapor diffusion method under the same crystallization conditions used for the free enzyme.**

**Eight** peptidic Michael acceptor compounds (SG74, SG75, SG81, SG82, SG83, SG84, SG85, and SG98) were dissolved in dimethyl sulfoxide (DMSO). Aliquots of EV68 3C protease in 25 mM Tris–HCl (pH 8.0), 200 mM NaCl, 5 mM DTT were incubated with inhibitor solution at a molar ratio of 1:5 for 30 min at room temperature and then subjected to cocrystallization using the sitting-drop vapor diffusion method under the same crystallization conditions used for the free enzyme.

No cryoprotectant was added for data collection, since the reservoir solutions contained sufficient amounts of glycerol. All diffraction data were collected at 100 K from a single crystal for each inhibitor complex at beamline BL14.1, BESSY (Berlin, Germany), using an MX225 CCD detector (Rayonics). The data were processed with MOSFLM (17) and reduced and scaled using the SCALA (18) program from the CCP4 suite (19).

**Structure determination.** The crystal structure of the EV68 3C protease was determined by molecular replacement (20) using MOLREP (21) and the atomic coordinates of human rhinovirus-2 3Cpro (8) as the search model. The initial model was built using Coot (22), and the program Refmac5 (19, 23) was used for model refinement. The complex structures were determined by molecular replacement using the structure of the free enzyme and refined as mentioned above to resolutions between 1.80 and 2.65 Å. Refinement statistics are summarized in Table 1.

The stereochemistry of the structures was assessed using PROCHECK (24). The quality of the structure-factor data and their agreement with the atomic model was evaluated with the program SFCHECK (25). The atomic coordinates and structure factors for all structures have been deposited with the RCSB Protein Data Bank (PDB); the PDB accession codes are listed in Table 1. All figures were generated using PyMOL (Schrödinger).

**Determination of substrate specificity and enzyme kinetics.** Eight dodecapeptide substrates (Fig. 2) for the high-pressure liquid chromatography (HPLC)-based cleavage assay were purchased from GL Biochem, Ltd. (Shanghai, China). These substrates represent the eight putative cleavage sites of the 3Cpro in the EV68 polypeptide. At their N and C termini, the peptides carried tryptophan residues for easy detection by UV spectroscopy, as described earlier for peptide substrates of the severe acute respiratory syndrome (SARS)-coronavirus main protease (26). To determine the substrate specificity of the protease, 50 μM concentrations of each peptide (dissolved in water) were incubated with 1 μM protease in buffer A containing 20 mM Tris (pH 7.3), 100 mM NaCl, and 1 mM EDTA at room temperature for 24 h. Reactions were terminated by adding 0.1% TFA, and the samples were analyzed using a reverse-phase HPLC C₁₂ column (Jupiter 4u Proteo 90Å; Phenomenex) with detection at 280 nm. The peak areas of the products were integrated to calculate the reaction rate of each substrate under the catalysis of the enzyme. The optimum substrate with the fastest reaction rate was selected for determination of the enzyme kinetics using fluorescence spectrophotometry. The fluorescence resonance energy transfer (FRET) peptide used as the substrate, Dabcyl-KEALFQ **↓** GPPQFE-Edans amide (95% purity; Biosyntan), contained the 2C3A cleavage site (indicated by the arrow).

Kinetic measurements were performed in the same buffer as for the HPLC test. The enhanced fluorescence due to the cleavage of this substrate as catalyzed by the enzyme was monitored at 490 nm with excitation at 340 nm, using a Cary Eclipse fluorescence spectrophotometer. The experiments were performed in a fluorescence cuvette (volume, 1 ml) with a light path of 1 cm for excitation and a light path of ~2 mm for emission, at a 90° angle. Slit widths were 5 nm in both cases. The photomultiplier voltage was 600 V, and the assay volume was 400 μl. The enzyme concentration for measuring Km was 0.2 μM, and the concentrations of the FRET peptide were varied from 2 to 20 μM. The initial rate, within 10% of the substrate consumption, was used to calculate the kinetic parameters using Michaelis–Menten equation fitting with the Origin program (OriginLab). Owing to solubility limitations, the FRET substrate could not be used at concentrations higher than 20 μM; the Km value was therefore estimated by nonlinear regression analysis as described previously (27). By using only relatively low substrate concentrations, we also avoided potential inner-filter effects caused by the substrate.

**Enzyme inhibition assay.** The FRET-based assay was also used for the determination of the inhibition constants of the α,β-unsaturated esters. At the outset, we experimentally characterized the inhibitors with respect to possible inner-filter effects. The absorption spectra of all of them were measured at a concentration of 100 μM in DMSO in the range from 260 to 600 nm. Only compound SG84 displayed a significant absorption above 260 nm and up to 310 nm. However, even from the latter compound, no absorption was registered around 340 nm, the excitation wavelength used in the fluorimetric assay. Also, at the emission wavelength used for the fluorimetric assay (i.e., 490 nm), none of the compounds showed significant absorption. Finally, fluorescence emission spectra were also recorded for all inhibitors, using the same excitation wavelength (340 nm) as for the fluorimetric assay. None of the compounds displayed a significant fluorescence signal around 490 nm, the emission wavelength used in the enzymatic test system. Thus, potential inner-filter effects did not have to be taken into account in our experiments.

The principle of the assays and more details have been described elsewhere (27, 28). Briefly, time-dependent progress curves were fitted to a first-order exponential (see equation 1) to obtain an observed first-order inhibition rate constant (kobs) (29). F is the product fluorescence (measured in arbitrary units), F₀ is the initial velocity, t is the time, and D is a displacement term accounting for the non-zero emission at the start of data collection. Since in the case of fast inactivation, the measurement of K₁ and k₂ tends to be difficult, kobs/[I] was used as an approximation of the pseudo-second-order rate constant, in order to evaluate all inhibitors for easy comparison.

\[
F = \frac{F_0}{k_{obs}} \cdot (1 - \exp(-k_{obs} \cdot t)) + D
\]

Reactions were performed with 0.2 μM protease and 20 μM FRET peptide in buffer A (20 mM Tris [pH 7.3], 100 mM NaCl, 1 mM EDTA) by the addition of the enzyme to the reaction buffer containing substrate and inhibitor. Three to five different inhibitor concentrations were tested that varied over a range from 0.1 to 100 μM. The data from the continuous assays were analyzed with the nonlinear regression analysis program Origin (OriginLab) to obtain k_{obs} for enzyme inactivation at each inhibitor.
### TABLE 1 Data collection and refinement statistics for crystals of EV68 3Cpro and its complexes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>EV68 3Cpro</th>
<th>EV68 3Cpro/SG74</th>
<th>EV68 3Cpro/SG75</th>
<th>EV68 3Cpro/SG81</th>
<th>EV68 3Cpro/SG82</th>
<th>EV68 3Cpro/SG83</th>
<th>EV68 3Cpro/SG84</th>
<th>EV68 3Cpro/SG85</th>
<th>EV68 3Cpro/SG98</th>
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<td>P3,21</td>
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<td>P3,21</td>
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<td>56.10, 56.10, 170.41</td>
<td>56.49, 56.49, 170.36</td>
<td>56.31, 56.31, 170.35</td>
<td>56.30, 56.30, 170.35</td>
<td>55.87, 55.87, 170.15</td>
<td>56.20, 56.20, 170.23</td>
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<td>Estimated solvent content (%)</td>
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<td>66.55</td>
<td>66.65</td>
<td>66.90</td>
<td>67.10</td>
<td>66.89</td>
<td>66.88</td>
<td>66.33</td>
<td>66.74</td>
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<td>Wavelength (Å)</td>
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<td>0.9184</td>
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<td>Resolution range (Å)</td>
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<td>34.11–2.05 (2.16–2.05)</td>
<td>36.92–2.20 (2.32–2.20)</td>
<td>48.77–2.65 (2.79–2.65)</td>
<td>34.07–2.00 (2.11–2.00)</td>
<td>36.99–2.25 (2.37–2.25)</td>
<td>20.417 (29.073)</td>
<td>40.150 (20.152)</td>
<td>55.740 (36.767)</td>
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<td>No. of reflections measured</td>
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<td>183,050 (26,814)</td>
<td>118,096 (17,334)</td>
<td>172,402 (25,467)</td>
<td>200,417 (29,073)</td>
<td>140,050 (20,152)</td>
<td>255,740 (36,767)</td>
<td>95,365 (13,852)</td>
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<td>29,927 (4,268)</td>
<td>11,333 (1,606)</td>
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<td>100 (100)</td>
<td>100 (100)</td>
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<td>0.089 (0.455)</td>
<td>0.086 (0.434)</td>
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<td>0.064 (0.445)</td>
<td>0.128 (0.554)</td>
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<td>19.3/22.8</td>
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<td>19.9/23.8</td>
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<td>1.660</td>
<td>1.622</td>
<td>1.969</td>
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*Values in parentheses are for the highest resolution shell where applicable.

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43. Matthews (43).

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44. Rmerge = Σᵢ [I(hkl) - ⟨I(hkl)⟩]/Σᵢ [I(hkl)], where ⟨I(hkl)⟩ is the average intensity over all equivalent reflections, Rcryst = Σᵢ [Fᵢ(hkl) - Fᵢ(hkl)]/Σᵢ Fᵢ(hkl). Rfree was calculated for a test set of reflections (5%) omitted from the refinement.
concentration. The slope of a graph of \( k_{obs} \) versus [I] was calculated using Origin and is reported as \( k_{obs}/[I] \) in Table 2.

**Cells and viruses.** A derivative of human hepatocellular carcinoma cells, constitutively expressing a T7 RNA polymerase (Huh-T7) (30), was grown in Dulbecco modified minimal essential medium supplemented with 2 mM glutamine, 100 U of penicillin/ml, 100 \( \mu \)g of streptomycin sulfate/ml, 400 \( \mu \)g of Geneticin (G-418 sulfate)/ml, and fetal calf serum (FCS; 10% in growth medium and 2% in maintenance medium). Other cells used were Vero A cells, a derivative of Vero cells (African green monkey kidney cells), BGM (buffalo green monkey kidney cells), and RD (rhabdomysosarcoma cells), which were grown in MEM Rega (Invitrogen) supplemented with 10% FCS for regular subculturing and 2% FCS for antiviral assays (Integro), 5 ml of 200 mM L-glutamine, and 5 ml of 7.5% sodium bicarbonate. HeLa Rh cells, a HeLa cell clone selected for susceptibility to HRV replication, were grown in minimal essential medium (catalog no. 41965-039, 500 ml/bottle; Invitrogen) supplemented with 10% FCS for regular subculturing and 2% FCS for antiviral assays (Integro), 5 ml of 200 mM L-glutamine, and 10 ml of 1 M HEPES (Spool no. 15630-056; Invitrogen).

Echovirus 11 (ECHO11; strain Gregory) and rhinoviruses (HRV types 2, 14, 29, 70, and 95) were kindly provided by K. Andries (Johnson & Johnson). Enterovirus 71 (EV71; strain BrCr) was provided by F. van Kuppeveld (Nijmegen, Netherlands). Poliovirus 1 (Sabin) was obtained from J. Martin, NIBSC, Hertfordshire, United Kingdom.

**Viral replicons and transfection.** Subgenomic replicon DNAs of poliovirus (PV; pRluc31 (31]) and hepatitis A virus (HAV; p18F-Luc (32]) encoded a full-length copy of poliovirus (strain Mahoney) and hepatitis A virus replicon DNA pRLuc31, pACYC-EV71-RLuc, and p18f-Luc, as well as \( 4.5 \mu \)l of Lipofectamine 2000 (Invitrogen) in 300 \( \mu \)l of Opti-MEM (final volume). To transfect replicon RNAs, RLuc31, EV71-RLuc, and 18F-Luc, the DMRIE-C reagent (3 \( \mu \)l/\mu g of purified RNA) was used as recommended (Invitrogen). After 4 to 5 h of incubation at 37°C, the transfection mixtures were replaced with growth medium containing different concentrations of the candidate inhibitor to be tested (40 \( \mu \)M in preliminary screening experiments or increasing concentrations, from 0 to 40 \( \mu \)M, when studying the concentration dependence). After 24 h of incubation, the cells were washed with 1 ml of phosphate-buffered saline and lysed in 0.15 ml of passive lysis buffer (Promega) at room temperature for 10 min. After freezing (–80°C) and thawing (room temperature), the cell debris was removed by centrifugation (13,000 rpm, 1 min), and the supernatant (10 or 20 \( \mu \)l) was assayed for firefly or Renilla luciferase activity (Promega) using an Anthos Lucy-3 luminescence plate reader (An- thos Labtec instruments). All experiments were carried out in triplicate or quadruplicate, and the results are presented as mean values with the standard deviations (SD).

**Cell toxicity.** A CellTiter 96 AQueous One Solution cell proliferation assay (MTS test; Promega) was used to determine the cytotoxic effect of inhibitors. In brief, the confluent monolayer of cells was incubated overnight under the pressure of different concentrations of the tested inhibitor in a total volume of 100 \( \mu \)l/well. A total of 100% of cell lysis was achieved by incubation with 0.1% Triton X-100 (positive control). After incubation, 50 \( \mu \)l of medium was discarded, and 10 \( \mu \)l of freshly prepared single-assay reagent was added to the cells. The color reaction was measured at 490 nm using the Anthos Lucy-3 luminescence plate reader (see above) after 1 or 2 h of cell incubation at 37°C. In addition to detect subtle differences in cytotoxicity of the tested compounds toward Huh-T7 cells, analysis of cell death was performed by using a ToxiLight nondestructive cytotoxicity bioassay kit as recommended by the manufacturer (Lonza, Rockland, ME).

**Antiviral assays in virus-infected cells.** Compound dilutions (dilution factor of 5, four-point dose-response curve for initial screening; dilution factor of 2, eight-point dose-response curve for further refinement) were prepared in the respective assay medium (see above) and added to empty wells of a 96-well assay plate by a liquid-handling robot (EVO200; Tecan). Subsequently, 50 \( \mu \)l of a 4\( \times \) virus dilution in assay medium (supplemented with 15 ml of 1.0 M MgCl\(_2\) (Sigma) in the case of HRV) was added, immediately followed by the addition of 50 \( \mu \)l of the respective cell suspension in assay medium, yielding a total assay volume of 200 \( \mu \)l. The assay plates were returned to the incubator (35°C for HRV and 37°C for the other viruses) for 3 to 4 days, the time at which a maximal cytopathic effect is observed.

For the evaluation of the antiviral effect, the assay medium was aspirated by a plate washer (Hydroflex; Tecan), replaced with 75 \( \mu \)l of a 5% MTS (Promega) solution in phenolsulfonphthalein-free medium (EVO200; Tecan), and incubated for 1.5 h (37°C, 5% CO\(_2\), 95 to 99% relative humidity). The absorbance (with the optical density [OD] cell culture condition varying between an OD of 0.6 and an OD of 0.8) was measured at a wavelength of 498 nm (Safire® 2; Tecan), and the OD values were converted to the percentage of untreated controls and compiled into antiviral dose-response curves.

Analysis of the raw data and calculation, if possible, of the EC\(_{50}\) and CC\(_{50}\) values were performed using a custom-made data processing software package (Accelrys). The EC\(_{50}\) (derived from the dose-response curve) represents the concentration at which 50% inhibition of viral replication would be observed. The CC\(_{50}\) (derived from the dose-response curve) represents the concentration at which the metabolic activity of the cells is reduced to 50% of the metabolic activity of untreated cells. All assay wells yielding a percent inhibition larger than 50 were inspected microscopically and scored for inhibition of virus-induced cytopathic effects, as well as evaluated for minor adverse effects on the host cells.

**RESULTS**

Substrate specificity and kinetics of EV68 3C\(^{pro}\). The substrate peptides used in the present study corresponded to the presumable...
processing sites of EV68 3Cpro within the viral polyprotein but carried N-terminal and C-terminal tryptophan residues for easy UV-spectroscopic detection of cleavage products at 280 nm. The individual peptides used were WHAITQ2GVPTYW (VP2↓VP3), WPDI-AQ2LDHLDW (VP3↓VP1), WDAMEQ2GITDYW (2A↓2B), WYVPRQ2SESWLW (2B↓2C), WEALFQ2GPPQFW (2C↓3A), WFAGIQ2GPYTGW (3A↓3B), WTAKVQ2GP-GFWDW (3B↓3C), and WFTDTQ2GEIVSW (3C↓3D). All of these peptides, with the exception of VP3↓VP1, contain a Gln followed by a small amino acid residue (Gly or Ser) framing the cleavage site. Overall, Gly in the P1 site leads to higher cleavage activities than Ser or Leu (Fig. 2). We observed the highest cleavage rates for 3B↓3C and 2C↓3A; the latter was chosen for kinetic studies. Consequently, the peptide substrate used for the FRET assay was Dabcyl-KTEALFQ↓GPPQFE-Edans amide. Since Lee et al. (12) reported that coxsackievirus B3 (CVB3) 3Cpro exhibits higher activity with the

<table>
<thead>
<tr>
<th>Name</th>
<th>Molecular structure</th>
<th>EV68 3Cpro inhibition (k_{obs}/[I] [M^{-1} s^{-1}])</th>
<th>Inhibition of replicons (EC_{50} [μM])</th>
<th>Toxicity (CC_{50} [μM])</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PV</td>
<td>EV71</td>
</tr>
<tr>
<td>SG74</td>
<td></td>
<td>ND**</td>
<td>&gt;40</td>
<td>ND</td>
</tr>
<tr>
<td>SG75</td>
<td></td>
<td>3.246 ± 107</td>
<td>7.5 ± 1.5</td>
<td>7.0 ± 1.1</td>
</tr>
<tr>
<td>SG81</td>
<td></td>
<td>42 ± 3</td>
<td>&gt;40</td>
<td>&gt;40</td>
</tr>
<tr>
<td>SG82</td>
<td></td>
<td>43,225 ± 1,850</td>
<td>ND</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>SG83</td>
<td></td>
<td>174,500 ± 9,245</td>
<td>1.0 ± 0.3</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>SG84</td>
<td></td>
<td>9,225 ± 423</td>
<td>ND</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>SG85</td>
<td></td>
<td>202,200 ± 8,103</td>
<td>0.5 ± 0.1</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>SG98</td>
<td></td>
<td>11,045 ± 661</td>
<td>4.0 ± 1.4</td>
<td>2.5 ± 0.5</td>
</tr>
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</table>

* ND, not determined.
SARS-CoV M\textsuperscript{pro} substrate (SAVLQ↓SGFRK) than with the best CVB3 3C\textsuperscript{pro} substrate peptide, we also tested this substrate against EV68 3C\textsuperscript{pro}, but we could not detect any activity. The $k_{\text{cat}}$ and $K_m$ values of the protease using the substrate corresponding to the 2C↓3A cleavage site were determined as 0.54 s\textsuperscript{-1} and 22.4 $\mu$M, respectively (not shown).

Crystal structures of EV68 3C\textsuperscript{pro} and its complexes. EV68 3C\textsuperscript{pro} was cloned into the pOPINE vector, which codes for an extra Lys residue and a His\textsubscript{tag} tag at the C terminus. We decided to have these extra residues at the C terminus of the enzyme, as in case of the picornaviral 3C proteases, the C terminus is oriented away from the globular protein and additional residues are unlikely to influence the structure or catalytic activity. This is supported by Kuo et al. (11), who showed that C-terminally tagged EV71 3C\textsuperscript{pro} has the same catalytic activity as the tag-free enzyme. The expression yield was ~5.0 mg of soluble protein per liter of medium. The protein was concentrated to 10.0 mg/ml for crystallization. Crystals grew from 0.07 M sodium acetate (pH 4.6), 5 to 15% PEG 4000, and 10 to 30% glycerol. For both the free enzyme and its inhibitor complexes, these crystals belonged to space group P3\textsubscript{1}2\textsubscript{1}1, with one 3C\textsuperscript{pro} monomer per asymmetric unit (Table 1).

In solution, EV68 3C\textsuperscript{pro} is a monomer as well, as indicated by gel filtration and dynamic light scattering (not shown). The crystal structure of the free enzyme was determined by molecular replacement using the structure of rhinovirus 3C protease (8) as a search model, and refined to a 2.4-Å resolution. All amino-acid residues were defined by electron density, including four histidines of the C-terminal His\textsubscript{tag}. Alternative conformations were detected in the electron density for the side chains of active-site His\textsubscript{40} and for Cys60. The final R-factor for this crystal structure was 20.7% ($R_{\text{free}} = 23.8\%$). The complex structures were determined based on the crystal structure of the free enzyme, at resolutions between 1.80 and 2.65 Å. The inhibitors could all be easily modeled according to the $F_o-F_c$ difference density. More details can be found in Table 1.

Structure of EV68 3C\textsuperscript{pro}. EV68 3C\textsuperscript{pro} adopts a chymotrypsin-like fold consisting of two β-barrel domains. These two domains comprise residues 15 to 77 and residues 99 to 173, respectively, and pack against each other to form a shallow substrate-binding cleft (Fig. 3A) that harbors the catalytic triad of Cys147, His\textsubscript{40}, and Glu71. There are two conformations for the side chain of His\textsubscript{40}. One conformation, with side chain torsion angles χ\textsubscript{1} = 69.0° [(+)-synclinal = +sc] and χ\textsubscript{2} = −98.7° (anticlinal = ac), corresponds to the catalytically competent form, in which the sulfur atom of Cys147 is in plane with the imidazole of His\textsubscript{40} and the carboxylate group of Glu71. In this conformation, the distance between Cys147 S and His\textsubscript{40} N\textsubscript{ε} is 3.3 Å, and the one between His\textsubscript{40} N\textsubscript{ε} and the carboxylate of Glu71 is 2.7 Å. In the other conformation, with side chain torsion angles χ\textsubscript{1} = 179.7° (anti-periplanar = ap) and χ\textsubscript{2} = 86.6° (+sc), the imidazole of His\textsubscript{40} is exposed to the solvent, with a distance of 6.8 Å between Cys147 S and His\textsubscript{40} N\textsubscript{ε}. A likely reason for the occurrence of this second conformation is the low pH (4.6) of crystallization, at which part of the His\textsubscript{40} side chains may be protonated, unable to interact with the sulphydryl of Cys147. Unfortunately, crystallization trials at higher pH invariably led to precipitation of the protein and equilibrating low-pH crystals in buffer of higher pH resulted in destruction of the crystals.

Substrate hydrolysis by cysteine and serine proteases occurs through a covalent tetrahedral intermediate resulting from attack of the active-site nucleophile onto the carbonyl carbon of the scissile bond. The developing oxyanion is stabilized by hydrogen bonds donated by amide groups of the enzyme. In EV68 3C\textsuperscript{pro}, the oxyanion hole is formed by the main-chain amides of Gly145 and the active-site Cys147. In the structure of the free enzyme, the oxyanion hole is occupied by a water molecule that interacts with the first of these amides.

Comparison of EV68 3C\textsuperscript{pro} and related picornaviral proteases. A sequence alignment (Fig. 3B) shows that the identity between the EV68 3C\textsuperscript{pro} and the corresponding enzymes of HRV2, EV71, CVB3, EV93, and PV is 49, 53, 67, 67, and 66%, respectively, whereas the 3C proteases of HAV and foot-and-mouth disease virus (FMDV) are more distantly related (sequence identities of 25 to 26%). In terms of three-dimensional structure, the closest relatives of the EV68 3C\textsuperscript{pro} are the enzymes of HRV2 (8), with a root mean square deviation (RMSD) of 0.58 Å for 170 superimposed Ca atoms, and PV (33), a member of subgroup C of human enteroviruses (HEV-C; RMSD of 0.64 Å for 173 Ca atoms). In contrast to the amino acid sequence comparisons (see above), the superimpositions of the three-dimensional 3C\textsuperscript{pro} structures reflect the classification into different subgroups of enteroviruses. Thus, the enzymes of HEV-B members CVB3 (12; J. Tan et al., unpublished data) and EV93 (34) exhibit RMS distances from EV68 3C\textsuperscript{pro} of 0.78 Å (for 173 Ca atoms) and 0.81 Å (for 159 Ca atoms), respectively. The 3C\textsuperscript{pro} of EV71 (35), a member of the HEV-A subgroup, exhibits an RMSD of 0.74 Å for 174 Ca atoms. The relatively large distance between the enteroviruses on the one hand and hepatitis A virus (HAV), as well as FMDV, on the other is reflected in high RMS distances between the EV68 3C\textsuperscript{pro} and the corresponding proteases of these viruses (36, 37), with RMSD values of 2.04 Å for 166 Ca atoms and 1.93 Å for 170 Ca atoms, respectively. The intermediate position of the EV68 3C\textsuperscript{pro} between the proteases of human rhinoviruses and those of other enteroviruses suggested that compounds optimized for inhibition of the EV68 enzyme might exhibit activities against a wide spectrum of picornaviruses.

Peptidic α,β-unsaturated esters: design, crystal structures of complexes with the target enzyme, and inhibitory activities. (i) General strategy. It was reported that several α,β-unsaturated esters acted as Michael acceptors with excellent inhibitory activity against HRV 3C protease. The compounds exhibited encouraging antiviral activity, stability in the presence of nonenzymatic thiols, and low cellular toxicity and were relatively easy to synthesize (8). Based on the substrate sequence (2C/3A site) and the three-dimensional structure of the EV68 3C\textsuperscript{pro} (see above), we designed and synthesized a series of α,β-unsaturated ester compounds of increasing length directed at the enzyme [Fig. 1]. Immediately after synthesis, new inhibitor candidates were cocrystallized with the target protease and had the X-ray structures of their complexes determined, in order to visualize details of ligand binding as a basis for the subsequent round of inhibitor design. Using the above-mentioned FRET-peptide substrate for the kinetic assay, the inhibitory activities of these compounds have been determined. Being Michael acceptors, these inhibitors initially form a reversible encounter complex with the 3C\textsuperscript{pro} and then undergo a chemical step (nucelophilic attack by Cys147 leading to irreversible covalent-bond formation). The observed second-order rate constant for inactivation ($k_{\text{obs}}/[I]$) depends on both the equilibrium binding constant, $K_i = k_{\text{cat}}/k_{\text{in}}$, and the chemical rate
FIG 3 (A) Three-dimensional structure of EV68 3C\textsuperscript{pro}. The catalytic triad consisting of Cys147, His40, and Glu71 is shown in stick mode. Hydrogen bonds between these residues are indicated by dashed lines, with the corresponding distances given in Å. (B) 2\textit{Fo}-\textit{Fc} electron density (contoured at 1\textit{H}9268\textsuperscript{above the mean}) for the catalytic center. Note the two alternative conformations for His40; only one of these interacts with Cys147. (C) Structure-based sequence alignment of 3C proteases of various picornaviruses. The secondary structure (as found in the crystal structure of the EV68 enzyme) is shown at the top, invariant residues are highlighted with red background, and conserved residues are shown in red.

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for covalent bond formation, $k_3$. For fast irreversible protease inhibitors, $K_i$ tends to be very difficult to obtain; therefore, usage of $k_{\text{obs}}/[I]$ is more suitable (8, 29). In this case, a higher value of $k_{\text{obs}}/[I]$ indicates that the inhibitor is more active.

(ii) **P1 position.** The P1 residue was not altered in our series of inhibitor candidates. A five-membered lactam was chosen as a surrogate for the glutamine side chain amide in this position, since the relatively rigid lactam side chain will likely lead to a reduced loss of conformational entropy upon binding to the S1 pocket, compared to the more flexible Gln, and therefore might lead to tighter binding of the inhibitor to the 3C protease. This can cause an increase of $k_{\text{obs}}/[I]$ by a factor of 10, compared to the Gln-containing compound (8). In fact, in all of our inhibitor complex structures, the lactam occupies the S1 subsite (Fig. 4), which involves residues Thr142, Arg143mc (“mc” stands for main chain), Ala144, Cys147, His161, and Gly163. At the bottom of the site, the

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**FIG 4** Binding-mode analysis by X-ray crystallography of α,β-unsaturated ethyl ester inhibitors. (A to H) Binding modes of SG74, SG75, SG81, SG82, SG83, SG84, SG85, and SG98, respectively. The catalytic triad is shown in yellow, while other residues involved in the binding site are shown in pink. Inhibitors are shown in green along with an omit $2F_o-F_c$ map (blue) contoured at 1.0σ above the mean. A stereo view of this figure can be found in the supplemental material.
Ne2 atom of His161 donates a hydrogen bond to the oxygen atom of the lactam; the length of this hydrogen bond among all of the complexes is 2.8 Å, indicating that this is a very stable and conserved interaction.

(iii) **P2 position.** The first compound to be synthesized, SG74 (Boc-GlnLactam-CH = CH-COOEt), just spans positions P2-P1′. Its P2-Boc group partly fills the S2 pocket. This compound does not show any inhibition of the EV68 3C\textsuperscript{pro} (Table 2). However, it still binds to the enzyme and features clear electron density in the crystal structure (Fig. 4A), an interesting demonstration of the sensitivity of X-ray crystallography.

On the basis of the structure with SG74, we decided that the S2 subsite should be occupied by a larger hydrophobic residue, and kept Phe invariant in the P2 position in all subsequent inhibitor candidates. The S2 subsite is formed by the catalytic His40..,Glu71 pair on one side, and Leu127 and Gly128 on the other, and closed off by Thr130 and Asn69 “at the top” (referring to the orientation shown in Fig. 4H). The pocket is hydrophobic in nature, explaining the preference of the EV68 3C\textsuperscript{pro} for hydrophobic residues such as Phe, Leu, or Val in position P2 of the substrates.

In order to fill the S2 pocket (see Fig. 4H), but moves toward S1′, in concert with the unusual orientation of His40. In order to fill the S2 pocket, the χ₁ torsion angle of the phenylalanine side chain should be in the −sc range, but in fact, it is ap in the structures of the complexes described here.

(iv) **P3 position.** The first inhibitors to contain P2 = Phe, SG75 (Cbz-Phe-GlnLactam-CH = CH-COOEt) and SG81 (Boc-Phe-GlnLactam-CH = CH-COOEt), span positions P3 to P1′. Carrying a Cbz cap in the P3 position, SG75 exhibits significant inhibition ($k_{\text{obs}}$/[I] = 3,246 M\textsuperscript{-1} s\textsuperscript{-1}), whereas SG81, which has a Boc cap in the same position, is not an inhibitor ($k_{\text{obs}}$/[I] = 42 M\textsuperscript{-1} s\textsuperscript{-1}) (Table 2). From the crystal structures of the SG75 and SG81 complexes (Fig. 4B and C), it can be seen that both the Cbz and Boc protecting groups follow the binding path of the main chain, rather than binding in the orientation usually taken by a P3 side chain, and the bulky Cbz moiety even partially extends into the S4 subsite. In addition, the carbonyl oxygen of the protecting group of SG75 accepts a 3.3-Å hydrogen bond (3.4 Å in case of SG81) from the NH of Gly164. When we increased the lengths of the compounds by one more amino acid residue, i.e., having the protecting group in P4 and an amino acid in P3, orientation of the side chain of the latter toward the solvent was observed. The EV68 3C\textsuperscript{pro} (like its homologues from other viruses) has no S3 pocket.

(v) **P4 position.** The S4 pocket can only tolerate small side chains, with alanine being the most favored one by the 3C\textsuperscript{pro} of...
Since our inhibitors have much larger groups at this position, such as Cbz in SG82, SG83, and SG85, Fmoc (9-fluorenylmethoxy carbonyl) in SG84, and Boc in SG98, these groups cannot penetrate into the S4 pocket; rather, they follow the path of the substrate main-chain and protrude partly into S5. There is no clear electron density for Fmoc in SG84 (Fig. 4F), and the compound, which was an intermediate in the synthesis of other inhibitors, is both toxic and has low in vitro activity (\(k_{\text{obs}}/|I| = 9.225 \text{ M}^{-1} \text{s}^{-1}\)) compared to SG83 or SG85. In contrast, the Cbz and Boc caps of SG82, SG83, SG85, and SG98 can be easily modeled into the Fo-Fc density. The P4-Cbz group makes strong hydrophobic interactions with residues Leu125, Asn165, Phe170, and Tyr122 of the S4 site (Fig. 4D, E, and G). A comparison of SG85 [Cbz-Ser(OtBu)-Phe-Gln-Lactam-CH=CH-COOEt] with SG98 [Boc-Thr(OtBu)-Phe-Gln-Lactam-CH=CH-COOEt; \(k_{\text{obs}}/|I| = 11.045 \text{ M}^{-1} \text{s}^{-1}\)] suggests that Cbz in P4 leads to stronger hydrophobic interactions (Fig. 4G and H) and explains the much higher inhibitory activity of SG85.

**Inhibitory effect on subgenomic replicons.** Using enterovirus (PV and EV71) and hepatovirus (HAV) replicons, initial evaluation of the inhibitory activity of our compounds was performed at a 40 \(\mu\text{M}\) concentration (Fig. 5). When examined microscopically, SG84 exhibited strong cellular toxicity and was therefore not studied further. However, at a concentration of 100 \(\mu\text{M}\), none of our compounds reduced by >10% (Table 2 and see Fig. S1 in the supplemental material) the metabolic activity of Huh-T7 cells and their viability according to cell toxicity assays (see Materials and Methods). SG81 did not result in inhibition of replicon replication and was therefore excluded as well. SG75, SG83, SG85, and SG98 efficiently inhibited the replication of the PV and EV71 replicons, but none inhibited the replication of the HAV replicon (Fig. 5). The HAV 3C protease is an outlier among the picornaviral 3C proteases, with significant deviations in the specificity-determining subsites (overall RMSD from EV68 3Cpro of 2.04 Å), probably explaining this lack of activity. The HAV replicon was consequently excluded from further investigation. From the dose-response curves presented in Fig. 6, SG85 and SG83 appear to be the most potent inhibitors of PV replicon replication (EC_{50} = 0.5 and 1.0 \(\mu\text{M}\), respectively). SG98 and SG75 were markedly less effective (4.0 and 7.5 \(\mu\text{M}\), respectively). Comparable results were obtained with the EV71 replicon (Fig. 7), where SG85 and SG83 proved more potent than SG98 and SG75. Direct comparison of the results of DNA and RNA replicon transfection in Huh-T7 cells excluded the involvement of the cellular T7 RNA polymerase in inhibition, implying that mainly replicon genome translation and replication steps were targeted by the inhibitors (see the supplemental material). Moreover, based on the observed predominant inhibition of the replication-competent (up to 95%) compared to the replication-deficient (20 to 50%) replicon (see the supplemental text and Fig. S2), the picornavirus 3C protease, the proteolytic activity of which is a prerequisite for the formation of a functional replication complex (1, 2), was confirmed as the likely in vivo target of our compounds.

**Antiviral activity of peptidic \(\alpha,\beta\)-unsaturated esters against picornaviruses.** The effect of most compounds was also evaluated on the replication of various picornaviruses in live-virus–cell-based assays using ECHO virus 11, EV71, multiple HRV types, and PV1. Both SG74 and SG81 proved to be inactive against these viruses.
viruses at the highest concentration tested or to produce high-μM EC_{50}. Interestingly, and despite high EC_{50}, both compounds were shown to perfectly inhibit virus-induced cytopathic effects for at least one concentration in the HRV29 assay (Table 3) without any adverse effects on the host cell. Compound SG75, with Cbz in the P3 position (as opposed to SG81, which has Boc in this position), exhibited potent and selective antiviral activity against all viruses included in the test panel, except for PV1, for which it showed a high EC_{50}. Most potent and same order-of-magnitude activity (low-μM EC_{50}) was observed against the different rhinovirus types (order of susceptibility: HRV70 > HRV14 > HRV2 > HRV29 > HRV85). Upon comparison, SG82, SG83, and SG85 showed a similar antiviral potency for each individual virus. Throughout the data set and similar to SG75, SG82, SG83, and SG85 were shown to completely protect host cells from virus-induced cytopathic effects except in the PV1 assay and to some extent in the HRV2 assay. Compared to SG75, activity was significantly more pronounced, as evident from the lower EC_{50}. For most rhinoviruses, on average, even high nM values were reached. Activity against ECHO11 and PV1 was markedly less pronounced. Most of the results of the cell-based assays show the same trends as those from the enzymatic assay and the replicons, except for SG82 that proved somewhat less active at the enzymatic level compared to SG83 and SG85.

DISCUSSION

It has been observed that enterovirus 68 shares properties with the rhinoviruses and the enteroviruses (40–42). This is in line with our X-ray crystallographic analysis of the EV68 3C protease. The three-dimensional structure of the enzyme is closely related to those of its homologues from rhinovirus 2 and poliovirus, whereas the structures of the 3C^{pro} of enterovirus 71, enterovirus 93, and coxsackievirus B3 are somewhat more distantly related and those of HAV and FMDV are markedly different.

We have shown that the EV68 3C protease is a target for anti-EV68 drug discovery and, beyond that, is very useful for the design of broad-spectrum anti-picornavirus inhibitors. A series of peptidic α,β-unsaturated ethyl esters of growing lengths has been designed on the basis of the crystal structure of the EV68 3C^{pro} and synthesized. In agreement with earlier reports on short inhibitors of the EV71 3C^{pro} (11), SG74 (Boc-GlnLactam-CH = CH-COOEt), a compound containing only residues P2 to P1, had no inhibitory activity against the enzyme of EV68. In spite of that, we could identify clear electron density for the compound in its complex with the enzyme. SG81, with Boc as the P3 cap, was an intermediate compound and did not show good inhibition. Again, however, it did bind to the substrate-binding pocket in the crystal structure. With introduction of the Cbz group at the P3 position (as in SG75), the inhibitory activity increased. This can be explained by the hydrophobic contacts that the Cbz group makes by occupying the S3 binding site for the substrate’s main chain and, beyond that, by partly extending into S4.

A comparison of SG82, SG83, SG84, SG85, and SG98, which comprise residues P4 to P1, reveals variable inhibitory potency and cytotoxicity. SG82 has a larger group (GluOtBu) in P3 compared to SG83 and SG85; it showed lower inhibition and some cytotoxicity in Huh-T7 cells. Upon shortening the side chain by one methylene group to yield AspOtBu as in SG83, the cytotoxicity disappeared completely. In contrast, SG84 has a fluorescein group in P4 and was cytotoxic. After replacing this group by Boc, as in SG98, cytotoxicity was no longer observed. However, with the Boc group in P4, the inhibitory activity of SG98 was by far not as good as that of SG85, which has SerOBU in P3 and Cbz in P4. Thus far, SG85 proved to be the best inhibitor against the EV68 3C protease. We have tested all of these compounds as inhibitors of picornaviral replicons and also against infection by EV71, poliovirus, ECHO virus 11, and various rhinovirus types at the cellular level. Again, SG85 showed the best inhibitory activity with (sub)micromolar EC_{50} in the PV and EV71 replicons. SG83 and SG85 proved to be the most potent inhibitors of the series in terms of inhibition of rhinovirus replication and, together with SG82, are about equipotent as inhibitors of EV71 replication. Our next aim is to optimize our lead compound, SG85, further, to yield antivirals with improved stability, bioavailability, and activity against a broad spectrum of picornaviruses.

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