More-powerful virus inhibitors from structure-based analysis of HEV71 capsid-binding molecules

Luigi De Colibus1,8, Xiangxi Wang2,8, John A B Spyrou1, James Kelly3, Jingshan Ren1, Jonathan Grimes1,4, Gerhard Puerstinger5, Nicola Stonehouse3, Thomas S Walter1, Zhongyu Hu6, Junzhi Wang6, Xuemei Li2, Wei Peng2, David J Rowlands3, Elizabeth E Fry1, Zihe Rao2,7 & David I Stuart1,4

Enterovirus 71 (HEV71) epidemics in children and infants result mainly in mild symptoms; however, especially in the Asia-Pacific region, infection can be fatal. At present, no therapies are available. We have used structural analysis of the complete virus to guide the design of HEV71 inhibitors. Analysis of complexes with four 3-(4-pyridyl)-2-imidazolidinone derivatives with varying anti-HEV71 activity pinpointed key structure-activity correlates. We then identified additional potentially beneficial substitutions, developed methods to reliably triage compounds by quantum mechanics–enhanced ligand docking and synthesized two candidates. Structural analysis and in vitro assays confirmed the predicted binding modes and their ability to block viral infection. One ligand (with IC50 of 25 pM) is an order of magnitude more potent than the best previously reported inhibitor and is also more soluble. Our approach may be useful in the design of effective drugs for enterovirus infections.

The large Picornaviridae family includes pathogens with major impacts on human and animal health. However there are, as yet, no approved therapies for picornavirus infections. The enteroviruses compose the largest picornavirus genus. Of these, human enterovirus 71 (HEV71) presents perhaps the greatest threat to public health after the human rhinoviruses responsible for most cases of the common cold. HEV71 is responsible for periodic disease outbreaks throughout the world, and in recent years there have been regular major epidemics in South Asia. These are associated with outbreaks of mild childhood exanthema, herpangina and hand, foot and mouth disease. However, especially in the Asia-Pacific region, fatal neurological and cardiovascular disorders can ensue.

Picornaviruses are small positive-stranded RNA viruses with non-enveloped icosahedral capsids comprising 60 copies of proteins VP1–VP4. Proteins VP1–VP3 each adopt a β-barrel configuration and are arranged with icosahedral symmetry such that VP1 surrounds the five-fold axes, and VP2 and VP3 alternate about the two- and three-fold axes (with VP4 being internal). Canyon-like depressions encircling the five-fold axes in enteroviral capsids are frequently the sites of receptor attachment. Uncoating, whereby the capsid opens to release the viral genome into the host-cell cytosol in order to replicate, is key to picornavirus infection. Like most enteroviruses, HEV71 contains within its capsid 60 copies of a hydrophobic ‘pocket factor’, a natural lipid (sphingosine), buried in a pocket lying at the base of the capsid protein VP1 (Fig. 1a). Expulsion of this molecule after binding of the virus to its receptor triggers a cascade of structural rearrangements, which open the capsid to facilitate genome release. Because expulsion of the pocket factor is required for infection, a tight replacement binder could be a useful antiviral agent acting on the virus capsid. Pleconaril and BTA798 are two examples of several classes of low-molecular-weight hydrophobic compounds identified to inhibit viral uncoating by such stabilization of the capsid.

Although no antipicornavirus drug has yet been licensed, the two aforementioned compounds have completed phase II clinical trials. BTA798 (refs. 9,10), developed by Biota Holdings, continues to show promise for asthmatic patients with rhinovirus infections. From the skeletons of Pleconaril and related molecules, a new class of imidazolidinones has been synthesized with anti-HEV71 activity (half-maximal inhibitory concentration (IC50) in the range of 0.001–25 µM (refs. 12,13), and the crystal structure of the HEV71 particle4,14,15 now provides an opportunity for the rational improvement of such inhibitors. The use of crystal structures of protein–ligand complexes in combination with in silico methods to guide antiviral inhibitor design is now well established as an effective strategy, with a notable early example being development of the first useful anti-influenza drug16. Nevertheless the use of whole viruses as targets has not been routinely integrated into pharmaceutical pipelines, and it is well known that existing in silico methods have usually struggled to rank compounds by binding affinity.

1Division of Structural Biology, University of Oxford, Oxford, UK. 2National Laboratory of Macromolecules, Institute of Biophysics, Chinese Academy of Science, Beijing, China. 3School of Molecular and Cellular Biology, Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, UK. 4Diamond Light Source, Didcot, UK. 5Department of Pharmaceutical Chemistry, University of Innsbruck, Innsbruck, Austria. 6National Institutes for Food and Drug Control, No. 2, Tiantan Xili, Beijing, China. 7Laboratory of Structural Biology, School of Medicine, Tsinghua University, Beijing, China. 8These authors contributed equally to this work. Correspondence should be addressed to D.I.S. (dave@strubi.ox.ac.uk) or Z.R. (raozh@xtal.tsinghua.edu.cn).

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We set out to design more-effective small-molecule antivirals targeting HEV71, on the basis of structural information. To this end, we analyzed the experimentally derived high-resolution structures of HEV71 bound to four 3-(4-pyridyl)-2-imidazolidinone derivatives (Fig. 1b)\(^2\), using robust \textit{in silico} docking methods to rank potential new inhibitors. We designed a number of compounds, triaged them \textit{in silico} and synthesized two. We used efficient \textit{in situ} crystallography\(^1\) to determine inhibited capsid structures, demonstrating that the compounds bound as expected to the virus and further showing that they inhibited infection. One of these compounds is an order of magnitude more potent than the previous best inhibitor.

**RESULTS**

\textbf{Structural basis of 3-(4-pyridyl)-2-imidazolidinone activity}

We determined the structures of HEV71 in complex with the uncoating inhibitors GPP2, GPP3, GPP4 and GPP12 (3-(4-pyridyl)-2-imidazolidinone derivatives; defined in Figs. 1 and 2 and Table 1). Because these, and most pocket-factor analogs, are rather insoluble, we dissolved them in DMSO before soaking them into preformed HEV71 crystals (Online Methods). We collected data at room temperature in crystallization plates at the Diamond Light Source\(^1\), producing structures at between 2.65- and 2.8-Å resolution (Online Methods and Table 1). As seen from the electron density maps, the compounds replace the natural pocket factor (modeled as sphingosine), with only small shifts (0.1 Å–0.4 Å) in the backbone of the residues lining the pocket, thus reflecting their shape similarity with sphingosine (Supplementary Fig. 1). The surface area accessible to solvent, calculated by Areaimol\(^19\), is 8 Å\(^2\) for GPP3, 11 Å\(^2\) for GPP2, 12 Å\(^2\) for GPP4, 9 Å\(^2\) for GPP12 and 9 Å\(^2\) for the natural pocket factor. These results demonstrate that all of these molecules are essentially fully buried, with GPP3 perhaps inserted slightly deeper into the pocket. All compounds bind with their pyridine ring close to the entrance of the pocket, with the carbonyl oxygen of the imidazole moiety hydrogen-bonding to the backbone nitrogen of residue Ile113, as seen with sphingosine, and the phenoxy ring sandwiched between two phenylalanines (Phe135 and Phe155) (Fig. 2). The introduction of a methyl group in the GPP3 linker region results in an order of magnitude tighter binding compared to that of GPP2 (Figs. 1 and 3a).

\textbf{In silico docking}

We assembled a database of published inhibition data for 47 HEV71 inhibitors\(^12,13,20\) (Supplementary Table 1) and generated correlation plots between the published IC\(_{50}\) values\(^12,13,20\) and the energy of interaction computed from the inhibitors’ docking poses in the VP1 pocket for quantum mechanics—polarized ligand docking (QMPLD)\(^21\) implemented in the Schrödinger suite (http://www.schrodinger.com/). We tested several docking methods (details in Supplementary Note and Supplementary Fig. 2), and of these the QMPLD\(^21\) method provided a compelling correlation of 0.81 (Fig. 3b).

The quantum-mechanical optimized procedure reliably predicted the experimentally observed poses (Supplementary Fig. 3). In particular, the predicted docking poses of GPP2 and GPP3 had r.m.s. deviations of less than 2 Å from the crystal structures (Supplementary Fig. 3). We determined the template structure used for the docking at 2.65-Å resolution, and to test the robustness of the method we repeated some of these experiments by independent structure determinations, both at a similar resolution\(^15\) (2.7 Å) and also at much lower resolution\(^14\) (3.7 Å). The results demonstrated that even...
a structure of rather low resolution can accurately reproduce the correct binding mode (Supplementary Fig. 4).

Plate-based inhibitor characterization

Using inhibitors GPP3 and GPP2, we next validated particle thermostability as a measure of compound potency by using a plate-based high-throughput thermofluor assay (particle stability thermal release assay (PaSTRy))22 developed to assess viral stability and the dynamics of uncoating. For a negative control, we used 1-((2-chlorophenoxo)methyl)-4-[(2,6-dichlorophenyl)ethoxy]-benzene (GPV13), a compound similar to SCH47820, which shows strong activity against poliovirus type 2 and several echoviruses and Coxackieviruses but only weak activity against HEV71 (ref. 9).

The temperature at which purified virus releases its RNA genome ($T_R$) is ~58 °C. However, after incubation with 200 µg/ml GPV13, GPP3 or GPP2 for 72 h at room temperature, the $T_R$ was raised to 60–61 °C (Fig. 3c), indicative of stabilization of the particle. At the lower concentration of 20 µg/ml, GPP3 and GPP2 still showed increased particle stability after 24-h incubation, whereas GPV13 had little effect even after 72 h (Fig. 3d). These results are consistent

![Figure 3](https://example.com/figure3.png)

**Figure 3** GPP3 bound to VP1 and thermal stabilization by GPP2, GPP3 and GPV13. (a) VP1 is shown as a cartoon (blue); side chains of hydrophilic residues at the pocket entrance and hydrophobic residues surrounding the methyl moiety of GPP3 are shown as sticks. Residues contacting the methyl group are shown: 4.8 Å to Ala133, 4.2 Å to Met253 and 4.0 Å to Phe131. (b) Correlation plot of binding affinities of 3-(4-pyridyl)-2-imidazolidinone derivatives predicted by GMPLD versus experimental $-\log(1/C_{50})$ (pIC$_{50}$) values. Red dots show the calculated pIC$_{50}$ for the new ligands; experimental values are in yellow. (c) First derivatives of the fluorescence curves (described in Online Methods). Gray line indicates control virus incubated with SYTO9 dye to detect the release of RNA. Cyan, red and blue lines indicate HEV71 virions incubated with 200 µg/ml GPV13, 200 µg/ml GPP3 or 200 µg/ml GPP2, respectively, with 72-h room-temperature (RT) incubation including SYTO9 dye. (d) As in c, using SYTO5, but with HEV71 virions incubated with 20 µg/ml GPV13 for 72 h at RT (cyan line), HEV71 virions incubated with 20 µg/ml GPP2 (blue line) or GPP3 (red line) for 24 h at RT, or control virus (gray line). (e,f) First derivative of the fluorescence curve for control sample (gray line) with dye SYPRO Red to detect protein unfolding (e) and for virions incubated at RT for 72 h with 200 µg/ml GPP2 (blue lines) or GPP3 (red lines) (f).

### Table 1 Data collection and refinement statistics

| Protein   | HEV71-GPP2 | HEV71-GPP3 | HEV71-GPP4 | HEV71-GPP12 | HEV71-NLD | HEV71-ALD |
|-----------|------------|------------|------------|-------------|-----------|-----------|
| No. crystals (positions) | 38 (41)    | 28 (31)    | 14 (17)    | 13          | 53 (55)   | 46        |
| Space group | I23        | I23        | I23        | I23         | I23       | I23       |
| a, b, c (Å) | 599.8, 599.8, 599.8 | 599.7, 599.7, 599.7 | 599.7, 599.7, 599.7 | 599.7, 599.7, 599.7 | 600.3, 600.3, 600.3 | 600.3, 600.3, 600.3 |
| Resolution (Å) | 50.0–2.65  | 50.0–2.80  | 50.0–2.80  | 50.0–2.80   | 50.0–2.75 | 50.0–2.75 |
| Rmerge  | 0.488      | 0.535      | 0.517      | 0.539       | 0.484     | 0.510     |
| Completency (%) | 84.8 (57.3) | 69.2 (30.0) | 61.5 (59.1) | 67.4 (66.5) | 67.2 (57.4) | 60.3 (49.9) |
| Redundancy | 2.5 (1.5) | 2.1 (1.3) | 1.7 (1.7) | 1.9 (1.8) | 1.7 (1.5) | 1.5 (1.4) |
| Refinement | | | | | | |
| Resolution (Å) | 50.0–2.65  | 50.0–2.80  | 50.0–2.80  | 50.0–2.80   | 50.0–2.75 | 50.0–2.75 |
| No. reflections | 797,620 / 41,800 | 551,890 / 29,091 | 478,258 / 25,074 | 532,325 / 27,945 | 551,343 / 29,185 | 494,155 / 26,036 |
| $R_{merge}$ / $R_{free}$ | 0.245 / 0.246 | 0.278 / 0.282 | 0.283 / 0.285 | 0.270 / 0.272 | 0.291 / 0.295 | 0.302 / 0.308 |
| No. atoms | Protein | 6,506 | 6,506 | 6,506 | 6,506 | 6,506 |
| Ligand/ion/water | 132 | 96 | 82 | 143 | 93 | 101 |
| B-factors | Protein | 21 | 20 | 25 | 22 | 22 | 19 |
| Ligand/ion/water | 17 | 23 | 27 | 19 | 21 | 21 |
| r.m.s. deviations | Bond lengths (Å) | 0.008 | 0.008 | 0.006 | 0.007 | 0.009 | 0.007 |
| Bond angles (°) | 1.5 | 1.5 | 1.4 | 1.4 | 1.5 | 1.4 |

Values are in parentheses are for highest-resolution shell. The $R_{free}$ is of limited significance, owing to the considerable noncrystallographic symmetry.

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with the half-maximal effective concentration (EC_{50}) values reported for GPP3, GPP2 and GPV13 of 10 nM, 100 nM and 40 μM, respectively. (Among the compounds tested, the only previously measured IC_{50}, for GPP2, was 1 nM (ref. 12).) Interestingly, the protein melting of untreated virus occurred in two distinct steps with melting-temperature (T_m) values of ~58 °C and ~65 °C. Taken together with T_R, this indicates a two-stage transition in protein conformation, with the lower-temperature transition corresponding to virus expansion and the release of RNA (Fig. 3e) and the higher-temperature transition corresponding to the protein melting. In contrast, only the higher-temperature transition occurred after incubation with 200 μg/ml GPP3 or GPP2, and the T_m peaks were sharper (Fig. 3f).

**Figure 4** VP1 pocket and docking of the new ligands. (a) A GRID map showing the interaction energies between the probe and the explored region within the VP1-binding pocket. The hotspot for binding a primary amine is shown by the yellow surface, which is drawn at −15 kcal mol^{-1}. (b,c) Molecular docking of NLD (b) and ALD (c) in the VP1 pocket. Both the ligands are shown as sticks. NLD hydrogen-bonds with main chain nitrogen of Gin202, and ALD establishes hydrogen-bond interactions with the side chain of Asp112, as shown by dotted lines. The side chain of Ile113 is hidden.

### Design of potentially improved HEV71 inhibitors

With these tools for assessing in silico docking and thermostability, we next used the structural data to steer the design of more-potent compounds. The binding pocket is more exposed in HEV71 than in most other picornaviruses\(^4\)\(^5\)\(^4\). We postulated, on the basis of inspection of the pocket entrance at the bottom of the canyon, that introducing a functional group such as an amine or amide on the pyridine ring (Fig. 3a) might simultaneously increase the solubility of the compound and enhance its affinity for the virion by allowing the formation of hydrogen bonds with polar residues (for instance, Q202 or D112) (Fig. 3a).

To test this hypothesis, we scanned the pocket surface in silico with a collection of probes (such as hydrophobic, amine cation, ester oxygen atom, methyl group and amine with lone pair), using GRID\(^2\)\(^3\) to identify binding hotspots. An amine with a lone-pair probe identified a hotspot around residue D112 with an overall energy of interaction of −15 kcal mol^{-1}. This minimum became more pronounced (−17 kcal mol^{-1}) when the probe was an amine cation. The region around Q202 is also a hotspot for binding the amine cation, with an interaction energy of −20 kcal mol^{-1} (Fig. 4a). These results support the hypothesis that introducing an amino group on the pyridine ring would increase the overall binding energy, owing to the formation of a hydrogen bond with residues on the canyon floor. On this basis, we generated modifications of the GPP3 molecule in silico, exploring the effect of multiple substituents on the pyridine ring and replacing the pyridine with alternative moieties of different size. We generated several dozen possibilities (with PRODRG\(^2\)\(^4\)) and triaged them by visual inspection. We selected some six compounds including furan, isoxazole, pyrrole and amine-thiazole derivatives to take forward to the next stage of in silico ranking and chose four to be synthesized. Of these, two were produced readily, and MS confirmed that they were highly pure (Supplementary Fig. 5).

We conducted in silico docking with the QMPLD method described above (Supplementary Note and Online Methods) for the two molecules synthesized, termed NLD and ALD (Fig. 4b,c). NLD (molecular weight 448 Da) is a variant of GPP3 in which position 2 of the pyridine ring has been replaced with a primary amine (Fig. 4b). The docking pose shows this molecule engaged with its amino group and forming a hydrogen bond to the carbonyl group of Q202, thereby increasing the number of virus capsid–ligand interactions. To achieve this, docking flips the pyridine ring by 180° compared to its orientation in the HEV71–GPP3 complex. The second molecule, ALD (molecular weight 476 Da), has position 2 of the pyridine ring replaced with an amide (Fig. 4c). The docking pose suggests that this substituent will form hydrogen bonds with the side chain of D112. The predicted IC_{50} values were 2.6 μM and 0.8 μM for NLD and ALD, respectively (Fig. 4b). The logP (logarithm of the n-octanol–water partition coefficient) for GPP3 is calculated to be 3.8, whereas for NLD and ALD this decreased to 3.6 and 3.0, respectively, thus suggesting that these compounds are rather more soluble than previous inhibitors. In addition, the theoretical absorption, distribution, metabolism and excretion (ADME-tox) properties (calculated with QikProp v3.6 in the Schrödinger suite and reported in Supplementary Table 2) appear favorable.

**ALD and NLD readily replace the natural pocket factor**

To establish whether and how ALD and NLD bound, we soaked them into HEV71 crystals. The compounds were indeed more soluble than the GPP series, and the soaking protocol used led to rapid degradation of the crystals. We therefore reduced the concentration of the compounds 550-fold to allow diffraction data to be collected and determined room-temperature structures at 2.75-Å resolution\(^1\). Both ligands maintain the key useful interactions described above (Fig. 5a,b). The presence of the amide group on the pyridine moiety allows ALD to establish hydrogen bonds with the side chain of D112, exactly as predicted by in silico docking (Fig. 5b). However for NLD, the crystal structure shows that the amine group on the pyridine moiety, rather than interacting with the peptide carbonyl moiety of Q202 as predicted by in silico docking, is rotated by almost 180° to interact instead with the side chain of D112, in a similar way...
Figure 5 Characterization of newly designed capsid binders bound to the VP1 pocket of HEV71. Crystal structures of the compounds bound to the virus. (a, b) Single-round real-space averaged |F|=|F| omit maps (green mesh) of NLD ligand in the HEV71–NLD complex (a) and ALD ligand in the HEV71–ALD complex (b). (c, d) First derivatives of the fluorescence curves for the PaSTRy assay. (c) The yellow line represents the control virus incubated with SYPRO RED to detect RNA release. The cyan, gray, red and blue lines represent HEV71 virions incubated with 200 µg/ml ALD, NLD, GPP12 or GPP4, respectively, with 24 h incubation at room temperature. (d) The yellow line represents the control virus incubated with SYPRO RED to detect the exposure of hydrophobic protein surfaces. The cyan, gray, red and blue lines represent HEV71 virions incubated with 200 µg/ml ALD, 200 µg/ml NLD, 200 µg/ml GPP12 or 200 µg/ml GPP4, respectively, with 24 h incubation at room temperature. (e) HEV71 samples titrated via TCID₅₀ in the presence of a range of concentrations of NLD (black), GPP3 (green) or ALD (cyan). Nonlinear regression was used to determine the IC₅₀ value. The IC₅₀ is the point at which the TCID₅₀ value is reduced by 50%. For clarity, the curves are represented on a logarithmic scale. Each point represents the average of three measurements. Error bars, s.e.m.

to that observed for ALD (Fig. 5b). With the exception of this reorientation, the experimental results indicate that the predicted docking poses (r.m.s. deviation <2 Å). To investigate the NLD docking, we used LigPrep (http://www.schrodinger.com/), which suggested that at pH 7 the pyridine nitrogen could be protonated. Redocking NLD with a protonated pyridine produced an essentially correct pose of similar energy (r.m.s. deviation of 0.5 Å in comparison to experimental result).

NLD and ALD are powerful HEV71 inhibitors in vitro

PaSTRy analysis confirmed that NLD and ALD are potent capsid stabilizers that enhance stabilization at moderate concentrations relative to those of known tight binders such as GPP3 (Fig. 5c,d). Quantification for such tight binders is difficult because the concentrations of binding sites (60 times the virus concentration of 0.2 µM) and competing pocket factor (at least equal to the concentration of binding sites) are far above the binding constant for NLD and ALD. The concentration needed to produce thermal stabilization will therefore underestimate the IC₅₀ by orders of magnitude⁵. We therefore also compared the inhibitory activities of ALD and NLD to those of GPP3 and GPP4 by in vitro half-maximal tissue-culture infectious dose (TCID₅₀) assays in Vero cells. We used ten-fold serial dilutions of virus in the presence of different concentrations of the compounds and exposed control wells to the equivalent concentration of solvent (DMSO) to ensure no cytopathic effect on uninfected cells or on virus titer. NLD was the most effective inhibitor, with an IC₅₀ of ~0.025 nM, inhibiting the viral titer to below 5% at concentrations over 0.05 nM. GPP3 was the next most effective inhibitor, with an IC₅₀ of 0.319 nM. ALD has an IC₅₀ of 8.54 nM (although the TCID₅₀ results suggested a more complex biphasic effect). GPP4 did not display inhibitory effects at concentrations up to 1,000 nM (Fig. 5c). Finally, we performed a further set of experiments to determine the IC₅₀ value by nonlinear regression. The IC₅₀ is the point at which the TCID₅₀ value is reduced by 50%. For clarity, the curves are represented on a logarithmic scale. Each point represents the average of three measurements. Error bars, s.e.m.

DISCUSSION

It has proved notoriously difficult to find useful therapies for picornaviral infections such as the common cold. At present, replacing the hydrophobic-pocket factors expelled from many picornaviruses as they uncoat the genome⁴ by more robust binders²⁶,²⁷ is the most promising point for therapeutic intervention⁵,⁶. To direct the discovery process, we determined crystal structures of HEV71 in complex with four ligands with a broad range of affinities. GPP4, the shortest of the four, only partially occupies the binding pocket and has the poorest EC₅₀. This relationship reveals that there is an optimal drug size, which correlates with the efficiency of binding—molecules of the right length fill the pocket better and are better inhibitors. The inhibitors that satisfy this requirement also offer an aromatic moiety at the correct point to occupy a hydrophobic trap formed by Phe135 and Phe155. Indeed, in all crystal structures of picornavirus–inhibitor complexes a pair of hydrophobic residues are found at positions equivalent to those occupied by these aromatic residues in HEV71 (refs. 28,29). Thus, structures of rhinovirus 14 in complex with Pleconavir³⁰ and poliovirus 2 in complex with a Schering-Plough compound³¹ show the antiviral agent located between the structurally equivalent residues Tyr128 and Tyr152, and Phe134 and Tyr159, respectively (Supplementary Fig. 6). The presence of this hydrophobic trap constrains the extent of penetration of such inhibitors and hence the length of the molecule. Increasing the length of the inhibitor causes a misalignment of the phenoxy group with respect to Phe135 and Phe155, thus decreasing the binding energy and undermining the inhibitory effect. Conversely, in GPP4, which is shorter (bearing just an iodine atom at position 6 on
the phenoxyl group), the trap locks the molecule to result in a partially filled cavity (Fig. 2d). Nearby, a methyl group in the linker region of GPP3 in large part fills a hydrophobic subpocket; consequently, GPP3 has the lowest EC50 value (10 nM). The hydrophobic residues (Phe131, Ala133 and Met253) lining the subpocket leave some space that could possibly accommodate a slightly bigger substituent (Fig. 3a). However, Chang et al.20 have shown that phenyl, dimethyl, ethyl and propyl groups cannot fit, because they decrease the affinity. Similarly, in rhinovirus bulkier substituents are unfavorable32, so a methyl group is probably close to optimal.

We have also demonstrated the utility of an extremely rapid plate-based fluorescence assay for inhibitor binding, which replicates the rank order of previously reported in vitro assays. In addition, by measuring RNA accessibility alongside protein unfolding, we have found that potent inhibitors elevate the capsid conformational transition associated with genome release to the point at which the capsid proteins melt.

The QMPLD method23, with guidance from the observed crystal structures, provided reliable docking results and predictions of binding strength (correlation coefficient 0.81 against a database of 47 prior results). This method, used with care, predicted correct docking poses from a virus structure determined at only 3.7-Å resolution. The power of the method is presumably partly due to its use of quantum mechanics to take into account the ligand polarization of the protein environment during the docking process.

Using experimental structural data together with in silico mapping of the pocket entrance for additional polar interactions, we designed optimized inhibitors (Fig. 4b,c) bearing hydrophilic substituents, which offered the additional benefit of increasing the solubility33. The generated docking poses for two of these inhibitors, ALD and NLD, confirmed additional putative hydrogen bonds with the virus and suggested that they were likely to bind more tightly to HEV71 than do any previously reported compounds (Fig. 4b).

Synthesized ALD and NLD were soluble and highly reactive with the virus crystals, and they replaced the pocket factor very effectively. Both contain flexible linker regions allowing them to adapt well to the shape of the binding pocket. ALD bound as predicted by QMPLD docking, whereas a portion of NLD assumed an alternative conformation. However, careful analysis of the protonation state of the molecule led to a revised docking that recapitulated the observed binding mode. In vitro analysis with the PaSTRy assay confirmed that both NLD and ALD are powerful capsid stabilizers, with NLD being more potent than the previous gold-standard compound, GPP3 (Fig. 5c,d). IC50 values from cell-based assays underlined the extraordinary potency of NLD, which was able to protect cells from HEV71 infection at a concentration of 25 pM (Fig. 5e). Furthermore the compounds showed good activity against all HEV71 subtypes and against CVA16, results suggesting that such compounds might be broadly effective against the disease (Supplementary Table 3). This is explained by the conservation of Asp112 of VP1, which forms a key

In summary, using the complete virus capsid as a target, we have used a combined experimental and computational approach, starting from a prior compound with limited solubility and nanomolar-range effective concentrations, to obtain, in a single round of design, a next-generation picomolar-range inhibitor that is broadly effective and relatively soluble and has many drug-like properties. (The calculated ADME-tox properties of NLD (Supplementary Table 2) indicate that the molecules are predicted to have generally acceptable pharmacokinetic properties.) Previous experience shows that such inhibitors can generate drug-resistant mutations34. If this were to occur with these compounds, more work would be required—perhaps a further round of design to build in resilience to common mutations (as performed in Hopkins et al.35). In conclusion, we propose that the approach we describe might facilitate the design of more-efficient inhibitors targeted at other enteroviruses, such as rhinoviruses, polioviruses and Coxackieviruses.

METHODS
Methods and any associated references are available in the online version of the paper.

Accession codes. Coordinates and structure factors for the six complexes have been deposited in the Protein Data Bank under accession codes 4CDQ (HEV71–GPP2), 4CDU (HEV71–GPP3), 4CDW (HEV71–GPP4), 4CDX (HEV71–GPP12), 4CEY (HEV71–NLD) and 4CEW (HEV71–ALD).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
D.I.S. and Z.R. supervised and coordinated the project; J.W., Z.H., X.L., G.P. and W.P. made samples available; X.W. purified and crystallized the samples and performed thermofluor experiments; G.P. and J.G. provided the PPI ligands; L.D.C. designed ALD and NLD; L.D.C. and J.A.B.S. ran the in silico docking and analyzed data under supervision by D.I.S.; L.D.C. and T.S.W. soaked crystals for data collection, which was performed by L.D.C., J.A.B.S., J.R. and E.E.F.; L.D.C., J.A.B.S., J.R. and D.I.S. contributed to data processing, structure determination and model building; J.K. performed the in vitro TCID50 assay together with N.S. and D.J.R. analyzed the data. L.D.C., E.E.F. and D.I.S., in discussion with J.R., D.J.R. and Z.R., wrote the manuscript. All authors read and approved the manuscript.

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ONLINE METHODS

Virus purification and crystallization. Cells were cultured and virus stocks prepared and crystallized as described previously4 in nanoliter vapor-diffusion Greiner CrystalQuick X plates36,37. Cubic crystals emerged in 2 weeks. GPP2, GPP3, GPP4 and GPP12 were dissolved in 100% DMSO with concentrations of 19 mg/ml, 18.5 mg/ml, 24 mg/ml and 68 mg/ml, respectively; GPP3 and GPP2 stock solutions were mixed with Crystal Screen 1 (Hampton Research) condition 13 in a ratio of 1:2 and further diluted to give a solution containing ~2 mg/ml ligand, ~7% PEG 400, 44 mM trisodium citrate and 22 mM Tris-HCl pH 8.5. GPP4 and GPP12 stock solutions were diluted 55 times in water supplemented with 18% of condition 13 of Crystal Screen 1 (Hampton Research). About 0.5 μl of this solution was added to the 0.2-μl crystallization drops 1–2 weeks before data collection (1 week was sufficient to allow binding to the virus). For ALD and NLD, the protocol was modified: they were dissolved in 100% DMSO with concentrations of 140 mg/ml and 260 mg/ml. These solutions were diluted 100 times in 100% DMSO and then further diluted 55 times in water supplemented with 18% of condition 13 of Crystal Screen 1 (Hampton Research). 1 μl of soaking was sufficient to allow full replacement of the pocket factor.

PaSTRy assay. Thermofluor experiments were performed as previously described22. 50-μl reactions were set up in a thin-walled PCR plate (Agilent) containing 0.5–1.0 μg of HEV71, 5 μM SYTO9 and 3 μM SYPRO Red in PBS, pH 7.4, and the temperature ramped from 25 °C to 99 °C, with fluorescence recorded in triplicate at 1 °C intervals. In order to replace the sphinogosine with HEV71 inhibitors completely, different concentrations (20 μg/ml and 200 μg/ml) and incubation times (72 or 24 h) at room temperature were used. The sphinogosine was expected to have a slow off-rate, and the assay was performed at equilibrium. 5% DMSO was used throughout. The melting temperature, Tm, was taken as the minimum of the negative first derivative of the curve.

Structure determination. Data were collected in sitethe18 on beamlines I24 and I03 at Diamond light source. Diffraction images of 0.05° or 0.1° rotation were recorded on a Pilatus 6M detector using an unattenuated beam of 0.05 × 0.05 mm2 at I24 or 0.10 × 0.06 mm2 at I03, with exposure times of 0.1 s per image. Owing to radiation damage in the microcrystals, data collection was limited to 3–10 frames per crystal. Data processing was performed with the HKL2000 package38. Reflections with fractional partialities of >0.7 or >0.5 were scaled to full intensity (program POST: D.I.S. and J. Diprose, unpublished program).

LogP calculations. LogP values were calculated with Virtual Computational Chemistry Laboratory (http://www.vcclab.org/)39.

Synthesis of ALD. Methyl4-[3-(5-[4-[[ethoxyimino)methyl]phenoxoy]-3-methylpentyl)-2-oxoimidazolidin-1-yl]pyridine-2-carboxylate (45 mg, 0.10 mM, 1.00 equiv) methanol (5 mL), NH3•H2O (10 mL) and NH4Cl (1.7 mg, 0.33 equiv) were placed in a 100-mL three-necked round-bottom flask. The resulting solution was stirred overnight at 40 °C and then concentrated under vacuum. The residue was diluted with 15 mL of water. The resulting solution was extracted with 3 × 10 mL of ethyl acetate and the organic layers combined. The mixture was dried over anhydrous sodium sulfate and concentrated under vacuum. The residue was washed with 2 × 5 mL of ether/hexane (1:1). This yielded 30 mg of ALD as a white solid.

Syntnes of NLD. A solution of tert-butyl 1-[4-[3-(5-[4-[[ethoxyimino)methyl]phenoxoy]-3-methylpentyl)-2-oxoimidazolidin-1-yl]pyridin-2-yl]carbamate (200 mg, 0.38 mM, 1.00 equiv) in TFA/CH2Cl2 (1:1) (20 mL) was placed in a 50-mL round-bottom flask. The resulting solution was stirred for 6 h at room temperature and then concentrated under vacuum. The crude product was purified by Flash-Prep–HPLC. This yielded 30 mg of NLD as a light-yellow solid.

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