Identification of a conserved virion-stabilizing network inside the interprotomer pocket of enteroviruses

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Enteroviruses pose a persistent and widespread threat to human physical health, with no specific treatments available. Small molecule capsid binders have the potential to be developed as antivirals that prevent virus attachment and entry into host cells. To aid with broad-range drug development, we report here structures of coxsackieviruses B3 and B4 bound to different interprotomer-targeting capsid binders using single-particle cryo-EM. The EM density maps are beyond 3 Å resolution, providing detailed information about interactions in the ligand-binding pocket. Comparative analysis revealed the residues that form a conserved virion-stabilizing network at the interprotomer site, and showed the small molecule properties that allow anchoring in the pocket to inhibit virus disassembly.
The group B coxsackieviruses (CVBs) are a major source of both acute and chronic diseases in humans. Age and immune status are thought to be the main determinants of morbidity and mortality, with infants, young children, and immunocompromised individuals being particularly susceptible to serious and sometimes life-threatening infections. Coxsackievirus B3 (CVB3) can cause cardiac arrhythmias and acute heart failure. Additionally, CVB3 infections during pregnancy have been linked to an increase in neurodevelopmental delays, fetal myocarditis, and spontaneous abortions. Coxsackievirus B4 (CVB4) appears to elicit or enhance certain autoimmune disorders such as type 1 diabetes as the virus has been isolated from individuals diagnosed with rapid onset type 1 diabetes, and these isolates were then shown to cause diabetes in mice models. Dotta et al. have provided arguably the most direct support for CVB4 as a viral trigger of diabetes via immunohistochemical detection and sequencing of virus from the pancreatic tissue of individuals diagnosed with rapid onset type 1 diabetes, and these isolates were then shown to cause diabetes in mice models. The emergence of drug-resistant viruses recently, we discovered that the site of binding is located at a conserved pocket such as a cysteine residue in VP1 (C73) and hydrophobic residues (VP1 F76 and VP3 F236) that interact with the benzene ring of the compound, contributing to the overall binding energy. In addition, there are other contributions inside the pocket such as an oxygen in the side chain engaging in a hydrogen bond with virus uncoating, and indicate that it is worthwhile to focus on developing therapies that include a synergistic combination of binders to potentially improve efficacy, alleviate side effects, and shorten treatment of enteroviral infections.

Results and discussion

CP17 bound to the interprotomer pocket of CVB3. CP17 is a benzenesulfonylamine derivative that potently inhibits the CVB3 Nancy strain in cells (EC50 0.7 ± 0.1 µM) via a direct interaction with the capsid that increases virion thermostability by 1.5 and 2.1 log10 TCID50/mL at 46 and 49 °C, respectively. A 4.0 Å cryo-EM structure of CP17 in complex with CVB3 Nancy (EMD-0103) revealed that the site of binding is located at a conserved VP1–VP3 interprotomer interface, but the low resolution of the map prevented identification of the detailed interactions within the pocket. We reprocessed the raw data (EMPIAR-10199) using RELION 3.0 and the resolution improved to 2.8 Å. The cryo-EM map shows pronounced backbone features for the four structural proteins of CVB3, and well-defined density for CP17 on the surface of the capsid. Importantly, the resolution is now sufficient for describing specific ligand–protein interactions (Fig. 1c). The interprotomer site is located between adjacent asymmetric units “protomers” in a narrow opening formed at the intersection of neighboring VP1 β-barrels and the C terminus of a proximally situated VP3 molecule. Three residues that play a key role in binding CP17 are conserved across enteroviruses. In CVB3 Nancy they are Arg219 (VP1), Arg234 (VP1), and Gln233 (VP3) (Fig. 1c). The Arg residues, which come from neighboring VP1 polypeptide chains, are situated in the deepest part of the pocket, and in the high-resolution structure, we observed that their guanidinium groups form salt bridges with the carboxylic end of CP17 (Fig. 1e). The Gln residue from the C terminus of VP3 is positioned at the entrance of the drug site, where the oxygen in the side chain engages in a hydrogen bond interaction with the NH located in the elbow region of the inhibitor. In addition, there are other contributions inside the pocket such as a cysteine residue in VP1 (C73) and hydrophobic residues (VP1 F76 and VP3 F236) that interact with the benzene rings of the compound, contributing to the overall binding energy and specificity of CP17 (Fig. 1c, e). The binding energy (sum of the van der Waals and electrostatics) for CP17 is −74 kcal/mol based on the NAMD energy plugin in VMD. The predicted value is comparable to potent inhibitors of the VP1 hydrophobic pocket, namely the capsid binders GPP3 (~66 kcal/mol) and NLD (either ~69 or ~64 kcal/mol depending on protonation state).
CP48 within the CVB4 virion. To further investigate and confirm the activity and structural basis for how inhibitors bind at the interprotomer pocket, we determined a structure of CVB4 in the presence of a commercially available analog, which we refer to as CP48. This particular inhibitor was found to be active against all six serotypes of CVBs, and completely inhibited poliovirus type 1 replication at a concentration of 144 μM. We chose to work with CVB4 because no structure exists for this important human pathogen. First, we confirmed that addition of CP48 increases CVB4 thermal stability (Fig. 2 and Supplementary Data). Then, purified virus was incubated with a saturating concentration of CP48 (virus:drug molar ratio of 1:2500), applied to grids, and flash-frozen for cryo-EM. After image processing, a subset of 18,626 particles yielded a 2.7 Å reconstruction using the FSC 0.143 threshold criterion. The outer surface of the virus particle is similar to that of other entroviral capsids, with major features including the fivefold star-shaped mesas, threefold propeller-like protrusions, and twofold depressions (Fig. 3a). In addition, there is stable and well-defined density for the inhibitor at the interprotomer site (Fig. 3a, c, d). The control structure of CVB4 incubated without compound revealed no additional density inside the pocket (Fig. 3b, e). We did not detect a conformational change induced by the presence of CP48 (RMSD for native versus CP48-bound virus: 0.45 Å). Modeling confirmed the critical role of the conserved pocket side chains: two Arg residues on the inner surface and a Gln residue at the entrance (Fig. 3d). Similar to how CP17 is anchored to CVB3, CP48 is stabilized by stacking interactions with two hydrophobic residues, Y67 in VP1 and F236 in VP3 (Fig. 3d, f). The C64 residue in VP1, though not involved in the direct interaction with CP48, is in close vicinity to

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**Fig. 1** Cryo-EM structure of CP17-bound CVB3. **a** Three-dimensional reconstruction of CVB3 after incubation with a saturating amount of capsid binder. The virion is viewed along the icosahedral twofold axis and colored according to radial distance in Å from the particle center. Density for CP17 is shown in orange. The map was resolved to 2.8 Å upon reprocessing raw data from a previous publication (see ref. 19; EMPIAR-10199). **b** CP17 binds in a pocket between neighboring protomers. VP1, green; VP2, dark blue; VP3, light blue. **c** Inhibitor and pocket residues at a display contour level of 2.5σ (σ is the standard deviation of the density map). **d** CP17 shown in density contoured to 1.6σ. **e** Ligand interactions diagram for CP17 generated by Schrödinger Maestro v12.02.
drug design. Here, we used high-resolution cryo-EM to reveal how a new class of capsid binders makes stabilizing contacts inside the interprotomer pocket. The network comprises about 15 residues with 3 highly conserved amino acids forming the core of the binding site. These three residues, each from a different polypeptide chain, dictate the size and shape of interprotomer-targeting compounds, as well as the mechanism of action (Fig. 4a). Other interactions that define the structure–activity relationship within the pocket include a pair of hydrophobic residues that stably position the benzene scaffolds of the inhibitors, and a cysteine residue that can hydrogen bond with the carboxylic end (Fig. 4b, c). When an inhibitor is stably anchored to the network (60 sites per a single virion), it interferes with motion transmission such that the virus particle cannot undergo expansive conformational changes in the interprotomer region, and hence is unable to uncoat the genome at precisely the right time in infection.

**Conclusion**

Despite decades of research on WIN antiviral compounds with, e.g., EC$_{50}$ value for pleconaril against human rhinovirus B1 reported as 0.2 µM, no drugs have been approved for use against enteroviruses.26 Recently, a new class of broad-spectrum capsid binders was described, which inhibit a variety of enteroviruses by occupying a positively charged surface depression in the interprotomer zone with, e.g., EC$_{50}$ value for CP17 against CVB3 as 0.7 µM. Structure-guided in vitro assays involving CVB3 and CP17 indicated that this class of capsid binders increases particle stability, which we have observed to be the case here for CVB4 and CP48. Virus variants with reduced susceptibilities to compounds targeting either pocket can be selected under pressure, with concomitant reduced viability.21,27 Reverse engineering mutation experiments revealed that four interprotomer mutants in CVB3 Nancy were not viable: VP1 Q160G, VP1 R234G, VP3 F236G, and VP3 Q233G (VP1 R219 was not tested).21 We were unable to perform similar experiments with CVB4 here because there is no infectious clone available. Nevertheless, structural alignments and experimental data suggest a conserved virion-stabilizing network within the interprotomer pocket that is less tolerant to mutations, a promising result for efforts to develop antivirals. Interestingly, Duyvesteyn et al.28 recently published a 1.8 Å resolution X-ray structure of bovine enterovirus F3 (EV-F3) with glutathione (GSH) positioned in a similar way within the pocket. The antioxidant engages the same virion-stabilizing network as CP17 and CP48, which is not surprising given that these molecules have strikingly similar geometrical and chemical features (Supplementary Fig. 2). Specifically, GSH adopts a hook-shaped structure with a carboxylic end and a sulfur-containing elbow region. The overall size approximates that of inhibitors that occupy the interprotomer site. The carboxylic group interacts with the two Arg residues from neighboring VP1 polypeptides inside the pocket, while the sulfur atom interacts with the oxygen in the Gln side chain of VP3. It is believed that for CVB3 and CVB4, GSH makes strong interactions with adjacent protomers to facilitate intracellular assembly of progeny virions.29–31 Further work is necessary to understand how these molecules, which share similar shape and chemistry, modulate stability at the pocket to either prevent uncoating or facilitate assembly. Outside of structural efforts, it will be important to assess the influence of cellular cues and factors on their different modes of action. Accordingly, we foresee the development of improved binders, as well as an enhanced understanding of the biological significance of the interprotomer site, by building on the information provided by these new structures which show molecules inside the pocket.
Methods

Virus culture and purification. BGM cells were a kind gift from the Rega Institute for Medical Research in Leuven. No authentication of the cell line was done. Cell supernatant was routinely tested as mycoplasma negative using the Eurofins Genomics Company mycoplasma testing service. Cells were cultured in Eagle’s minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1× nonessential amino acids, 1% GlutaMAX, and 1% antibiotic–antimycotic solution in a chamber environment adjusted to 37 °C and 5% CO2. To produce virus particles for the study, 30 confluent T175 flasks were inoculated with CVB4 (GenBank: AF311939.1) at a multiplicity of infection of ~0.5 in serum-free medium. Additionally, the infection medium contained 20 mM HEPES (pH 7.0). At 3 days post-infection, widespread viral cytopathic effect was evident, and the contents of each flask were collected, freeze–thawed three times, and centrifuged at 4000 r.p.m. and 4 °C for 10 min to remove cellular debris. The supernatant was then carefully removed and concentrated using a Centricon centrifugal filter device (100 kDa cut-off). Virus particles were purified by centrifuging through a CsCl gradient (top density 1.25 g/cm3 and bottom density 1.48 g/cm3) at 30,000 r.p.m. and 4 °C for 19 h. The gradient/exchange buffer consisted of 10 mM HEPES (pH 7.0), 150 mM NaCl, 2 mM MgCl2, and 2 mM CaCl2. Bands containing intact virions were collected and the CsCl was removed by buffer exchange.

Thermostability assay. Approximately 5 × 104 TCID50 units of CVB4 strain E2 was mixed with 20 μM concentration of CP48 in six tubes (reaction volume 52 μL) and incubated over a range from 37 to 52 °C for 2 min, followed by rapid cooling on ice. The infectious virus load in the samples was estimated by an end-point titration assay using BGM cells. Specifically, serial 10 log dilutions were prepared in infection medium and applied to BGM cell monolayers on 96-well plates arranged one day before use by seeding 2 × 104 cells per well. Two days after infection, the BGM cell monolayers were examined for cytopathic effects and TCID50/mL was estimated using Kärber–Spearman formula. The experiment was repeated with four independent biological replicates for each measurement. The detection limit is 102 TCID50/mL.

Cryo-EM sample preparation and data collection. The compound 4-[(6-propoxy-2-naphthyl)sulfonyl]amino]benzoic acid (CP48) was ordered from a commercial supplier (www.specs.net) and dissolved in DMSO at a concentration of 10 mg/mL. We further diluted (10× dilution) the compound in a gradient/exchange buffer. Purified CVB4 and CP48 were then mixed at a molar ratio of 1:2500, which yielded a final capsid binder concentration of 0.17 mg/mL. The mixture was incubated at 37 °C for 1 h. For cryo-EM sample preparation, 3.0 μL samples of CVB4–CP48 were applied to glow-discharged grids (Ted Pella product No. 01824).

Fig. 3 Cryo-EM of the CP48–CVB4 complex in comparison to CVB4 alone. a Visualization of CVB4 in the presence of CP48. The view is along the twofold axis with radial coloring, and the inset shows clear inhibitor density at the interprotomer site near the fivefold axis of symmetry. Density for the 385.44 Da CP48 is displayed in magenta. b The corresponding region of the cryo-EM density of the CVB4 control. c CP48 fits well into the additional density detected in the cryo-EM map of the CP48–CVB4 complex. d A close-up view of the capsid binder (magenta) within the interprotomer pocket. e A close-up view of the interprotomer pocket in the cryo-EM map of CVB4 alone shows no density for the compound. d, e Color coding for the viral proteins VP1, VP2, and VP3 is the same as in Fig. 1. f Interaction diagram of CP48 with CVB4 viral proteins generated in Schrödinger Maestro software v12.02.
Fig. 4 Stabilization inside the interprotomer pocket of enteroviruses. a Structural alignment of the three key residues that form the core of the virion-stabilizing network inside the interprotomer pocket. Atomic models used for the alignment are listed in C. b Alignment of PDB IDs 6ZCK, 6ZCL, and 1EV1 with the same view as a but with CP17/48-stabilizing hydrophobic residues of the interprotomer pocket added to the visual. c Conservation of anchor residues in the pocket based on the structural data presented in this study. The three residues that are highly conserved are in bold whereas the other major elements (hydrophobic and cysteine) vary. Enterovirus A, B, C, D, and F and Rhinovirus A, B, and C species are indicated in column 1 along with the wwPDB IDs. The numbering of the residues in columns 2–6 were taken from the wwPDB files listed in column 1. (*) not modeled in the coordinates for 1HXS.

Image processing. Defocus values of CVB4–CP48 micrographs were determined by Gctf34. A total of 31,436 particles were picked from 4377 micrographs using a Gatan K2 Summit direct electron detection camera at a nominal magnification of ×130,000, giving a pixel size of 1.24 Å per pixel. The total electron dose was approximately 46 electrons per Å2 fractionated into 30 frames. Frame images in each movie were aligned using MotionCor2 (ref.33). A control dataset of CVB4 without CP48 was collected within the Instruct-ERIC Center Finland at the University of Helsinki using a Talos Arctica equipped with a Falcon III direct electron detection camera (Table 1). A total of 8860 movies were acquired at a nominal magnification of ×120,000, giving a pixel size of 1.24 Å per pixel. The accumulated electron dose was approximately 36 electrons per Å2 fractionated into 30 frames. MotionCor2 was used to produce a single micrograph from aligned and averaged movie frames (Supplementary Fig. 3).

ResMap images, map cross-sections, and FSC curves for the three structures are included in Supplementary Fig. 4 (ref. 37).

Modeling. An initial template for CVB4 capsid proteins VP1–VP4 was derived from a homology-based model calculated by I-TASSER38. The UCSF Chimera Build Structure tool was used to translate the Simplified Molecular Input Line Entry Specification (SMILES) string for CP48 into a three-dimensional structure and parameterization was completed using SwissParam39,40. Structures for viral proteins and drug were docked into the EM density using UCSF Chimera, followed by iterative manual adjustment and real-space refinement using COOT41. Sequence assignment was guided by bulky amino acid residues such as Phe, Tyr, Trp, and Arg, and featureful density allowed placement of the ligand. The optimized model for CVB4–CP48 was then subjected to end-stage refinement using the molecular dynamics flexible fitting program originally developed by Klaus Schulten and coworkers42. Harmonic restraints were applied to prevent overfitting during simulations. Capsid proteins for the CVB4 virion without drug were modeled using a similar protocol and comparison to the CP48 refinement did not induce conformational changes in the virion. The RMSD between CP48–CVB4 and CP48 alone was 0.45 Å. The binding energy for CP48 inside the interprotomer pocket was obtained using the NAMD energy plugin in VMD43,44. We used the same procedure to refine atomic coordinates for CVB3–CP17 (PDB ID code 6GZV) into the newly determined CVB3–CP17 2.8 Å map. The structural alignment of PDB files was done using the MatchMaker feature of UCSF Chimera. Ligand Interactions diagrams for the compounds in the interprotomer pockets were generated by Schrödinger Maestro v12.02 (Schrödinger Release 2019-4: Maestro v12.2, Schrödinger, LLC, New York, NY, 2020).

Statistics and reproducibility. The thermostability assay was analyzed as a box and whisker plot from n = 4 independent experiments. The summary of the cryo-EM data collection, refinement, and validation statistics are shown in Table 1 (ref. 35).
| Table 1 Cryo-EM data collection, refinement, and validation statistics. |
|-------------------------------------------------------------|
| **CVB4–CP48 (EMD-11165)** | **CVB3–CP17 (EMD-11166)** | **CVB4 (EMD-11300)** |
| (PDB 6ZCK) | (PDB 6ZCL) | (PDB 6ZMS) |
| Magnification | 130,000 | 130,000 | 120,000 |
| Voltage (kV) | 300 | 300 | 200 |
| Electron exposure (e⁻/Å²) | 47 | 47 | 30 |
| Defocus range settings (µm) | −0.6 to −3.0 | −0.6 to −3.0 | −0.1 to −2.0 |
| Pixel size (Å) | 1.06 | 1.06 | 1.24 |
| Symmetry imposed | 12 | 12 | 12 |
| Initial particle images (no.) | 31,436 | 17,300 | 96,985 |
| Final particle images (no.) | 18,626 | 13,252 | 46,627 |
| Map resolution (Å) | 2.7 | 2.8 | 3.4 |
| FSC threshold | 0.143 | 0.143 | 0.143 |
| Map resolution range (Å) | 999–2.12 | 999–2.12 | 999–2.48 |
| Refinement | | | |
| Map sharpening B factor (Å²) | −70 | −77 | −90 |
| Model composition | | | |
| Non-hydrogen atoms | 6443 | 6370 | 6397 |
| Protein residues | 800 | 798 | 800 |
| Ligands | 2 | 2 | 0 |
| R.m.s. deviations | | | |
| Bond lengths (Å) | 0.91 | 0.86 | 0.90 |
| Bond angles (°) | 1.13 | 1.01 | 1.01 |
| Validation | | | |
| MolProbity score | 1.4 | 1.16 | 1.52 |
| Clashscore | 0 | 0 | 0 |
| Poor rotamers (%) | 3.9 | 2.4 | 1.7 |
| Ramachandran plot | | | |
| Favored (%) | 93 | 94 | 91 |
| Allowed (%) | 5 | 5 | 6 |
| Disallowed (%) | 2 | 1 | 3 |

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Author contributions
J.W.F., A.D., and S.J.B. conceived the idea and designed the experiments. J.W.F., A.D., and A.L.S. carried out the experiments. J.W.F., A.D., A.L.S., and S.J.B. contributed to interpretation of results and writing of the manuscript.

Competing interests
The authors declare no competing interests.

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