An orally available 3-ethoxybenzisoxazole capsid binder with clinical activity against human rhinovirus

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S1 Materials and Methods

^{1}H NMR spectra were recorded on a Bruker AC-200, AC-300 or AV-400 spectrometer in $CDCl_3$ and the spectra were referenced to the residual protonated solvent signal. Thin layer chromatography (TLC) was performed on E. Merck Kieselgel 60 F-254 plates and flash chromatography was carried out using Merck silica gel 60, 230-400 mesh. Low-resolution mass spectra were recorded on a VG micromass 70 or a VGTRIO-1 mass spectrometer with an ion source temperature of 200^{o}C and electron impact energy of 70eV.

S1.1 Synthetic Chemistry: Synthesis of BTA798 6

S1.1.1 Preparation of 2-hydroxy-4-methoxybenzohydroxamic acid

A solution of hydroxylamine was prepared by the addition of aqueous sodium hydroxide (393 mg, 9.82 mmol) in water (1.6 mL) to a stirred solution of hydroxylamine hydrochloride (292 mg, 4.21 mmol) in water (3.5 mL). Immediately a solution of methyl 2-hydroxy-4-methoxybenzoate (511mg, 2.81 mmol) in 1,4-dioxane (1.5 mL) was slowly added and the resulting mixture stirred under argon at room temperature for 18 h. The

reaction mixture was concentrated on a rotary evaporator to half the original volume and the product precipitated by the addition of concentrate hydrochloric acid while keeping the sample on ice. The resulting suspension was filtered to give 2-hydroxy-4-methoxybenzohydroxamic acid (476 mg, 92%) as a pale brown solid. ^{1}H NMR δ (ppm): 3.72 (s, 3H); 6.36 (m, 2H); 7.41 (d, 1H).

S1.1.2 Preparation of 3-hydroxy-6-methoxy-1,2-benzisoxazole

A solution of carbonyldiimidazole (1.07 g, 6.57 mmol) in anhydrous THF (8 mL) was added to a stirred boiling solution of the hydroxamic acid (602 mg, 3.29 mmol) in THF (6 mL). The resulting solution was heated at reflux for 8 to 10 h then allowed to cool to room temperature and stirred overnight under argon. Analysis of the sample by TLC (silica, 1:1 hexane/ethyl acetate) showed minimal starting material and a new nonpolar substance. The solution was evaporated on a rotary evaporator to give an orange oil. Water (6 mL) was added and the contents cooled on ice and acidified to pH 2 with concentrated hydrochloric acid. The crude, damp 3-hydroxy-6-methoxy-1,2-benzisoxazole precipitated as an orange cream solid (645 mg). ^{1}H NMR δ (ppm): 3.82 (s, 3H); 6.73 (fd, 1H); 6.80 (dd, 1H); 7.52 (d, 1H); MS (ESI) 166 $(M+1)^+$.

S1.1.3 Preparation of 3-ethoxy-6-methoxy-1,2-benzisoxazole

A sample of dry 3-hydroxy-6-methoxy-1,2-benzisoxazole (193 mg, 1.17 mmol) was dissolved in anhydrous THF (4 mL) with ethanol (75 μL, 1.29 mmol) and triphenylphosphine (460 mg, 1.75 mmol) and cooled to 0º. Diisopropylazodicarboxylate (345 μL, 1.75 mmol) was added slowly and after 15 min the reaction was removed from ice and stirred at room temperature overnight under argon. The solution was evaporated to dryness and the residue pre-absorbed onto silica then chromatographed on silica (19 g) commencing with an eluent of hexane (300 mL) and increasing the polarity to 10-30% ethyl acetate/hexane to give 3-ethoxy-6-methoxy-1,2-benzisoxazole (101 mg, 44%) as white crystals. ^{1}H NMR δ (ppm): 1.50 (t, 3H); 3.87 (s, 3H); 4.47 (q, 2H); 6.86 (m, 2H); 7.47 (d, 1H); MS (ESI) 194 $(M+1)^+$.

S1.1.4 Preparation of 3-ethoxy-6-hydroxy-1,2-benzisoxazole

A solution of boron tribromide (1.0 M in dichloromethane, 1.39 mL, 1.39 mmol) was added to a stirred solution of 3-ethoxy-6-methoxy-1,2-benzisoxazole (179 mg, 928 μmol) in dichloromethane (4 mL) at -78º under argon. The reaction mixture was gradually warmed to room temperature over 2 h and stirred overnight. Analysis by TLC (silica, 2:1 hexane/ethyl acetate) showed some unreacted starting material as well as new polar material in the sample. Water (5 mL) and ice were added and the aqueous phase was neutralized by addition of saturated $NaHCO_3$ solution and then saturated with NaCl. The aqueous phase was extracted into dichloromethane (3 x 60 mL) then the organic extracts combined and washed with brine (10 mL) and dried ($NaSO_4$). The product 3-ethoxy-6-hydroxy-1,2-benzisoxazole was purified by chromatography on silica (18 g; eluent 2.5%, 5% then 15% ethyl acetate/hexane) eluting as the second compound (108 mg, 65%) following unreacted 3-ethoxy-6-methoxy-1,2-benzisoxazole (46 mg). ^{1}H NMR δ (ppm): 1.45 (t, 3H); 4.40 (q, 2H); 6.74 (m, 2H); 7.38 (m, 1H); MS (ESI) 180 $(M+1)^+$.

S1.1.5 Preparation of BTA798 (BTA798) ***6***

A mixture of 3-ethoxy-6-hydroxy-1,2-benzisoxazole (28 mg, 156 μmol), 2-[-1-(6-methyl-3-pyridazinyl)-4-piperidinyl]ethanol (42 mg, 188 μmol) and polymer-supported triphenylphosphine (145 mg, 234 μmol) in anhydrous THF (3 mL) was cooled (0º) and stirred under argon. Neat diisopropylazodicarboxylate (46 μL, 234 μmol) was added slowly and the reaction mixture allowed to warm to room temperature and stirred overnight. The reaction mixture was filtered then pre-adsorbed onto silica and chromatographed on silica (5 g) using firstly 2:1 hexane/ethyl acetate as eluent then gradually increasing the polarity to 70% ethyl acetate/hexane to afford BTA798 **6** (44 mg, 73%) as a white powder. Anal. ($C_{21}H_{26}N_4O_3$) calc C 65.95, H 6.85, N 14.65; found C 65.94, H 6.79, N 14.58; ^{1}H NMR (400 MHz) δ (ppm): 1.36 (m, 2H); 1.49 (t, *J* = 7.1 Hz, 3H); 1.81 (m, 5H); 2.52 (s, 3H); 2.91 (m, 2H); 4.07 (t, *J* = 6.2 Hz, 2H); 4.33 (m, 2H); 4.45 (q, *J* = 7.1 Hz, 2H); 6.84 (m, 3H); 7.04 (d, *J* = 9.3 Hz, 1H); 7.46 (d, *J* = 9.2 Hz, 1H); ^{13}C NMR (100 MHz) δ (ppm): 14.6, 21.1, 31.6, 33.1, 35.5, 45.7, 66.0, 93.6, 107.5, 113.3, 113.7, 121.1, 127.9, 150.6, 158.8, 161.8, 165.7, 166.3; MS (ESI) 383 $(M+1)^+$.

S1.2 Virus Culture

HeLa Ohio cells were seeded in 225 cm^2 flasks (Corning Labware, Corning, NY,) in DMEM-F12 supplemented with 10% (v/v) FBS at a concentration of 6.5 x 10^6 cells per flask. After an overnight incubation at 37ºC in a humidified 5% CO_2 atmosphere (Sanyo MCO-17AIC incubator; Quantum Scientific, Milton, Australia), the monolayer of cells was approximately 60% confluent. The supernatant was removed, 50 mL of assay media added and the cells were inoculated with HRV 2 at an approximate multiplicity of infection (MOI) of 0.005. Flasks were incubated at 33ºC in a humidified 5% CO_2 atmosphere until greater than 80% CPE were observed. Virus was then harvested and transferred to -80ºC for storage.

S1.3 CPE Assays

BTA798 free base, pleconaril, pirodavir, BTA188 and AG7088 were prepared as 4 or 10 mg mL^{-1} stocks in cell culture grade DMSO. Working stocks were generated by dilution in DMEM/F12 Assay media (Gibco, Australia), supplemented with 2% (v/v) FBS (Gibco, Australia) and 10 mM $MgCl_2$. BTA798 or the control compounds were evaluated in triplicate with serial threefold dilutions of compound in assay media. Untreated infected cells (compound replaced with an equal volume of assay media) and cells with no virus were also included in triplicate in each test. HeLa Ohio cells in suspension were prepared at a concentration of 1.5 x 10^5 cells per mL in assay media. A volume of 100 µL of cell suspension (final concentration of 1.5 x 10^4 cells/well) containing HRV at the appropriate MOI was added to test, control article and untreated control HRV infected wells bringing the final volume in the wells up to 200 µL. A volume of 100 µL of cells without virus was added to the cell-only no infection control wells. Following infection, assay plates were incubated at 33˚C in a humidified 5% CO_2 atmosphere for five days. Cell viability was tested with an MTT assay and the protective effect of compounds on virus-induced cell death assessed by comparison of the control and compound samples[1].

S1.4 Low Inoculum/High Passage Selection of Pleconaril-resistant HRV Variants

HeLa Ohio cells were seeded in 25 cm^2 flasks (Corning Labware, Corning, NY) in DMEM-F12 supplemented with 10% (v/v) FBS at a concentration of 6.5 x 10^5 cells per flask. After an overnight incubation at 37˚C in a humidified 5% CO_2 atmosphere, the monolayer of cells was approximately 80% confluent. The supernatant was removed, fresh media containing pleconaril (10 mL) was added to the flasks and the cells were inoculated with either HRV 2 or HRV 14 at an MOI of 0.002 prepared in assay medium containing 2% (v/v) FBS and 10 mM $MgCl_2$. The pleconaril concentration (30 ng mL^{-1}, 79 nM) approximated the EC_{50} value in the first passage and was doubled with each subsequent passage. For passage 2 through to passage 6, 80 to 90 µL of the virus harvested from the previous passage was used to inoculate fresh cells. Flasks were incubated at 33˚C in a humidified 5% CO_2 atmosphere until greater than 80% CPE were observed. Pleconaril-resistant virus was harvested by freeze-thawing and centrifugation for 10 minutes at 4°C at 5000 g and then frozen in a dry ice bath and transferred to -80˚C for long-term storage. Virus from each passage was assessed for mutations in the VP1 region by DNA sequencing.

The shift in susceptibility (relative resistance) for HRV variants resistant to pleconaril was measured by determining the EC_{50} value for a compound the resistant variant and comparing it to the EC_{50} value of the wild type strain in control experiments conducted under the same conditions and run at the same time (Table S1). A relative resistance greater than 10 was taken to indicate reduced viral susceptibility of the viral variant to the compound compared with wild type virus.

S1.5 Preclinical Pharmacokinetic Studies

All animal experimentation was conducted with the approval of the regulatory authorities in the United Kingdom under the Animals (Scientific Procedures) Act 1986. Dogs (Beagles, 3 of each sex) and rats (lister hooded strain, 28 males and 27 females) received a single oral dose by gavage of BTA798 in a solution of 1% carboxymethylcellulose at a

nominal dose level of 50 mg free base per kg bodyweight. [^{14}C]-BTA798 (IDT Laboratories) was included in the solution to provide a level of radioactive dose of 5 MBq.kg^{-1}. Blood samples were collected from each animal at 0.25, 0.5, 1, 3, 4, 6, 8, 12, 16, 24, 48, and 72 hours after dose administration and the levels of BTA798 determined by measurement of the radioactivity of the samples in a Packard Tri Carb liquid scintillation counter (Canberra Packard).

S1.6 Purification of Virus

Samples of purified virus were produced by adaptation of a published method[2].Virus stocks grown in HeLa Ohio cells underwent three cycles of freeze-thawing (-70ºC/ 37ºC), then the stocks were pooled and centrifuged at 2000g at 4ºC for 20 min in Beckman-Coulter Avanti J-25 centrifuge to remove the cell debris. Polyethlene glycol 6000 (Merck) and sodium chloride (Sigma) were added to the clarified supernatant to a final concentration of 7% (w/v) and 380 mM, respectively. The mixture was then incubated overnight at 4ºC. The precipitated virions were centrifuged at 10,000g for 15 min at 4ºC, then were resuspended in 400 µL TNE buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl and 1 mM EDTA) containing 1% (v/v) Nonidet P-40. The suspension was clarified at 12,000g for 3 min in an Eppendorf microcentrifuge prior to loading onto a 15-45% (w/v) linear sucrose (Sigma) gradient in TNE buffer, which was assembled in a 14 mL centrifuge tube (Beckman Ultra-Clear). The sucrose gradient loaded with the clarified virion suspension was centrifuged in a TH64I Rotor in a Beckman L8-70M Ultracentrifuge overnight at 100,000g at 4ºC. Gradients were fractionated and fractions containing virus were identified by SDS-PAGE and pooled. The pooled fractions were centrifuged at 200,000g for 2 hr at 4ºC and the viral pellet was resuspended in a small volume of TNE buffer. The purity of HRV 2 produced for crystallography was assessed by SDS-PAGE analysis of a sample of the purified virus (Figure S1). Electrophoresis of purified virus revealed four bands, corresponding to the molecular weights of the four capsid proteins VP1, VP2, VP3 and VP4.

S1.7 Crystallization

The crystallization protocol was adapted from a previously described method[3]. The protein concentration of the purified virus preparation was estimated from the SDS-PAGE results, then the virus was concentrated using centrifugal concentrators (Millipore, NSW, Australia) to a protein concentration of approximately 1 mg mL^{-1} in 50 mM Tris-HCl buffer, pH 7.5. A stock of BTA798 was made by dissolving 1 mg BTA798 in 200 µL pure DMSO (Sigma, NSW, Australia). An aliquot of 1 µL of the BTA798 solution was mixed with 25 µL of virus stock solution and incubated at 4ºC overnight. Crystallization was performed using the hanging drop vapor diffusion method in 24-well Linbro culture plates (ICN, Biochemicals Inc., Ohio, USA). Crystallization drops were prepared by pipetting 2 µL of HRV/BTA798 mixture and 2 µL of reservoir solution on siliconized cover slips (Hampton Research, California, USA). The drops were equilibrated against 1 mL of the reservoir solution at 22ºC. Crystals of HRV 2 formed readily in solutions containing 0.4 to 0.7 M of ammonium sulfate as a precipitant. The crystals were consistent in appearance across the different precipitant and pH ranges investigated, being roughly cubic and a maximum size of ~ 0.07 mm in each dimension.

S1.8 Collection of Diffraction Data and Data Analysis

Single crystals of HRV 2 complexed with BTA798 were removed from the mother liquor in the crystallization plates using custom-made nylon cryoloops. The crystals were transferred into a drop of mother liquor containing 30% (v/v) glycerol as a cryoprotectant. After one minute in the cryoprotectant, the crystals were mounted on the endstation of the MX1 beamline at the Australian Synchrotron[4], where they were then snap frozen at -100ºC. The frozen crystals were exposed to an X-ray beam and diffraction data were collected on a Rigaku imaging plate detector (Rigaku Corporation, Tokyo, Japan). Multiple datasets were obtained using different crystals with typical oscillation angles of 0.1º. Diffraction from the HRV 2 crystals was weak but clear with well defined diffraction spots observed. Indexing of the data showed that the crystals belonged to space group $P2_122_1$, with cell parameters a = 309.2 Å, b = 345.4 Å and c = 377.4 Å. This is the same space group with closely similar cell dimensions as has been reported for HRV 2 complexed with a fragment of its cellular receptor[3] but differs from the space groups found previously for HRV 2 alone[5, 6].

Datasets were processed using XDS[7, 8] and PHENIX[9]. The structure was solved by molecular replacement with Phaser[10] using a pentamer (5 copies of the protomer containing the VP1, VP2, VP3 and VP4 protein chains, forming one of the 5-fold symmetric capsid substructures) of a published[5] HRV 2 crystal structure (PDB id:1FPN). The top solution contains 6 pentamers (a total of 30 protomers) derived from 4 different adjacent capsids (Figure S2). NCS symmetry was applied and TLS refinement used with 5 TLS groups per protomer determined using the TLSMD server[11, 12] followed by several rounds of refinement at a resolution of 3.2Å with PHENIX[9]. Visual inspection of the partially refined structure and comparison to that for other HRV subtypes indicated that the structure of VP4 contained an error, propagated by molecular replacement from an error in 1FPN, with part of VP4 being built into electron density arising from the N-terminus of VP1. VP4 was removed from the partially refined structure and the N-terminal residues of VP1 added using Coot[13]. VP4 was then manually rebuilt on the basis of the VP4 structures from HRV14 (1HRI[14]), HRV16 (1AYM[15]) HRV3 (1RHI[16]) and HRV1A (2HWD[17]). Further rounds of refinement in PHENIX with modified NCS and TLS constraints incorporating the revised VP1 and VP4 proteins gave the final apo capsid structure. Regions of clear electron density were visible in the space corresponding to the volume within the capsid, probably arising from bases of the viral RNA genome, but no attempt was made to build any RNA into the model. A molecule of BTA798 **6** was built into the unsatisfied electron density present in each protomer's ligand binding site followed by further rounds of refinement to generate the final complex structure. Both the shape and the extent of the electron density are definitive for BTA798 occupying the ligand binding site (Figure 1b). The compound was refined with occupancy of 1, resulting in consistent B-factors for BTA798 **6** and the surrounding residues. The final protomer structure has been deposited in the PDB (PDB ID: 3VDD).

The HRV2 – BTA798 complex structure consists of 23880 amino acids, 360 waters and 30 molecules of BTA798 **6** (Table S2). Assessment of the structure by Molprobity[18] indicates that the structure is of acceptable quality, with a score of 3.43 indicating

acceptable, but slightly lower than usual, quality for all structures at this resolution. This score is unsurprising considering the nature of the capsid structure being far larger and more complex than the usual globular proteins used as the basis for the scoring function. Comparison of the HRV2 – BTA798 complex structure with that of the published HRV2 structure containing lauric acid in the ligand binding canyon[5] shows that the differences between the two structures are minor, with a backbone RMSD between the 1FPN and HRV2 – BTA798 protomers of 0.3 Å. The compound is bound in the predominantly hydrophobic pocket in VP1, where other capsid-binding rhinovirus inhibitors have been identified (reviewed in De Palma *et al.* 2008[19]), consisting of residues Ile99, Asn100, Leu101, Gln102, Phe119, Ser121, Ile123, Tyr143, Met144, Tyr145, Ala167, Val169, Phe180, Leu182, Leu185, Tyr191, Tyr192, Met193, Thr207, Thr210, Asn211, Met213, Leu216, His237 and His259.

S2 Supplementary Data

S2.1 Table S1 Cross resistance of Pleconaril-resistant HRV variants to BTA798, BTA188 and Pirodavir

HRV serotype	Designation	Amino acid mutation[a]	Relative resistance[b] to:			
			BTA798	BTA188	Pirodavir	Pleconaril
HRV 2	2-35-P3	I99F	17	74	159	63
	2-35-P6	I99F	137	131	256	> 101
HRV 14	14-35-P5	A150V, C199R, E276K[c]	24	> 2500	148	> 48
	14-35-P6	A150V, E276K[c]	67	> 2500	> 227	> 48

[a] Residues of VP1 numbered from 1 to 289 from amino end

[b] Relative resistance calculated as the EC_{50} value for the resistant variant divided by the EC_{50} value for the wild type strain. A relative resistance greater than 10 indicates reduced viral susceptibility of the variant to the test article. The values represent the mean from two to three experiments

[c] Single variant containing two or more single-point mutations

S2.2 Table S2 Data collection and refinement statistics

Data collection	
Space group	$P2_122_1$
Cell dimensions	
a, *b*, *c* (Å)	309.2, 345.4, 377.4
Resolution (Å)	3.20
No. of crystals	4
No. of observations	7032884
No. of unique reflections	660200
R_{merge} [a]	25.2
I / σ_I [b]	7.0 (12.0)
Completeness (%)[b]	99.7 (100)
Redundancy [b]	10.6 (10.0)
Refinement	
Resolution (Å)	3.20
No. reflections	660200
No. atoms	
Protein	190200
Water	360
BTA798	840
R_{work} [c] / R_{free} [d] (%)	25.2 / 28.4
R.m.s. deviations	
Bond lengths (Å)	0.02
Bond angles (°)	1.9
B-factors (Å^2)	
Main-chain	60.4
Side-chain	61.2
Water	27.0
Residues in most favoured region of Ramachandran plot (%)	94.8

[a] $R_{merge} = \Sigma_{hkl}\Sigma_i|I_i - \langle I\rangle|/|\langle I\rangle|$, where I_i is the intensity for the *i*th measurement of an equivalent reflection with indices *h,k,l.*

[b] The values in parentheses are for the highest resolution bin.

[c] $R_{work} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes respectively.

[d] R_{free} was calculated with 5% of the diffraction data that were selected randomly and not used throughout refinement.

S2.3 Figure S1

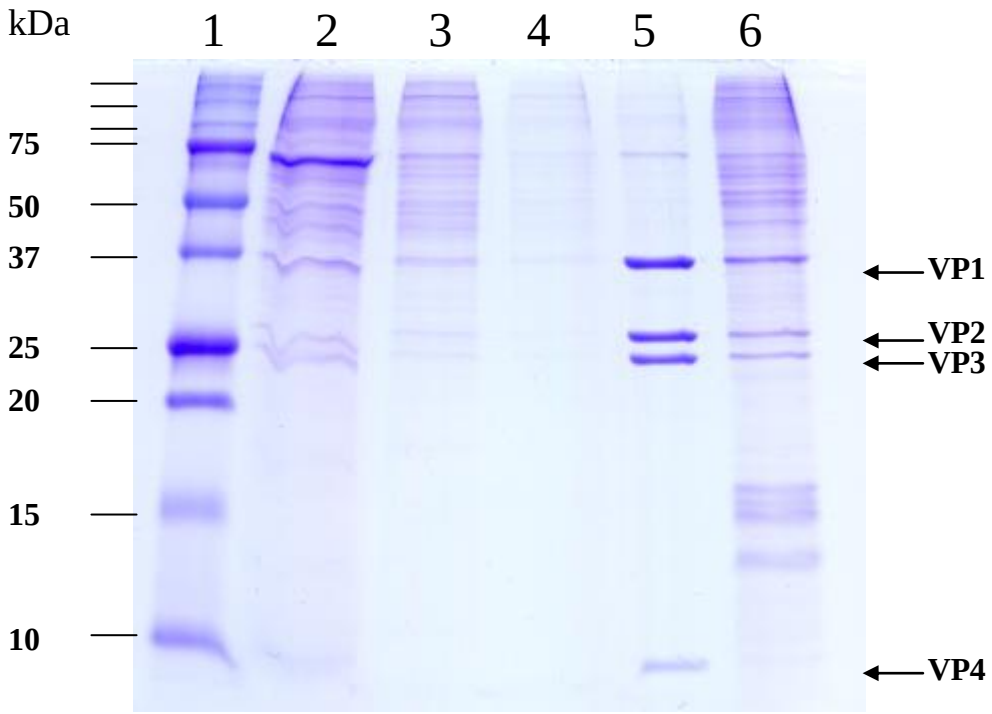

Figure S1 SDS-PAGE of HRV 2 purified by sucrose gradient centrifugation for crystallography. The 15% acrylamide gel was stained with Coomassie blue. Marker molecular weights in Kilodaltons (kDa) are supplied on the left. The expected positions of the viral capsid proteins VP1 to VP4 on the gel are indicated by arrows to the right of the gel. Lane 1: Molecular weight marker (Precision Plus, Biorad); Lane 2 and 3: Supernatants from virus pelleting steps; Lane 4: Final wash supernatant; Lane 5: Purified virus. Lane 6: Resuspended pellet after purification step. The purification protocol has separated the intact virions (Lane 5) from the damaged viral particles which remain in the pellet (Lane 6).

S2.4 Figure S2

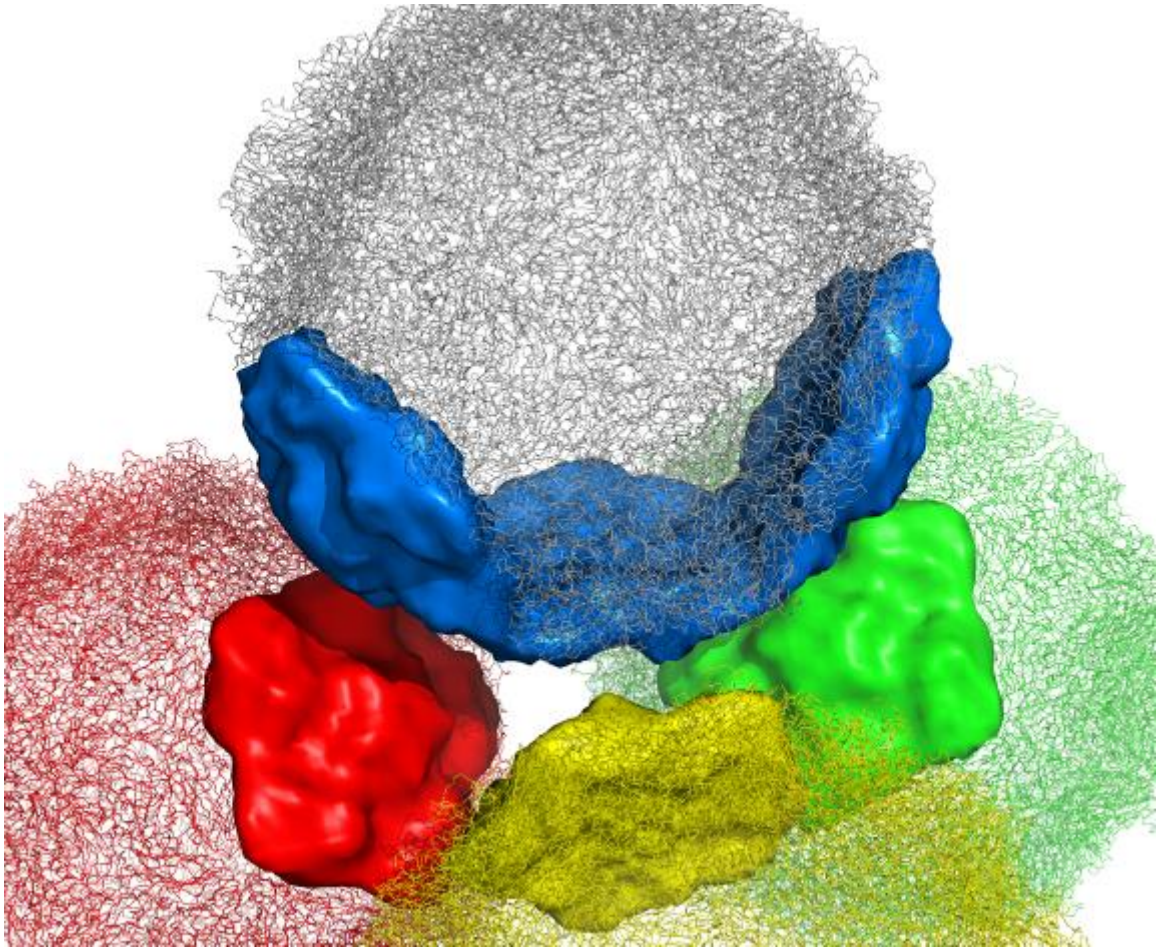

Figure S2 Diagrammatic representation of the packing of four adjacent viral particles in the crystal structure of HRV2 complexed with BTA798. Each viral particle is shown as a backbone trace colored by particle except for the 6 pentamers forming the asymmetric unit, which are shown as molecular surfaces. Figures S2 and S3 were prepared with PyMOL[20].

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