Supplementary Material for

X-ray screening identifies active site and allosteric inhibitors of SARS-CoV-2 main protease

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This PDF file includes:

- Materials and Methods
- Supplementary Text
- Figs. S1 to S9
- Tables S2, S5, and S6
- References

Other Supplementary Material for this manuscript includes the following:
(available at science.sciencemag.org/content/science.abf7945/DC1)

- Tables S1, S4, and S7 as separate Excel files
- Table S3 as a separate .pdf file
- MDAR Reproducibility Checklist
**Materials and Methods**

**Protein production and purification**

The protein was overexpressed in *E. coli* and purified for subsequent crystallization according to previously published protocols and plasmid constructs(1). Lysis was carried out in 20 mM HEPES buffer supplemented with 150 mM NaCl using ultrasound for cell disruption. After separation of the cell fragments and the dissolved protein, a subsequent nickel NTA column was used to extract the M₆₆₆₆-histidine-tag fusion protein. The cleavage of the histidine tag was achieved by a 3C protease during an overnight dialysis step. The histidine tag and the 3C protease were removed using a nickel NTA column, and as a final step gel filtration was performed with an S200 Superdex column.

**Crystallization experiments**

Co-crystallization with the compounds was achieved by mixing 0.23 μL of protein solution (6.25 mg/mL) in 20 mM HEPES buffer (pH 7.8) containing 1 mM DTT/TCEP (respectively), 1 mM EDTA, and 150 mM NaCl with 0.22 μL of reservoir solution consisting of 100 mM MIB, pH 7.5, containing 25% (w/w) PEG 1500 and 5% (v/v) DMSO, and 0.05 μL of a micro-seed crystal suspension using an Oryx4 pipetting robot (Douglas Instruments). This growth solution was equilibrated by sitting drop vapor diffusion against 40 μL reservoir solution.

Prior to crystallization, 125 nL droplets of 10 mM compound solutions from the two libraries in DMSO were applied to the wells of SwissCI 96-well plates (2-well or 3-well low profile, respectively) and subsequently dried in vacuum. Taking the crystallization drop volume into account this resulted in a final compound concentration of 2.5 mM and a molar ratio of ~13.6 of compound to protein. To obtain well-diffracting crystals in a reproducible way micro-seeding was used(30). Crystals appeared within a few hours and reached their final size (~200×100×10 μm³) after 2 - 3 days. Crystals were manually harvested and cryo-cooled in liquid nitrogen for subsequent X-ray diffraction data collection. We aimed at harvesting two crystals per crystallization condition as a compromise between through-put and increasing the probability to collect data from well diffracting crystals.

**Data collection**

Data collection was performed at beamlines P11, P13 and P14 at the PETRA III storage ring at DESY in Hamburg over a period of four weeks. Exclusive use of DESY beamline P11 was generously granted by the DESY directorate for the project. EMBL also provided special access to beamlines P13 and P14.

**Data processing and structure refinement**

An automatic data processing and structure refinement pipeline “xia2pipe” was written specifically to support this project. Raw diffraction images from the PETRA III beamlines were processed using three crystallographic integration software packages: XDS(31), autoPROC(32), followed by staraniso(33), and DIALS via xia2(34, 35). Diffraction data quality indicators for all datasets and the 43 hits are summarized in Fig. S9. The datasets had an average resolution of 2.12 Å (criterion: CC1/2 > 0.5), CC1/2 of 0.97, and Wilson B of 27.8 Å² (Fig. S9). Crystallographic data of all structures submitted to the PDB are summarized in table S4.

For clustering and hit identification, all datasets were integrated and merged to a resolution of 1.7 Å. In order to reduce the influence of noise for lower resolution datasets, the following
processing was applied to standardize the Wilson plot for each dataset: the datasets were split into equally sized bins, each covering 1000 reflections, and a linear fit was applied to the logarithm of the average intensities in each shell. The residual between the data and the Wilson fit was calculated, considering sequentially one additional bin from low to high resolution until the residual exceeded 10%, if applicable. The intensities in all higher resolution bins beyond this point were scaled to fit the calculated Wilson B factor.

The results of each dataset were then automatically refined using Phenix(36). Refinement began by choosing one of two manually refined starting models (differing in their unit cell, table S4), followed by [1] rigid body and ADP refinement, [2] simulated annealing, ADP, and reciprocal space refinement, [3] real-space refinement, and [4] a final round of reciprocal space refinement as well as TLS refinement, with each residue pre-set as a TLS group. This procedure was hand-tuned on 5 test datasets; the procedure and parameters were manually adjusted to minimize Rfree until deemed satisfactory for the continuation of the project. All processing and refinement results were logged in a database, which enabled comparison between methods and improvement over time. All code and parameters needed to reproduce this pipeline are available online(37).

**Hit finding: cluster4x and PanDDA analysis**

The resulting model structure Ca positions were then ingested into cluster4x(10), which briefly (a) computes a correlation coefficient between each structure over the position of all Ca atoms, (b) performs a principle component analysis on the resulting correlation matrix, (c) presents 3 chosen principal components to a human, who then manually annotates clusters. Clusters were ordered chronologically and separated into groups of 1500 and subsequently clustered into groups of approximately 60-120 datasets based on a combination of reciprocal and Ca-atom differences using cluster4x. In an earlier version of the software, structure factor amplitudes were used for clustering instead of refined Ca positions, and both methods were applied for hit finding. The resulting clusters were then analyzed via PanDDA(11) using default parameters. The resulting PanDDA analyses were manually inspected for hits which were recorded.

**Manual structure refinement**

Identified hits were further refined by alternating rounds of refinement using refmac(38), phenix.refine(36) or MAIN(39), interspersed with manual model building in COOT(40).

**In silico screening of compound libraries**

To enable a preselection of potentially promising compounds to support the experimental X-ray screening effort and to get an idea about the most promising compounds, we pursued a virtual screening workflow consisting of the selection of a representative ensemble of binding site conformations, non-covalent molecular docking and rescoring. We performed this study with 5,575 compounds of the Fraunhofer IME Repurposing Collection. UNICON(41) was applied to prepare the library compounds. To consider binding site flexibility, we used multiple receptor structures. We applied SIENA(42) to extract five representative binding site conformations for the active site of Mpro. We chose the structures with the PDB IDs 5RFH, 5RFO, 6W63, 6Y2G and 6YB7. The SIENA-derived aligned structures were used and the proteins were preprocessed using Protoss(43) to determine protonation states, tautomeric forms, and hydrogen orientations. The binding site was defined based on the active site ligand of the structure with the PDB ID 6Y2G (ligand ID O6K). A 12.5 Å radius of all ligand atoms was chosen as binding site
definition. The new docking and scoring method JAMDA was applied with default settings for the five selected binding sites\(^{44}\). Subsequently, HYDE\(^{45}\) was used for a rescoring of all predicted poses of the library compounds. The 200 highest ranked compounds according to the HYDE score were extracted. For 70 of these compounds, well-diffracting crystals were obtained in the X-ray screening. Intriguingly, only calpeptin, a known cysteine protease inhibitor, could be co-crystallized and was found on rank 3 (table S7).

**Mass Spectrometry**

\(\text{M}^{\text{pro}}\) was prepared for native MS measurements by buffer-exchange into ESI compatible solutions (300 mM NH\(_4\)OAc, 1 mM DTT, pH 7.5) by five cycles of centrifugal filtration (Vivaspin 500 columns, 30,000 MWCO, Sartorius). Inhibitors were dissolved to 1 mM in DMSO. Then inhibitors and \(\text{M}^{\text{pro}}\) were mixed to final concentrations of 50 \(\mu\)M and 10 \(\mu\)M, respectively, and incubated for 16 h at 4 °C. For putative covalent ligands, compounds were incubated at 1 mM with 100 \(\mu\)M \(\text{M}^{\text{pro}}\) in 20 mM Tris, 150 mM NaCl, 1 mM TCEP, pH 7.8, for 16 h prior to buffer exchange. Buffer exchange was carried out as described above and samples were diluted tenfold prior to native MS measurements. All samples were prepared in triplicate. Nano ESI capillaries were pulled in-house from borosilicate capillaries (1.2 mm outer diameter, 0.68 mm inner diameter, filament, World Precision Instruments) with a micropipette puller (P-1000, Sutter instruments) using a squared box filament (2.5 × 2.5 mm\(^2\), Sutter Instruments) in a two-step program. Subsequently capillaries were gold-coated using a sputter coater (CCU-010, safematic) adding 5.0 × 10\(^{-2}\) mbar, 30.0 mA, 100 s, 3 runs to vacuum limit 3.0 × 10\(^{-2}\) mbar argon.

Native MS was performed using an electrospray quadrupole time-of-flight (ESI-Q-TOF) instrument (Q-TOF2, Micromass/Waters, MS Vision) modified for higher masses\(^{46}\). Samples were ionized in positive ion mode with voltages of 1300 V applied at the capillary and 130 V at the cone. The pressure in the source region was kept at 10 mbar throughout all native MS experiments. For desolvation and dissociation, the pressure in the collision cell was adjusted to 1.5 × 10\(^{-2}\) mbar argon. Native-like spectra were obtained at an accelerating voltage of 30 V. To calibrate raw data, CsI (25 mg/ml) spectra were acquired. Calibration and data analysis were carried out with MassLynx 4.1 (Waters) software. In order to determine each inhibitor binding to \(\text{M}^{\text{pro}}\), peak intensities of zero, one or two bound ligands were analyzed from three independently recorded mass spectra at 30 V acceleration voltage. Results are shown in table S6. Samples deemed to be technical failures were excluded e.g. inconsistent nanoESI due to unstable electrospray or clogging of a capillary. Further, we excluded data that were defined as non-observable binding under the tested conditions. Our definition of a non-observable inhibitor binding included mass spectra which show less than 10% intensity fraction of the first ligand.

Small-molecule mass spectrometry.

Analytical LC-MS data were obtained using a Waters Alliance 2795-HT LC-MS system equipped with a Phenomenex Kinetex column (2.6 \(\mu\)m, C18, 100 Å, 4.6 x 100 mm\(^2\)) and a Phenomenex Security Guard precolumn (Luna, C5, 300 Å) at a flow rate of 1 mL/min at 30°C. Detection was carried out by a diode array detector (Waters 2998) in the range 210 to 600 nm together with a Waters Quatro-Micro mass detector, operating simultaneously in ES+ and ES− modes between 100 and 1000 m/z. Solvents were: A, HPLC grade H\(_2\)O containing 0.05% formic acid; B, HPLC grade CH\(_3\)CN containing 0.045% formic acid. The gradient was as follows: 0 min, 10% B; 10 min, 90% B; 12 min, 90% B; 13 min, 10% B; 15 min, 10% B. Samples (20 \(\mu\)L) were made up in a mixture of buffer and CH\(_3\)CN between 10 and 1000 \(\mu\)g/mL.
Compounds incubated with buffer or cell medium were treated the same by adding an equivalent volume of acetonitrile followed by centrifugation for 30 minutes at 10,000xg. For reference, HEAT, tolperisone, and isofloxythepin were incubated at a concentration of 100 µM in 100 mM MIB buffer at pH 6.0, 7.5, and 9.0 for 48 h, respectively. The compounds exposed to the cell medium were incubated for 48 h at two concentrations of 100 µM and at the experimentally determined CC_{50} (HEAT 30 µM, tolperisone 100 µM, and isofloxythepin 17 µM). The cell medium with CC_{50} concentration was incubated with and without Vero E6 cells. As an additional control 2.5 mM isofloxythepin was incubated in 100 mM MIB buffer at pH 7.5.

The decay of HEAT and tolperisone is detectable by LC-MS in both the crystallization solution and the cell medium in the presence and absence of Vero E6 cells (Fig. S7). For HEAT the mass spectra indicate that the parent compound elutes at 4.1 minutes and the fragment at 7.3 minutes. The respective mass spectra indicate the expected MH+ masses. For isofloxythepin, the proposed decay product modeled in the X-ray structure is not directly detectable in LC-MS experiments. In the control experiment with 2.5 mM isofloxythepin, two peaks appear at 10.9 and 11.1 minutes elution time, indicating two of its oxidation products. One of these is a compound characterized in the literature (ZINC0000346322) with a UV-vis absorption peak at 333 nm, which can also be observed for these compounds.

Antiviral assays

Compounds. All compounds were diluted to a 50 mM concentration in 100% DMSO and stored at -80°C.

Cytotoxicity assays. Vero E6 cells (ATCC CRL-1586) were seeded at 3.5 × 10^4 cells/well in 96-well plates. After 24 h, the cell culture media was changed and 2-fold serial dilutions of the compounds were added. Cell viability under 42 h compound treatment was determined via the Cell Counting Kit-8 (CCK-8, Sigma-Aldrich #96992) following the manufacturer’s instructions.

Antiviral activity assays. Vero E6 cells (ATCC CRL-1586) seeded at 3.5 × 10^4 cells/well in 96-well plates were pretreated 24 h later with twofold serial dilutions of the compounds. After 1 h incubation with the compounds, SARS-CoV-2 (strain SARS-CoV-2/human/DEU/HH-1/2020) was subsequently added at a MOI of 0.01 and allowed absorption for 1 h. The viral inoculum was removed, cells were washed with PBS without Mg^{2+} / Ca^{2+} and fresh media containing the compounds (final DMSO concentration 0.5% (v/v)) was added to the cells. Cell culture supernatant was harvest 42 h post infection and stored at -80°C. Viral RNA was purified from the cell culture supernatant using the QIAamp Viral RNA Mini Kit (QIAGEN #52906) in accordance with the manufacturer’s instructions. Quantification of vRNA was carried out by the interpolation of RT-qPCR (RealStar SARS-CoV-2 RT-PCR Kit, Altona Diagnostics #821005) results onto a standard curve generated with serial dilutions of a template of known concentration. Titers of infectious virus particles were measured via immunofocus assay. Briefly, Vero E6 cells (ATCC CRL-1586) seeded at 3.5 × 10^4 cells/well in 96-well plates were inoculated with 50 µl of serial tenfold dilutions of cell culture supernatant from treated cells. The inoculum was removed after 1 h and replaced by a 1.5% methylcellulose-DMEM-5% FBS overlay. Following incubation for 24 h, cells were inactivated and fixed with 4.5% formaldehyde. Infected cells were detected using an antibody against SARS-CoV-2 NP (ThermoFischer, PA5-81794). Foci were counted using an AID ELISpot reader from Mabtech. The cytotoxic concentrations that reduced cell growth by 50% (CC_{50}) and the effective concentrations that reduced infectious particles or vRNA by 50% (EC_{50}) were calculated by fitting the data to the sigmoidal function using GraphPad Prism version 8.00 (GraphPad Software, La Jolla California
Supplementary Text

In the following, we discuss those compounds that did not show significant antiviral activity but for which we could determine the binding pose based on the crystal structures.

**Active site, covalent**

**Isofl Roxithepin** binds as a degradation product (Fig. 3C). Here, the piperazine group is not found in the crystal structure but the dibenzothiepin moiety (9-Fluoranyl-3-propan-2-yl-5,6-dihydrobenzo[b][1]benzothiepine) is observed in the active site, bound as a thioether to Cys145. The tricyclic system stretches from the S1 across to the S1’ pocket. According to the electron-density maps, two orientations of the molecule are possible, with either the fluorine or the isopropyl group placed inside the S1 pocket. Degradation of the drug with piperazine as the leaving group has been previously reported (47). Isofl Roxithepin is an antagonist of dopamine receptors D1 and D2 and has been tested as a neuroleptic in phase II clinical trials.

**Leupeptin** is a well-known cysteine protease inhibitor and was therefore included in our screening effort as a positive control (48). Structurally, it is highly similar to calpeptin. Indeed, this peptidomimetic inhibitor also forms a thiohemiacetal and occupies the substrate pocket, much like calpeptin (Fig. S3A and 3E). The binding mode is identical to the recently released room-temperature structure of Mpro with leupeptin (49).

**Maleate** was observed covalently bound in seven structures during hit finding. In all cases maleate served as the counter ion of the applied compound. In these crystal structures the maleate, rather than the applied compound, forms a thioether with the thiol of Cys145, modifying it to succinyl-cysteine. The thiol of Cys145 undergoes a Michael-type nucleophilic attack on the C2 of maleate. A similar adduct has been described for maleate isomerase (50) as an intermediate structure in the isomerization reaction. The covalent adduct is further stabilized by hydrogen bonds to the backbone amide of Gly143 and Cys145 to the carboxylate group (C1) of succinate. The terminal carboxylate (C4) is positioned by hydrogen bonds to the side chain of Asn142 and a water-bridged hydrogen bond to the side chain of His163 (Fig. S3B).

**TH-302** (Evofosfamide) is covalently linked to Cys145 through nucleophilic substitution of the bromine, leading to thioether formation (Fig. S3C). The other bromine-alkane chain occupies the S1 pocket while the nitro-imidazole stretches into pocket S2. The substitution of chlorine or hydroxyl for bromines in TH-302 has been demonstrated in cell culture (51). Our mass spectrometry analysis suggested the loss of a bromine atom (Fig. S6C).

**Zinc pyrithione** was already demonstrated to have inhibitory activity against SARS-CoV-1 Mpro (52). The pyrithione chelates the Zn2+ ion which coordinates the thiolate and imidazole of the catalytic dyad residues Cys145 and His41 (Fig. S3D). The remaining part of the ionophore protrudes out of the active site. This tetrahedral binding mode of zinc has previously been described for other zinc-coordinating compounds in complex with HCoV-229E Mpro (53). Interestingly, antiviral effects against a range of corona- and non-coronaviruses have already...
been ascribed to zinc pyrithione, although its effect had been attributed to inhibition of RNA-dependent polymerase (54). Zinc pyrithione exhibits both antifungal and antimicrobial properties and is known in treatment of seborrheic dermatitis. We tested zinc pyrithione in initial antiviral assays but it showed high toxicity and was not followed up further.

**Active site, non-covalent**

**Adrafinil** binds mainly through van der Waals interactions to M\(^{pro}\). In particular, its two phenyl rings are inserted into pockets S1’ and S2 (Fig. S3E). A hydrogen bond is formed between the backbone amide of Cys145 and the hydroxylamine group. The side chain of Met49 is wedged between the two phenyl rings.

**Fusidic acid** interacts with M\(^{pro}\) mainly through hydrophobic interactions, especially through the alkene chain within pocket S2 and the tetracyclic moiety packing against Ser46 (Fig. S3F). Moreover, the carboxylate group forms indirect hydrogen bonds, mediated via two water molecules, to the main chain of Thr26, Gly143 and Cys145. In addition, the same carboxylate group forms a hydrogen bond to an imidazole molecule from the crystallization conditions. This imidazole occupies pocket S1’ and mediates hydrogen bonds to the backbone of His41 and Cys44. These indirect interactions offer opportunities for optimization of compounds binding to M\(^{pro}\). Fusidic acid is a well-known bacteriostatic compound, with a steroid core structure.

**LSN-2463359** binds mainly to M\(^{pro}\) by interaction of the pyridine ring with the S1 pocket (Fig. S3G). Besides van der Waals interactions with the β-turn Phe140-Ser144, contributing to the pocket, it also forms a hydrogen bond to the side chain of His163.

**SEN1269** binds only to the active site of one protomer in the native dimer. This causes a break in the crystallographic symmetry, leading to a different crystallographic space group (table S4). The central pyrazine ring forms a hydrogen bond to Gln189 (Fig. S3H). The terminal dimethylaniline moiety sits deep in pocket S2 which is enlarged by an outwards movement of the short α-helix Ser46-Leu50 by 1.7 Å (Ser46 Cα-atom) compared to the native structure. This includes a complete reorientation of the side chain of Met49 which now points outside of the S2 pocket. Additionally, the C-terminus of a crystallographic neighboring M\(^{pro}\) protomer is trapped between SEN1269 and part of the S1 pocket, including a hydrogen bond between Asn142 and the backbone amide of Phe305 and Gln306 of the C-terminus.

**Tretazicar** binds at the active site entrance at pocket S3/S4 (Fig. S3I). The amide group forms hydrogen bonds to the backbone carbonyl of Glu166, the adjacent nitro group forms hydrogen bonds to the side chain of Glu192 and the backbone amide of Thr190.

**UNC2327** binds to active site of M\(^{pro}\) by stacking its benzothiadiazole ring against the loop Glu166-Pro168 that forms the shallow pocket S3 (Fig. S3J). This is stabilized by a hydrogen bond between the benzothiadiazole and the main chain carbonyl of Glu166. The piperidine ring and adjacent carbonyl are inserted into pocket S1’ and interact with Thr25 and His41.

**Allosteric site I**

**Ifenprodil, PD-168568** and **RS-102895** all exhibit an elongated structure, consisting of two aromatic ring systems separated by a linker containing a piperidine or piperazine ring (Fig S4A-C). All three binders exhibit a distance of at least 12 Å between the terminal aromatic rings. Thus, this binding mode is unlikely to be identified through fragment screening. The hydrophobic pocket in the helical domain is covered by the side chain of Gln256. In our complex structures, this side chain adopts a different conformation exposing Ile213, and generating the hydrophobic pocket. Similar to pelitinib, one of the terminal aromatic ring systems is inserted
into the hydrophobic groove in the dimerization domain. The linker moiety stretches across the
native dimer interface and the second aromatic ring is positioned close to Asn142, adjacent to the
active site loop where residues 141-144 contribute to the pocket S1. In particular, in the case of
RS-102895, two hydrogen bonds are formed to the side and main chains of Asn142. In contrast
to ifenprodil, RS-102895 and PD-168568 do not exhibit selective antiviral activity (SI<5).

Tofoglflozin binds to the same hydrophobic pocket as pelitinib, ifenprodil, RS-102895, and
PD-168568 but no antiviral activity was observed at 100 µM, the highest concentration tested. In
contrast to the other four compounds, it does not reach across to the opposing protomer in the
native dimer. Its main interaction with Mpro is via its iso-benzofuran moiety that occupies the
hydrophobic pocket (Fig. S4D).

Covalent binder to Cys156

In the aurothioglucose/Mpro crystal structure, the strong nucleophile Cys145 becomes
oxidized to a sulfinic acid. The initial reaction is the disproportionation of aurothioglucose into
Au(0) and a disulfide dimer of thioglucose. This is followed by a cascade of redox reactions of
thioglucose, its disulfide and sulfenic acid. A disulfide linkage to thioglucose is only observed at
Cys156 on the surface of Mpro (Fig. S5A). Here the thioglucose moiety is located between
Lys100 and Lys102.

Glutathione isopropyl ester binds to the surface-exposed Cys156 via a disulfide linkage
(Fig. S5B). Additionally, the ester forms a hydrogen bond to the backbone amide of Tyr101,
while the amine of the other arm of the molecule is interacting with the side chain amine of
Lys102.

Surface pockets

AR-42 binds with its phenyl ring to a small hydrophobic pocket in the dimerization domain
formed by residues Gly275, Met276, Leu286 and Leu287 (Fig. S5C). Additionally, the central
amide forms a hydrogen bond to the backbone carbonyl of Leu272.

AZD6482 binds to a pocket on the back of the catalytic domain, away from the native
dimer interface (Fig. S5D). The nitrobenzene ring is inserted in a pocket formed by His80,
Lys88, Leu89 and Lys90. The central aromatic system and morpholine ring lie flat on the surface
of Mpro. Furthermore, Asn63 forms a hydrogen bond to the keto-group in the pyrimidine ring.

Climbazole binds in a shallow surface pocket, wedged between two crystallographic
symmetry-related molecules (Fig. S5E). Only van der Waals interactions are observed. One
monomer contributes with residues Phe103, Val104, Arg105 and Glu178 to this binding site,
while the other monomer contributes Asn228, Asn231, Leu232, Met235 and Pro241.

Clonidine also sits between two crystallographic, symmetry-related molecules and binds
through van der Waals interactions (Fig. S5F). Here one protomer mainly forms the binding site,
by contributing Asp33, Asp34 and Ala94. The other protomer contributes Lys236, Tyr237 and
Asn238. The amine ring of clonidine forms a loose ring stacking interaction to Tyr237, while a
hydrogen bond between the backbone carbonyl of Lys236 and the ring connecting amine of
clonidine is formed. The side chain of Lys236 is flipped to the side to make room for the
chlorine containing ring system.

Ipidacrine is in contact with two different Mpro protomers (Fig. S5G). The tricyclic ring
system is packed against a surface loop, including residues Pro96 and Lys97 as well as Lys12. It
also interacts with the end of an α-helix including residues Gln273, Asn274 and Gly275.
**Tegafur** binds in a shallow surface pocket generated by residues Asp33, Pro99, Lys100 and Tyr101. The main interaction is through π-stacking of the aromatic ring of Tyr223. The side chain of Lys100 flips away and generates space for the compound (Fig. S5H).
Fig. S1.
Characterization of repurposing screening library and results from X-ray screening. (A) Normalized histograms of molecular weight distributions of two commonly used fragment screening libraries F2X-Universal (median 193.2 Da) and DSiP (a version of the “poised library” (7), 211.2 Da), the two combined repurposing libraries used in the present effort (Fraunhofer IMG, 371.3 Da, Dompé “Safe-in-man” 316.3 Da, combined 366.5 Da), and the resulting hits from our X-ray screen (403.6 Da). Normal distributions are indicated by solid lines in corresponding colors. Compounds with a molecular weight above 1000 Da are not shown. (B) Flow chart with overview of analyzed compounds and identified hits compounds including classification of binding sites and antivirally active compounds found at those sites.
Fig. S2

X-ray hit compounds were tested in a non-toxic range for inhibition of SARS-CoV-2 replication in Vero E6 cells. The vRNA yield (grey bars) and cell viability (red circles) were determined by RT-qPCR and the CCK-8 method, respectively, for 37 of 43 hits in the X-ray screen. All data are mean ± SD of three independent replicates in one experiment. Upper and lower boundaries of yellow bars represent one and two log reduction in vRNA level. Twofold serial dilutions of compounds were used to treat cells for 42 hours, where 100 µM was used as the highest concentration for all compounds except remdesivir (10 µM), cinanserin HCl (125 µM), HEAT HCl (25 µM), Zn pyrithione (1 µM), pelitinib (12.5 µM), zaldaride (50 µM), isofloxythepin (25 µM) and RS-102895 HCl (50 µM). Control is DMSO without compound. Remdesivir and cinanserin were used as positive controls. Colistimethate and fluorometholone were initially classified as X-ray hits and, thus, tested for antiviral activity. After a second more detailed inspection of the electron-density maps, they are not classified as hits any longer.
Fig. S3

The structures of inactive compounds bound at active site. Compounds are depicted as colored sticks. $M^\text{pro}$ is shown as a grey cartoon model with residues important for ligand binding shown as stick models and hydrogen bonds are indicated by dashed lines. Ligands binding covalently to the active site residue Cys145: (A) leupeptin, (B) maleate, (C) TH-302. Ligands binding through coordination: (D) zinc pyrithione. Ligands binding non-covalently to the active site: (E) adrafinil, (F) fusidic acid, (G) LSN-2463359, (H) SEN1269 (C-terminus of neighboring $M^\text{pro}$ protomer shown as pink stick model), (I) tretazicar, (J) UNC2327.
**Fig. S4**

The structures of compounds bound at allosteric site 1. Compounds are depicted as colored sticks. M\textsuperscript{pro} is shown as a grey cartoon model with residues important for ligand binding shown as stick models and hydrogen bonds are indicated by dashed lines. The other protomer of the M\textsuperscript{pro} dimer is shown in white and residues are labeled with an asterisk. Ligands binding to allosteric site 1: (A) ifenprodil, (B) RS-102895, (C) PD-168568, (D) tofogliflozin.
Fig. S5

The structures of inactive compounds bound at neither allosteric nor active site.
Compounds are depicted as colored sticks. Mpro is shown as a grey cartoon model with residues
important for ligand binding shown as stick models and hydrogen bonds are indicated by yellow
dashed lines. Interacting residues are depicted as grey sticks. Residues contributing Ligands
binding covalently to Cys156: (A) aurothioglucose, (B) glutathione isopropyl ester. Other
surface pockets: (C) AR-42, (D) AZD6482, (E) climbazole (F) clonidine, (G) ipidacrine, (H) tegafur.
**Fig. S6**  
**Binding of compounds confirmed by native mass-spectrometry.** Main mass spectra of M_pro with compounds. (A), Triglycidyl isocyanurate, (B) calpeptin, (C) TH-302 and (D) HEAT-HCl. Insets depict main charge state signals with native M_pro (0) binding to one (1) or two (2) compounds, exhibiting the molecular mass of the complete compound (A and B) or a fragment (C and D). Mass spectra were recorded after the inhibitor was washed out (A and C) or in presence of fivefold excess of compound (B and D). Average compound masses are given and charged states are labelled. Representative spectra are shown.
Fig. S7
Decomposition of tolperisone and HEAT under crystallization and cell culture conditions.
Diode array absorption (black) and ES+ spectrum (blue) of LC-MS experiment with HEAT (A) and tolperisone (E) at pH 7.5 after 48 h (ES+ spectrum is time corrected by 0.15 minutes). Grey subpanels show mass spectra of respective time points: (A) 4.1 min left, 7.3 min right; (E): 4.1 min left, 8.1 min right). (B) – (D) Mass spectra of peak at 7.3 minutes of respective experiment with HEAT. HEAT with CC$_{50}$ concentration, incubated in cell medium without cells (B) and with cells (C). HEAT incubated with 100 µM in cell culture medium without cells (D).
Subpanels of B)-D) show an extract of the ES+ spectrum of the peak of the expected degradation product. (F) –(H) Mass spectra of peak at 8.1 minutes of respective experiment with tolperisone. Tolperisone with CC50 concentration, incubated in cell medium without cells (F) and with cells (G). Subpanels of (F) and (G) show an extract of the ES+ spectrum of the peak of the expected degradation product. Representative data for one of two technical replicates is shown.
**Fig. S8**

**Comparison of human coronavirus M\(^\text{pro}\) sequences.** Sequence alignment of β- (SARS-CoV-1&2, MERS-CoV, HCoV-OC43 and HCoV-HKU1) and α- (HCoV-NL63, HCoV-229E) coronaviruses. SARS-CoV-2 M\(^\text{pro}\)-residues interacting with compounds in allosteric site 1 (yellow) and 2 (blue) are indicated in colored boxes.
Fig. S9

Data quality of X-ray screening diffraction data. Distribution of data quality indicators of all collected X-ray diffraction datasets (upper panel) and of datasets with identified compound (lower panel): diffraction resolution (left), CC1/2 of the datasets (middle), and Wilson B-factor (right).
Table S1.

**Screened drug repurposing libraries.** All tested compounds from the “Fraunhofer IME Repurposing Collection” and the “Safe-in-man” library, including information about tested crystals, obtained high-quality datasets and identified hits.

Table provided as separate file.
Table S2.

**Overview of the 43 hit compounds from the X-ray screen.** List of all identified hit compounds, marking the 37 unique hits and the compounds subjected to antiviral testing. PC = positive control.

| Hit | Compound name             | Unique binder | Tested in antiviral assay | PDB deposition |
|-----|---------------------------|---------------|---------------------------|----------------|
| #1  | adrafinil                 | +             | +                         | 7ANS           |
| #2  | AR-42                     | +             | +                         | 7AXO           |
| #3  | AT7519                    | +             | +                         | 7AGA           |
| #4  | aurothioglucose           | +             | -                         | 7ARS           |
| #5  | AZD6482                   | +             | +                         | 6YVF           |
| #6  | bromemic acid             | +             | -                         | -              |
| #7  | calpeptin                 | +             | +                         | 7AKU           |
| #8  | climbazole                | +             | +                         | 7AOL           |
| #9  | clonidine                 | +             | +                         | 7AWW           |
| #10 | dexrazoxane               | +             | +                         | 7A1U           |
| #11 | fusidic acid              | +             | +                         | 7AY7           |
| #12 | glutathione isopropyl ester | +           | -                         | 7AX6           |
| #13 | glycine                   | +             | +                         | -              |
| #14 | HEAT                      | +             | +                         | 6YNQ           |
| #15 | ifenprodil                | +             | +                         | 7AQI           |
| #16 | ipidacrine                | +             | +                         | 7AF0           |
| #17 | isofloxythepin            | +             | +                         | 7AYI           |
| #18 | LSN-2463359               | +             | -                         | 7AWU           |
| #19 | methazolamide             | +             | +                         | 7AWU           |
| #20 | MUT056399                 | +             | +                         | 7AP6           |
| #21 | necrostatin-1             | +             | +                         | -              |
| #22 | PD-168568                 | +             | +                         | 7AMJ           |
| #23 | pelitinib                 | +             | +                         | 7AXM           |
| #24 | polydatin                 | +             | +                         | -              |
| #25 | quipazine maleate         | +*            | +                         | 7AHA           |
| #26 | RS-102895                 | +             | +                         | 7ABU           |
| #27 | SEN1269                   | +             | -                         | 7AVD           |
| #28 | succinylcholine           | +             | +                         | -              |
| #29 | SUN-B-8155                | +             | -                         | -              |
| #30 | tegafur                   | +             | +                         | 7AWR           |
| #31 | TH-302                    | +             | +                         | 7AWS           |
| #32 | tofogliflozin             | +             | +                         | 7APH           |
| #33 | tolperisone               | +             | +                         | 7ADW           |
| #34 | tretazicar                | +             | +                         | 7AK4           |
| #35 | triglycidyl isocyanurate  | +             | +                         | 7AQJ           |
| #36 | UNC-2327                  | +             | +                         | 7AQE           |
| #37 | zinc pyrithion            | +             | +                         | 7B83           |
| #38 | BP-554 maleate            | *             | +                         | -              |
| #39 | cinepazide maleate        | *             | +                         | -              |
| #40 | HTMT maleate              | *             | +                         | -              |
| #41 | tremebutine maleate       | *             | +                         | 7AOL           |
| #42 | vicriviroc maleate        | *             | +                         | 7AK4           |
| #43 | zaldaride maleate         | *             | +                         | -              |
| PC  | leupeptin                 | n/a           | -                         | 7NEV           |

*) binding of maleate observed for seven compounds containing maleate as counterion. Thus, only quipazine maleate was counted as unique hit, reducing the number of unique hits from 43 to 37.
Table S3.
**Comprehensive summary sheets of hit compounds.** Summary showing electron-density maps, compound interactions with M\(^{pro}\), detailed compound information, biochemical and cell-based antiviral reduction data.

Table provided as separate file.
Table S4.
Summary of X-ray crystallographic data processing and refinement statistics.

Table provided as separate file.
**Table S5.**

*In vitro* antiviral activity, cytotoxicity and selectivity of selected compounds against SARS-CoV-2. EC\(_{50}\)- half-maximal effective concentration; EC\(_{90}\)- effective concentration at 90% inhibition; CC\(_{50}\)- half-maximal cytotoxic concentration. Viral titers, vRNA yield and cell viability were determined by RT-qPCR, immunofocus assays, and the CCK-8 method, respectively. Samples that showed at least one hundredfold reduction in infectious particles in combination with either selectivity indices greater than five or no cytotoxicity in the tested concentration range are considered antivirally active and are marked with an asterisk. Values were calculated from three independent replicates in one experiment.

| Compound            | Infectious particles EC\(_{50}\) [µM] | Infectious particles EC\(_{90}\) [µM] | vRNA copies EC\(_{50}\) [µM] | vRNA copies EC\(_{90}\) [µM] | Cytotoxicity CC\(_{50}\) [µM] | Selectivity Index CC\(_{50}\)/EC\(_{50}\) |
|---------------------|--------------------------------------|--------------------------------------|-----------------------------|-----------------------------|-----------------------------|---------------------------------|
| AT7519*             | 25.16                                | 47.84                                | 43.95                       | 78.6                        | > 100                       | > 3.98                          |
| calpeptin*          | 0.072                                | 0.17                                 | 0.035                       | 0.158                       | > 100                       | > 1389                          |
| HEAT                | 24.05                                | 27.54                                | 23.97                       | 0.17                        | 55.42                       | 2.3                             |
| ifenprodil*         | 46.86                                | 74.13                                | 49.32                       | 66.07                       | > 100                       | > 2.13                          |
| isofloxxythepin      | 4.8                                  | 7.52                                 | 4.92                        | 7.2                         | 17                          | 3.54                            |
| MUT056399*          | 38.24                                | 59.22                                | 36.73                       | 65.34                       | > 100                       | > 2.62                          |
| pelitinib*          | 1.25                                 | 2.34                                 | 1.24                        | 2.13                        | 13.96                       | 11.17                           |
| quipazine maleate   | 31.64                                | 69.18                                | 43.22                       | 56.23                       | > 100                       | > 3.16                          |
| RS102895            | 19.8                                 | 54.98                                | 16.73                       | 23.24                       | > 100                       | > 2.78                          |
| tolperisone*        | 19.17                                | 34.47                                | 20.79                       | 30.99                       | > 100                       | > 5.22                          |
| triglycidyl isocyanurate* | 30.02                  | 54.03                                | 31.51                       | 54.67                       | > 100                       | > 3.33                          |
Table S6.
Native MS verified binding of compounds to M<sup>pro</sup>. The table shows compounds and their molecular weight. Mass spectra of compounds and M<sub>pro</sub> (final conc. 50 µM and 10 µM) were analyzed by converting peak intensities into intensity fractions for zero, one and two ligands (0/1/2 ligands in %) bound per M<sup>pro</sup> dimer. Values represent mean of biological triplicates. Mass of the fragmented compounds is given when the observed mass is deviating from the expected mass. Type and mass of counterion is indicated if applicable.

| Compound (counter ion) | Compound/counter ion mass [Da] | Intensity fraction (0/1/2 compounds per M<sup>pro</sup> dimer) [%] | Mass of fragmented compounds [Da] |
|------------------------|--------------------------------|-------------------------------------------------|---------------------------------|
| calpeptin              | 362.5                          | 5 / 27 / 68                                    |                                 |
| triglycidyl isocyanurate | 297.3                          | 8 / 38 / 53                                    |                                 |
| zinc pyrithione        | 317.7                          | 11 / 34 / 55                                   | 128                             |
| HEAT (HCl)             | 295.4 / 35                     | 39 / 37 / 24                                   | 181                             |
| HTMT (dimaleate)       | 382.4 / 2*116.1                | 55 / 24 / 21                                   | 131                             |
| dextrazoxan            | 268.3                          | 56 / 29 / 15                                   |                                 |
| adrafinil             | 289.35                         | 58 / 28 / 14                                   |                                 |
| TH-302                | 449.04                         | 59 / 30 / 10                                   | 365                             |
| ifenprodil (hemitartrate) | 325.2 / 74.0                   | 61 / 24 / 15                                   | 126                             |
| AZD6482               | 408.5                          | 62 / 29 / 9                                    |                                 |
| glutathione-isopropyl-ester | 349.41                       | 62 / 28 / 10                                   | 126/188                         |
| AT7519                | 382.24                         | 65 / 28 / 7                                    |                                 |
| cinepazide (maleate)  | 417.5 / 116.1                  | 72 / 21 / 7                                    |                                 |
| UNC2327               | 319.4                          | 76 / 24 / 0                                    |                                 |
| fusidic acid          | 516.7                          | 78 / 18 / 5                                    |                                 |
| AR-42                 | 312.4                          | 78 / 18 / 4                                    | 580                             |
| PD-168568 (HCl)       | 440.41 / 2*35                  | 81 / 16 / 3                                    | 350                             |
| tofogliflozin (hydrate) | 404.45                         | 82 / 18 / 0                                    | 380                             |
| MUT056399             | 293.27                         | 83 / 17 / 0                                    |                                 |
| colistimethate (Na)   | 1735.82 / 23                   | 83 / 15 / 2                                    |                                 |
| vicriviroc (maleate)  | 533.6 / 116.1                  | 88 / 12 / 0                                    | 535                             |
| pelitinib             | 467.92                         | 88 / 12 / 0                                    |                                 |
Table S7.

*In silico screening of repurposing library against M^\text{pro}*. The highest ranked 200 compounds of the virtual screening. The names and HYDE scores of the top ranked molecules are given. The yellow background highlights compounds for which high-quality X-ray data was obtained in the X-ray screening. The green background highlights compounds that were detected in the active site in the X-ray screen. Compounds highlighted in light green show a similar binding mode to the fragment with the PDB ligand ID K0G in complex with M^\text{pro} (PDB ID 5R83). Compounds highlighted in light yellow were reported as being active in other screening studies.

Table provided as separate file.
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