Acrylamide fragment inhibitors that induce unprecedented conformational distortions in enterovirus 71 3C and SARS-CoV-2 main protease

Bo Qin\textsuperscript{a,†}, Gregory B. Craven\textsuperscript{b,†}, Pengjiao Hou\textsuperscript{a,‡}, Julian Chesti\textsuperscript{c}, Xinran Lu\textsuperscript{c}, Emma S. Child\textsuperscript{b}, Rhodri M.L. Morgan\textsuperscript{b}, Wenchao Niu\textsuperscript{d}, Lina Zhao\textsuperscript{d}, Alan Armstrong\textsuperscript{c}, David J. Mann\textsuperscript{b,*}, Sheng Cui\textsuperscript{a,*}

\textsuperscript{a}NHC Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100076, China
\textsuperscript{b}Department of Life Sciences, Imperial College London, South Kensington Campus, London SW7 2AZ, UK
\textsuperscript{c}Department of Chemistry, Imperial College London, Molecular Sciences Research Hub, White City Campus, Wood Lane, London W12 0BZ, UK
\textsuperscript{d}CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, Institute of High Energy Physics, University of Chinese Academy of Sciences, Beijing 100049, China

Received 26 February 2022; received in revised form 26 May 2022; accepted 1 June 2022

**KEY WORDS**
SARS-CoV-2; Protease inhibitors; Covalent fragments; Allosteric inhibition; EV71

**Abstract** RNA viruses are critically dependent upon virally encoded proteases to cleave the viral polyproteins into functional proteins. Many of these proteases exhibit a similar fold and contain an essential catalytic cysteine, offering the opportunity to inhibit these enzymes with electrophilic small molecules. Here we describe the successful application of quantitative irreversible tethering (qIT) to identify acrylamide fragments that target the active site cysteine of the 3C protease (3C\textsuperscript{P90}) of Enterovirus 71, the causative agent of hand, foot and mouth disease in humans, altering the substrate binding region. Further, we re-purpose these hits towards the main protease (M\textsuperscript{P90}) of SARS-CoV-2 which shares the 3C-like fold and a similar active site. The hit fragments covalently link to the catalytic cysteine of M\textsuperscript{P90} to inhibit its activity. We demonstrate that targeting the active site cysteine of M\textsuperscript{P90} can have profound allosteric effects, distorting secondary structures to disrupt the active dimeric unit.

*Corresponding authors. Tel./fax: +86 10 67828669 (Sheng Cui); +44 20 75945302 (David J. Mann).
E-mail addresses: D.mann@imperial.ac.uk (David J. Mann), cui.sheng@ipb.pumc.edu.cn (Sheng Cui).
†These authors made equal contributions to this work.
Peer review under responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences

https://doi.org/10.1016/j.apsb.2022.06.002
2211-3835 © 2022 Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
1. Introduction

RNA viruses cause significant morbidity and mortality in human and animal hosts\(^2\). For example, enteroviruses (EV) include many important human pathogens with the best characterized being enterovirus 71 (EV71), rhinovirus (HRV), coxsackievirus B3 (CVB3), enterovirus D68 (EV-D68) and poliovirus (PV). EV71 is one cause of hand, foot and mouth disease (HFMD) in humans and is associated with severe neurological disease with considerable mortality\(^3\). Vaccines against EV71 have been developed and approved\(^4\) but outbreaks persist and there are no antiviral drugs available for treating EV71\(^5\)\(^6\).

Like many other RNA viruses, EV71 relies on proteases to cleave a polyprotein precursor into individual functional mature proteins. For enteroviruses, the majority of this proteolytic processing utilizes the 3C protease (3C\(^\text{pro}\))\(^7\). Given the essential role of virally-encoded proteases in viral life-cycles, numerous protease inhibitors have been developed for potential clinical use\(^8\). These include a large collection of picornaviral 3C\(^\text{pro}\) inhibitors, such as an HRV 3C\(^\text{pro}\) inhibitor Rupintrivir (AG7088)\(^9\) that failed to show patient benefit in phase II clinical trials\(^10\). To date, no 3C\(^\text{pro}\) inhibitors have been approved for clinical use.

With the global rise of SARS-CoV-2, scientific attention has focused on the causative agent, SARS-CoV-2\(^11\). At the end of 2021, the emergence of the heavily mutated omicron variant of SARS-CoV-2 (B.1.1.529) put the world on alert. This variant is classified as the fifth variant of concern (VOC) for its alarming SARS-CoV-2 (B.1.1.529). Co-crystals of 3C\(^\text{pro}\) fragments demonstrated the occupancy of a novel, cryptic pocket in 3C\(^\text{pro}\). Furthermore, when repurposed towards M\(^\text{pro}\), the covalent fragments also preferentially targeted the active site cysteine, inhibiting the enzyme activity and, in one case, additionally disrupting the quaternary structure of M\(^\text{pro}\).

2. Results and discussion

2.1. Covalent fragment screening against EV71 3C\(^\text{pro}\) by quantitative irreversible tethering (qIT)

To target cysteine C147 on EV71 3C\(^\text{pro}\), we constructed a 1040-member covalent fragment library using a combination of in-house parallel synthesis and commercial vendors (Fig. 1A and B). Fragment-like core scaffolds were functionalized with cysteine-reactive chemical groups, with the majority (>95%) being acrylamides. Acrylamide “warheads” are featured in several clinically approved covalent drugs and are favored for their mild electrophilic reactivity, minimizing potential non-specific reactivity and associated toxicity\(^12\). In line with the generally accepted fragment-based drug design (FBDD) guidelines, the library was designed to maximize scaffold diversity and to conform to the “rule of 3”: MW \(\leq 300\), clogP \(\leq 3\), H-bond donors/acceptors \(\leq 3\) (Fig. 1C).

We applied our fluorescence-based covalent fragment screening platform (qIT) to identify fragments which covalently bind to C147 on EV71 3C\(^\text{pro}\) (Fig. 2A). To determine the rate of reaction between a cysteine thiol and an acrylamide fragment, the cysteine quantitation probe CPM is employed to measure the degree of cysteine modification at a series of timepoints. The CPM probe competes with the acrylamide for modification of the cysteine residue such that the fluorescence signal is inversely proportional to the extent of acrylamide-cysteine labelling, allowing the rate of reaction (\(v\)) to be determined by exponential regression analysis in high-throughput. Our workflow uses GSH as a control cysteine-containing biomolecule and hit fragments are those that react significantly faster with 3C\(^\text{pro}\) than with GSH (Fig. 2B). The selectivity of the fragment towards the 3C\(^\text{pro}\) is quantified by the rate enhancement factor (REF) which was used to identify and prioritize hit fragments (Fig. 2C).

The 1040-member acrylamide fragment library was screened at 500 \(\mu\text{M}\)/L against EV71 3C\(^\text{pro}\) (5 \(\mu\text{M}\)/L) or GSH (5 \(\mu\text{M}\)/L) in parallel and the fluorescence intensity measured over 24 h. The majority of the library (61%) displayed measurable reactivity with EV71 3C\(^\text{pro}\) over the 24-h time course, with roughly half of those fragments showing selectivity over GSH (REF >1) (Fig. 3A). There were 13 fragments which had a REF greater than three standard deviations (1SD = 2.5) over the geometric mean...
(geomean REF = 1.0), for example acrylamide 1, and these were taken forward for repeat qIT testing and mass spectrometry validation (Supporting Information Fig. S1). Pleasingly, four of those fragments both had reproducible qIT profiles and clearly mono-modified EV71 3Cpro by intact protein mass spectrometry (Fig. 3B–D and Supporting Information Fig. S2). The glutathione selectivity of the four validated hit fragments ranged from REF = 8.5–24.3 and the compounds shared some common chemical features: acrylamides 1 and 2 both contain the same 1,3-thiazole core and methylene linker while acrylamides 3 and 4 have similar isoxazole motifs with more extended linkers.

Encouragingly, all four hits are alkyl acrylamides which typically are associated with low levels of off-target reactivity and this is supported by their slow reactivity with glutathione26.

2.2. The hit fragments covalently bind to residue C147 of EV71 3Cpro and accommodate a novel cryptic pocket

To reveal the binding site of the cysteine-reactive fragments, we labelled recombinant EV71 3Cpro with acrylamide fragments 1–4 and subjected the resulting complexes to crystallization trials. Unfortunately, the WT EV71 3Cpro-fragment complexes did not

Figure 1  Cysteine-reactive covalent fragment library composition. (A) Covalent fragment library composition. (B) Representative structures of library members. (C) Physicochemical properties of the library: MW = molecular weight; HBA = hydrogen-bond acceptor; HBD = hydrogen-bond donor; tPSA = total polar surface area.

Figure 2  Quantitative irreversible tethering (qIT) screening platform. (A) Assay overview: The target thiol (5 µmol/L), EV71 3Cpro or glutathione, is reacted with acrylamide fragments (0.5 mM) under pseudo-first order conditions. Reaction progress is followed by discrete measurements of free target thiol concentration using the fluorogenic probe CPM and the rate of reaction (v) is determined by exponential regression analysis. (B) Fluorescence intensity is converted into percentage cysteine modification by normalizing to DMSO control (0%, no thiol) to GSH (100%). Fragments are characterized as (i) non-reactive, (ii) reactive but non-selective or (iii) reactive and selective by comparing the reactivity profiles between EV71 3Cpro and GSH. (C) Kinetic selectivity is quantified by the rate enhancement factor (REF) which is used to identify and prioritise hit compounds.
yield crystals so we employed a 3C\textsuperscript{pro}\ mutant construct bearing the H133G to expedite the structural studies. The H133G mutant has WT-level protease activity, containing a WT-like catalytic triad, and harbors the H133G mutation at the hinge region of the catalytic triad, which improves the flexibility of the \(\beta\)-ribbon\textsuperscript{2}. Using the H133G mutant, we were able to determine crystal structures of 3C\textsuperscript{pro}\, the H133G mutant to expedite the structural studies. The H133G mutant was solved to the resolution of 1.2\,Ă, indicating that the geometry of the Ser-His-Asp catalytic triad was disrupted. Displacement of the NH group of G145 was 6.4\,Ă, indicating the geometry of the oxyanion hole could not form. (2) The upper wall of the S1 pocket (the most important pocket for substrate recognition) collapsed, and the size of the pocket became too narrow to accommodate the P1 residue. (3) The leaving group side pockets S1’ and S2’ disappeared, and a previously unobserved cryptic S’ pocket was generated. Residues constituting the cryptic S’ pocket involve I104, T106, H108, M109, M112, V114, F140, T142, A144, G145 and Q146. We marked the location of these residues constituting S’ pocket in a multiple sequence alignment of various picornavirus 3C\textsuperscript{pro}, most of which are conserved (Supporting Information Fig. S3).

2.4. The hit acrylamide fragments inhibit EV71 3C\textsuperscript{pro} and SARS-CoV-2 M\textsuperscript{pro} activity in vitro

To investigate which structural features of the thiazole-acrylamide fragments are key to binding, we tested analogues 5, 6 and 7 for their kinetic binding profiles against 3C\textsuperscript{pro} using qIT (Table 1). Benzothiazole 5 retained potency (REF = 13.5) with a similar kinetic profile to thiazole 1, further indicating that the thiazole motif drives the binding. Conversely, the N=H acrylamides 6 and

---

**Figure 3**  Screening cascade and hit validation. (A) Primary qIT screen summary: Pie chart shows number of fragments characterized as non-reactive, reactive but non-selective or reactive and selective. Hits have REF >3 standard deviations over the geometric mean. (B) Illustrative qIT data for acrylamide 1 (0.5 mmol/L) in reaction with EV71 3C\textsuperscript{pro} or glutathione (5\,μmol/L). (C) Summary of screening cascade. (D) Illustrative intact protein mass spectrometry data for modification of EV71 3C\textsuperscript{pro} (5\,μmol/L) reacting with acrylamide 1 (0.5 mmol/L for 750 min).
fragment growth towards the canonical binding groove. The features of fragments may be introduced here and based on the crystal structure of cyclopropane ring of acrylamide indicates that larger substituents may be required for efficient binding. Indeed, tolerance of the fragments. With the emergence of SARS-CoV-2 as a threat to global public health, we sought to determine if our 3Cpro-selective fragments could be re-purposed towards this second plus strand RNA virus. Given that 3Cpro and Mpro are both cysteine proteases that share similar chymotrypsin-folds, we hypothesized that our acrylamide fragments might also be effective against Mpro. The first examples of covalent SARS-CoV-2 Mpro inhibitors have recently been disclosed, but novel acrylamide-based Mpro inhibitor scaffolds remain highly desirable. Accordingly, we incubated each fragment with SARS-CoV-2 Mpro and used intact protein mass spectrometry to check for covalent modification (Table 1 and Supporting Information Fig. S4). Encouragingly, acrylamides 1, 3 and 4 showed partial modification while fragments 2 and 5 both labelled Mpro to completion. Using an Mpro activity assay, we validated these results and found that the inhibitory potency against Mpro is overall greater than 3Cpro (IC₅₀ = 10–60 μmol/L), with acrylamides 2 and 5 being the most potent against either proteases. To test selectivity of compounds 1–5, we investigated their potency against another SARS-CoV-2 cysteine protease, the papain-like protease (PLpro). PLpro contains a catalytic triad C111–H272-D286 at the active site, but its substrate binding pockets are distinct from Mpro or 3Cpro. The S1–S2 subpockets of PLpro are very narrow which can only accommodate glycine residues; hence, it presents a good control for compound selectivity assessment. Only fragment 4 exhibits moderate efficacy against PLpro (IC₅₀ = 33.47 μmol/L), and all other fragments were ineffective. Of note, fragments 2 and 5 potent against Mpro cannot inhibit PLpro, suggesting evident selectivity. Although other covalent fragment inhibitors of Mpro have recently been disclosed, to our knowledge, our fragments represent the first examples of acrylamide-based fragment inhibitors of Mpro. Acrylamide-based electrophiles offer low pharmacological risk as indicated by their widespread clinical use, emphasizing the development potential of fragments 2 and 5. To better understand what role the acrylamide warhead plays in the fragment binding, we synthesized analogues of fragment 2 in which the acrylamide warhead was substituted with a vinyl sulfonamide, chloroacetamide or acrylate electrophile (Supporting Information Fig. S5). Interestingly, although these three alternative warheads have higher intrinsic reactivity than acrylamides, they did not increase the potency, suggesting that the acrylamide plays a key structural role in the covalent modification.

2.5. Mechanism of the fragment efficacy against Mpro

To reveal the inhibitory mechanism of these fragments against Mpro, we determined the crystal structure of Mpro with 2 and with 5 (Fig. 6). The crystals of Mpro–5 complex diffracted to 2.3 Å, had a P2₁2₁2₁ space group and contained one Mpro dimer in the
asymmetric unit (ASU). Structural comparison of two monomers in ASU gave a root-mean-squared deviation (RMSD) of 0.73 Å. The crystals of the Mpro$_2$ complex diffracted to 1.8 Å, had a C2 space group and contained a single Mpro molecule in the ASU. It formed a typical Mpro dimer with the symmetry mate (-x, -y, -z), suggesting two Mpro protomers have identical conformation.

We identified electron density of compound 5 connecting to the active site cysteine C145 in both Mpro monomers in ASU. For the Mpro$_2$ complex, we observed electron density of 2 connecting to the active site residue C145. We generated the polder maps for the above structures with the compound 2 or 5 omitted (Supporting Information Fig. S6). Positive densities clearly delineated the structure of compounds 2 and 5, confirming the presence of the fragments.

In the active site, the acrylamide moiety of 5 forms a covalent bond with C145 (Fig. 6A and B). While the R’ group (benzothiazole) of 5 is accommodated in the deep S2 pocket of Mpro, the cyclopropane group is exposed to solvent. The benzothiazole/S2 pocket interaction is mainly hydrophobic, involving residues H41, M49, Q189 and M165. The stacking of the H41 imidazole side chain with the benzothiazole moiety stabilizes the fragment.

Similarly, the acrylamide moiety of 2 is covalently linked to residue C145 (Fig. 6C and D). Owing to the high resolution of Mpro$_2$ structure and unambiguous electron density for the fragment, we were able to build the fragment more accurately. We measured the length of the S-C bond between C145 and compound 2 to be 1.8 Å, very close to the average length of single S-C bond, 1.82 Å. The trifluoromethyl thiazole moiety of compound 2 is also accommodated by the S2 pocket. While the trifluoromethyl group touches the apex of the pocket and the thiazole ring π-stacks with the side chain of H41. Both two and five occupy only the S1’ and S2 subsites, implying substantial opportunity to develop these fragments.

While picornavirus 3Cpro functions as a monomer, coronavirus Mpro is an obligate dimer. An additional C-terminal domain in Mpro stabilizes dimerization and the dimerization interface is essential to maintain the active conformation. We next investigated the oligomerization state of the five Mpro-fragment complexes using size-exclusion chromatography (Supporting Information Fig. S7). As expected, Mpro$_2$, Mpro$_3$ and Mpro$_4$ eluted as dimers with calculated molecular masses of 45.7, 45.7 and 47.9 kDa, respectively. Interestingly, however, Mpro$_5$ eluted as a monomer. The calculated molecular mass of Mpro$_5$ is 25.3 kDa, whereas the theoretical molecular mass of Mpro monomer is 33.7 kDa. The retention volume of Mpro$_2$ lies between the monomeric and dimeric forms, with a calculated...
Table 1  Biochemical characterization of acrylamide fragments.

| Compd. | Structure | EV71 3C<sub>pro</sub> qIT (K<sub>IC50/I</sub>)<sup>1</sup> (L/mol·s) | REF | In vitro inhibition IC<sub>50</sub> (μmol/L) | SARS-CoV-2<sup>1</sup> <br> M<sub>pro</sub><br> Covalent labelling by MS after 18 h | In vitro inhibition IC<sub>50</sub> (μmol/L) | PL<sub>pro</sub><br> In vitro inhibition IC<sub>50</sub> (μmol/L) |
|--------|----------|---------------------------------|-----|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| GSH    | EV71 3C  | 0.009                           | 0.207 | 24.3                           | 63.6                           | 93% +235 Da | 21.4                           | >100                           |
| 1      |          | 0.012                           | 0.103 | 8.5                            | 40.7                           | 100% +251 Da | 10                             | >100                           |
| 2      |          | 0.010                           | 0.110 | 10.5                           | 224.6                          | 18% +284 Da | 56.6                           | >100                           |
| 3      |          | 0.014                           | 0.230 | 16.3                           | 68.3                           | 75% +224 Da | 20.2                           | 33.5                           |
| 4      |          | 0.016                           | 0.222 | 13.5                           | 30.1                           | 100% +260 Da | 17                             | >100                           |
| 5      |          | <0.001                          | 0.004 | na                             | _                             | _ | _ |
| 6      |          | <0.001                          | <0.001 | na                             | _                             | _ | _ |

Figure 6  Structure of SARS-CoV-2 M<sup>pro</sup> complexed with compounds 2 and 5. (A) Surface plot of SARS-CoV-2 M<sup>pro</sup> complexed by compound 5 (cyan). Compound 5 occupies pockets S1<sup>0</sup> and S2. (B) Ribbon model of SARS-CoV-2 M<sup>pro</sup>—5. Residues surrounding the benzothiazole moiety of five are shown with stick model. (C) Surface plot of SARS-CoV-2 M<sup>pro</sup> complexed with 2 (cyan). The trifluoromethyl thiazole moiety of 2 occupies the S2 pocket. (D) Ribbon model of SARS-CoV-2 M<sup>pro</sup>—2. Residues surrounding the trifluoromethyl thiazole moiety are shown with stick model.
molecular mass of 37.6 kDa. This suggests the dimerization was partially impaired.

2.6. Unique inhibitory mechanism of compound 5

To further validate the effects of 5 on Mpro dimerization, we tested the oligomerization state of two Mpro mutants, C145A and C156W in the absence and presence of ligand. As well as the active site cysteine (C145), C156 is also surface exposed and potentially reactive so the C156W mutant served as a control mutation. Indeed, mutant C156W behaved similarly to the wild-type enzyme: apo-Mpro C156W eluted as dimers in size-exclusion chromatography, whilst labelling with 5 retarded Mpro elution to that expected for a monomer (Fig. 7A). By contrast, mutant C145A remained dimeric irrespective of the presence of 5. Given that the C145A mutation prevents the labelling of the active site cysteine, these results clearly indicate that the labelling of C145 by acrylamide 5 drives dimer disruption.

Our crystallographic data provides further insights into the inhibition mechanism. Although Mpro 5 forms dimers in crystal lattices, these are notably different from authentic Mpro dimers: (1) Most published Mpro dimers have 2-fold symmetry between two protomers, but Mpro 5 protomers exhibit marked difference, RMSD = 0.73 Å. The structure of each Mpro 5 protomer is also notably different from the free enzyme (PDB ID: 6YB7), r.m.s.d = 0.71–0.78 Å. In this regard, Mpro 2 is more similar to the free enzyme. The structure of Mpro 2 dimer has 2-fold symmetry and each protomer is highly similar to the free enzyme, r.m.s.d = 0.22 Å. (2) The binding of 5 enlarged the substrate binding pockets and affected the nearby regions (Fig. 7B). Compared to the free enzyme, the loops surrounding compound 5 have expanded to make room for the benzothiazole motif. This induced conformational changes of several residues and regions at the dimerization interface. In the chain A of the Mpro 5 dimer, the extreme C-terminal region at the dimer interface is disordered, which is likely caused by the labelling of compound 5 on C145. The fragment induced conformational alterations may contribute to the destabilization of Mpro 5 dimers. In summary, we found that 5 has at least two mechanisms of action to inhibit Mpro: (1) covalently linking to the catalytically essential cysteine and occupying the substrate binding pockets; (2) destabilizing the dimerization of Mpro.

To further elucidate how Mpro dimerization is disrupted by modification with compound 5, we ran molecular dynamics simulations comparing WT Mpro and WT Mpro 5 system (Supporting Information Fig. S8 left). We found that compound 5 destabilizes the steady-state dimer interaction energy by 80.6 kcal/mol (Fig. S8 right).

2.7. Data availability

Final coordinates and structure factors of EV71 3Cpro complexed by compounds 2 and 1 and SARS-CoV-2 Mpro complexed by compounds 2 and 5 have been deposited in the Protein Data Bank under the accession codes: 7WYL, 7WYM, 7WYO and 7WYP.

3. Conclusions

We have identified acrylamide fragments that target both the EV71 3Cpro and SARS-CoV-2 Mpro, and inhibit their activity by covalently reacting with their catalytic cysteines. Importantly, some of these hit fragments cause profound structural rearrangements of each protease: in the case of EV71 3Cpro a new subsite pocket is formed at the expense of the normal active site architecture whilst with Mpro key structural features required for dimerization are distorted preventing formation of the active dimeric unit. The discovery of these conformational change-based mechanisms of action on covalent fragment binding demonstrates the utility of solution-based...
screening methodologies as an alternative to crystallographic fragment screening in which structural rearrangements are unlikely. The fragment-like nature of the inhibitors described herein enables them to bind diverse targets in different modes as exemplified by the distinct binding poses of compound 2 in complex with EV71 3Cpro and after repurposing against SARS-CoV-2 Mpro. As such, these molecules lack specificity and without further development are anticipated to have significant off-target activity and toxicity in more complex systems. Nevertheless, these hit ligands provide excellent candidates for development of potent protease inhibitors by structure-based design.

Acknowledgments

This work was supported by grants from Chinese Academy of Medical Sciences (CAMS) Innovation Fund for Medical Sciences (2021-12M-1-037, China), National Key Research and Development Program of China (2016YFD0500300) and the CRP-ICGEB Research Grant 2019 (CRP/CHN19-02, China). This work was supported by grants from the Institute of Chemical Biology (Imperial College London, UK), the UK Engineering and Physical Sciences Research Council (Studentship award EP/F500416/1, UK) and The Imperial College COVID19 Research Fund. The crystallization facility at Imperial College was funded by BBSRC (BB/D524840/1, UK) and the Wellcome Trust (202926/Z/16/Z, UK). Finally, we also thank Ms Liu siqi for help with EV71 3C protein expression and crystallization.

Author contributions

Sheng Cui and David J. Mann designed the study. Sheng Cui, Gregory B. Craven and Pengjiao Hou analyzed the data and performed experiments. Sheng Cui, David J. Mann, Bo Qin et al. Severe enterovirus A71 associated hand, foot and mouth disease, Vietnam, 2018: preliminary report of an impending outbreak. Euro Surveill 2018;23:1800590.

Lin JY, Kung YA, Shih SR. Antivirals and vaccines for enterovirus A71. J Biomed Sci 2019;26:65.

Sun D, Chen S, Cheng A, Wang M. Roles of the Picornaviral 3C protease in the viral life cycle and host cells. Viruses 2016;8:82.

Wang HM, Liang PH. Picornaviral 3C protease inhibitors and the dual 3C protease/coronavirus 3C-like protease inhibitors. Expert Opin Ther Pat 2010;20:59–71.

Patrick AK, Binford SL, Brothers MA, Jackson RL, Ford CE, Diem MD, et al. In vitro antiviral activity of AG7088, a potent inhibitor of human rhinovirus 3C protease. Antimicrob Agents Chemother 1999;43:2444–50.

Hayden FG, Turner RB, Gwatney JM, Chi-Burris K, Gersten M, Hsyu P, et al. Phase II, randomized, double-blind, placebo-controlled studies of ruprintrivir nasal spray 2-percent suspension for prevention and treatment of experimentally induced rhinovirus colds in healthy volunteers. Antimicrob Agents Chemother 2003;47:3907–16.

Hu B, Guo H, Zhou P, Shi ZL. Characteristics of SARS-CoV-2 and COVID-19. Nat Rev Microbiol 2021;19:141–54.

Classification of Omicron (B.1.1.529): SARS-CoV-2 variant of concern. Available from: https://www.who.int/news/item/26-11-2021-classification-of-omicron-(b.1.1.529)-sars-cov-2-variant-of-concern.

Anand K, Ziebuhr J, Wadhwani P, Mesters JR, Hilgenfeld R. Coronavirus main protease (3CLpro) structure: basis for design of anti-SARS drugs. Science 2003;300:1763–7.

Ratia K, Saikatendu KS, Santarsiero BD, Barretto N, Baker SC, Stevens RC, et al. Severe acute respiratory syndrome coronavirus papain-like protease: structure of a viral deubiquitinating enzyme. Proc Natl Acad Sci U S A 2006;103:5717–22.

Singh J, Petter RC, Baillie TA, Whitty A. The resurgence of covalent drugs. Nat Rev Drug Discov 2011;10:307–17.

Lee CS, Rattu MA, Kim SS. A review of a novel, Bruton’s tyrosine kinase inhibitor, ibrutinib. J Oncol Pharm Pract 2016;22:92–104.

Butterworth S, Cross DAE, Finlay MRV, Ward RA, Waring MJ. The structure-guided discovery of osimertinib: the first U.S. FDA approved mutant selective inhibitor of EGFR T790M. MedChemComm 2017;8:820–2.

Jackson PA, Widen JC, Harki DA, Brummond KM. Covalent modifiers: a chemical perspective on the reactivity of a,b-unsaturated carbonyls with thiol via hetero-michael addition reactions. J Med Chem 2017;60:839–85.

Douangamath A, Fearn D, Geurts P, Krojer T, Lukacik P, Owen CD, et al. Crystallographic and electrophilic fragment screening of the SARS-CoV-2 main protease. Nat Commun 2020;11:5047.

Craven GB, Affron DP, Allen CE, Matthies S, Greener JG, Morgan RML, et al. High-throughput kinetic analysis for target-directed covalent ligand discovery. Angew Chem Int Ed Engl 2018;57:5257–61.

Craven GB, Affron DF, Kiesel T, Wong TLM, Jhoti H, et al. Multiparameter kinetic analysis for covalent fragment optimization by using quantitative irreversible tethering (qITT). ChemBioChem 2020;21:3417–22.

Graham F. Daily briefing: pfizer’s COVID pill looks promising. Nature 08 November 2021. Available from: https://doi.org/10.1038/d41586-021-03379-5.

Zhai Y, Fang C, Zhang Q, Zhang R, Zhao X, Duan Y, et al. Crystal structure of SARS-CoV-2 main protease in complex with protease inhibitor PF-07321332. Protein Cell 2022;13:689–93.

Wu KD, Chen GS, Liu JR, Hsieh CE, Chern JW. Acrylamide functional group incorporation improves drug-like properties: an example with EGFR inhibitors. ACS Med Chem Lett 2019;10:22–6.

Congreve M, Carr R, Murray C, Jhoti H. A ‘rule of three’ for fragment-based lead discovery?. Drug Discov Today 2003;8:876–7.
characterization of covalent reactive groups for the prospective design of irreversible inhibitors. *J Med Chem* 2014;57:10072–9.

27. Wang J, Fan T, Yao X, Wu Z, Guo L, Lei X, et al. Crystal structures of enterovirus 71 3C protease complexed with rupintrivir reveal the roles of catalytically important residues. *J Virol* 2011;85:10021–30.

28. Dai W, Zhang B, Jiang XM, Su H, Li J, Zhao Y, et al. Structure-based design of antiviral drug candidates targeting the SARS-CoV-2 main protease. *Science* 2020;368:1331–5.

29. Hoffman RL, Kania RS, Brothers MA, Davies JF, Ferre RA, Gaijwala KS, et al. Discovery of ketone-based covalent inhibitors of coronavirus 3CL proteases for the potential therapeutic treatment of COVID-19. *J Med Chem* 2020;63:12725–47.

30. Trinajstić N. Calculation of carbon-sulphur bond lengths. *Tetrahedron Lett* 1968;9:1529–32.

31. Khan A, Heng W, Wang Y, Qiu J, Wei X, Peng S, et al. *In silico and in vitro* evaluation of kaempferol as a potential inhibitor of the SARS-CoV-2 main protease (3CLpro). *Phytother Res* 2021;35:2841–5.

32. Khan A, Umbreen S, Hameed A, Fatima R, Zahoor U, Babar Z, et al. *In silico* mutagenesis-based remodelling of SARS-CoV-1 peptide (ATLQAIAS) to inhibit SARS-CoV-2: structural-dynamics and free energy calculations. *Interdiscip Sci* 2021;13:521–34.