Coronavirus

SARS-CoV-2 3CL-pro mutations selected in a VSV-based system confer resistance to nirmatrelvir, ensitrelvir, and GC376

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Protease inhibitors are among the most powerful antiviral drugs. Nirmatrelvir is the first protease inhibitor specifically developed against the SARS-CoV-2 protease 3CLpro that has been licensed for clinical use. To identify mutations that confer resistance to this protease inhibitor, we engineered a chimeric vesicular stomatitis virus (VSV) that expressed a polyprotein composed of the VSV glycoprotein (G), the SARS-CoV-2 3CLpro, and the VSV polymerase (L). Viral replication was thus dependent on the autocatalytic processing of this precursor protein by 3CLpro and release of the functional viral proteins G and L, and replication of this chimeric VSV was effectively inhibited by nirmatrelvir. Using this system, we applied nirmatrelvir to select for resistance mutations. Resistance was confirmed by retesting nirmatrelvir against the selected mutations in additional VSV-based systems, in an independently developed cellular system, in a biochemical assay, and in a recombinant SARS-CoV-2 system. We demonstrate that some mutants are cross-resistant to ensitrelvir and GC376, whereas others are less resistant to these compounds. Furthermore, we found that most of these resistance mutations already existed in SARS-CoV-2 sequences that have been deposited in the NCBI and GISAID databases, indicating that these mutations were present in circulating SARS-CoV-2 strains.

Introduction

In late 2019, the zoonotic transmission of a new coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), into the human population (1) led to worldwide efforts to find effective treatments against the various pathologies caused by the virus. Inhibitors of viral enzymes, such as proteases, have proven to be highly potent drugs in the treatment of HIV and hepatitis C virus infections. However, resistant viruses rapidly emerge unless the protease inhibitors are given in combination with other directly acting antivirals (2, 3). SARS-CoV-2 encodes two proteases. The 3-chymotrypsin-lik e protease (3CLpro) cleaves 11 sites in the viral polyproteins pp1a and pp1ab and is also referred to as the main protease or non-structural protein 5, indicating that it cleaves more sites than the second protease and its location within the polyproteins, respectively (4). The second viral protease, papain-like protease (PLpro), cleaves three additional sites in pp1a and pp1ab (5). Thus, both proteases are essential for viral replication and are therefore interesting drug targets.

Recently, the 3CLpro inhibitor nirmatrelvir was approved for clinical use. Nirmatrelvir acts as a peptidomimetic, covalent inhibitor binding to the catalytic site cysteine (C145), thereby blocking its function (6-8). Nirmatrelvir has been authorized in combination with ritonavir by the U.S. Food and Drug Administration for emergency use in high-risk SARS-CoV-2–infected individuals under the trade name Paxlovid [emergency use authorization (EUA) 105 Pfizer Paxlovid, 22.12.2021]. In the studies leading to the Paxlovid (nirmatrelvir/ritonavir) EUA, mouse hepatitis virus (MHV) 3CLpro was used as a surrogate for SARS-CoV-2 3CLpro to generate resistance data, which may not be the ideal system. In addition, very recently, several preprints have described nirmatrelvir resistance mutations in authentic SARS-CoV-2, either generated de novo (9, 10), found in isolates (11, 12), or modeled in silico (13). Working with SARS-CoV-2 requires biosafety level 3 (BSL-3) installations because of its virulence (14). Even more so, performing SARS-CoV-2 antibody or antiviral resistance studies demands utmost caution to avoid biosafety breaches and subsequent spread of mutant variants.

To address these caveats, we describe in this study a BSL-2 system based on vesicular stomatitis virus (VSV) that allows the selection of resistance mutations in the SARS-CoV-2 3CLpro. Several mutations identified were validated in cell-based, biochemical, and recombinant SARS-CoV-2 assays, and two mutations were found to be identical to (L167F) or at the same residue (Q192) as those described in the other manuscripts characterizing resistance in authentic SARS-CoV-2. We furthermore showed that some mutations selected by one 3CLpro inhibitor can confer cross-resistance to other inhibitors.

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RESULTS
MHV 3CL\textsuperscript{pro} is less sensitive to nirmatrelvir than SARS-CoV-2 3CL\textsuperscript{pro}

We compared the sensitivity of SARS-CoV-2 and MHV 3CL\textsuperscript{pro} to the active component of Paxlovid, nirmatrelvir, using the gain-of-signal variant of a VSV-based 3CL\textsuperscript{pro} measurement assay shown in fig. S1 (A and B) and described in detail recently (17). Briefly, the coronavirus 3CL\textsuperscript{pro} proteases flanked by autocatalytic sites were cloned into the phosphoprotein (P protein) gene of VSV. This recombinant P:3CL\textsuperscript{pro} is expressed in cells, which are then infected with a red fluorescent protein (DsRed) expressing VSV lacking the native P protein. The P protein with the internal 3CL\textsuperscript{pro} is therefore essential for viral genome replication and DsRed expression. In the absence of protease inhibitor, the P:3CL\textsuperscript{pro} protein is autocatalytically cleaved, and DsRed is not expressed. In the presence of a protease inhibitor, the P protein is functional, VSV replicates, and DsRed is expressed. Using this system, we found that MHV 3CL\textsuperscript{pro} showed a weaker response to nirmatrelvir than SARS-CoV-2 3CL\textsuperscript{pro} (Fig. 1A). The sequence identity of the two proteins is only 50% (fig. S2), whereas the structures of the SARS-CoV-2 and MHV 3CL\textsuperscript{pro} enzymes are strongly conserved. However, the interaction site of nirmatrelvir (a distance of 5 Å or less from the compound) shows seven amino acid differences between the two enzymes, namely, H164Q, M165L, P168S, R188A, T190V, and A191V (counting from the first residue (serine) after the glutamine of the N-terminal cleavage site). We therefore suggest that MHV 3CL\textsuperscript{pro} is not an optimal proxy to study SARS-CoV-2 resistance mutations.

A VSV-based non–gain-of-function system was generated to predict SARS-CoV-2 3CL\textsuperscript{pro} mutations

To generate a safer alternative to selection of drug-resistant SARS-CoV-2 strains for studying mutants, we engineered a chimeric VSV variant, where the intergenic region between the glycoprotein (G) and polymerase (L) genes were replaced by the 3CL\textsuperscript{pro} gene of SARS-CoV-2 (fig. S3A). Upon translation, G, 3CL\textsuperscript{pro}, and L form a surrogate polypeptide, which must be processed by 3CL\textsuperscript{pro} to generate the functional viral proteins G and L. This surrogate polypeptide mimics the polypeptide that is produced by SARS-CoV-2 because dimerization of 3CL\textsuperscript{pro} is obligate for its function (18, 19) and cleavage of the cognate 3CL\textsuperscript{pro} N- and C-terminal motifs must occur for successful VSV replication. By applying an appropriate protease inhibitor (+PI), this processing is disturbed, and, therefore, viral replication cannot occur (fig. S3B). Through passaging the chimeric VSV variant in the presence of suboptimal concentrations of a protease inhibitor, 3CL\textsuperscript{pro} mutations that are generated by the error-prone viral polymerase (20, 21) are selected for resistance to the inhibitor (fig. S3C).

In a first proof-of-concept study, we selected a mutant against the inhibitor GC376, which acquired an amino acid change in the 3CL\textsuperscript{pro} from phenylalanine to leucine at position 305 (F305L) in the...
autocatalytic cleavage motif at the C terminus of the protease. This virus gained a mildly faster replication kinetic and produced higher titers in the presence of GC376 and nirmatrelvir compared with the parental virus (Fig. 1, B and C). Related coronaviruses have leucine at position 305 as a preferred cleavage motif (Fig. 1D); therefore, the likely mechanism of the selection of F305L is autocleavage site optimization. We used the wild-type (WT) VSV-3CL for subsequent mutation selection studies with nirmatrelvir. We also included the F305L as parental virus for further selection experiments because the F305L mutation has been found in regional outbreaks (mostly in England) and has been deposited in the Global Initiative on Sharing All Influenza Data (GISAID) database (22–24) with three different codon usages to obtain leucine instead of phenylalanine (Fig. S4A). The mutants were variants of Delta, mainly the sublineage AY.4 (Fig. S4B). We therefore assumed that F305L could be an advantageous mutation that, in combination with further mutations, may give rise to protease inhibitor–resistant lineages.

**Nirmatrelvir-resistant 3CL\textsuperscript{pro} mutants were selected for in the VSV-3CL\textsuperscript{pro} system**

We next used the WT and F305L mutant viruses to select for nirmatrelvir-resistant 3CL\textsuperscript{pro}. Baby hamster kidney 21 (BHK-21) cells in a 96-well plate were infected at a low multiplicity of infection (MOI; 0.01). Where cytopathic effects were visible in the first passage (25 of 48 wells from parental WT and 17 of 48 wells from parental F305L), supernatants were used for passaging individual wells with increasing concentrations [WT infection: 30 μM (initial), 40 μM (second round), and 50 μM (third round); F305L infection: 50 μM (initial), 75 μM (second round), and 100 μM (third round)] of nirmatrelvir. At every passage, where cytopathic effects were observed again, supernatants were collected from the cell culture of individual 96 wells and transferred to individual new wells of a 96-well plate. At every passage, each well was sequenced individually, the target region being 3CL\textsuperscript{pro} and adjacent parts of G and L. We only counted mutants from unambiguous chromatogram peaks (as exemplified in fig. S3C). If in the first or second passage there were still overlapping peaks, then we sequenced the well again after the next passage. By this continuous selection pressure, the fittest mutant virus variant became dominant over the WT (and potential other mutants) in each well and made up the entirety of the genomic RNA, cDNA, and subsequent polymerase chain reaction (PCR) fragment. To lastly exclude minority mutant populations that were not visible in a Sanger sequencing chromatogram but could contribute to the resistance phenotype, we later reintroduced mutations into 3CL\textsuperscript{pro} measurement systems individually. We found 39 distinct mutations within 3CL\textsuperscript{pro} by Sanger sequencing. Viruses carried from one dominant mutation up to four mutations. The mutations were distributed over the entire sequence of 3CL\textsuperscript{pro} (Fig. 2A and table S1). We categorized them into catalytic site, near-catalytic site, dimerization interface, and autocleavage site mutants. A fourth category for all mutations not fitting the first three was chosen as “allosteric” mutants. The mutants Y54C, L141F, L167F, and Q192R occurred in residues in very close proximity [within 5 Å of Protein Data Bank (PDB) 3CL\textsuperscript{pro} structure 7vh8; Fig. 2B and table S2] to nirmatrelvir. We searched for the mutants in the National Center for Biotechnology Information (NCBI) Virus database (25) and GISAID EpiCoV (22–24) and found most of the mutations, or at least the same residue with a different mutation, in deposited sequences with varying coverage (Fig. 2A and table S1). We further subdivided GISAID entries into depositions made before and after the emergency use authorization of Paxlovid (nirmatrelvir/ritonavir) on 22 December 2021 (table S3). An update of the Paxlovid EUA (18 March 2022) included 3CL\textsuperscript{pro} mutants that were retrieved from patients treated with Paxlovid (nirmatrelvir/ritonavir; Fig. 2A). The update stated that it was unclear whether these mutations had clinical relevance (26).

**Replication kinetics and dose response were analyzed for selected 3CL\textsuperscript{pro} mutants**

To confirm these potential resistance mutations, we chose six virus samples to perform replication kinetics and dose-response experiments. Mutants were selected for further testing on the basis of two criteria. First, we chose virus variants with catalytic site mutations because alterations in drug binding residues (direct or indirect) are more likely to alter efficacy. Second, we chose the most frequently recovered mutant outside of the catalytic site. Four samples were derived from WT VSV-G–3CL\textsuperscript{pro}, and two were derived from the F305L variant. Supernatants for the replication kinetic experiments were collected at indicated time points after infection, and supernatants for the virus nirmatrelvir dose-response experiments were collected 24 hours after infection. The replication kinetics revealed that all variants were still capable of replicating to high titers, suggesting that resistance mutations did not result in a strong negative effect on 3CL\textsuperscript{pro} activity (Fig. 3). The dose responses showed that WT VSV-G–3CL\textsuperscript{pro} replication was inhibited by 10\textsuperscript{6}-fold at 100 μM nirmatrelvir (Fig. 3A). We tested two L167F variants because this mutant arose twice independently. The similarity of their dose responses (Fig. 3, B and C), as well as the low variation of the biological replicates, suggests that the differences in the degree of resistance that we observed between the mutants were not artifacts. We tested additional single mutants, namely, the catalytic site mutant Y54C (Fig. 3D), a mutant from the mutation cluster shown in Fig. 2B, N203D (Fig. 3E), and the autocleavage site mutant F305L (Fig. 3F). To test whether mutants that were selected from the F305L background had increased resistance, we also tested double mutants G138S/F305L (Fig. 3G) and Q192R/F305L (Fig. 3H). We observed the strongest resistance phenotype in the double mutant Q192R/F305L, replicating to high viral titers with a pronounced cytopathic effect (fig. S5) even in the presence of 100 μM nirmatrelvir.

**Reintroduction of 3CL\textsuperscript{pro} mutations confirms their resistance phenotype**

As shown in table S1, VSV-induced 3CL\textsuperscript{pro} mutations were observed after the first passage when nirmatrelvir was applied. To validate the resistance data of replication-competent VSV-3CL\textsuperscript{pro} and, at the same time, exclude the effects of potential additional mutations arising within the dose-response experiment, we reintroduced some of the catalytic center mutations (Y54C, L167F, and Q192R) into a recently developed protease activity measurement tool based on replication-incompetent VSV (fig. S1, A and B) (17). Briefly, the protease activity measurement tools comprise replication-incompetent VSV-DsRed variants missing either the viral phosphoprotein (ΔP) or polymerase (ΔL). These viruses are complemented with either an INTRAmolecular-3CL\textsuperscript{pro}-tagged phosphoprotein or IN- TErmolecular green fluorescent protein (GFP)–3CL\textsuperscript{pro}–L fusion protein, respectively. The P.3CL\textsuperscript{pro} or GFP-3CL\textsuperscript{pro}–L proteins are...
**Fig. 2. Sequencing of 3CLpro escape mutants and comparison to databases and Paxlovid EUA information.**

(A) Mutants were recovered from VSV-G-3CLpro-L WT (*) and the F305L variant (red, **). Autocleavage site mutants are colored in turquoise. Catalytic site mutants are in green. Near-catalytic site mutants are in light green, dimerization interface mutants are in yellow, and allosteric mutants are in white. Viruses with more than one mutation are displayed above in a gray box and named a to f. The number of mutated sequences in the databases from NCBI and GISAID is displayed below the mutations in gray. If specific mutations were not present in the database, then the residue is displayed with any mutation that occurred at this position. Multiple such different amino acid changes that were not selected in our virus are displayed with X (N203X and V204X). Mutations from the Paxlovid EUA are divided into mutations found in cell culture and mutations sequenced from treated patients. The coverage of mutation entries was obtained on 2 June 2022.

(B) Visualizations of mutation-affected residues are shown. Residues that were mutated one time are highlighted in yellow, two times in light orange, three times in dark orange, and four times in red. The 3CLpro protease dimer with bound nirmatrelvir (purple) was visualized in ChimeraX from the PDB structure 7vh8 (33). Catalytic center mutations are within a range of 5 Å as visualized in dark green.

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expressed in cells from transfected plasmids. The cells are then infected with the replication-incompetent VSV-DsRed variant and treated with inhibitors. An intramolecular 3CLpro tag in combination with VSV-ΔP-DsRed constitutes a gain-of-signal or "on" assay. An intermolecular 3CLpro tag in combination with VSV-ΔL-DsRed constitutes a loss-of-signal or "off" assay. We found that the identified single catalytic center mutations conferred partial resistance against nirmatrelvir of the 3CLpro from the Wuhan-1 and the Omicron SARS-CoV-2 variants (Omicron signature mutation in 3CLpro P132H; Fig. 4, A to D), which could be further enhanced by the introduction of a second mutation in the autocleavage site (F305L; Fig. 4, E to H). The mutation Q192R arose in the F305L parental virus. Introducing Q192R alone reduced 3CLpro activity mildly, as we observed by increased basal signal in 3CLpro-OnQ192R at low nirmatrelvir concentrations. Adding F305L as the second mutation, thereby restoring the original combination from the double mutant virus, rescued this phenotype (Fig. 4G). A randomly selected combination of catalytic center mutations led to a strong loss in enzymatic activity (fig. S6, A and B). We further introduced two mutants (A194S and G138S) into the 3CLpro measurement assays, which also conferred resistance to nirmatrelvir (Fig. 4, I and J).

Fig. 3. Replication kinetics and nirmatrelvir dose responses of parental VSV-G-3CLpro-L and mutant variants. (A to H) Replication kinetics and dose responses are shown for WT (A), L167F (B), L167F-2 (C), Y54C (D), N203D (E), F305L (F), G138S/F305L (G), and Q192R/F305L (H) VSV-G-3CLpro-L. Supernatants for replication kinetics were collected at indicated time points. Supernatants for virus nirmatrelvir dose response were collected 24 hours after infection (n = 2 biologically independent replicates per condition, with individual data points shown and connecting lines of mean values). neg, without nirmatrelvir; TCID₅₀, median tissue culture infectious dose.
Nirmatrelvir and GC376 react differently to 3CLpro mutants

Comparing GC376 with nirmatrelvir in the 3CLpro Y54C and L167F mutants directly revealed that these mutants react differently to the compounds. Y54C confers a similar resistance to GC376 as to nirmatrelvir (Fig. 5A). GC376 and nirmatrelvir interact similarly with the residue Y54, whereas L167 and Q192 are distant to GC376 and close to nirmatrelvir (within 5 Å; Fig. 5B). L167F and Q192R appeared to affect the activity of GC376 less than nirmatrelvir (Fig. 5, C and D, and tables S4 and S5). Nirmatrelvir IC50 values were especially high in the 3CLpro-On construct. We sought to improve the assay sensitivity by changing the readout method from a Fluorospot to a flow cytometry-based readout. With this approach, we could decrease the IC50 of the WT 3CLpro-On to 0.91 μM nirmatrelvir (Fig. S6C and table S6).
Fig. 5. Nirmatrelvir and GC376 react differently to mutants. (A) Gain-of-signal assay results are shown for single mutants Y54C and L167F versus WT tested with GC376 and nirmatrelvir (Y54C: n = 4; L167F: n = 3 biologically independent replicates per condition). (B) GC376 (PDB: 7k0g) and nirmatrelvir (PDB: 7vh8) 3CL<sup>pro</sup> crystal structures are shown with GC376 in green (and colored by heteroatom) and nirmatrelvir in light blue (and colored by heteroatom) and proximal residues in orange (within zone of 5 Å). Compound-to-residue distances are shown with purple dotted lines. (C) Fitting of gain-of-signal assay results is shown for single mutants Y54C and L167F versus WT tested with GC376 and nirmatrelvir. (D) Fitting of gain-of-signal assay results is shown for single mutant Q192R versus WT tested with GC376 and nirmatrelvir. Data in (C) and (D) are presented as the SDs of n = 4 biologically independent replicates per condition.
Confirmation of resistance mutations in a second cell-based assay system, biochemical assay, and with recombinant SARS-CoV-2

The resistance phenotype of L167F observed with gain- and loss-of-signal assays was confirmed using another recently published cellular system (27). In this complementary assay, a polyprotein of Ssrc, 3CLpro with N- and C-terminal autocleavage sites, HIV Tat, and luciferase was used to repress transcription when 3CLpro was active (fig. S7). Bona fide chemical inhibitors blocked 3CLpro activity and restored luciferase signal in a dose-dependent manner (Fig. 6, A to C). Similar to the results described above, L167F was more resistant to nirmatrelvir than GC376 (Fig. 6, A and C, and table S7). Furthermore, this mutant was most resistant to ensitrelvir, a recently developed compound in clinical trials in both the Src-3CLpro-Tat-Luc (Fig. 6B and table S7) (28) and our assay (fig. S8 and table S8).

For further confirmation of resistance phenotypes, we purified recombinant enzymes (fig. S9, A to C). We tested catalytic activity with a substrate dose-response kinetic experiment with purified WT 3CLpro versus mutants Y54C, L167F, and Q192R with the substrate Ac-Abu-Tle-Leu-Gln-LmCA releasing the fluorogenic molecule 7-amino-4-methylcoumarin (fig. S10A). The ratio between the catalytic constant or turnover rate ($k_{cat}$) and the Michaelis-Menten constant ($K_m$), displayed as $k_{cat}/K_m$, showed that some of the mutants partially lose catalytic activity, most notably Q192R, which was in line with the cellular assays (Fig. 6D and table S9). For further resistance confirmation, we applied a biochemical fluorescence resonance energy transfer assay, which uses a quencher (DABCYL) and a fluorogenic substrate (EDANS) that are connected by a peptide (KTSAVLQSGFRKME) that is recognized and (DABCYL) and a fluorogenic substrate (EDANS) that are connected by a peptide (KTSAVLQSGFRKME) that is recognized and cleaved by 3CLpro (fig. S10B). Upon cleavage, fluorescence of EDANS increases. All three mutant 3CLpro enzymes were more resistant to nirmatrelvir than the WT 3CLpro (Fig. 6E and table S10).

Last, we confirmed our findings in recombinant SARS-CoV-2 viruses expressing a reporter gene (fig. S10C) (29, 30). The recombinant SARS-CoV-2 variant expressing mCherry used for mutagenesis, aside from its transgene, was sequence identical to the Wuhan-1 variant (data file S1) (29, 30). Viruses carrying L167F alone and in combination with F305L were able to replicate but replicated slower than WT virus and produced smaller plaques (Fig. 6, F and G). The mutations introduced into recombinant SARS-CoV-2-mCherry had been found in clinical samples before our study (data file S2). As expected, both L167F single and L167F/F305L double mutants were more resistant to nirmatrelvir than the WT (Fig. 6H and table S11).

Structural modeling of mutant 3CLpro variants

To explore potential mechanisms of resistance, we performed molecular modeling with an in silico alanine mutation scanning and resistance mutation scanning with MOE suite (16) and with the Robetta service (31). MOE modeling was based on the PDB structure 7rfw (32), and Robetta modeling was based on 7vh8 (33), both of which are 3CLpro structures with high nirmatrelvir occupancy. Experimental alanine scanning (34, 35) and in silico alanine screening (36, 37) are routinely used to evaluate the impact of single amino acid mutations on protein structure, and the models can provide plausible explanations for the structural basis of nirmatrelvir resistance.

Figure 7A shows that the most important losses of binding affinity are primarily related to mutation of residues whose side chains directly contact the ligand, such as H41, M49, N142, H163, M165, and Q189, and secondarily to other residues lining the binding site, such as Y54, H164, E166, P168, D187, and Q192. Residues with hydrophobic side chains, such as L27, Y54, F140, L167, and F181, seem to have a pivotal role in the structural integrity of the binding site (Fig. 7B) despite having a negligible impact on the variation of binding affinity.

The primary effect of Y54C in these models is the disruption of a stabilizing interprotein hydrogen bond to the backbone oxygen of D187 and disruption of additional weak but stabilizing interactions with surrounding hydrophobic residues, such as a π-charge interaction between the phenyl ring of Y54 and the guanidinium group of R40. No major direct interaction to the 3.5 Å distant C20 methyl group of nirmatrelvir exists (Fig. 7C and fig. S11A). However, loss of the critical hydrogen bond between loop regions 43 to 55 and the adjacent loop around D187 allows for a structural rearrangement destabilizing the distal part of the binding site, likely increasing the inherent plasticity of this protein region.

Residue G138 lies in a solvent-accessible loop, with backbone torsion angles in the β sheet region. Replacing it with a polar serine (G138S) while maintaining the same backbone conformation led to the Cβ of S138 pointing into the protein interior, and all of the preferred rotamers led to unfavorable interactions or required a rearrangement of the affected region. Formation of new hydrogen bonds, for example, with a backbone hydrogen of F140 and the sulfur of C128 (Fig. 7D) likely led to a rearrangement of the S1 subpocket, which is responsible for hosting the terminal carbamidemoeity that mimics the P1 glutamine in natural peptide substrates.

Supervised molecular dynamics simulations of the nirmatrelvir-3CLpro recognition process revealed how L141 is one of the first residues that is contacted during the approach of nirmatrelvir into the binding site (38). In the L167F mutant, the larger side chain of phenylalanine cannot be accommodated without a structural rearrangement, which likely leads to repulsive interaction between the trifluoromethyl (CF₃) moiety of nirmatrelvir and weakening its interactions with other proximal residues such as N142, which are thought to play a pivotal role in maneuvering the ligand entrance in the catalytic pocket (38). As anticipated in the alanine scan, the L167F mutation seems to have an indirect effect on the binding affinity by alteration of the β sheet that constitutes the lower portion of the binding site, where a set of hydrogen bonds are established between nirmatrelvir and the backbone of both H164 and E166 (fig. S11B). A similar distortion of the binding pocket by the bulkier phenylalanine has also been described recently (10).

Last, polar Q192 stabilizes a solvent-exposed loop participating in hydrogen bonds to backbone oxygen and nitrogen of V186, backbone oxygen of R188, and a stabilizing contact to the CF₃ group of nirmatrelvir. Replacement with positively charged R192 disrupted this network, which likely results in a structural rearrangement and altered binding to nirmatrelvir (Fig. 7E). The slight increase in binding affinity of Q192R concurrent with protease destabilization (Fig. 7, A and B) could be explained by recontouring of the subpocket hosting the negatively polarized CF₃ moiety of nirmatrelvir and interacting with positively charged R192 (Fig. 7E and fig. S11C). Despite the predicted, marginally more favorable interaction with nirmatrelvir, an overall unfavorable effect of the mutation could still be possible because of altered sequestration of nirmatrelvir and the destabilization of the loop region lining the S2 and S4...
subpockets of the catalytic site, where important residues such as Q189 are located.

DISCUSSION

In our study, we selected mutations in the main protease 3CL\textsuperscript{pro} of SARS-CoV-2 against the protease inhibitor nirmatrelvir with a non–gain-of-function system based on VSV. The selected mutations were confirmed in two cellular assays and in one biochemical assay, along with confirmation using recombinant SARS-CoV-2. For the catalytic site mutations, a resistance mechanism was postulated on the basis of mapping the mutations onto the cocrystal structure of 3CL\textsuperscript{pro}-nirmatrelvir and generating mutant models with Robetta (15) and MOE (16).
In previous initial resistance studies leading to emergency use authorization of Paxlovid (nirmatrelvir/ritonavir), the 3CL\textsuperscript{pro} of a related coronavirus, MHV, was used to select for resistance mutations. The 3CL\textsuperscript{pro} of SARS-CoV-2 and MHV share 50% sequence identity. In this study, we compared the activity of 3CL\textsuperscript{pro} of SARS-CoV-2 and MHV and found that MHV 3CL\textsuperscript{pro} responded only mildly to nirmatrelvir in our gain-of-signal assay (17). Although the structures of SARS-CoV-2 and MHV 3CL\textsuperscript{pro} are conserved, we propose that the low amino acid sequence identity alters the binding pocket affinity to nirmatrelvir sufficiently to reduce the sensitivity against the inhibitor. Key corresponding residues of the binding pocket (within 5 Å or less) are different.

**Fig. 7. Structural modeling of mutant 3CL\textsuperscript{pro} variants.** (A) Colorimetric mapping of the dAffinity value (kcal/mol) by virtual alanine scanning with MOE suite. Residues within 5 Å of the nirmatrelvir position are displayed. Colors range from blue (negative values, indicating increased protein-ligand affinity) to red (positive values, indicating decreased protein-ligand affinity). The nirmatrelvir (NV) structure is shown in light blue. (B) Colorimetric mapping of the dStability value (kcal/mol), computed as above for (A). Colors range from blue (negative values, indicating increased protein stability) to red (positive values, indicating decreased protein stability). (C) The catalytic center of 3CL\textsuperscript{pro} from PDB structure 7vh8 is shown with nirmatrelvir bound. Y54 (top) forms a strong hydrogen bond (HB; highlighted with a blue dashed line) with D187, whereas nirmatrelvir is at a distance of 3.5 Å (yellow dashed line). The exchange of Y54 with C (bottom) leads to a loss of the hydrogen bond to D187 and makes room in the nirmatrelvir binding pocket due to the smaller side chain of cysteine versus tyrosine. (D) G138 (top) contacts H172 with a hydrogen bond. S138 (bottom) forms several new hydrogen bonds with the backbone hydrogen of F140, backbone oxygen of K137, and the sulfur of C128. (E) Q192 (top) forms hydrogen bonds with the oxygen and nitrogen of V186 and the oxygen of R188 and stabilizes the polar contact to the CF\textsubscript{3} group of nirmatrelvir. R192 (bottom) disrupts this hydrogen bond network; subsequent rearrangement could form additional interactions with the CF\textsubscript{3} group.
namely, H164Q, M165L, P168S, V186R, R188A, T190V, and A191V. Furthermore, amino acid changes that occurred in our selection experiments, Y126F and F305L, are already present in the MHV 3CL\textsuperscript{pro} sequence. Together, we argue that MHV 3CL\textsuperscript{pro} was not an optimal proxy for resistance studies.

Recently, chimeric VSV variants with SARS-CoV-2 spike were used to predict spike protein immune escape mutations by selecting against neutralizing serum (39–41). The fast occurrence of mutations was facilitated in those studies by the high error rate of the VSV polymerase (20, 21). In a similar approach, we exploited this high error rate in a recombinant VSV expressing 3CL\textsuperscript{pro} to select for 3CL\textsuperscript{pro} mutations that confer resistance against protease inhibitors. The 3CL\textsuperscript{pro} was used to replace the function of an intergenic region between the viral glycoprotein (G) and the polymerase (L). The intergenic regions of VSV are responsible for separate gene expression, which, in other viruses, is accomplished by a polyprotein and proteases. Although this polyprotein of VSV-G-3CL\textsuperscript{pro}-L is only a surrogate to the one in SARS-CoV-2, the cognate cleavage sites, the requirement for dimerization of the protease for proper function (18, 19), and the context of a replicating virus in the cell make this approach an attractive proxy.

Initially, we selected the 3CL\textsuperscript{pro} mutant F305L using GC376, which showed reduced sensitivity to GC376 as well as to nirmatrelvir. This mutation lies in the 3CL\textsuperscript{pro} cleavage site that flanks 3CL\textsuperscript{pro} at its C terminus. The leucine-glutamine (LQ) motif found in the mutated site is known to be preferred over phenylalanine-glutamine (FQ) as a target motif for 3CL\textsuperscript{pro} (42–44), which may explain the reduced sensitivity of the F305L mutant to the protease inhibitors. We then selected both WT and the F305L mutant against nirmatrelvir. We also used F305L as parental virus because we found that this variant existed already in regional outbreaks (mainly in England), underlining the viability of this mutation and its potential replicative advantage. These clusters were mainly of the Delta subvariant AY.4. Delta was replaced gradually by Omicron, which may have ended the spread of the Delta F305L. Nevertheless, we also found combinations of the Omicron signature mutation P132H with F305L. We were therefore interested in finding potential combinations of F305L with further protease inhibitor resistance mutations, assuming that the combination would show a higher degree of resistance than single mutations, which we did observe. Mutations from both WT and F305L were selected, which ultimately allowed the mutants to escape the inhibitor. Resistance phenotypes were confirmed by dose–response experiments and reintroduction of mutations into recently developed protease activity measurement systems (17) and alternative methods such as biochemical (45) and cellular assays (27).

We collected a total of 39 unique mutations, of which Y54C, L167F, N203D, and D216Y occurred twice independently. F305L was selected with both GC376 and nirmatrelvir. Six of 39 occurred in the catalytic site, 2 near the catalytic site, 7 at the dimer interface, 3 in the autocleavage sites, and 21 in the rest of the 3CL\textsuperscript{pro} sequence (which we called allosteric mutations). First, we confirmed catalytic site mutants (Y54C, G138S, L167F, and Q192R), where the resistance mechanism is likely straightforward: the steric disturbance of nirmatrelvir binding. Then, we tested the near-catalytic site substitution A194S, which is more prevalent than the previous catalytic site mutants in virus isolates. In GISAID, this particular mutation can be found in over 800 sequence depositions in the variants of concern Alpha, Gamma, Delta, Lambda, and Omicron. Changes of the residue A194 in general are frequent, with over 3000 entries. Although it is not known whether this mutant was selected by the use of nirmatrelvir in patients, the fact that it is a resistant mutant and prevalent in virus sequences makes it a variant worth tracking. We further combined L167F and Q192R with the autocleavage site mutation F305L, which further increased the resistance. The combination of Y54C or L167F with the Omicron signature mutation P132H also conferred increased resistance, highlighting the potential relevance of these mutations for the Omicron variant. The substitution F305L was described as a resistance mutation in this study. An adjacent mutant, T304L, was found in nirmatrelvir selection experiments with authentic SARS-CoV-2 (9), and the suggested mechanism was autocleavage site optimization. Given that F305L is also likely an autocleavage site optimizing mutant, we did not test it further in a biochemical assay because such assays use mature protease in which autoprocessing does not play a role. Lacking an appropriate method, we therefore did not investigate the mechanism of F305L. Nevertheless, such mutants merit further study in assays that can elucidate the mechanism of action.

One technical particularity in the 3CL\textsuperscript{pro}.On construct is that the nirmatrelvir IC\textsubscript{50} values are higher than those generally reported in the literature. However, IC\textsubscript{50} values are generally higher in cell-based assays than in biochemical assays, as we described previously (17). Briefly, in the excess of an inhibitor and constant renewal of protease fusion protein, signals are expected to plateau later than in a biochemical assay with a fixed amount of enzyme. Furthermore, the screening method used in this study to assess mutants was originally developed as a high-throughput screening tool for 3CL\textsuperscript{pro} inhibitors using a FluoroSpot reader that allows fast sampling (17). We improved the assay sensitivity by changing the readout method from FluoroSpot to flow cytometry–based sampling. Flow cytometry sampling is not only more sensitive but also more time consuming. Flow cytometry readouts captured milder degrees of inhibition and resulted in a more gradual signal increase; therefore, this resulted in lower IC\textsubscript{50} values in 3CL\textsuperscript{pro}.On assay [0.91 μM nirmatrelvir, which is closer to the published range of 74.5 (66.5 to 83.4) nM] (46).

We cross-validated several of our mutants in different assays. We confirmed the resistance data of L167F with a previously published cellular assay (27) and the mutants Y54C, L167F, and Q192R with a biochemical assay (47). We showed also in a biochemical assay that the kinetic metrics of the mutants Y54C, L167F, and Q192R are attenuated to varying degrees. However, the VSV-chimeric viruses containing resistant 3CL\textsuperscript{pro} showed little fitness loss. In three recent preprints, L167F and various mutants at Q192 were identified to be resistance mutations in authentic SARS-CoV-2 (9, 10, 12).

Last, we confirmed the viability and resistance of the single mutant L167F and in combination with F305L in a previously published recombinant SARS-CoV-2 expressing a reporter gene (29, 30), confirming the validity of our mutation prediction tool based on VSV and the resistance mutations identified. Genetically modifying highly pathogenic viruses such as SARS-CoV-2 can be considered so-called gain–of–function experiments if the recombinant virus is more apt to cause disease or if treatment is made more difficult than the WT variant. We therefore applied several safety measures such as using a virus for mutagenesis that was sequence similar to the Wuhan-1 variant. Therefore, compared with currently circulating viruses, it has not undergone extensive evolution and, if
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set free, would unlikely be able to compete with current Omicron variants. Other previously described antivirals approved for use in humans have been shown to inhibit viral replication of this Wuhan-1 strain. Moreover, current vaccines used in humans to protect against SARS-CoV-2 have been developed on the basis of the sequence of the spike glycoprotein of this, or a similar, Wuhan-1 strain. Thus, neutralizing antibody responses induced by these vaccines will be able to protect against these recombinant viruses. Second, the plaques the mutant recombinant viruses formed in Vero E6 cells were found to be smaller than that of WT recombinant virus, and replication kinetics also indicate mild attenuation. Third, the mutations introduced into recombinant SARS-CoV-2–mCherry had been found in clinical samples already.

In this study, we identified several mutations such as Y54C, G138S, L167F, Q192R, A194S, and F305L in the SARS-CoV-2 3CLpro that confer resistance to the 3CLpro inhibitors nirmatrelvir, ensitrelvir, and GC376. To understand these mutations in light of the Omicron variant, we combined two of our most intensively studied mutations, Y54C and L167F, with the Omicron 3CLpro signature mutation P132H. These results showed that the mutations are functional, thereby confirming their potential relevance in this context of the Omicron variant.

Complementary structure modeling approaches based on Robetta and the MOE reveal potential effects of the catalytic site mutants Y54C, G138S, L141F, L167F, and Q192R. Y54C and G138S seem not to directly affect nirmatrelvir binding but may lead to a restructuring of the catalytic site, thereby indirectly affecting inhibitor binding. L141F is likely affecting the early sampling of the catalytic pocket by nirmatrelvir. L167F may distort the distal region of the binding pocket. Last, Q192R could strengthen a polar interaction with nirmatrelvir, which may alter sequestration of the compound in an unfavorable position concurrent with destabilization of a loop containing important nirmatrelvir-catalytic site interaction partners such as Q189.

Our study has limitations. The mutations generated in VSV occurred in an artificial polyprotein, which, like pp1a or pp1ab, comprises precursors for large protein subunits and requires 3CLpro dimerization for autocleavage. Nevertheless, the polyprotein structure is different, which could result in mutations not relevant in authentic SARS-CoV-2. Along the same line, in this artificial polyprotein, only autocleavage or cis-cleavage occurs, whereas in SARS-CoV-2, the mature 3CLpro additionally cleaves distant or trans-cleavage sites. It has been shown that autocleavage of 3CLpro in coronavirus is a stepwise process with distinct N- and C-terminal autocleavage binding pocket confirmations (48, 49), where the C-terminal autocleavage occurs after N-terminal autocleavage and might resemble a matured structure as in the trans-cleavage confirmation. Even so, this system could, in theory, disregard trans-cleavage–specific mutants, if such exist. Last, we did not elucidate the exact mode of resistance of the different mutants described in this study. Although we modeled catalytic site mutations and describe a plausible mechanism for autocleavage site mutants, solving crystal structures was beyond the scope of this work and remains for future studies.

In conclusion, our findings argue for a highly selective application of protease inhibitors to patients at increased risk of severe disease because extensive, unselective use is expected to rapidly lead to emergence of drug resistance. Furthermore, the combination of different drugs is a proven strategy to avoid resistance mutations, as has been shown for HIV (3) and hepatitis C virus (2) therapy. As more compounds became available, combinations including classes of inhibitors targeting distinct viral functions, such as protease and polymerase inhibitors, may be an effective strategy. However, as we observed in this study, 3CLpro mutants can react differently to specific compounds. Therefore, even the combination of different protease inhibitors could lower the risk of viral escape.

MATERIALS AND METHODS
Study design
The overall rationale of the study was to develop a mutation selection tool based on VSV and to describe mutants as proof of concept for that tool. The study was performed on cell lines and in silico, and no animal husbandry or human participants were involved. Human and monkey cell lines with replicating BSL-1, BSL-2 and BSL-3 viruses were treated with protease inhibitors to observe resistance phenotypes in appropriate facilities. Viral titers were determined using median tissue culture infectious dose (TCID50) and plaque assays. Measurement readouts were fluorescence and luminescence based and detected by flow cytometry, an enzyme-linked immunosorbent spot (ELISpot) reader with fluorescence-detection capacity (FluoroSpot), and fluorescence and luminescence multiwell readers. Autofluorescent fibers were excluded automatically and manually from spot counting in the FluoroSpot readout. Experiments were neither blinded nor randomly distributed to experimenters. We chose sample sizes empirically on the basis of experience from former studies. At least two and up to four biologically independent replicates were performed per condition. Biologically independent meant distinct wells with the same condition, not multiple measurements of the same wells (technical replicates). Resistance phenotypes were reproduced at least twice, usually more often and in different combinations (comparing single mutants to each other and the WT or WT to single and double mutants). Representative measurements were chosen to compile graphs and figures.

Cloning strategies
The chimeric VSV variant with 3CLpro instead of the intergenic region between G and L was cloned by Gibson assembly [New England Biolabs (NEB)] (50). A VSV-G plasmid (51) was digested with KpnI and Hpal (NEB), removing a C-terminal part of G, the intergenic region, and a small N-terminal part of L. Insert fragments were generated as follows. Missing C-terminal G with an additional overhang to the N-terminal cleavage site of 3CLpro was amplified with primers 33n–before–KpnI–for and G-cut1-rev. 3CLpro with its N- and C-terminal cleavage sites, and a C-terminal overhang to L was amplified from Wuhan-1 (NCBI Reference Sequence: NC_045512.2) cDNA with primers cut1-for and cut2-L-rev. The N-terminal missing L sequence was amplified with primers cut2-L-for and 33n–after–Hpal–rev. For subsequent Gibson assembly, the fragments were ligated in a fusion PCR using the outer primers 33n–before–KpnI–for and 33n–after–Hpal–rev with all three fragments as templates. The cloning primers for VSV vectors are shown in table S12, and the annotated sequence is shown in data file S4.

3CLpro-Off and 3CLpro-On point mutants were generated by mutagenic Gibson assembly on parental plasmids (GenBank accession codes: 3CLpro-Off: ON262564 and 3CLpro-On: ON262565).
For 3CL\textsuperscript{pro}-Off mutants, a lentiviral expression plasmid expressing VSV L (identical sequence as blasticidin 3CL\textsuperscript{pro}-Off plasmid without GFP and 3CL\textsuperscript{pro}) was digested with HpaI, which removed the central polypurine tract/central termination sequence (cPPT/CTS) and cytomegalovirus (CMV) promoter sequences and a small N-terminal part of L. This missing sequence was replaced with the identical sequence from 3CL\textsuperscript{pro}-Off, with the addition of the N-terminal 3CL\textsuperscript{pro} sequence up to the respective mutation site with primers blasticidin-for and 3CL\textsuperscript{pro}*-mut-x*-rev, where *mut-x* is the mutation of interest. The C-terminal part of 3CL\textsuperscript{pro} and the small missing fragment of L were generated by PCRs on parental vectors with primers 3CL\textsuperscript{pro}*-mut-x*-for and 33n–after–HpaI–rev.

For 3CL\textsuperscript{pro}-On mutants, a lentiviral hygromycin vector (modified from Addgene pLenti CMVie-ires-BlasR accession #119863) was digested with Nhel and Pacl. N-terminal 3CL\textsuperscript{pro} insert fragments with vector overhangs were generated with hygro-P-for and 3CL\textsuperscript{pro}*-mut-x*-rev. C-terminal 3CL\textsuperscript{pro} insert fragments with vector overhangs were generated with 3CL\textsuperscript{pro}*-mut-x*-for and P-hygro-rev. Double mutants were cloned by repeating the site-directed mutagenesis with a second primer pair in combination with Gibson assembly on an already mutant-bearing plasmid. Cloning primers for 3CL\textsuperscript{pro}-Off and 3CL\textsuperscript{pro}-On mutant variants are shown in table S13.

Cell lines

BHK-21 cells [American Type Culture Collection (ATCC)] were cultured in Glasgow minimum essential medium (Lonza) supplemented with 10% fetal calf serum (FCS), 5% tryptose phosphate broth, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) (P/S; Gibco). The 293T cells (293tsA1609neo, ATCC) and 293-VSV cells (293 expressing N, P-GFP, and L of VSV) (52) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS, P/S, 2% glutamine, 1x sodium pyruvate, and 1x non-essential amino acids (Gibco). Vero E6 cells (ATCC, CRL-1586) were cultured in DMEM supplemented with 5% FCS (VWR) and 1% P/S-glutamine (PSG) solution (Corning). A549-hACE2 (Biomedical Resource Ontology, NR-53821) were grown in DMEM supplemented with 4 mM l-glutamine, glucose (4500 mg/liter), 1 mM sodium pyruvate, sodium bicarbonate (1500 mg/liter), 10% FCS, 1x nonessential amino acid solution (Gibco), and blasticidin (100 µg/ml; Gibco).

Virus recovery

VSV-G-3CL\textsuperscript{pro}-L was rescued in 293T cells by CaPO\textsubscript{4} transfection of whole-genome VSV plasmids together with T7 polymerase and N, P, M, G, and L expression plasmids as helper plasmids (53). Briefly, genome and helper plasmids were transfected into 293T in the presence of 10 µM chloroquine to avoid lysosomal DNA degradation. After 6 to 16 hours, chloroquine was removed, and cells were cultured until cytopathic effects occurred. M and G proteins were used as helper plasmids; although these proteins are optional in the recovery of VSV, they were chosen here as a precaution to support the rescue of a potentially attenuated virus variant. After the rescue, viruses were passaged on 293-VSV cells and plaque-purified twice on BHK-21 cells. AP and ΔL VSV variants expressing DsRed were produced on replication supporting 293-VSV cells. VSV-G-3CL\textsuperscript{pro}-L was fully replicon competent and produced on BHK-21 cells.

**Replication kinetics, TCID\textsubscript{50} assays, and dose responses**

Initial replication kinetics (WT versus F305L) were performed as single-step kinetics. BHK-21 cells (10\textsuperscript{5} per well) were seeded in 24-well plates 1 day before infection. Cells were infected in duplicate with an MOI of 5 of VSV 3CL\textsuperscript{pro} WT or the F305L variant. One hour after infection, the medium was removed, cells were washed with phosphate-buffered saline (PBS), and fresh medium was added. Supernatant was collected at the indicated time points and stored at −80°C until further analysis. For quantification, TCID\textsubscript{50} assays were performed as described previously (54). In short, 100 µl of serial dilutions of virus were added in octuplicates to 10\textsuperscript{3} BHK-21 cells seeded in a 96-well plate. Six days after infection, the TCID\textsubscript{50} values were read out, and titers were calculated according to the Kaerber method (55).

For WT versus different mutant replication kinetics, multistep growth kinetics were performed. BHK-21 cells (10\textsuperscript{5} per well) were seeded in 24-well plates 1 day before infection. Cells were infected in duplicates with an MOI of 0.5 of VSV 3CL\textsuperscript{pro} WT or mutant variants.

For initial dose-response experiments, 5 × 10\textsuperscript{4} BHK-21 cells per well were seeded in 48-well plates 1 day before infection. Cells were infected in duplicates with an MOI of 0.05 of VSV 3CL\textsuperscript{pro} WT or mutant variants, and indicated concentrations of nirmatrelvir were added to the wells. After 48 hours, supernatants were collected and titrated to determine the TCID\textsubscript{50}.

For mutant comparing dose-response experiments, 5 × 10\textsuperscript{4} BHK-21 cells per well were seeded in 48-well plates 1 day before infection. Cells were infected in duplicates with an MOI of 0.05 of VSV 3CL\textsuperscript{pro} WT or mutant variants, and indicated concentrations of nirmatrelvir were added to the wells. To prevent initial escape or further mutation in WT or already mutation-bearing viruses (“intra-assay mutants”), respectively, supernatants of all viruses were collected after the first mutant (Q192R and F305L) showed a massive cytopathic effect at 100 µM nirmatrelvir (at about 24 hours after infection). Initial dose responses (WT versus F305L) were performed as described above, but the supernatant was collected after 48 hours.

**Viral RNA isolation and 3CL\textsuperscript{pro} sequencing**

VSV-G-3CL\textsuperscript{pro}-L RNA was isolated with the E.Z.N.A. Viral RNA Kit (Omega Bio-Tek Inc.) or the NucleoSpin RNA Virus (Macherey-Nagel GmbH). BHK-21 cells were infected with VSV-G-3CL\textsuperscript{pro}-L WT and the F305L (3CL\textsuperscript{pro}) variant in 96-well plates. Virus-containing supernatants were collected from individual 96 wells, and the RNA was purified from the supernatants according to the manufacturers’ instructions. Then, cDNA was synthesized from isolated viral RNA by the RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific). 3CL\textsuperscript{pro} sequence was amplified by PCR with primers (forward: CTCAGGTGGTCCAGAACATCCCTCAC and reverse: GATGTTGGGATGGGATTGCG) and sent for sequencing (Microsynth AG). Obtained sequences were mapped to the 3CL\textsuperscript{pro} WT (Wuhan-1) reference sequence in Geneious Prime 2022.0.2 and examined for mutations.

**Mutation selection assay**

BHK-21 cells (10\textsuperscript{4} per well) in a 96-well plate were seeded 1 day before infection with WT VSV-G-3CL\textsuperscript{pro}-L or VSV-G-3CL\textsuperscript{pro}-L-F305L at an MOI of 0.01 and indicated nirmatrelvir doses. Each virus variant occupied 48 wells of the 96-well plate. Wells that
displayed cytopathic effect after 2 days (25 of 48 from parental WT and 17 of 48 from parental F305L) were further passaged with increasing concentrations of nirmatrelvir with each passage (WT: 30, 40, and 50 μM; F305L: 50, 75, and 100 μM). Table S1 indicates at which passage a pure mutant virus could be distinguished by Sanger sequencing such that only one base pair peak appeared in the chromatogram instead of a mixture with the parental virus. Only pure mutants are displayed in Fig. 2 and table S1.

Expression and purification of His-tagged 3CL\textsuperscript{PRO} and point mutations
Plasmids containing cDNA of SARS-CoV-2 main protease 3CL\textsuperscript{PRO} [pMCSG92 (56)] and mutants thereof were prepared as described in the following. Plasmids were cloned by site-directed mutagenesis with primers of table S13 on pMCSG92. One hundred nanograms of each plasmid were applied to 50 μl of thawed competent BL21(DE3) Tuner Escherichia coli (Merck) on ice in 1.5-ml tubes. Bacterial suspensions containing the plasmids were flicked and incubated for 30 min on ice. Subsequently, bacteria were heated to 42°C for 90 s in a thermomixer without shaking and put back on ice for 5 min. Four hundred microliters of NZCYM (NZ amine, casamino acids, yeast extract, MgSO\textsubscript{4}, NaCl, and agar) medium produced in-house [10.0 g of NZ amine (Art.-Nr. CP76.1, Roth), 5.0 g of NaCl, 1.0 g of casamino acids (Gibco), 5.0 g of yeast extract (Art. Nr. 2363.2, Roth), 2.0 g of MgSO\textsubscript{4} × H\textsubscript{2}O, and 1 liter of double-distilled water (ddH\textsubscript{2}O, adjusted to pH 7.4)] were added to each bacterial suspension, and the bacteria were amplified for 1 hour at 37°C in a bacterial shaker in 1.5-ml tubes. Meanwhile, LB agar plates containing selection antibiotics (ampicillin) were prepared and incubated at room temperature. Two hundred microliters of bacterial culture were cut out on individual plates and incubated overnight at 37°C. Single colony formation was observed the following day.

Individual colonies were picked and placed in 5 ml of NZCYM medium supplemented with selection antibiotics and amplified overnight at 37°C. The next day, overnight cultures were amplified in 1 liter of NZCYM medium supplemented with selection antibiotics to an optical density of 0.2, after which protein expression was induced by applying 1 mM isopropyl-β-D-thiogalactopyranoside. After 5 hours, bacteria were harvested by centrifugation, and the supernatant was discarded. Bacterial pellets were frozen at −20°C for further use.

Pellets were suspended in 10 ml of Ni–nitritolriacetic acid (NTA) running buffer (20 mM Tris-HCl, 300 mM NaCl, and 10 mM imidazole, adjusted to pH 7.4) and transferred into 50-ml tubes. Bacteria were lysed using an ultrasonic probe on ice. The homogenates were centrifuged at 10,000g for 10 min, and the supernatant was filtered using 0.45- and 0.22-μm syringe filter units. After preparing a Ni-NTA agarose column (Invitrogen, Ni-NTA Agarose R90115) and washing with 30 ml of Ni-NTA running buffer, the filtered homogenate was applied to the column, and the flow-through was collected. The column was again washed with 3 × 10 ml of Ni-NTA running buffer. The His-tagged protein bound to the Ni-NTA resin and was then eluted with 3 × 1 ml of Ni-NTA elution buffer (20 mM Tris-HCl, 300 mM NaCl, and 200 mM imidazole, adjusted to pH 7.4). The obtained protein solutions were dialyzed at 4°C overnight against a storage buffer [1 mM Tris-HCl, 4 mM NaCl, 2.2 mM KCl, 0.04 (v/v) Tween 20, 3 mM dithiothreitol (DTT), and 20.2 (v/v) glycerol, adjusted to pH 8].

Eluted protein samples were further purified using size exclusion chromatography with fast protein liquid chromatography (ÄKTA Pure FPLC System, Superdex 200 10/300 GL). At each step of the protocol, samples for SDS–polyacrylamide gel electrophoresis (SDS-PAGE) analysis were obtained, and the successful expression of the 3CL\textsuperscript{PRO} proteins was monitored by SDS-PAGE and Coomassie R staining. The final degree of protein purity was estimated to be greater than 90% on the basis of Coomassie R staining, similar among the different preparations of 3CL\textsuperscript{PRO} WT and mutant forms.

Screening assay with FluoroSpot readout
293T cells (3 × 10\textsuperscript{5} per well) were seeded in six-well plates, transfected 1 day after seeding with 3CL\textsuperscript{PRO} plasmids using TransIT-\textsuperscript{PRO} (Mirus Bio LLC), and incubated overnight. Then, cells were trypsinized and seeded into a 96-well plate with 2 × 10\textsuperscript{4} cells per well in 50 μl of complete growth medium. Directly after seeding, compounds and virus (MOI: 0.1) were added in 50 μl of complete growth medium to wells. After 48 to 72 hours, supernatants were removed, and fluorescent spots were counted in a ELI/Fluoro/ImmuNoSpot counter (Cellular Technology Limited Europe GmbH). Longer incubation times of 72 hours increased the overall signal and were chosen to achieve a clear signal of the more resistant double mutants, which, as expected, have a lower signal output in 3CL\textsuperscript{PRO}-On assays. The manufacturer-provided software CTL switchboard 2.7.2 was used to scan 90% of each well area concentrically to exclude reflection from the well edges, and counts were normalized to the full area. Automatic fiber exclusion was applied while scanning. The excitation wavelength for DsRed was 570 nm, and the D\textsubscript{F} R triple band filter was used to collect fluorescence. In addition, manual quality control for residual fibers was performed. To increase comparability between 3CL\textsuperscript{PRO}-On and 3CL\textsuperscript{PRO}-Off signals, we normalized DsRed events with the following strategies. In 3CL\textsuperscript{PRO}-On, the highest compound concentrations did not reach equal values due to the different responsiveness of each mutant. Therefore, we normalized to the highest mean of the experiment, which was the WT signal. In 3CL\textsuperscript{PRO}-Off, untreated wells reached the same signal yield in WT and mutants. Therefore, we normalized the signal to each individual highest mean of the construct.

Screening assay with flow cytometry readout
293T cells (3 × 10\textsuperscript{5} per well) were seeded in six-well plates and transfected with 3CL\textsuperscript{PRO} plasmids using TransIT-\textsuperscript{PRO} (Mirus Bio LLC) and incubated overnight. Then, cells were trypsinized and seeded into a 96-well plate with 2 × 10\textsuperscript{4} cells per well in 50 μl. Compound and virus (MOI: 0.1) were added in 50 μl to reach desired concentrations. After 2 days, cells were detached with 0.05% trypsin-EDTA (Gibco) and transferred to a 96-well round-bottom plate ( TPP Techno Plastic Products AG) for automatic sampling by flow cytometry using a BD FACSCanto II. Gates were set to distinguish live and dead cells and to exclude doublets. Singlet cells were divided into DsRed positive and negative based on reference to samples, which were infected but not treated with inhibitor (17). Samples were analyzed using BD FACSDiva 8.0.1 (BD Biosciences).

Cross-validation with orthologous cellular Src-3CL\textsuperscript{PRO}-Tat-Luc assay
293T cells (3 × 10\textsuperscript{6} per well) were seeded in a six-well dish. Twenty-four hours later, they were transfected with 2 μg of the WT Src-3CL\textsuperscript{PRO}.Tat-Luc or mutants thereof with TransIT-\textsuperscript{LT} (Mirus,
catalog number MIR 2304). Four hours after transfection, cells were washed with PBS, trypsinized, resuspended in medium, and counted. Cells (2 × 10^6 per well) were seeded in 50 μl of medium in a flat-bottom 96-well plate (Greiner). Inhibitor dilution series were added in twofold excess to required concentrations in 50 μl of medium. After 44 hours, medium was removed, and 50 μl of Bright-Glo reagent (Promega) was added to each well. Cells were incubated for 5 min in the dark and then transferred to a white flat 96-well plate (CLS3600, Corning) for measuring luminescence on a Synergy H1 plate reader (Agilent). The percent inhibition was calculated with the following formula

\[
\% \text{ inhibition} = 100 - \left(\frac{100}{\text{relative luminescence}}\right)
\]

3CLpr enzymatic activity

WT and variant proteases were produced in-house as described in the "Expression and purification of His-tagged 3CLpr and point mutations" section. Solutions of WT 3CLpr (85 ng) and variants (170 ng) were prepared in 30 μl of appropriate buffer [20 mM Tris/HCl (pH 8), 150 mM NaCl, bovine serum albumin (0.1 mg/ml), and 1 mM DTT], and these 30 μl were added to each well in a black 96-well plate (BPS Biosciences). The substrate Ac-Abu'-Tle-Leu-Gln[MCα [acetyl-l-α-aminobutyroyl-l-tert-leucyl-l-leucyl-l-glutamine α-(4-methylcoumaryl-7-amide)] was purchased from Peptide Inc. and resuspended in dimethyl sulfoxide at 5 mM concentration. Twenty microliters of buffer with diluted substrate were then added to the protein solutions at different concentrations. The plate was immediately placed inside a GloMax Explorer reader (Promega). Fluorescence was measured by excitation at 365 nm and detected with a 415-445 nm emission filter.

To determine enzymes' initial velocities, we plotted RFU (in relative fluorescent units) on the y axis and time (in min) on the x axis. We performed a simple linear regression analysis. Fitting values from 0 up to 60 min were used in a range that had a linear increase. The resulting slopes represented the initial velocity expressed as RFU/min for each protein variant at each substrate concentration. Slope values were plotted (y axis) against the substrate concentration (x axis). Last, the obtained values were fitted using the "Michaelis-Menten" equation built into GraphPad Prism 9 to extrapolate the kinetic parameters \(K_m\) and \(V_{max}\)

\[Y = \frac{V_{max} \cdot X}{K_m + X}\]

\(k_{cat}\) was calculated by dividing \(V_{max}\) by \([E]\), where \([E]\) is the given enzyme concentration. WT and variant 3CLpr catalytic efficiencies were determined as \(k_{cat}/K_M\).

Cross-validation with biochemical 3CLpr inhibition assay

The biochemical assay used to confirm mutations was based on the 3CLpr activity assay from BPS Biosciences, catalog number 78042-2. The 3CLpr in the kit was replaced by an in-house produced 3CLpr and mutants thereof, as described in 3CLpr purification. Solutions of WT 3CLpr and mutants at 5 ng/μl in 30 μl of buffer (composition described above) were prepared according to the kit’s manual. Ten microliters of fivefold excess to tested nirmatreirvir concentrations were added to the 30 μl of 3CLpr solution and incubated for 30 min. Then, 10 μl of fluorogenic substrate (DABCYL-KTSAVLQSGFRKME-EDANS) were added (generating in total a 1:5 dilution of the excess nirmatreirvir and, therefore, final concentrations) and incubated for 4 hours. Fluorescence was measured by excitation at 365 nm and detected with a 460 nm emission filter in the GloMax Explorer. Blank (assay buffer plus substrate) values were subtracted from sample values.

Replication kinetics with recombinant SARS-CoV-2 expressing mCherry

Monolayers of Vero E6 cells (six-well plate, 10^6 cells per well, triplicates) were infected with the indicated viruses at an MOI of 0.01. After viral adsorption for 1 hour, the supernatant was discarded, the cells were washed three times with PBS, and postinfection medium (3 ml per well) was added. At the indicated time points, the supernatant (300 μl per well) was collected and titrated by plaque assay (29).

Cross-validation with recombinant SARS-CoV-2 (rWA1) expressing mCherry

A monolayer of A549-hACE2 cells was infected with 300 plaque-forming units of the indicated viruses in quadruplicates at 37°C. After viral adsorption for 1 hour, the supernatant was discarded, and the cells were washed twice with PBS. Then, phenol red-free postinfection medium (DMEM + 2% fetal bovine serum + 1% PSG) containing the indicated concentrations of nirmatreirvir was added to each well. The mCherry intensity was determined at 48 and 72 hours postinfection under a Synergy LX Multimode Reader (Agilent). Wells without drug or virus were used as negative controls or baseline signal. Positive controls were wells with virus but no drug. Infection percentages of wells with different amounts of inhibitor were calculated by subtracting the negative control (mean of wells without virus or drug) and then dividing by the positive control (mean of wells with virus but without drug). Data were analyzed in GraphPad Prism 9, and IC_{50} values were calculated as the highest dilution of the nirmatreirvir-containing sample that prevents 50% plaque formation in infected cells, determined by a sigmoidal dose-response curve (see the “Statistical analysis” section).

IC_{50} and EC_{50} calculations

In this study, different assay systems were used to generate resistance data, namely, VSV-based cellular assays with Fluorospot and flow cytometry readsouts, an orthologous cell-based assay with a luciferase readout, as well as a biochemical assay and SARS-CoV-2–mCherry assay with fluorescence readsouts. Although the magnitudes of resistance are different in these assays, the tendencies agree. We expected the dynamic range of the 3CLpr cellular assays to be greater than in a biochemical assay, where there is a fixed amount of enzyme. In cells, the continuous renewal of protease-viral fusion proteins in an excess of inhibitor likely led to a later plateauing of the signal. At lower concentrations, compound molecules are depleted and the signal plateaus. In Fluorospot readsouts, the 3CLpr. On assay data were normalized to the highest mean value in an experiment. 3CLpr. Off data were normalized to the highest value of each construct in an experiment. In the flow cytometry experiments, 3CLpr. On assay data were also normalized to the highest value of each construct in an experiment. For purified WT and mutant enzymes, IC_{50} values were determined using the biochemical assay “3CL Protease, Untagged (SARS-CoV-2)” from BPS Biosciences with the assay substrate DABCYL-
KTSAVLQSGFRKME-EDANS. IC$_{50}$ and EC$_{50}$ calculations and statistical analysis for all assays were performed with GraphPad Prism 9 (see the "Statistical analysis" section).

**Nanopore sequencing of recombinant SARS-CoV-2 (rWA1) expressing mCherry**
To validate the sequence of the recombinant SARS-CoV-2 (rWA1) expressing mCherry, we used the Nanopore sequencing "Midnight protocol," version 6 (57). Primer pools generating 1200–base pair (bp) overlapping amplicons were purchased from Integrated DNA Technologies, as referenced in the abovementioned protocol. The sequencing reactions were prepared using the Rapid Barcoding Kit SQK-RBK110.96 (Oxford Nanopore Technologies) and were performed in a sequencer (MinION Mk1B) using a proprietary flow cell (R9.4.1, Oxford Nanopore Technologies). Electrical signals were translated into nucleotide sequences (basecalling). Sequenced reads were sorted into separate files for each sample (demultiplexing). Demultiplexing was done using the super high accuracy model in Guppy 6.1.5. Output sequences generated so-called fastq files, and sequences below 200 and above 1200 bp were removed. Sequences between 200 and 1200 bp were assembled with the algorithm epityme-labs/wf-artic v0.3.18 in Nextflow 22.04.4. The SARS-CoV-2 lineage pangolin 4.1.1 was used to map the sequences. A visualization application (Nextclade 2.4.0) was used to check mutations.

**Protein structure preparation for molecular modeling**
The three-dimensional structure of the SARS-CoV-2 3CL$_{pro}$ complexed with nirmatrelvir was retrieved from the PDB [PDB code: 7RFW; method: x-ray diffraction; resolution: 1.73 Å (32)] and prepared for molecular modeling evaluations exploiting several tools implemented in the MOE 2022.02 suite (16). Specifically, the "Structure Preparation" tool was used to assign each protein residue with alternative conformations to the one characterized by the highest occupancy value, and the "Proteon 3D" program was exploited to assign each titratable amino acid to the most appropriate protonation state at a pH of 7.4. Last, the coordinates of hydrogen atoms were energy-minimized using the AMBER10:EHT force field (58) until a gradient of 0.1 kcal mol$^{-1}$ Å$^{-2}$ was reached.

**In silico alanine and resistance mutation scanning**
An in silico evaluation of the impact of SARS-CoV-2 3CL$_{pro}$ mutations on both the stability of the protein and the affinity toward nirmatrelvir was conducted using the "Protein Design" module of MOE using the previously mentioned complex structure. In particular, the "Alanine Scan" and "Resistance Scan" tools were used to perform two virtual mutagenesis experiments.

First, we applied the Alanine Scan interface, in which each of the 612 amino acids composing the dimeric SARS-CoV-2 3CL$_{pro}$ was mutated into an alanine residue, calculating at each given time the energy difference between the mutated protein and the WT form concerning both the potential energy of the protein itself (dStability) and the affinity toward nirmatrelvir (dAffinity). Values were then color-plotted on the crystal complex of nirmatrelvir and SARS-CoV-2 3CL$_{pro}$ using UCSF Chimera (59). Then, we used the Resistance Scan interface to investigate the impact of a selected pool of mutations: Y54C, G138S, L141F, L167F, and Q192R. For both types of calculations, the conformational sampling was carried out through LowModeMD (60) using the AMBER10:EHT forced field coupled with the generalized Born implicit solvent model (61); the dAffinity value was determined through the GBVI/VSA (62) method.

**Statistical analysis**
Raw and normalized data are provided in data file S3. Dose-response data points of 3CL$_{pro}$-On, 3CL$_{pro}$-Off, and biochemical assays were fitted using a four-parameter logistic regression (sigmoid, 4PL, $X$ is the concentration). IC$_{50}$ values were extrapolated as the concentration value at which the signal was 50% between the top and bottom plateaus of each subdataset. Dose-response curves of the Src-3CL$_{pro}$-Tat-Luc–based assay were fitted with the same regression, setting $X$ as 2 for IC$_{50}$ extrapolation

$$Y = \frac{\text{Top} - \text{Bottom}}{1 + (\text{IC}_{50}/X)^{\text{HillSlope}}} + \text{Bottom}$$

Data obtained with flow cytometry were normalized and fitted using the nonlinear regression function "[agonist] versus normalized response." The EC$_{50}$ values were extrapolated as the medium value between the top and bottom plateaus of each sub-dataset

$$Y = \frac{100 \times X}{\text{EC}_{50} + X}$$

Nirmatrelvir dose-response curves of recombinant SARS-CoV-2 expressing mCherry were normalized and fitted using the nonlinear regression function "log(inhibitor) versus normalized response – variable slope"

$$Y = \frac{100}{1 + 10^{\log(\text{IC}_{50} - X)} \cdot \text{HillSlope}}$$

Kinetic parameters and catalytic activity of WT and mutant 3CL$_{pro}$ enzymes were calculated as described in the corresponding method section. The 95% confidence intervals were generated by the described fittings and are provided in supplementary tables together with IC$_{50}$ and EC$_{50}$ values. All statistical analyses were performed with GraphPad Prism 9.

**Supplementary Materials**
This PDF file includes:
Fig. S1 to S11
Table S1 to S13

Other Supplementary Material for this manuscript includes the following:
MDAR Reproducibility Checklist
Data files S1 to S4

View/request a protocol for this paper from Bio-protocol.

**REFERENCES AND NOTES**
1. P. Zhou, X.-L. Yang, X.-G. Wang, B. Hu, L. Zhang, W. Zhang, H.-R. Si, Y. Zhu, B. Li, C.-L. Huang, H.-D. Chen, J. Chen, Y. Luo, H. Guo, R.-D. Jiang, M.-Q. Liu, Y. Chen, X.-R. Shen, X. Wang, X.-S. Zheng, K. Zhao, Q.-J. Chen, F. Deng, L.-L. Liu, B. Yan, F.-X. Zhan, Y.-Y. Wang, G.-F. Xiao, Z.-L. Shi, A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* **579**, 270–273 (2020).
2. R. Voelker, Combination drug for HCV infection. *JAMA* **318**, 790 (2017).
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3. M. Harrington, C. C. J. Carpenter, Hit HIV-1 hard, but only when necessary. Lancet 355, 2147–2152 (2000).

4. K. Fan, P. Wei, Q. Feng, S. Chen, C. Huang, L. Ma, B. Lai, J. Pei, Y. Liu, J. Chen, L. Lai, Bio-
synthesis, purification, and substrate specificity of severe acute respiratory syndrome co-
ronavirus 3C-like protease. J. Biol. Chem. 279, 1637–1642 (2004).

5. B. H. Harcourt, D. Jukneliene, A. Kanjanahaluethai, J. Bechill, K. M. Severson, C. M. Smith,
A. Padhi, T. Tripathi, Hotspot residues and resistance mutations in the nirmatrelvir.

6. A. M. Kaufer, T. Theis, K. A. Lau, J. L. Gray, W. D. Rawlinson, Laboratory biosafety
measures involving SARS-CoV-2 and the classification as a Risk Group 3 biological agent.

7. S. Khar e, C. Gurry, L. Freitas, M. B. Schultz, G. Bach, P. A. Rota, S. C. Baker, Identification of severe acute respiratory syndrome coronavirus replicate products and characterization of papain-like protease activity. J. Virol. 78, 13630–13612 (2004).

8. S. E. Greasley, S. Noell, O. Plotnikova, R. Ferre, W. Liu, B. Bolanos, K. Fennell, J. Nicki, T. Craig,
and confer drug resistance to nirmatrelvir. bioRxiv 2022.06.28.497978 [Preprint]

9. D. E. Kim, D. Chivian, D. Baker, Protein structure prediction and analysis using the Robetta
inhibitors of cysteine proteases containing the nitrile warhead: Recent advancement in the
nirmatrelvir against SARS-CoV-2 variants. J. Biol. Chem. 298, 101972 (2023).

10. S. Iketani, H. Mohri, B. Cullbertson, S. J. Hong, D. Man, L. M. Luck, K. M. Annawajaya, Y. Guo,
L. Silvestrini, N. Belhaj, L. Coomez, Y. Gerelli, A. Lauria, V. Liberà, P. Mariani, P. Marzullo,
and confer resistance to nirmatrelvir. bioRxiv 2022.08.07.503098 [Preprint]

11. Y. Hui, E. M. Lewandowski, H. Tan, R. T. Morgan, X. Zhang, L. M. C. Jacobs, G. B. Butler,
J. Morrison, G. A. Weiss, Combinatorial alanine-scanning. Curr. Opin. Chem. Biol. 5, 302–307 (2001).

12. D. E. Kim, D. Chivian, D. Baker, Protein structure prediction and analysis using the Robetta
nirmatrelvir to resistance of nirmatrelvir. bioRxiv 2022.06.28.497978 [Preprint]

13. A. K. Padhi, T. Tripathi, Hotspot residues and resistance mutations in the nirmatrelvir-
2023.02.11, 1010–1780 (2018).

14. M. J. Gray, W. D. Rawlinson, Laboratory biosafety measures involving SARS-CoV-2 and the classification as a Risk Group 3 biological agent. Pathology 52, 790–795 (2020).

15. J. R. Bristow, Virus Variation Resource - improved response to emergent viral outbreaks.

16. M. Rut, K. Groborz, L. Zhang, X. Sun, M. Zmudzinski, B. Pawlik, X. Wang, D. Chivian, J. Neyts, W. Młynarski, R. Hilgenfeld, M. Drag, SARS-CoV-2 Mpro inhibitors and activity-based probes for patient-sample imaging. Nat. Chem. Biol. 17, 222–228 (2021).

17. A. Manandhar, V. Sinivasulu, M. Harmand, H. Tanazi, H. Omar, D. J. Colussi, J. Gordon,
W. Childers, M. L. Klein, T. H. Al-Tel, M. Abou-Gharbia, K. M. Eloey, Discovery of novel small-
improving viruses. Proc. Natl. Acad. Sci. U.S.A. 118, e211193118 (2021).

18. C. Ye, K. Chiem, J.-Q. Park, J. A. Silvas, D. Morales Vasquez, J. Sour苒nt, M. J. Lin, A. L. Greninger, R. K. Plumper, J. B. Terrelles, J. J. Kobie, R. M. Walter, J. C. de la Torre, L. Martín-Sobrido, Analysis of SARS-CoV-2 infection dynamics in vivo using reporter-ex-
1022.02.11, 1010–1780 (2018).

19. L. Silvestrini, N. Belhaj, L. Coomez, Y. Gerelli, A. Lauria, V. Liberà, P. Mariani, P. Marzullo,
and confer resistance to nirmatrelvir. bioRxiv 2022.06.28.497978 [Preprint]

20. J. R. Bristow, Virus Variation Resource - improved response to emergent viral outbreaks.

21. M. Rut, K. Groborz, L. Zhang, X. Sun, M. Zmudzinski, B. Pawlik, X. Wang, D. Chivian, J. Neyts, W. Młynarski, R. Hilgenfeld, M. Drag, SARS-CoV-2 Mpro inhibitors and activity-based probes for patient-sample imaging. Nat. Chem. Biol. 17, 222–228 (2021).

22. A. Manandhar, V. Sinivasulu, M. Harmand, H. Tanazi, H. Omar, D. J. Colussi, J. Gordon,
W. Childers, M. L. Klein, T. H. Al-Tel, M. Abou-Gharbia, K. M. Eloey, Discovery of novel small-
improving viruses. Proc. Natl. Acad. Sci. U.S.A. 118, e211193118 (2021).

23. C. Ye, K. Chiem, J.-Q. Park, J. A. Silvas, D. Morales Vasquez, J. Sour苒nt, M. J. Lin, A. L. Greninger, R. K. Plumper, J. B. Terrelles, J. J. Kobie, R. M. Walter, J. C. de la Torre, L. Martín-Sobrido, Analysis of SARS-CoV-2 infection dynamics in vivo using reporter-ex-
inhibitors of cysteine proteases containing the nitrile warhead: Recent advancement in the
nirmatrelvir against SARS-CoV-2 variants. J. Biol. Chem. 298, 101972 (2023).

24. S. Khar e, C. Gurry, L. Freitas, M. B. Schultz, G. Bach, P. A. Rota, S. C. Baker, Identification of severe acute respiratory syndrome coronavirus replicate products and characterization of papain-like protease activity. J. Virol. 78, 13630–13612 (2004).

25. A. K. Padhi, T. Tripathi, Hotspot residues and resistance mutations in the nirmatrelvir-
2023.02.11, 1010–1780 (2018).

26. M. J. Gray, W. D. Rawlinson, Laboratory biosafety measures involving SARS-CoV-2 and the classification as a Risk Group 3 biological agent. Pathology 52, 790–795 (2020).

27. S. A. Moghadasi, M. A. Esler, Y. Otsuka, J. T. Becker, S. N. Maroae, C. B. Anderson, S. Chakamakui, C. Belica, C. Wick, D. A. Harki, D. W. Young, L. Scampavia, T. P. Spicer, K. Shi, H. Ahara, W. L. Brown, R. S. Harris, Gain-of-signal assays for probing inhibition of SARS-CoV-2 Mpro/C3CL19β in living cells. MBio 13, e0078422 (2022).

28. H. Muke, H. Yotsuyanagi, N. Omigari, Y. Doi, T. Imamura, T. Sonoyama, T. Fukuura,
and confer drug resistance to nirmatrelvir. bioRxiv 2022.06.28.497978 [Preprint]

29. J. R. Bristow, Virus Variation Resource - improved response to emergent viral outbreaks.

30. M. Rut, K. Groborz, L. Zhang, X. Sun, M. Zmudzinski, B. Pawlik, X. Wang, D. Chivian, J. Neyts, W. Młynarski, R. Hilgenfeld, M. Drag, SARS-CoV-2 Mpro inhibitors and activity-based probes for patient-sample imaging. Nat. Chem. Biol. 17, 222–228 (2021).

31. A. Manandhar, V. Sinivasulu, M. Harmand, H. Tanazi, H. Omar, D. J. Colussi, J. Gordon,
W. Childers, M. L. Klein, T. H. Al-Tel, M. Abou-Gharbia, K. M. Eloey, Discovery of novel small-
improving viruses. Proc. Natl. Acad. Sci. U.S.A. 118, e211193118 (2021).

32. C. Ye, K. Chiem, J.-Q. Park, J. A. Silvas, D. Morales Vasquez, J. Sour苒nt, M. J. Lin, A. L. Greninger, R. K. Plumper, J. B. Terrelles, J. J. Kobie, R. M. Walter, J. C. de la Torre, L. Martín-Sobrido, Analysis of SARS-CoV-2 infection dynamics in vivo using reporter-ex-

molecule inhibitors of SARS-CoV-2 main protease as potential leads for COVID-19 treatment. J. Chem. Inf. Model. 61, 4745–4757 (2021).

46. D. R. Owen, C. M. N. Allerton, A. S. Anderson, L. Aschenbrenner, M. Avery, S. Berritt, B. Boras, R. D. Cardin, A. Carlo, K. J. Coffman, A. Dantonio, L. Di, H. Eng, R. Ferre, K. S. Gajiwala, S. A. Gibson, S. E. Greasley, B. L. Hurst, E. P. Kadar, A. S. Kalgutkar, J. C. Lee, J. Lee, W. Liu, S. W. Mason, S. Noell, J. J. Novak, R. S. Obach, K. Ogilvie, N. C. Patel, M. Pettersson, D. K. Rai, M. R. Reese, M. F. Sammons, J. G. Sathish, R. S. P. Singh, C. M. Steppan, A. E. Stewart, J. B. Tuttle, L. Updyke, P. R. Verhoest, L. Wei, Q. Yang, Y. Zhu, An oral SARS-CoV-2 Mpro inhibitor clinical candidate for the treatment of COVID-19. Science 374, 1586–1593 (2021).

47. A. Manandhar, B. E. Blass, D. J. Colussi, I. Almi, M. Abou-Gharbia, M. L. Klein, K. M. Eleyoke. Targeting SARS-CoV-2 M33Cpro by HCV NS3/4a inhibitors: In silico modeling and in vitro screening. J. Chem. Inf. Model. 61, 1020–1032 (2022).

48. M.-F. Hsu, C.-J. Kuo, K.-T. Chang, H.-C. Chang, C.-C. Chou, T.-P. Ko, H.-L. Shy, G.-G. Chang, A.-H. J. Wang, P.-H. Liang. Mechanism of the maturation process of SARS-CoV 3CL protease. J. Biol. Chem. 280, 31257–31266 (2005).

49. T. Muramatsu, C. Takemoto, Y.-T. Kim, H. Wang, W. Nishii, T. Terada, M. Shirouzu, S. Yokoyama. SARS-CoV 3CL protease cleaves its C-terminal autoprocessing site by novel subite cooperativity. Proc. Natl. Acad. Sci. 113, 12997–13002 (2016).

50. D. G. Gibson, L. Young, R.-Y. Chuang, J. C. Venter, C. A. Hutchison, H. O. Smith, Enzymatic sequencing of SARS-CoV-2 using 1200bp tailed amplicons and Oxford Nanopore Rapid Science resistance of human ovarian cancer to VSV-GP oncolytic viral therapy. C. Marth, J. Kimpel, D. VonLaer, Application of interferon modulatorsto overcome partial Naunyn Schmiedebergs Arch. Exp. Pathol. Pharmakol. 16021(2016).

51. M. J. Schnell, L. Buonocore, M. A. Whitt, J. K. Rose, The minimal conserved transcription stop-start signal promotes stable expression of a foreign gene in vesicular stomatitis virus. J. Virol. 70, 2318–2323 (1996).

52. D. Panda, P. X. Dinh, L. K. Beaus, A. K. Pattnaik, Induction of interferon and interferon signaling pathways by replication of defective interfering particle RNA in cells constitutively expressing vesicular stomatitis virus replication proteins. J. Virol. 84, 4826–4831 (2010).

53. S. E. Witko, C. S. Kotash, R. M. Nowak, J. E. Johnson, L. A. C. Boutillier, K. J. Melville, S. G. Heron, D. K. Clarke, A. S. Abramovitz, R. M. Hendry, M. S. Sidhu, S. A. Udem, C. L. Parks, An efficient helper-virus-free method for rescue of recombinant paramyxoviruses and rhinoviruses from a cell line suitable for vaccine development. J. Virol. Methods 135, 91–101 (2006).

54. C. Dold, C. Rodríguez Urbiola, G. Wollmann, L. Egerer, A. Muik, L. Bellmann, H. Fieg!, C. Marth, J. Kimpel, D. Von Laer, Application of interferon modulators to overcome partial resistance of human ovarian cancers to VSV-GP oncolytic viral therapy. Mol. Ther. Oncolytics 3, 16021 (2016).

55. G. Kärber, Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Naunyn Schmiedebergs Arch. Exp. Pathol. Pharmacol. 160, 480–483 (1931).

56. L. Zhang, D. Lin, X. Sun, U. Curth, C. Drosten, L. Sauererhing, S. Becker, K. Rox, R. Hilgenfeld, Crystal structure of SARS-CoV-2 main protease provides a basis for design of improved a-ketoamide inhibitors. Science 368, 409–412 (2020).

57. N. E. Freed, M. Vlkova, M. B. Faisal, O. K. Silander, Rapid and inexpensive whole-genome sequencing of SARS-CoV-2 using 1200 bp tiled amplicons and Oxford Nanopore Rapid Barcoding. Biol. Methods Protoc. 5, bpa014 (2020).

58. D. A. Case, T. Darden, T. E. Cheatham III, C. Simmerling, J. Wang, R. E. Duke, R. Luo, M. Crowley, R. Walker, W. Zhang, K. M. Merz, B. Wang, S. Hayik, A. Rottberg, G. Seabra, I. Kolossvary, K. F. Wong, F. Paesani, J. Vanicek, W. Xu, S. R. Brozell, T. Steinbrecher, H. Gohlke, L. Yang, C. Tan, J. Morgan, V. Hornak, G. Cui, D. H. Mathews, M. G. Seetin, C. Sagui, V. Babine, P. A. Kollman, Amber 10: User’s Manual (University of California, San Francisco, 2008).

59. E. F. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng, T. E. Ferrin, UCSF Chimera—A visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612 (2004).

60. P. Labute, LowModeMD—Implicit low-mode velocity filtering applied to conformational search of macrocycles and protein loops. J. Chem. Inf. Model. 50, 792–800 (2010).

61. A. Onufriev, D. A. Case, D. Bashford, Effective Born radii in the generalized Born approximation: The importance of being perfect. J. Comput. Chem. 23, 1297–1304 (2002).

62. P. Labute, The generalized born/volume integral implicit solvent model: Estimation of the free energy of hydration using London dispersion instead of atomic surface area. J. Comput. Chem. 29, 1693–1698 (2008).

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