Cite as: J. Qiao *et al.*, *Science*
10.1126/science.abf1611 (2021).

SARS-CoV-2 M^{pro} inhibitors with antiviral activity in a transgenic mouse model

Jingxin Qiao^{1*}, Yue-Shan Li^{1*}, Rui Zeng^{1*}, Feng-Liang Liu^{2,3*}, Rong-Hua Luo^{2,3*}, Chong Huang^{1*}, Yi-Fei Wang^{4*}, Jie Zhang¹, Baoxue Quan¹, Chenjian Shen¹, Xin Mao¹, Xinlei Liu¹, Weining Sun¹, Wei Yang¹, Xincheng Ni¹, Kai Wang¹, Ling Xu^{2,3}, Zi-Lei Duan^{2,3}, Qing-Cui Zou³, Hai-Lin Zhang^{3,5}, Wang Qu³, Yang-Hao-Peng Long³, Ming-Hua Li³, Rui-Cheng Yang¹, Xiaolong Liu¹, Jing You¹, Yangli Zhou¹, Rui Yao¹, Wen-Pei Li¹, Jing-Ming Liu¹, Pei Chen⁴, Yang Liu¹, Gui-Feng Lin¹, Xin Yang¹, Jun Zou¹, Linli Li⁴, Yiguo Hu¹, Guang-Wen Lu¹, Wei-Min Li¹, Yu-Quan Wei¹, Yong-Tang Zheng^{2,3†}, Jian Lei^{1,6†}, Shengyong Yang^{1†}

¹State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China. ²Key Laboratory of Animal Models and Human Disease Mechanisms of the Chinese Academy of Sciences, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China.

³Kunming National High-level Biosafety Research Center for Non-human Primates, Center for Biosafety Mega-Science, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650107, China. ⁴Key Laboratory of Drug Targeting and Drug Delivery Systems, Ministry of Education, West China School of Pharmacy, Sichuan University, Chengdu, Sichuan 610041, China. ⁵State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China. ⁶National Clinical Research Center for Geriatrics, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China.

*These authors contributed equally to this work.

†Corresponding author. Email: yangsy@scu.edu.cn (S.Y.); lejian@scu.edu.cn (J.L.); zhengyt@mail.kiz.ac.cn (Y.-T.Z.)

The COVID-19 pandemic caused by the SARS-CoV-2 virus continually poses serious threats to global public health. The main protease (M^{pro}) of SARS-CoV-2 plays a central role in viral replication. We designed and synthesized 32 new bicyclic proline-containing M^{pro} inhibitors derived from either Boceprevir or Telaprevir, both of which are approved antivirals. All compounds inhibited SARS-CoV-2 M^{pro} activity in vitro with IC₅₀ values ranging from 7.6 to 748.5 nM. The co-crystal structure of M^{pro} in complex with MI-23, one of the most potent compounds, revealed its interaction mode. Two compounds (MI-09 and MI-30) showed excellent antiviral activity in cell-based assays. In a SARS-CoV-2 infection transgenic mouse model, oral or intraperitoneal treatment with MI-09 or MI-30 significantly reduced lung viral loads and lung lesions. Both also displayed good pharmacokinetic properties and safety in rats.

The COVID-19 pandemic is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1–3). Despite intensive countermeasures implemented around the world, morbidity and mortality remain high with many countries facing a new wave of infections (4, 5). Due to limited antiviral agents available to combat SARS-CoV-2 infection, developing specific antiviral drugs against SARS-CoV-2 is urgently needed.

SARS-CoV-2 is an enveloped positive-sense single-stranded RNA (+ssRNA) virus, belonging to the genus *Betacoronavirus* (1–3, 6). This virus contains a ~30 kb RNA genome, encoding two large overlapping polyprotein precursors: pp1a and pp1ab, four structural proteins (Spike, Envelope, Membrane, and Nucleocapsid), and several accessory proteins (1, 2, 6). The cleavage of the two polyproteins (pp1a/pp1ab) into individual non-structural proteins is es-

sential for viral genome replication. This cleaving process is performed by two viral proteases: main protease (M^{pro}, also named 3CL protease) and papain-like protease (7). These viral proteases are thus important antiviral targets (8, 9). Notably, M^{pro} exclusively cleaves polypeptides after a glutamine (Gln) residue, and no any known human protease displays the same cleavage specificity as M^{pro} (9, 10). This may allow development of drugs that are specific to M^{pro} to reduce potential side effects.

Despite some SARS-CoV-2 M^{pro} inhibitors being reported (11–21) and a dipeptidyl inhibitor by Pfizer entering the Phase I clinical trials (14, 15, 22), previous literature on inhibitors of SARS-CoV-2 M^{pro} (11–22) has not included infection data in an animal model. Here we describe the design of 32 new SARS-CoV-2 M^{pro} inhibitors and two of them showing effective antiviral activity in mice.

The design of SARS-CoV-2 M^{pro} inhibitors was based on the reported crystal structures of SARS-CoV-2 M^{pro} (1I-13) and our co-crystal structures of SARS-CoV-2 M^{pro} in complex with the approved antivirals against hepatitis C virus (HCV) infection, Boceprevir (PDB entry: 7COM) and Telaprevir (PDB entry: 7C7P) (fig. S1). The active site of M^{pro} is composed of four sites (S1', S1, S2 and S4), which often accommodate four fragments (P1', P1, P2 and P3, respectively) of peptidomimetic inhibitors (8, 10). In our design of new inhibitors (Fig. 1), we fixed P1 as an optimal fragment, used P2 being derived from either Boceprevir or Telaprevir, and allowed P3 to change. First, an aldehyde was used as the warhead in P1 to form a covalent bond with the catalytic site Cys145, which is essential for the antiviral activity (13). Comparing with other bulky warheads, the small and high electrophilic aldehyde has been reported to be more potent (7, 10, 20, 22). However, the clinical safety of the generated aldehydes remains to be determined because of possible off-target effects due to the high electrophilicity of aldehyde (23). Second, we chose a 5-membered ring (γ -lactam) derivative of glutamine to occupy the S1 site of M^{pro}, which not only mimics the native P1 glutamine of the substrates but also increases the activity of inhibitors (24, 25). Third, we used a bicycloproline moiety, either (1R,2S,5S)-6,6-dimethyl-3-aza-bicyclo[3.1.0]hexane-2-formamide (P2 of Boceprevir) or (1S,3aR,6aS)-octahydrocyclopenta[c]pyrrole-1-formamide (P2 of Telaprevir), as a P2 fragment. This was inspired from our co-crystal structures of SARS-CoV-2 M^{pro} in complex with Boceprevir and Telaprevir (fig. S1), in which the two bicycloproline moieties suitably occupy the S2 pocket of M^{pro}. Particularly, the rigid and hydrophobic bicycloproline is expected to increase the drug exposure in vivo (26). Finally, by analyzing the characteristics of the S4 site of M^{pro}, we decided to use hydrophobic subgroups with medium size for P3, to enhance potency and pharmacokinetic (PK) properties. We thus designed and synthesized 32 compounds with various P3 fragments (fig. S2, MI-01 – MI-32). Among these compounds, MI-01 – MI-14 contain P2 of Boceprevir, while MI-15 – MI-32 include P2 of Telaprevir. Chemical structures (fig. S2), synthetic routes and characterization by NMR and ESI-MS spectra of these compounds are provided in supplementary materials.

Biochemical activities of the 32 compounds against SARS-CoV-2 M^{pro} were determined by a fluorescence resonance energy transfer (FRET) assay. For this, recombinant SARS-CoV-2 M^{pro} protein was prepared. The k_{cat}/K_m value of the recombinant protein was determined as $50656 \pm 4221 \text{ M}^{-1}\text{S}^{-1}$, similar to the previous result (11). In the FRET assay, all 32 compounds (MI-01 – MI-32) showed potent inhibitory activities on SARS-CoV-2 M^{pro}, with IC₅₀ values ranging from 7.6 to 748.5 nM (table S1). Of them, 24 compounds displayed two-digit nanomolar IC₅₀ values, and 3 exhibited single-digit

values (MI-21: 7.6 nM; MI-23: 7.6 nM; MI-28: 9.2 nM). The positive controls GC376 and 11b, two of the most potent SARS-CoV-2 M^{pro} inhibitors reported (13, 17), exhibited IC₅₀ values of 37.4 nM and 27.4 nM in the same assay, respectively. Next, a differential scanning fluorimetry (DSF) assay was performed to validate the direct binding between our compounds and SARS-CoV-2 M^{pro}. All the compounds displayed large thermal shifts (ΔT_m) ranging from 12.5°C to 21.7°C (table S1), indicating their tight binding to SARS-CoV-2 M^{pro}. It is worth mentioning that the two different bicycloproline moieties (P2) did not significantly impact the inhibitory activities and binding abilities (table S1 and fig. S2, e.g., MI-03 *vs* MI-21, MI-12 *vs* MI-28, and MI-14 *vs* MI-30).

To illustrate the detailed binding mode of our compounds with SARS-CoV-2 M^{pro}, we determined the 2.0 Å structure of M^{pro} in complex with one of the most active compounds, MI-23 (IC₅₀ = 7.6 nM) (Fig. 2, A to D). The crystal of M^{pro}-MI-23 complex belongs to the space group C2 (table S2) with one molecule per asymmetric unit. The biological dimer of M^{pro} is formed by an M^{pro} monomer and its symmetry-mate across the crystallographic two-fold axis (Fig. 2A). MI-23 binds to the active site of M^{pro} as expected (Fig. 2, C and D). The carbon of the warhead aldehyde interacts with the sulfur atom of catalytic residue Cys145 to form a 1.8 Å covalent bond (Fig. 2C). The oxygen of the aldehyde forms two hydrogen bonds with the main-chain amides of Cys145 and Gly143 (forming the “oxyanion hole”) (Fig. 2D). The P1 γ -lactam ring of MI-23 inserts deeply into the S1 pocket. The oxygen and nitrogen of lactam form two hydrogen bonds with the side chain of His163 (2.8 Å) and the main chain of Phe140 (3.3 Å), respectively. The main-chain amide of P1 makes a 2.9 Å hydrogen bond with the backbone O of His164. Based on the chemical property of proline (27), the rigid P2 bicycloproline of MI-23 adopts the *trans-exo* conformation with restricted N- α bond rotation (the ϕ torsion angle is about -61.8°). This causes the bicycloproline group to point to the hydrophobic S2 pocket, where it forms hydrophobic interactions with C γ of Met165, C β and C γ of Gln189 and His41, C ϵ of Met49 as well as the backbone C and C α of Asp187 and Arg188. The backbone oxygen of P3 interacts with the backbone amide of Glu166 with a 2.9 Å hydrogen bond. The 1-ethyl-3,5-difluorobenzene moiety of P3 shows an extended conformation and occupies the S4 site. This moiety forms hydrophobic interactions with C γ of Gln189, C α and backbone C of Leu167 and Pro168 (Fig. 2D). The benzene ring of P3 also forms a very weak hydrophobic interaction with Gly251 from an adjacent translational symmetry protomer due to crystal packing. Overall, the binding pattern of the representative compound MI-23 with M^{pro} is consistent with our design concept.

We then selected 20 compounds with IC₅₀ < 50 nM in

the enzyme inhibition assay to examine their cytotoxicity and cellular antiviral activity. First, the cytotoxicity of these compounds was evaluated using the CCK8 assay, and no compounds showed cytotoxicity ($CC_{50} > 500 \mu\text{M}$) in the cell lines tested, including Vero E6, HPAEpiC, LO2, BEAS-2B, A549 and Huh7 cells (tables S3 and S4).

Next, the cellular antiviral activity was examined by a cell protection assay. In this assay, the viability of SARS-CoV-2 infected Vero E6 cells with or without treatment with the compounds was assessed using CCK8. All the compounds dose-dependently protected cells from death with 50% effective concentration (EC_{50}) values ranging from 0.53 to 30.49 μM (table S4). Of note, six compounds, including MI-09 (0.86 μM), MI-12 (0.53 μM), MI-14 (0.66 μM), MI-28 (0.67 μM), MI-30 (0.54 μM), and MI-31 (0.83 μM), exhibited nanomolar or low micromolar EC_{50} values (Fig. 3A). We noticed that some compounds (e.g., MI-22 and MI-25) with high potency in the enzymatic assay showed marginal activity in the cell protection assay, perhaps due to relatively low lipophilic groups in P3 and the resulting poor cell membrane permeability (28). Real-time quantitative PCR (RT-qPCR) revealed that all of the six compounds inhibited SARS-CoV-2 virus replication in HPAEpiC cells with low nanomolar EC_{50} values (0.3 – 7.3 nM) (Fig. 3B). In the same CCK8 and RT-qPCR assays, the positive control GC376 showed EC_{50} values of 1.46 μM and 153.1 nM, respectively, and the corresponding values for 11b are 0.89 μM and 23.7 nM. To further corroborate the antiviral potency of these compounds, RT-qPCR was conducted in another cell line, Huh7. The six compounds showed antiviral EC_{50} values of 31.0 – 96.7 nM, while GC376 and 11b displayed EC_{50} values of 174.9 nM and 74.5 nM, respectively (fig. S5).

To identify which of the six compounds is suitable for in vivo antiviral studies, pharmacokinetic (PK) experiments were conducted in Sprague-Dawley (SD) rats. Two compounds MI-09 and MI-30 showed relatively good PK properties with oral bioavailability of 11.2% and 14.6%, respectively (table S5). As a compound with oral bioavailability of above 10% has a potential for development as an oral drug (29), MI-09 and MI-30 were selected for further in vivo antiviral study. The key PK parameters of MI-09 and MI-30 are briefly summarized in Fig. 4, A and B. When administered intravenously (i.v.) (10 mg/kg), intraperitoneally (i.p.) (20 mg/kg) and orally (p.o.) (20 mg/kg), MI-09 showed area under the curve (AUC) values of 7429 hours*ng/mL, 11581 hours*ng/mL, and 1665 hours*ng/mL, respectively, while MI-30 displayed AUC values of 9768 hours*ng/mL, 14878 hours*ng/mL, and 2843 hours*ng/mL, respectively. Following i.p. administration, MI-09 or MI-30 displayed a half-life ($T_{1/2}$) of 4.53 hours or 3.88 hours, a bioavailability of 78.0% or 76.2%, and a clearance rate (CL) of 22.67 mL/min/kg or 17.10 mL/min/kg, respectively. Based on the EC_{50}/EC_{90} val-

ues from HPAEpiC cells, a single i.p. dose of 20 mg/kg/d MI-09 or MI-30 maintained the plasma levels at the EC_{50} (1.2 nM for MI-09, 1.1 nM for MI-30) and EC_{90} (47.9 nM for MI-09, 58.8 nM for MI-30) for about 24 hours and 6 hours (fig. S3, A and B), respectively. Also, a single p.o. dose of 20 mg/kg/d MI-09 or MI-30 sustained the plasma levels at the EC_{50} and EC_{90} for about 10 hours and 6 hours (fig. S3, C and D), respectively. While, according to the EC_{50}/EC_{90} values from Vero E6 cells, with a single i.p. dose of 20 mg/kg/d MI-09 or MI-30, the duration of drug plasma levels at above EC_{50} (0.86 μM for MI-09, 0.54 μM for MI-30) and EC_{90} (3.62 μM for MI-09, 2.12 μM for MI-30) were about 3 hours and 2 hours, respectively. A single p.o. dose of 20 mg/kg/d MI-09 or MI-30 made the drug plasma concentrations reach to EC_{50} but not to EC_{90} in Vero E6 cells.

MI-09 and MI-30 were then evaluated for their toxicity in SD rats. In an acute toxicity experiment, no rats died after i.v. (40 mg/kg), i.p. (250 mg/kg) or p.o. (500 mg/kg) treatment with either MI-09 or MI-30 (table S6). In a repeated dose toxicity study, treatment with MI-09 or MI-30 by i.v. at 6 and 18 mg/kg/d, i.p. at 100 and 200 mg/kg/d or p.o. at 100 and 200 mg/kg twice daily for 7 consecutive days did not result in noticeable toxicity in the animals (table S6).

Further, we investigated the in vivo antiviral activity of our compounds in a human angiotensin-converting enzyme 2 (hACE2) transgenic mouse model, which is susceptible to SARS-CoV-2 (30). In our pilot study, hACE2 transgenic mice were intranasally inoculated with SARS-CoV-2 (2×10^6 TCID₅₀ virus/mouse), and treated with vehicle (control), MI-09 (50 mg/kg p.o. bid or 50 mg/kg i.p. qd) or MI-30 (50 mg/kg i.p. qd) starting at 1 hour prior to virus inoculation (Fig. 4C) and continuing until 5 days post infection (5 dpi). During the 6-day period, no abnormal behaviors and body weight loss were observed in any animals tested. On 1 dpi, the mean viral RNA loads in the lung tissues of the three treatment groups were significantly ($P < 0.05$, Student's *t*-test) lower than that of the control group (Fig. 4D). On 3 dpi and 5 dpi, the viral RNA loads in the lung tissues of treatment groups were almost undetectable, and those of control group were also very low (below the limit of detection, LOD), which might be due to the mild degree of infection.

We thus increased the virus challenge dose of SARS-CoV-2 to 5×10^6 TCID₅₀, which mimics a moderate infection. The mice were treated as described above, except that the doses increased to 100 mg/kg for both i.p. and p.o. administration of MI-09 and MI-30 (Fig. 4C). The higher dose of virus challenge led to a higher level of viral loads in the lungs of infected mice as expected. The mean viral RNA loads in the lung tissues of the three treatment groups were slightly on 1 dpi and significantly ($P < 0.05$, Student's *t*-test) on 3 dpi lower than those of the control group (Fig. 4D). On

5 dpi, the viral loads in the lung tissues were undetectable in the treatment groups, and low (around or below LOD) in the control group.

Histopathological analysis was performed for the lungs of mice infected with 5×10^6 TCID₅₀ SARS-CoV-2. On 3 dpi, the vehicle-treated mice showed moderate alveolar septal thickening and inflammatory cell infiltration, whereas all compound-treated animals exhibited slight alveolar septal thickening and mild inflammatory cell infiltration (Fig. 4E). To investigate if the compounds ameliorate lung damage by affecting host immune response, we studied the expression of inflammatory cytokines and chemokines as well as immune cell infiltration in the lungs. MI-09 or MI-30 reduced the levels of IFN- β , and CXCL10 (Fig. 4F). Also, fewer neutrophils and macrophages occurred in the lungs of compound-treated mice compared with the control mice (Fig. 4, G and H), suggesting inhibition of immune cell infiltration. Altogether, i.p. or p.o. administration of MI-09 or MI-30 could efficiently inhibit SARS-CoV-2 replication and ameliorate SARS-CoV-2-induced lung lesions in vivo, and they represent an important step toward the development of orally available anti-SARS-CoV-2 drugs.

REFERENCES AND NOTES

- 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). [doi:10.1038/s41586-020-2012-7](https://doi.org/10.1038/s41586-020-2012-7) Medline
- F. Wu, S. Zhao, B. Yu, Y.-M. Chen, W. Wang, Z.-G. Song, Y. Hu, Z.-W. Tao, J.-H. Tian, Y.-Y. Pei, M.-L. Yuan, Y.-L. Zhang, F.-H. Dai, Y. Liu, Q.-M. Wang, J.-J. Zheng, L. Xu, E. C. Holmes, Y.-Z. Zhang, A new coronavirus associated with human respiratory disease in China. *Nature* **579**, 265–269 (2020). [doi:10.1038/s41586-020-2008-3](https://doi.org/10.1038/s41586-020-2008-3) Medline
- Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, The species *Severe acute respiratory syndrome-related coronavirus*: Classifying 2019-nCoV and naming it SARS-CoV-2. *Nat. Microbiol.* **5**, 536–544 (2020). [doi:10.1038/s41564-020-0695-z](https://doi.org/10.1038/s41564-020-0695-z) Medline
- P. Smith, Covid-19 in Australia: Most infected health workers in Victoria's second wave acquired virus at work. *BMJ* **370**, m3350 (2020). [doi:10.1136/bmj.m3350](https://doi.org/10.1136/bmj.m3350) Medline
- M.-K. Looi, Covid-19: Is a second wave hitting Europe? *BMJ* **371**, m4113 (2020). [doi:10.1136/bmj.m4113](https://doi.org/10.1136/bmj.m4113) Medline
- Y. Chen, Q. Liu, D. Guo, Emerging coronaviruses: Genome structure, replication, and pathogenesis. *J. Med. Virol.* **92**, 418–423 (2020). [doi:10.1002/jmv.25681](https://doi.org/10.1002/jmv.25681) Medline
- R. Hilgenfeld, From SARS to MERS: Crystallographic studies on coronavirus proteases enable antiviral drug design. *FEBS J.* **281**, 4085–4096 (2014). [doi:10.1111/febs.12936](https://doi.org/10.1111/febs.12936) Medline
- K. Anand, J. Ziebuhr, P. Wadhwani, J. R. Mesters, R. Hilgenfeld, Coronavirus main proteinase (3CL^{pro}) structure: Basis for design of anti-SARS drugs. *Science* **300**, 1763–1767 (2003). [doi:10.1126/science.1085658](https://doi.org/10.1126/science.1085658) Medline
- L. Zhang, D. Lin, Y. Kusov, Y. Nian, Q. Ma, J. Wang, A. von Brunn, P. Leyssen, K. Lanko, J. Neyts, A. de Wilde, E. J. Snijder, H. Liu, R. Hilgenfeld, α -Ketoamides as broad-spectrum inhibitors of coronavirus and enterovirus replication: Structure-based design, synthesis, and activity assessment. *J. Med. Chem.* **63**, 4562–4578 (2020). [doi:10.1021/acs.jmedchem.9b01828](https://doi.org/10.1021/acs.jmedchem.9b01828) Medline
- T. Pillaiyar, M. Manickam, V. Namasivayam, Y. Hayashi, S.-H. Jung, An overview of severe acute respiratory syndrome–coronavirus (SARS-CoV) 3CL protease inhibitors: Peptidomimetics and small molecule chemotherapy. *J. Med. Chem.* **59**, 6595–6628 (2016). [doi:10.1021/acs.jmedchem.5b01461](https://doi.org/10.1021/acs.jmedchem.5b01461) Medline
- Z. Jin, X. Du, Y. Xu, Y. Deng, M. Liu, Y. Zhao, B. Zhang, X. Li, L. Zhang, C. Peng, Y. Duan, J. Yu, L. Wang, K. Yang, F. Liu, R. Jiang, X. Yang, T. You, X. Liu, X. Yang, F. Bai, H. Liu, X. Liu, L. W. Guddat, W. Xu, G. Xiao, C. Qin, Z. Shi, H. Jiang, Z. Rao, H. Yang, Structure of M^{pro} from SARS-CoV-2 and discovery of its inhibitors. *Nature* **582**, 289–293 (2020). [doi:10.1038/s41586-020-2223-y](https://doi.org/10.1038/s41586-020-2223-y) Medline
- L. Zhang, D. Lin, X. Sun, U. Curth, C. Drosten, L. Sauerherring, S. Becker, K. Rox, R. Hilgenfeld, Crystal structure of SARS-CoV-2 main protease provides a basis for design of improved α -ketoamide inhibitors. *Science* **368**, 409–412 (2020). [doi:10.1126/science.abb3405](https://doi.org/10.1126/science.abb3405) Medline
- W. Dai, B. Zhang, X.-M. Jiang, H. Su, J. Li, Y. Zhao, X. Xie, Z. Jin, J. Peng, F. Liu, C. Li, Y. Li, F. Bai, H. Wang, X. Cheng, X. Cen, S. Hu, X. Yang, J. Wang, X. Liu, G. Xiao, H. Jiang, Z. Rao, L.-K. Zhang, Y. Xu, H. Yang, H. Liu, Structure-based design of antiviral drug candidates targeting the SARS-CoV-2 main protease. *Science* **368**, 1331–1335 (2020). [doi:10.1126/science.abb4489](https://doi.org/10.1126/science.abb4489) Medline
- R. L. Hoffman, R. S. Kania, M. A. Brothers, J. F. Davies, R. A. Ferre, K. S. Gajiwala, M. He, R. J. Hogan, K. Kozminski, L. Y. Li, J. W. Lockner, J. Lou, M. T. Marra, L. J. Mitchell Jr., B. W. Murray, J. A. Nieman, S. Noell, S. P. Plankin, T. Rowe, K. Ryan, G. J. Smith 3rd, J. E. Solowiej, C. M. Steppan, B. Taggart, Discovery of ketone-based covalent inhibitors of coronavirus 3CL proteases for the potential therapeutic treatment of COVID-19. *J. Med. Chem.* **63**, 12725–12747 (2020). [doi:10.1021/acs.jmedchem.0c01063](https://doi.org/10.1021/acs.jmedchem.0c01063) Medline
- B. Boras et al., Discovery of a Novel Inhibitor of Coronavirus 3CL Protease as a Clinical Candidate for the Potential Treatment of COVID-19. *bioRxiv* **293498** [preprint]. 13 September 2020.
- M. Westberg et al., Rational design of a new class of protease inhibitors for the potential treatment of coronavirus diseases. *bioRxiv* **275891** [preprint]. 16 September 2020.
- C. Ma, M. D. Sacco, B. Hurst, J. A. Townsend, Y. Hu, T. Szeto, X. Zhang, B. Tarbet, M. T. Marty, Y. Chen, J. Wang, Boceprevir, GC-376, and calpain inhibitors II, XII inhibit SARS-CoV-2 viral replication by targeting the viral main protease. *Cell Res.* **30**, 678–692 (2020). [doi:10.1038/s41422-020-0356-z](https://doi.org/10.1038/s41422-020-0356-z) Medline
- M. D. Sacco, C. Ma, P. Lagarias, A. Gao, J. A. Townsend, X. Meng, P. Dube, X. Zhang, Y. Hu, N. Kitamura, B. Hurst, B. Tarbet, M. T. Marty, A. Kolocouris, Y. Xiang, Y. Chen, J. Wang, Structure and inhibition of the SARS-CoV-2 main protease reveal strategy for developing dual inhibitors against M^{pro} and cathepsin L. *Sci. Adv.* **6**, eabe0751 (2020). [doi:10.1126/sciadv.abe0751](https://doi.org/10.1126/sciadv.abe0751) Medline
- W. Zhu, M. Xu, C. Z. Chen, H. Guo, M. Shen, X. Hu, P. Shinn, C. Klumpp-Thomas, S. G. Michael, W. Zheng, Identification of SARS-CoV-2 3CL protease inhibitors by a quantitative high-throughput screening. *ACS Pharmacol. Transl. Sci.* **3**, 1008–1016 (2020). [doi:10.1021/acsptsci.0c00108](https://doi.org/10.1021/acsptsci.0c00108) Medline
- K. S. Yang, X. R. Ma, Y. Ma, Y. R. Alugubelli, D. A. Scott, E. C. Vatansever, A. K. Drelich, B. Sankaran, Z. Z. Geng, L. R. Blankenship, H. E. Ward, Y. J. Sheng, J. C. Hsu, K. C. Kratch, B. Zhao, H. S. Hayatshahi, J. Liu, P. Li, C. A. Fierke, C. K. Tseng, S. Xu, W. R. Liu, A quick route to multiple highly potent SARS-CoV-2 main protease inhibitors. *ChemMedChem* **15**, 1–8 (2020). [doi:10.1002/cmdc.202000000](https://doi.org/10.1002/cmdc.202000000) Medline
- A. D. Rathnayake, J. Zheng, Y. Kim, K. D. Perera, S. Mackin, D. K. Meyerholz, M. M. Kashipathy, K. P. Battaile, S. Lovell, S. Perlman, W. C. Groutas, K.-O. Chang, 3C-like protease inhibitors block coronavirus replication in vitro and improve survival in MERS-CoV-infected mice. *Sci. Transl. Med.* **12**, eabc5332 (2020). [doi:10.1126/scitranslmed.abc5332](https://doi.org/10.1126/scitranslmed.abc5332) Medline
- R. Cannalire, C. Cerchia, A. R. Beccari, F. S. Di Leva, V. Summa, Targeting SARS-CoV-2 proteases and polymerase for COVID-19 treatment: State of the art and future opportunities. *J. Med. Chem.* [acs.jmedchem.0c01140](https://doi.org/10.1021/acs.jmedchem.0c01140) (2020). [doi:10.1021/acs.jmedchem.0c01140](https://doi.org/10.1021/acs.jmedchem.0c01140) Medline
- C. Gampe, V. A. Verma, Curse or cure? A perspective on the developability of

- aldehydes as active pharmaceutical ingredients. *J. Med. Chem.* **63**, 14357–14381 (2020). [doi:10.1021/acs.jmedchem.0c01177](https://doi.org/10.1021/acs.jmedchem.0c01177) [Medline](#)
24. P. S. Dragovich, T. J. Prins, R. Zhou, S. E. Webber, J. T. Marakovits, S. A. Fuhrman, A. K. Patick, D. A. Matthews, C. A. Lee, C. E. Ford, B. J. Burke, P. A. Rejto, T. F. Hendrickson, T. Tuntland, E. L. Brown, J. W. Meador 3rd, R. A. Ferre, J. E. V. Harr, M. B. Kosa, S. T. Worland, Structure-based design, synthesis, and biological evaluation of irreversible human rhinovirus 3C protease inhibitors. 4. Incorporation of P₁ lactam moieties as L-glutamine replacements. *J. Med. Chem.* **42**, 1213–1224 (1999). [doi:10.1021/jm9805384](https://doi.org/10.1021/jm9805384) [Medline](#)
 25. R. P. Jain, H. I. Pettersson, J. Zhang, K. D. Aull, P. D. Fortin, C. Huitema, L. D. Eltis, J. C. Parrish, M. N. G. James, D. S. Wishart, J. C. Vederas, Synthesis and evaluation of keto-glutamine analogues as potent inhibitors of severe acute respiratory syndrome 3C^{pro}. *J. Med. Chem.* **47**, 6113–6116 (2004). [doi:10.1021/jm0494873](https://doi.org/10.1021/jm0494873) [Medline](#)
 26. Y. Yip, F. Victor, J. Lamar, R. Johnson, Q. M. Wang, D. Barket, J. Glass, L. Jin, L. Liu, D. Venable, M. Wakulchik, C. Xie, B. Heinz, E. Villarreal, J. Colacino, N. Yumibe, M. Tebbe, J. Munroe, S.-H. Chen, Discovery of a novel bicycloproline P2 bearing peptidyl α -ketoamide LY514962 as HCV protease inhibitor. *Bioorg. Med. Chem. Lett.* **14**, 251–256 (2004). [doi:10.1016/j.bmcl.2003.09.074](https://doi.org/10.1016/j.bmcl.2003.09.074) [Medline](#)
 27. H. K. Ganguly, G. Basu, Conformational landscape of substituted prolines. *Biophys. Rev.* **12**, 25–39 (2020). [doi:10.1007/s12551-020-00621-8](https://doi.org/10.1007/s12551-020-00621-8) [Medline](#)
 28. P. Bazzini, C. G. Wermuth, in *The Practice of Medicinal Chemistry*, C. G. Wermuth, D. Aldous, P. Raboisson, D. Rognan, Eds. (Academic Press, ed. 4, 2015), pp. 319–357.
 29. Y. C. Martin, A bioavailability score. *J. Med. Chem.* **48**, 3164–3170 (2005). [doi:10.1021/jm0492002](https://doi.org/10.1021/jm0492002) [Medline](#)
 30. S.-H. Sun, Q. Chen, H.-J. Gu, G. Yang, Y.-X. Wang, X.-Y. Huang, S.-S. Liu, N.-N. Zhang, X.-F. Li, R. Xiong, Y. Guo, Y.-Q. Deng, W.-J. Huang, Q. Liu, Q.-M. Liu, Y.-L. Shen, Y. Zhou, X. Yang, T.-Y. Zhao, C.-F. Fan, Y.-S. Zhou, C.-F. Qin, Y.-C. Wang, A mouse model of SARS-CoV-2 infection and pathogenesis. *Cell Host Microbe* **28**, 124–133.e4 (2020). [doi:10.1016/j.chom.2020.05.020](https://doi.org/10.1016/j.chom.2020.05.020) [Medline](#)
 31. M. S. Weiss, R. Hilgenfeld, On the use of the merging *R* factor as a quality indicator for X-ray data. *J. Appl. Crystallogr.* **30**, 203–205 (1997). [doi:10.1107/S0021889897003907](https://doi.org/10.1107/S0021889897003907)
 32. P. A. Karplus, K. Diederichs, Linking crystallographic model and data quality. *Science* **336**, 1030–1033 (2012). [doi:10.1126/science.1218231](https://doi.org/10.1126/science.1218231) [Medline](#)
 33. V. B. Chen, W. B. Arendall 3rd, J. J. Headd, D. A. Keedy, R. M. Immormino, G. J. Kapral, L. W. Murray, J. S. Richardson, D. C. Richardson, MolProbity: All-atom structure validation for macromolecular crystallography. *Acta Crystallogr. D* **66**, 12–21 (2010). [doi:10.1107/S0907444909042073](https://doi.org/10.1107/S0907444909042073) [Medline](#)
 34. W. Kabsch, XDS. *Acta Crystallogr. D* **66**, 125–132 (2010). [doi:10.1107/S0907444909047337](https://doi.org/10.1107/S0907444909047337) [Medline](#)
 35. P. R. Evans, G. N. Murshudov, How good are my data and what is the resolution? *Acta Crystallogr. D* **69**, 1204–1214 (2013). [doi:10.1107/S0907444913000061](https://doi.org/10.1107/S0907444913000061) [Medline](#)
 36. A. Vagin, A. Teplyakov, Molecular replacement with MOLREP. *Acta Crystallogr. D* **66**, 22–25 (2010). [doi:10.1107/S0907444909042589](https://doi.org/10.1107/S0907444909042589) [Medline](#)
 37. L. Zhu, S. George, M. F. Schmidt, S. I. Al-Gharabli, J. Rademann, R. Hilgenfeld, Peptide aldehyde inhibitors challenge the substrate specificity of the SARS-coronavirus main protease. *Antiviral Res.* **92**, 204–212 (2011). [doi:10.1016/j.antiviral.2011.08.001](https://doi.org/10.1016/j.antiviral.2011.08.001) [Medline](#)
 38. P. Emsley, B. Lohkamp, W. G. Scott, K. Cowtan, Features and development of Coot. *Acta Crystallogr. D* **66**, 486–501 (2010). [doi:10.1107/S0907444910007493](https://doi.org/10.1107/S0907444910007493) [Medline](#)
 39. O. Kovalevskiy, R. A. Nicholls, F. Long, A. Carlon, G. N. Murshudov, Overview of refinement procedures within REFMAC5: Utilizing data from different sources. *Acta Crystallogr. D* **74**, 215–227 (2018). [doi:10.1107/S2059798318000979](https://doi.org/10.1107/S2059798318000979) [Medline](#)
 40. G. Bricogne *et al.*, BUSTER version 2.10.3 (Global Phasing Ltd., 2017).
 41. Y. Liu, W. Kati, C.-M. Chen, R. Tripathi, A. Molla, W. Kohlbrenner, Use of a fluorescence plate reader for measuring kinetic parameters with inner filter effect correction. *Anal. Biochem.* **267**, 331–335 (1999). [doi:10.1006/abio.1998.3014](https://doi.org/10.1006/abio.1998.3014) [Medline](#)

ACKNOWLEDGMENTS

We thank Prof. Shile Huang (Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, Shreveport, LA 71130-3932, USA) for careful proofreading, and Dr. Hong-Yi Zheng, Ms. Xiao-Yan He, Mr. Wen-Wu Huang (Key Laboratory of Animal Models and Human Disease Mechanisms of the Chinese Academy of Sciences, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China) for their assistance in animal experiments. We also thank the staff of Shanghai synchrotron radiation facility (SSRF) beamline BL19U1 (Shanghai, China) and Dr. Johanna Hakanpää of PETRA III beamline P11 (Hamburg, Germany) for supports; **Funding:** This work was supported by the fast-track research fund on COVID-19 of Sichuan Province (2020YFS0006, 2020YFS0010), the fast-track grants of SARS-CoV-2 research from West China Hospital, Sichuan University (HX-2019-nCoV-053, HX-2019-nCoV-039), and partially by the National Natural Science Foundation of China (81930125 and 00402354A1028), 1.3.5 project for disciplines of excellence, West China Hospital, Sichuan University, the Fundamental Research Funds for the Central Universities (20822041D4060), and the National Key R&D Program of China (2020YFC0842000 to Y.-T.Z.); **Author contributions:** S.Y., J.L. and Y.-T.Z. conceived and supervised the research, and designed the experiments; S.Y., Y.-F.W., Y.-S.L. and J.L. performed the drug design; Y.-S.L., C.H., J. Zhang, B.Q., C.S., X.M., Xinlei Liu, R.-C.Y., Xiaolong Liu, J.Y. and S.Y. performed chemical syntheses, separation, purification, and structural characterizations; J.L., R.Z. and J.Q. with the assistance of K.W. and X.N. performed gene expression and protein purification, crystallization and diffraction data collection; J.L., R.Z. and S.Y. determined and analyzed the crystal structures; J.Q., Y.Z., R.Y., W.-P.L., J.-M.L., P.C., Y.L. and G.-F.L. performed enzymatic inhibition assays, DSF assays and cellular cytotoxicity assay; R.-H.L. and F.-L.L. with the assistance of L.X., Z.-L.D., Q.-C.Z., H.-L.Z., W.Q., Y.-H.-P.L. and M.-H.L. performed cellular antiviral assays and in vivo antiviral studies; W.S., W.Y. and J.Q. performed in vivo toxicity studies; L.L., Y.H., G.-W.L., W.-M.L. and Y.-Q.W. with the assistance of X.Y. and J. Zou analyzed and discussed the data; S.Y., J.L. and Y.-T.Z. with the assistance of J.Q., Y.-F.W., Y.-S.L. and L.L. wrote the manuscript; **Competing interests:** Sichuan University has applied for PCT and Chinese patents covering MI-09 and MI-30 as well as related compounds with a bicycloproline structure in P2 position. **Data and materials availability:** The coordinates and structure factors of SARS-CoV-2 M^{pro} in complex with MI-23, Boceprevir and Telaprevir have been deposited into PDB with accession numbers 7D3I, 7COM, and 7C7P, respectively. This work is licensed under a Creative Commons Attribution 4.0 International (CC BY 4.0) license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>. This license does not apply to figures/photos/artwork or other content included in the article that is credited to a third party; obtain authorization from the rights holder before using such material.

SUPPLEMENTARY MATERIALS

science.sciencemag.org/cgi/content/full/science.abf1611/DC1

Figs. S1 to S5

Tables S1 to S7

Materials and Methods

Supplementary Text

References (31–41)

MDAR Reproducibility Checklist

8 October 2020; accepted 12 February 2021

Published online 18 February 2021

10.1126/science.abf1611

Fig. 1. Schematic diagram of the design of novel SARS-CoV-2 M^{pro} inhibitors.

Fig. 2. Overall structure of SARS-CoV-2 M^{pro}-MI-23 complex. (A) The cartoon view of M^{pro} dimer (Molecule A, Cyan; Molecule B, purple). Three domains (I, II and III) of each monomer are marked. The catalytic dyad Cys145-His41 is located in the cleft between domains I and II. MI-23 in both molecules is shown in purple or in orange. The N and C termini of each M^{pro} are labeled. Labels for molecule B are in *italics*. (B) The chemical structure of MI-23. (C) The MI-23 binding pocket of M^{pro}. *Fo*-*Fc* density maps (grey mesh, $\sigma = 2.5$) are shown for MI-23 (purple) as well as the covalent bond formed by Cys145 and the warhead aldehyde. Cys145 and His41 are displayed in yellow and blue, respectively. (D) The interactions between M^{pro} and MI-23. The hydrogen bonds between them are shown in black dashed lines. Ser1 from molecule B interacts with Glu166 and Phe140 in molecule A (red dashed lines) to support S1 pocket formation. The warhead carbon is marked with a black asterisk in B, C and D. Figures (A, C and D) were prepared using the program PyMOL (<https://pymol.org>).

Vero E6 Cells

HPAEpiC Cells

Fig. 3. Antiviral activity of six compounds in cell-based SARS-CoV-2 antiviral activity assays. (A) Vero E6 cells were infected with SARS-CoV-2 at an MOI of 0.1 and treated with different concentrations of test compounds (MI-09, MI-12, MI-14, MI-28, MI-30 and MI-31). At 72h post infection, the cytopathic effect caused by SARS-CoV-2 infection was quantitatively analyzed using CCK8 (Beyotime) according to the manufacturer's protocol. Data are shown as mean $\pm$ SD, $n = 3$ biological replicates. (B) HPAEpiC cells were infected with SARS-CoV-2 at an MOI of 0.01 and treated with different concentrations of test compounds (MI-09, MI-12, MI-14, MI-28, MI-30 and MI-31). At 48 hours post infection, viral RNA copies (per mL) were quantified from cell culture supernatants by the RT-qPCR method. Data are shown as mean $\pm$ SD, $n = 2$ biological replicates.

Fig. 4 (preceding page). MI-09 and MI-30 reduced lung viral loads and lung lesions in a SARS-CoV-2 infection transgenic mouse model. (A and B) Chemical structures and summary of in vitro activity data and bioavailability of MI-09 and MI-30. (C) Overview of in vivo study design. (D) Viral loads in the lungs of SARS-CoV-2-infected hACE2 transgenic mice. Mice infected with the indicated dose of SARS-CoV-2 were treated with MI-09, MI-30 or vehicle solution, and then were sacrificed at the indicated time. Five lung lobes of each mouse were collected to determine viral loads. Data represent the median of five lung lobes of individual mice. The horizontal dotted line shows the viral load limit of detection (LOD) of $1.0 \log_{10}$ RNA copies. Data below the limit of detection are shown at the limit of detection. All results are shown as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ two-tailed, unpaired Student's t -test. (E) The representative images of lung histopathological changes from SARS-CoV-2 (5×10^6 TCID₅₀) infected hACE2 mice on 3 dpi. Magnified views of the boxed regions for each image are shown below. Black arrows indicate alveolar septal thickening, and red arrows point to inflammatory cell infiltration. The whole lung tissue scan images of the SARS-CoV-2 infected hACE2 mice on 3 dpi are provided in fig. S4. (F) Representative chemokine and cytokine assessment of the lung tissues ($n = 3$) of the indicated groups, as detected in the lung tissue homogenate on 3 dpi. The results are shown as mean $\pm$ SD * $P < 0.05$ and ** $P < 0.01$ when compared with the vehicle group. One-way ANOVA was used. (G and H) Infiltration analysis for neutrophils and macrophages in the lungs of SARS-CoV-2 (5×10^6 TCID₅₀) infected hACE2 mice on 3dpi. The percentages of macrophages and neutrophils in the lungs are shown in (G). Statistical significance was analyzed by unpaired Student's t tests. * $P < 0.05$, ** $P < 0.01$. The representative images of fluorescence staining are shown in (H). White triangles and arrows indicate macrophages and neutrophils, respectively. TCID₅₀, 50% tissue culture infectious dose.