Identification of proteasome and caspase inhibitors targeting SARS-CoV-2 M\textsuperscript{pro}

Dear Editor,

Since the beginning of 2020, the Coronavirus (CoV) Disease 2019 (COVID-19) pandemic has posed formidable challenges to public health security. The main protease (M\textsuperscript{pro}, 3CL\textsuperscript{pro}) of CoVs plays essential roles in viral replication, making them attractive targets for antiviral drug development\textsuperscript{1–3}. Dozens of SARS-CoV-2 M\textsuperscript{pro} inhibitors have been reported with some entering clinical trials, but none is approved for COVID-19 treatment to date\textsuperscript{1–3}. In this study, we discovered that the proteasome inhibitor MG132 and caspase inhibitors such as Z-VAD(OMe)-FMK are effective SARS-CoV-2 M\textsuperscript{pro} inhibitors.

We recently identified arsenic trioxide as an effective mutant p53 rescue compound functioning by increasing protein thermostability\textsuperscript{4}. Here we used protein thermostability as a readout to screen SARS-CoV-2 M\textsuperscript{pro}-thermostabilizing compounds from a library of 4198 chemical entities containing US Food and Drug Administration (FDA)-approved drugs and clinical-stage or known-target compounds (Supplementary Table S1). In the differential scanning fluorimetry (DSF) assay, Z-VAD(OMe)-FMK, MG132, boceprevir, thermopsine, and baicaline were identified as the top five SARS-CoV-2 M\textsuperscript{pro}-thermostabilizing compounds, which increased the melting temperature \((T_m)\) of M\textsuperscript{pro} by at least 2 °C (Fig. 1a, Supplementary Fig. S1a, and Supplementary Table S1). The destabilizing hits (Fig. 1a and Supplementary Fig. S1b) were not pursued further because of their potential promiscuity of binding or other undesirable properties. The top five M\textsuperscript{pro}-stabilizing compounds were validated in concentration titration, whereby Z-VAD(OMe)-FMK was consistently the most potent M\textsuperscript{pro}-stabilizing compound (Supplementary Fig. S1c, d).

We next predicted the M\textsuperscript{pro} binding modes of the five hits and performed a structure–activity relationship (SAR) analysis. The substrate-binding pocket of M\textsuperscript{pro}, containing four subsites (S1′, S1, S2, and S4), is highly conserved among all CoV M\textsuperscript{pro} homologs (Supplementary Fig. S2a, upper panel). We previously designed a series of M\textsuperscript{pro} inhibitors harboring a classic core structure, in which the four subsites (warhead, R1, R2, and R3) were assigned to occupy the four subsites of M\textsuperscript{pro}, respectively (Supplementary Fig. S2a, lower panel),\textsuperscript{5} as exemplified by N31, 11a, GC376, and MI-23\textsuperscript{6} (Supplementary Fig. S2b). Among the top five hits, baicaline and thermopsine are natural products with low molecular weight (Supplementary Fig. S1d), and thus they are unlikely to compactly occupy all four subsites. Boceprevir has been reported to function based on the classic core structure\textsuperscript{7}, and its M\textsuperscript{pro} binding mechanism has been elucidated\textsuperscript{8}. The remaining two hits, MG132 and Z-VAD(OMe)-FMK, structurally harbor a classic core structure and were thus proposed to bind to the active site of M\textsuperscript{pro} in a conventional binding mode (Supplementary Fig. S2c). Notably, Z-VAD(OMe)-FMK lacks an R2 subgroup in the proposed binding mode (Supplementary Fig. S2c). To test the proposed binding mode, we collected 34 commercially available compounds sharing high structural similarity with MG132 and Z-VAD(OMe)-FMK (Supplementary Fig. S2d). Among them, three caspase inhibitors and three proteasome inhibitors detectably stabilized M\textsuperscript{pro} (Fig. 1b, the 6 blue bars, \(\Delta T_m > 0.5 {^\circ}\text{C}\)). The 17 compounds (1–17) lacking cysteine-binding warhead (Supplementary Fig. S2d) failed to stabilize M\textsuperscript{pro} (Fig. 1b, \(\Delta T_m < 0.1 {^\circ}\text{C}\)). We next focused on caspase and proteasome inhibitors and collected 25 commercially available inhibitors (Supplementary Fig. S2e). The DSF results suggested: (1) harboring core structure is apparently a prerequisite for being a competent M\textsuperscript{pro} thermostabilizer (the 8 blue bars); (2) the top 5 potent compounds all contain a fluoromethyl ketone (FMK) warhead (the first 5 bars); and (3) small-sized R2 is apparently associated with high potency (comparison among the first 6 bars). In summary, the three most potent Z-VAD(OMe)-FMK, Z-DEVD-FMK, and Z-IETD-FMK contain a core structure with an FMK warhead and a small-sized R2.

To validate that M\textsuperscript{pro} is a direct target of MG132 on the atomic level, we solved a high-resolution crystal structure of M\textsuperscript{pro}-MG132 (Supplementary Table S2; 1.7 Å resolution). The two M\textsuperscript{pro} molecules formed a symmetry homodimer (Supplementary Fig. S3a). As proposed in Fig. S2c, MG132 binds to the active site based on the classic core structure (Supplementary Fig. S3b), whereby an aldehyde, side chain of Leu, side chain of the second Leu, and benzylxoycarbonyl (Cbz) act as the warhead, R1, R2, and R3, respectively (Fig. 1c). Mass spectrometry (MS) did not reveal an obvious molecular weight increase of M\textsuperscript{pro} upon incubation with MG132 (Supplementary Fig. S3c; eb selen was used as a control), which is presumably due to the highly dynamic and reversible bond between the MG132 aldehyde and the targeted cysteine. The mode of MG132 binding to SARS-CoV-2 M\textsuperscript{pro} significantly differs from that of its binding to the proteasomal 20S subunit because of the different shape of the binding pocket (Supplementary Fig. S3d). Nevertheless, it is similar to those of the reported classical core-structure-based M\textsuperscript{pro} inhibitors (Fig. 1d). Notably, MG132 subgroups R1, R2, and R3 are all derived from hydrophobic Leu and Cbz, which undergo extensive hydrophobic interactions with M\textsuperscript{pro} (Fig. 1e).

We next solved a high-resolution crystal structure of the M\textsuperscript{pro}-Z-VAD(OMe)-FMK complex (Supplementary Table S2; 1.8 Å resolution), wherein the asymmetric unit contains one molecule (Supplementary Fig. S4a). Z-VAD(OMe)-FMK binds to the active site of M\textsuperscript{pro} (Supplementary Fig. S4b), however, in an unexpected binding mode (Fig. 1f). The FMK group acts as a previously unreported Cys145-binding warhead, which was confirmed by MS (Supplementary Fig. S4c, d; Z-DEVD-FMK and Z-IETD-FMK with the FMK group were also confirmed to covalently bind to Cys145).

Received: 18 January 2021 Revised: 8 May 2021 Accepted: 11 May 2021 Published online: 01 June 2021

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FMK is frequently used as a warhead to tether cysteines of caspases. However, it has not been reported to be used in SARS-CoV-2 M<sub>pro</sub> inhibitors, to our knowledge. The binding mode of M<sub>pro</sub>-Z-VAD(OMe)-FMK differs significantly from that of caspase-1-Z-VAD-FMK, whereby the Z-VAD(OMe)-FMK molecule linearly occupies the long narrow pocket of caspase-1 (Supplementary Fig. S4e). Due to R3 re-orientation, the binding mode of Z-VAD (OMe)-FMK to M<sub>pro</sub> is completely different from that of previously reported M<sub>pro</sub> inhibitors (Fig. 1g). The four features of M<sub>pro</sub>-Z-VAD (OMe)-FMK binding (Fig. 1h)—FMK acting as the warhead, absence of R2, re-orientation of flexible hydrophobic R3, and compact occupation of the S2-S4 joint site—may contribute to the...
high potency of Z-VDAD(OMe)-FMK in thermostabilizing SARS-CoV-2 Mpro. During preparation of this manuscript, a crystal structure of Mpro-Z-VDAD(OMe)-FMK (PDB ID: 7C8B) was released in the PDB database by an independent group, confirming the observed unconventional binding mode.

The inhibitory activities of the three caspase inhibitors and MG132 against SARS-CoV-2 Mpro were determined in vitro using a fluorescence resonance energy transfer (FRET)-based assay. The three caspase inhibitors exhibited potent Mpro inhibitory effects with half-maximal inhibitory concentrations (IC50) in the nanomolar and low micromolar range (Fig. 1i, 0.59–280 μM), while MG132 displayed a higher IC50 (3.91 μM). Generally speaking, the three caspase inhibitors were comparable in their IC50 values with the reported rationally designed Mpro inhibitors (Supplementary Fig. S5a, 0.03–30.0 μM).

The antiviral activities of these four compounds against SARS-CoV-2 were next determined in Vero cells. At 24 h following SARS-CoV-2 infection, the three caspase inhibitors displayed potent half-maximal effective concentrations (EC50) in the nanomolar and low micromolar range in Vero cells (Fig. 1j, 0.64–1.88 μM). We noticed that Z-VDAD(OMe)-FMK exhibited higher potency in enzymatic activity assay, whereas lower potency in antiviral activity assay when compared to the other two caspase inhibitors. It may be associated with potential ‘off-target’ effect of Z-VDAD(OMe)-FMK when inhibiting Mpro in the cell-based antiviral assay, for example, promiscuously binding to multiple caspases and proteins of host cells. None of the tested caspase inhibitors caused cytotoxicity in Vero cells, exhibiting half-maximal cytotoxic concentrations (CC50) > 300 μM (Fig. 1j). MG132 is cytotoxic to this cell line3, and its EC50 could not be reliably determined (Fig. 1j). The extended cytotoxicity studies consistently suggested that these three caspase inhibitors were relatively non-toxic to cells (Supplementary Fig. S5b). Compared to the anti-SARS-CoV-2 activities of the reported Mpro inhibitors measured in the same Vero cells, the non-toxic caspase inhibitors were superior to those of N31, Boceprevir3, and GC-3763, comparable with the six most potent Mpro inhibitors (MI-09/12/14/28/30/31) reported by Qiao and colleagues2, but less potent than our previously optimized 11a/b2 (Supplementary Fig. S5a).

In summary, we identified MG132, Z-VDAD(OMe)-FMK and its structural analogs as direct Mpro-binding small molecules with antiviral activity against SARS-CoV-2. MG132 is widely recognized as a CoV inhibitor, with several reported and proposed targets. Our study provides evidence at single-atom resolution that Mpro is a direct target of MG132. In contrast to the reported rationally designed Mpro inhibitors, Z-VDAD(OMe)-FMK binds to the active site of Mpro in an unconventional binding mode, which may contribute to the observed high potency of Z-VDAD(OMe)-FMK in inhibiting SARS-CoV-2. Our studies provide compelling structural evidence to support an alternative strategy for the design of potent inhibitors against SARS-CoV-2 Mpro.

DATA AVAILABILITY
The datasets generated in this study are available from the corresponding authors upon reasonable request.

CODE AVAILABILITY
Atomic coordinates and structure factors of the reported crystal structures have been deposited with the worldwide Protein Data Bank (https://www.rcsb.org) under the PDB codes 7CUT and 7CUU.

ACKNOWLEDGEMENTS
M.L. was funded by the NSFC (82073292, 81622002, 8186130368), the SJTU Trans-med Awards Research, the Guangdong Clinical Medicine Grant (828318), the Shanghai Excellent Young Academic Leader Program (20XD1422700), the Shanghai Medical and Health Excellent Discipline Leader Development Plan (2018BR36), Shanghai Collaborative Innovation Center for Translational Medicine (TM201902), the Foundation of the National Facility for Translational Medicine (Shanghai) (TMFSK-2020-003), the Newton Advanced Fellowship, and the Samuel Waxman Cancer Research Foundation. H.Y. was funded by the Science and Technology Commission of Shanghai Municipality (20431900200) and the Department of Science and Technology of Guangxi Zhuang Autonomous Region (2020AB40007). L.Z. was funded by the NSFC (31970165). We thank the staff at the BL17U1/B19U1 beamlines of the National Center for Protein Science Shanghai (NCPSS) at Shanghai Synchrotron Radiation Facility (SSRF) and high-throughput screening core of the National Research Center for Translational Medicine (Shanghai).

AUTHOR CONTRIBUTIONS
M.L., H.Y., and L.Z. conceived the project and designed the experiments; Z.W. and Y.X. performed enzymatic activity assays; L.F. and C.P. performed the MS experiments; J.K. performed enzymatic activity assays; L.F. and C.P. performed the MS experiments; Y.Z. solved the crystal structure; M.L., H.Y., and L.Z. conceived the project and designed the experiments; Z.W. and Y.X. performed the screening and protein crystallization; Y.Z. solved the crystal structure; J.K. performed enzymatic activity assays; L.F. and C.P. performed the MS experiments; Q.W. performed the antiviral assays. Z.W., M.L., H.Y., and L.Z. wrote the manuscript.

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41392-021-00639-8.

Competing interests: The authors declare no competing interests.
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