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CORRESPONDENCE

Structure basis for inhibition of SARS-CoV-2 by the feline drug GC376

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Dear Editor,

The coronavirus pandemic has brought public health challenges worldwide. The main protease (M^{pro}), also called $3CL^{pro}$ (3C-like protease), is encoded by the gene of non-structural protein 5 (nsp5). It is an attractive antiviral drug target to halt the progress of severe acute respiratory syndrome coronavirus-2 (SARS-COV-2), the causative pathogen of coronavirus disease 2019 (COVID-19) [1]. After viral infection in host cells, the replicase gene encodes two polyproteins, pp1a (486 kDa) and pp1ab (790 kDa). They are then cleaved by papain-like protease 2 ($PL2^{pro}$) at 3 sites and M^{pro} at another 11 sites. Then, pp1a and pp1ab are processed to release a series of non-structural proteins (NSPs) that mediate viral replication and transcription [1]. The function of M^{pro} is indispensable to the viral life cycle. Thus, inhibiting the activity of M^{pro} can effectively impede the coronavirus replication. Over one 100 host-cell proteins that are involved in essential cellular processes have also been reported as substrates of SARS-COV-2 M^{pro}, suggesting that M^{pro} is a multifunctional viral factor [2].

To evaluate drugs that may target SARS-COV-2 M^{pro} for COVID-19 treatment, we purified SARS-CoV-2 M^{pro} protein and attempted to validate certain compounds that have been reported to inhibit coronavirus M^{pro} effectively [3]. The SARS-COV-2 M^{pro} was expressed in *Escherichia coli* cells and then purified via affinity and size-exclusion chromatography. The purified SARS-COV-2 M^{pro} showed a monodispersed peak with a molecular weight of ~33.8 kDa (Supplementary information, Fig. S1a). We selected nine drugs and performed thermal shift assay (TSA) to assess the effect of each drug on SARS-CoV-2 M^{pro}. Each drug (20 μM) was incubated with SARS-COV-2 M^{pro} and the melting profile was monitored based on the SYBRO orange reaction in the range of 25–99 °C. Only GC376, an antiviral drug used to treat feline coronavirus disease [4], displayed an obvious effect, shifting the melting curve of SARS-CoV-2 M^{pro} from 50.9 °C to 55.2 °C (+4.3 °C). Other drugs showed little effect (Fig. 1a, Supplementary information, Table S2). These results show that GC376 may bind to SARS-COV-2 M^{pro}. To determine if the process is titratable, the TSA experiment was repeated with different concentrations of GC376 (0–20 μM). The melting temperature of M^{pro} was found to increase in a dose-dependent manner (Fig. 1b). Taken together, our results showed that GC376 interacts directly with SARS-COV-2 M^{pro}.

GC376 is a dipeptidyl bisulfite adduct salt and has been reported to inhibit M^{pro} [4], suggesting that GC376 may be a broad-spectrum antiviral drug. To determine the structural basis of SARS-COV-2 M^{pro} inhibition by GC376, we determined the structure of GC376 bound SARS-COV-2 M^{pro} at the resolution of 2.35 Å. Like SARS-CoV M^{pro}, the SARS-CoV-2 M^{pro} forms a dimer with the two protomers vertically packed into each other (Fig. 1c and Supplementary information Table S1). The SARS-COV-2 M^{pro} monomer has three domains with domains I (residues 8–101) and II (residues 102–184) forming a barrel structure of six antiparallel β-strands. Between domain I and domain II is a cleft that forms the catalytic dyad C145-H41 and the substrate binding site, which is composed of three highly conserved sub-pockets S1, S2 and S4. Domain III (residues 201–303) comprises a globular cluster of five antiparallel α-helices, which is involved in the dimerization of M^{pro}. Domains II and III are connected through a loop (residues 185–200), while domain I contains a stretched out "N-finger" (NH_2-terminal) that is inserted into domain II of its adjacent protomer via interaction with F140 and G166. The surface charge distribution in the active site of SARS-CoV-2 M^{pro} displays a type of bipolar distribution that is suitable for the binding of peptide substrates (Fig. 1d). GC376 was found to occupy the M^{pro} substrate binding site (Fig. 1e) and form extensive hydrogen bonds with the surrounding residues of F140, G143, H163, E166, and H172 of M^{pro}. The glutamine surrogate ring and the leucine group of GC376 fit into the pockets in M^{pro} S1 and S2 and serve as conserved residents for substrate recognition. GC376 also uses an aldehyde bisulfite to covalently bind to the catalytic residue C145, suggesting its potentiality to block the catalytic dyad. This shows the potential of GC376 to act as a covalent inhibitor to prevent the binding and cleavage of substrates by SARS-CoV-2 M^{pro}. When we compared the structure of our GC376-bound M^{pro} complex to the previously reported SARS-CoV-2 M^{pro} structure [5] (Fig. 1f) and MERS-COV M^{pro}-GC376 structure [6] (Fig. 1g), we saw a good agreement. Comparison with the SARS-CoV-2 M^{pro} structure shows considerable conservation of domains I and II, with small discrepancies in domain III. Comparison with the MERS-COV M^{pro}-GC376 structure shows nearly overlapped conformation of the two GC376 molecules, indicating that the inhibition of these viruses by GC376 is mediated through a similar mechanism. We also compared our structure to the GC376-SARS-CoV-2 M^{pro} structure reported by Ma et al. [7]. (Fig. 1h) and found that the phenylmethyl group of GC376 in Ma et al.'s structure points deeper to S4 site in the pocket. The conformation of SARS-CoV-2 M^{pro}-GC376 reported by another group [8] is

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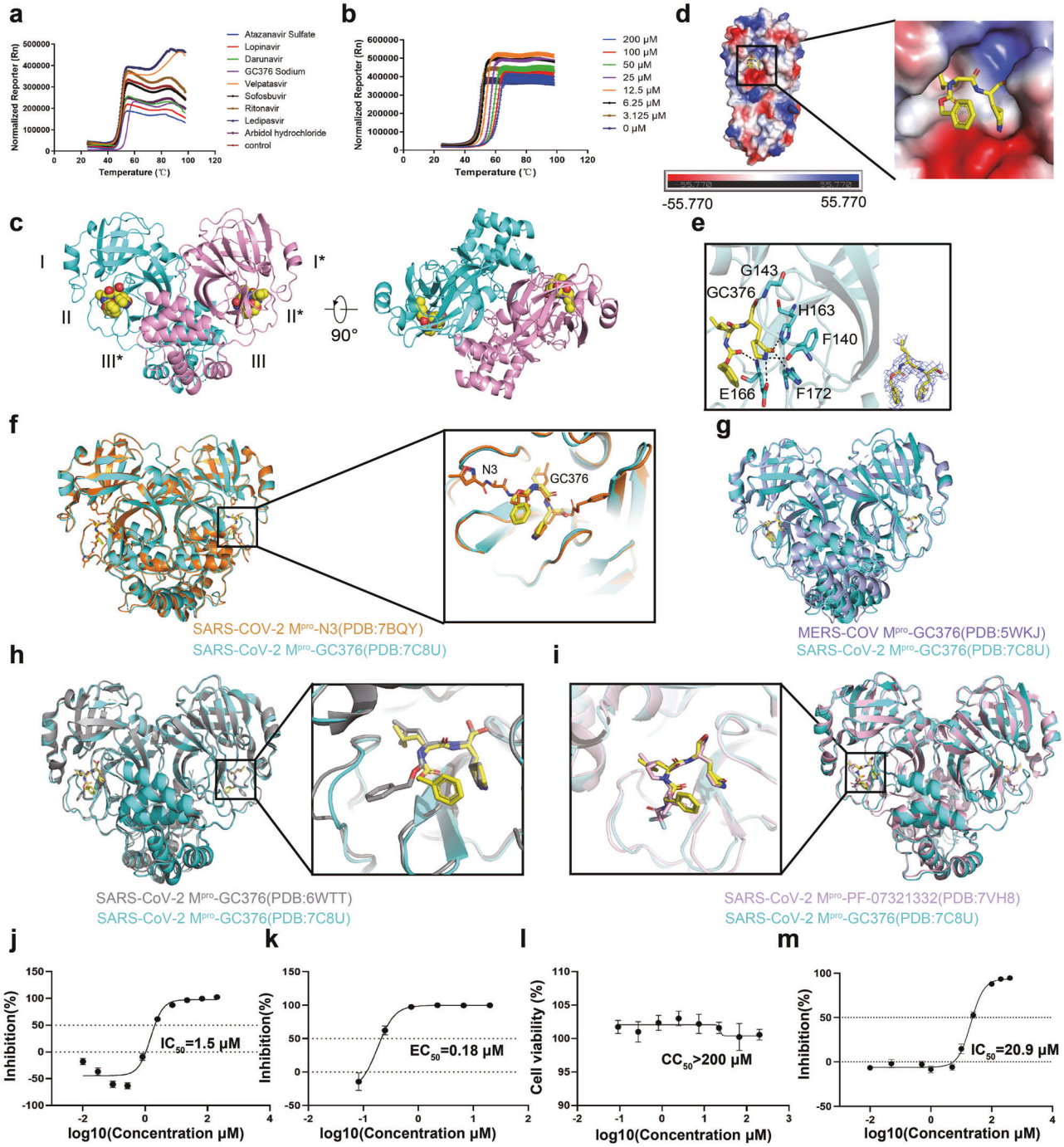

Fig. 1 Structure of SARS-CoV-2 M^{pro} and GC376 activity. a Thermal shift assay of SARS-CoV-2 M^{pro} with different drugs. **b** Thermal shift assay of SARS-CoV-2 M^{pro} with different concentrations of GC376. **c** Overall structure of SARS-CoV-2 M^{pro} in complex with GC376 in two different views. **d** Electrostatic surface of SARS-CoV-2 M^{pro}. Blue: positive charge potential; Red: negative charge potential. The value ranges from −55.77 (red) to 0 (white) to 55.77 (blue). **e** Binding of GC376 (yellow sticks) to the SARS-CoV-2 M^{pro} pocket with the electron density map for GC376. **f** Comparison between SARS-CoV-2 M^{pro}-GC376 and SARS-COV-2 M^{pro}-N3 crystal structures. **g** Comparison between SARS-CoV-2 M^{pro}-GC376 and MERS-COV M^{pro}-GC376. **h** Comparison of the SARS-COV-2-GC376 structure we reported (PDB: 7CBU) to the SARS-COV-2-GC376 structure reported by Ma et al. (PDB:6WTT). **i** Comparison of SARS-CoV-2 M^{pro}-GC376 and SARS-CoV-2 M^{pro}-PF-07321332. **j** IC_{50} of GC376 on the purified M^{pro} enzyme. **k** EC_{50} of GC376 on inhibition of SARS-Cov-2 in Vero E6 cells. **l** CC_{50} of GC376 on Vero E6 cells. **m** IC_{50} of GC376 on the purified M^{pro} P132H.

similar to our structure, with the phenylmethyl groups at slightly different positions (Supplementary information, Fig. S1b). We also compared our structure to GC376-SARS- CoV-2 Mpro structure reported by Fu et al. [1] and Vuong et al. [2] and also found a similar binding mode. These conformational differences may be caused by flexibility in the loop (Q189-A193) region near the site 4 (Supplementary information, Fig. S1c). We also compared our structure to the structure of the SARS-COV-2 M^{pro} in complex with the PF-07321332 [9], a SARS-CoV-2 M^{pro} inhibitor, which has been approved for emergency treatment of COVID-19, in combination with Ritonavir (brand name: Paxlovid) [10]. Comparison to the SARS-COV-2 M^{pro}-PF-07321332 structure showed that the glutamine surrogate rings of the GC376 and PF-07321332 fit into the S1 pocket well and form hydrogen bonds with the neighboring

residues in the binding pocket, indicating that the GC376 and PF-07321332 act through a similar mechanism.

In addition to GC376, non-covalent SARS-COV-2 M^{pro} inhibitors have also been developed [11]. To verify the effectiveness of GC376, we tested the inhibitory activity of GC376 for SARS-CoV-2 M^{pro}, with the 50% inhibition concentration (IC_{50}) of 1.5 μM (Fig. 1j). We also tested the ability of GC376 to inhibit SARS-CoV-2 infection in pre-seeded Vero E6 cells exposed to the authentic virus. GC376 showed a 50% maximal effect concentration (EC_{50}) of 0.18 μM (Fig. 1k). Meanwhile, GC376 showed a 50% cytotoxic concentration (CC_{50}) of more than 200 μM, indicating a strong safety profile (Fig. 1l). It has been reported that GC376 treatment showed little toxicity in K18-hACE2 mice, although it also had clearly beneficial effect against SARS-CoV-2 in vivo [12]. The Omicron sub-variants BA.1 and BA.2 of SARS-CoV-2 have become the dominant infective strains. The missense mutation P132H in M^{pro} is >95% prevalent in both Omicron sub-variants [13]. We also tested the ability of GC376 to inhibit M^{pro} P132H enzymatic activity with IC_{50} of 20 μM (Fig. 1m), suggesting that GC376 may act as a broad-spectrum drug for different variants of SARS-COV-2. A pre-IND (Investigational New Drug application) about usage of GC376 in COVID treatment has been submitted to the FDA [14]. All of these data suggest that GC376 is worthy of further testing in clinical trials. Both our structural analysis and our in vitro assays in this study indicate that GC376 is an effective and safe drug candidate suitable for use against COVID-19 by inhibiting SARS-CoV-2 M^{pro}.

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AUTHOR CONTRIBUTIONS

XDL performed the protein purification and structure analysis of M^{pro} and wrote the paper under the supervision of SYZ; BXC assisted in the work of XDL; WJS performed cell experiments under supervision of LKZ; WCY, YJ, and BXC participated in writing the manuscript and preparing figures; SYZ and HEX helped design the study and wrote the paper.

ADDITIONAL INFORMATION

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