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SARS-CoV-2 M<sub>pro</sub> inhibitors with antiviral activity in a transgenic mouse model

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Fig. 1. Schematic diagram of the design of novel SARS-CoV-2 M<sub>pro</sub> inhibitors.
routes, and characterization of these compounds by nuclear magnetic resonance and electrospray ionization mass spectrometry.

The 32 compounds’ biochemical activities against SARS-CoV-2 Mpro were determined by a fluorescence resonance energy transfer (FRET) assay. For this, recombinant SARS-CoV-2 Mpro protein was prepared. The turnover number (kcat)/Michaelis constant (Km) value of the recombinant protein was determined as 50,656 ± 4221 M⁻¹ s⁻¹, similar to a previous result (11). In the FRET assay, all 32 compounds (MI-01 to MI-32) showed potent inhibitory activities on SARS-CoV-2 Mpro, with 50% inhibitory concentration (IC50) values ranging from 7.6 to 748.5 nM (table S1). Of these, 24 compounds displayed two-digit nanomolar IC50 values, and three 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 Mpro 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 Mpro. All the compounds displayed large thermal shifts ranging from 12.5° to 21.7°C (table S1), indicating their tight binding to SARS-CoV-2 Mpro. It is noteworthy that the two different bicycloproline moieties (P2) did not affect the inhibitory activities and binding abilities (e.g., MI-03 versus MI-21, MI-12 versus MI-28, and MI-14 versus MI-30; table S1 and fig. S2).

To illustrate the detailed binding mode of our compounds with SARS-CoV-2 Mpro, we determined the 2.0-Å structure of Mpro in complex with one of the most active compounds, MI-23 (IC50 = 7.6 nM) (Fig. 2, A to D). The crystal structure of the Mpro–MI-23 complex belongs to space group C2 (table S2) with one molecule per asymmetric unit. The biological dimer of Mpro is formed by an Mpro monomer and its symmetry-mate across the crystallographic two-fold axis (Fig. 2A). MI-23 binds Qiao et al., Science 371, 1374–1378 (2021) 26 March 2021
to the active site of M^pro as expected (Fig. 2, C and D). The carbon of the warhead interacts with the sulfur atom of catalytic residue Cys^145 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 Cys^145 and Gly^143 (forming the “oxygen hole”) (Fig. 2D). The P1 γ-lactam ring of MI-23 inserts deeply into the S1 pocket. The horizontal dotted line shows the viral load limit of detection (LOD) of 1.0 log_{10} RNA copies. Data below the LOD are shown at the LOD. *P < 0.05, **P < 0.01 (two-tailed unpaired Student’s t test). (E) Representative images of lung histopathological changes from SARS-CoV-2–infected hACE2 mice (5 × 10^6 TCID_{50}) at 3 dpi. Magnified views of the boxed regions for each image are shown below. Black arrows indicate alveolar septal thickening; red arrows point to inflammatory cell infiltration. See fig. S4 for whole-lung tissue scan images of SARS-CoV-2–infected hACE2 mice at 3 dpi. (F) Representative chemokine and cytokine assessment of the lungs (n = 3) of the indicated groups, as detected in lung tissue homogenate at 3 dpi. Data are means ± SD. *P < 0.05, **P < 0.01 versus the vehicle group (one-way analysis of variance). (G) and (H) Infiltration analysis for neutrophils and macrophages in the lungs of SARS-CoV-2–infected hACE2 mice (5 × 10^6 TCID_{50}) at 3 dpi. (G) Percentages of macrophages and neutrophils in the lungs. *P < 0.05. **P < 0.01 (unpaired Student’s t test). (H) Representative images of fluorescence staining. White triangle and arrow indicate macrophage and neutrophil, respectively.

Next, the compounds’ cellular antiviral activity was examined by a cell protection assay. In this assay, the viability of SARS-CoV–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 (29). Quantitative reverse transcription polymerase chain reaction (RT-qPCR) revealed that all six compounds inhibited SARS-CoV-2 virus replication in HPaEpiC cells with low-nanomolar EC_{50} values (0.3 to 7.5 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 were 0.89 μM and 23.7 nM. To further corroborate the antiviral potential of these compounds, we conducted RT-qPCR in another cell line, Huh7. The six compounds showed antiviral EC_{50} values of 31.0 to 96.7 nM, whereas 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, we conducted PK experiments in Sprague-Dawley 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). Because a compound with oral bioavailability of >10% has 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 summarized in Fig. 4, A and B. When administered intravenously (i.v.) (10 mg/kg), intraperitoneally (i.p.) (20 mg/kg), MI-09 showed area under the curve (AUC) values of 7429 hours-ng ml^-1, 11,581 hours-ng ml^-1, and 1665 hours-ng ml^-1, respectively, whereas MI-30 displayed AUC values of 9768 hours-ng ml^-1, 14,878 hours-ng ml^-1, and 2843 hours-ng ml^-1, respectively. After i.p. administration, MI-09 or MI-30 displayed a half-life (T_{1/2}) of 4.53 hours, a bioavailability of 78.0%, and a clearance rate (CL) of 22.67 ml min^-1 kg^-1. The corresponding values for MI-30 were T_{1/2} = 3.88 hours, bioavailability = 76.2%, and CL = 17.10 ml min^-1 kg^-1. On the basis of the EC_{50}/EC_{90} values from HPaEpiC cells, a single i.p. dose of 20 mg kg^-1 day^-1 MI-09 or MI-30 maintained the plasma levels at EC_{50} of 1.2 nM for MI-09, 1.1 nM for MI-30, and EC_{90} of 47.9 nM for MI-09, 58.8 nM for MI-30 for ~24 hours and 6 hours (fig. S3, A and B), respectively. Also, a single p.o. dose of 20 mg kg^-1 day^-1 MI-09 or MI-30 sustained the plasma levels at EC_{50} and EC_{90} for ~10 hours and 6 hours (fig. S3, C and D), respectively. Moreover, according to the EC_{50}/EC_{90} values from Vero E6 cells, with a single i.p. dose of 20 mg kg^-1 day^-1 MI-09 or MI-30, the durations of drug plasma levels above EC_{50} (0.86 μM for MI-09, 0.54 μM for MI-30) and EC_{90} (3.02 μM for MI-09, 2.12 μM for MI-30) were ~3 hours and 2 hours, respectively. A single p.o. dose of 20 mg kg^-1 day^-1 MI-09 or MI-30 caused the drug plasma concentrations to reach EC_{90} but not EC_{50} in Vero E6 cells. MI-09 and MI-30 were then evaluated for their toxicity in 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^-1 day^-1, i.p. at 100 and 200 mg kg^-1 day^-1, 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 TCID_{50} (50% tissue culture infectious dose) virus per mouse] and were then treated with vehicle (control), MI-09 [50 mg/kg p.o. twice daily (bid) or 50 mg/kg i.p. once daily (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 or body weight loss were observed in any animals tested. At 1 dpi, the mean viral RNA loads in the lung tissues of the three treatment groups were significantly lower (P < 0.05, Student’s t test) than that of the control group (Fig. 4D). At 3 dpi and 5 dpi, the viral RNA loads in the lung tissues of treatment groups were almost undetectable, and those of the 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 TCID_{50}, 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 lower than those of the control group at 1 dpi and significantly lower (P < 0.05, Student’s t test) at 3 dpi (Fig. 4D). At 5 dpi, the viral loads in the lung tissues were undetectable in the treatment groups and were low (near or below LOD) in the control group.

Histopathological analysis was performed for the lungs of mice infected with SARS-CoV-2 at 5 × 10^6 TCID_{50}. At 3 dpi, the vehicle-treated mice showed moderate alveolar septum thickening and inflammatory cell infiltration, whereas all compound-treated animals exhibited slight alveolar septum thickening and mild inflammatory cell infiltration (Fig. 4E). To investigate whether 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 expression levels of IFN-β and CXCL10 (Fig. 4F). Also, fewer neutrophils and macrophages occurred in the lungs of compound-treated mice than in control mice (Fig. 4, G and H), suggesting inhibition of immune cell infiltration. Together, our results show that 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.

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SUPPLEMENTARY MATERIALS

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Supplementary Text
References (31–41)
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