Identification of diphenoxylate as an antiviral agent against severe acute respiratory syndrome coronavirus 2

Jin Soo Shin,[1] Eunhye Jung,[1] Yejin Jang,[1] Soo Bong Han,[1,2,*] Meehyein Kim[1,3,*]

[1]Infectious Disease Research Center, Korea Research Institute of Chemical Technology (KRICT), Daejeon 34114, Republic of Korea; [1]Department of Medicinal and Pharmaceutical Chemistry, University of Science and Technology, Daejeon 34114, Republic of Korea; [3]Graduate School of New Drug Discovery and Development, Chungnam National University, Daejeon 34134, Republic of Korea

Running title: Diphenoxylate inhibits SARS-CoV-2 infection

***Corresponding authors**

Meehyein Kim, Ph.D.

Address: Infectious Diseases Therapeutic Research Center, Korea Research Institute of Chemical Technology, 141 Gajeongro, Yuseong, Daejeon 34114, Republic of Korea

E-mail: mkim@krict.re.kr; Telephone: 82-42-860-7540

Soo Bong Han, Ph.D.

Address: Infectious Diseases Therapeutic Research Center, Korea Research Institute of Chemical Technology, 141 Gajeongro, Yuseong, Daejeon 34114, Republic of Korea

E-mail: sbhan@krict.re.kr; Telephone: 82-42-860-7133

Abstract

The antiviral activity of chemical derivatives of diphenylmethyl piperazine together with diphenylbutyl or diphenylpropyl piperidine against SARS-CoV-2 was examined. The results revealed that diphenoxylate has the most potent antiviral efficacy, with an EC_{50} value of 1.4 μM and a CC_{50} value of >100 μM, resulting in selectivity index >71.4. These data provide an insight into the treatment of SARS-CoV-2 infection using this opioid drug or into the development of antivirals via modification of diphenylpropyl piperidine.

Keywords: SARS-CoV-2, antiviral, diphenoxylate, diphenylpropyl piperidine

Main text

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a member of the genus *Betacoronavirus*, harbors a positive sense RNA genome of approximately 30 kb in length. The genome has 14 ORFs that encode 27 proteins (1). The virus was first detected after people attending a seafood market in Wuhan, China, in December 2019 suffered pneumonia-like symptoms; the virus then spread rapidly to other countries (2). Within 6 months, the global outbreak of SARS-CoV-2 had caused 7.2 million confirmed infections and 413 thousand deaths (3). Unfortunately, the only drug available to treat this viral infection is remdesivir, which has been approved pre-emptively in some countries, including the United States, Japan, South Korea and India, but not worldwide. Remdesivir (also named GS-5734) is a monophosphoramidate prodrug of an adenosine analogue; the drug shows broad-spectrum antiviral activity against RNA viruses, including Ebola virus, Marburg virus, and Middle East respiratory syndrome coronavirus (MERS-CoV) (4). The search for alternative antivirals and/or synergistic drugs for use as combination therapy with remdesivir poses a significant challenge with respect to screening of chemical libraries comprising clinically approved drugs that could be repurposed.

SARS-CoV-2 utilizes angiotensin converting enzyme 2 (ACE2) as a receptor for target cell recognition; this event relies on the interaction between the peptidase domain (PD) of ACE2 and the receptor binding domain of the viral S protein (5). A recent *in silico* prediction study proposed that structurally similar diphenylmethyl piperazine and diphenylbutyl piperidine compounds, e.g., buclizine and loperamide, could bind to the PD of ACE2, thereby antagonizing binding of the viral S protein to the host receptor (6). We wondered whether these two compounds, or their derivatives, could function as anti-SARS-CoV-2 inhibitory molecules. Therefore, our aim was to evaluate the antiviral activity of diphenyl compounds in virus-

infected cells and to identify the most potent one for therapeutic treatment or further chemical modification.

SARS-CoV-2, isolated from a patient, was provided by the Korea Centers for Disease Control and Prevention (hCoV/Korea/KCDC-03/2020) and amplified for three passages in Vero CCL-81 cells (American Type Culture Collection, Rockville, MD) cultured in Dulbecco's modified Eagle's medium (HyClone, South Logan, UT) at 37℃ in a biosafety level 3 (BSL3) laboratory. The viral titer was determined in a plaque assay, and stocks were stored at -80℃ before use. The control compound, remdesivir (purity, 99.74%), was purchased from MedChem Express (Princeton, NJ), while the test compounds, which included derivatives of diphenylmethyl piperazine, diphenylbutyl piperidine, and diphenylpropyl piperidine, were provided by the Korea Chemical Bank (KCB, Daejeon, Republic of Korea). These were dissolved in DMSO to yield a final concentration of 5 mM and were used for primary screening (Supplementary Figure 1). Solid diphenoxylate powder was also provided by KCB. The purity of the test compounds was verified as > 95% by liquid chromatography-mass spectrometry analysis.

We examined whether the *in silico* simulation-derived compounds buclizine and loperamide inhibited SARS-CoV-2 infection *in vitro*. Vero CCL-81 cells were seeded in 96-well plates (2×10^4 cells per well) and treated 24 h later with 3-fold serial dilutions of each compound (from 200 μM). Cells were then infected with an equal volume of virus at a multiplicity of infection (MOI) of 0.1. At 48 h post-infection, cells were fixed and permeabilized prior to immunofluorescence assays using a mouse anti-S-antibody (GeneTex, Irvine, CA) and Alex Fluor 488-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA). Cell viability was measured by counterstaining nuclei with 4′,6-diamidino-2-phenylindole

(DAPI; Invitrogen). The number of S-derived (green) and cell nuclei-derived (blue) signals detected from four spots per well was quantified in duplicate using the Operetta high content screening system (Perkin Elmer, Waltham, MA) and the built-in Harmony software. We found that buclizine had a 50% effective concentration (EC_{50}) of 32.1 μM and was not toxic at a maximum concentration of 100 μM [50% cytotoxic concentration (CC_{50}), >100.0 μM). By contrast, loperamide was more potent (EC_{50}, 7.0 μM) but relatively toxic (CC_{50}, 17.8 μM) (Table 1). Remdesivir had an EC_{50} of 7.6 μM at sub-toxic concentrations (CC_{50}, >100.0 μM), indicating the reliability of our image-based antiviral assay system (7). Although buclizine and loperamide were active against SARS-CoV-2, their selectivity indices (SI) (>3.1 and 7.2, respectively) were lower than that of remdesivir (>18.3).

We obtained diphenyl derivatives from a chemical library and identified 12 new compounds (Supplementary Figure 1). Seven buclizine derivatives were subjected to antiviral examination (Table 1 and Supplementary Figure 1A). Only manidipine showed anti-SARS-CoV-2 activity (SI, >3.7) as effective as buclizine; the other compounds showed reduced activity or higher cytotoxicity (SI, <2.5). Thus, an antiviral agent with markedly increased effects was not identified from these diphenylmethyl piperazine compounds. Therefore, we used an alternative approach to target five derivatives of loperamide that harbored diphenylbutyl or diphenylpropyl piperidine groups (Supplementary Figure 1B). The antiviral assay revealed that diphenoxylate inhibited SARS-CoV-2 infection with markedly improved antiviral activity (EC_{50}, 1.4 μM; CC_{50}, >100 μM and SI, >71.4). It is noteworthy that the potency of diphenoxylate is about 5-fold greater than that of remdesivir. Immunofluorescence analysis using a powder stock with a purity over 95% provided visual confirmation that diphenoxylate reduced expression of the viral S protein in a dose-dependent manner, but did

not affect the number of nuclei (Figure 1A and B).

To confirm that diphenoxylate inhibits SARS-CoV-2 infection, we needed to exclude the possibility that the results from the image-based assays were not due to a fluorescence quenching effect by the compound or to non-specific binding of the antibody to a host protein regulated by viral infection. We performed western blot analysis additionally to examine antiviral activity directly. Vero CCL-81 cells were incubated in 6-well plates (5×10^5 cells per well) at 37℃ for 1 day. They were infected with SARS-CoV-2 at an MOI of 0.1 for 1 h and then were treated with diphenoxylate or remdesivir at 10 and 100 μM for 2 days. The results revealed that diphenoxylate inhibits expression of the viral S protein with two molecular weights of approximately 70 and 90 kDa in a similar way observed in the remdesivir-treated samples (Figure 1C). Thus, diphenoxylate is a potent antiviral compound that selectively blocks SARS-CoV-2 infection *in vitro*.

Diphenoxylate is an opioid drug used to treat diarrhea in combination with atropine (8). To the best of our knowledge, this is the first report to show that diphenoxylate has antiviral activity against SARS-CoV-2. In the absence of CoV-specific antivirals, this finding is important because it shows that the antiviral efficacy of diphenoxylate is comparable to that of remdesivir at sub-toxic concentrations. Prior to clinical trials, the compound should be tested in animal models such as human ACE2-expressing transgenic mice or golden Syrian hamsters (9, 10) challenged with SARS-CoV-2. We plan to examine the synergistic effects of diphenoxylate when combined with remdesivir to evaluate its potential applicability as a treatment for COVID-19.

Acknowledgements

This work was supported by a National Research Foundation of Korea (NRF) grant, funded by the Korean government (MSIT) (NRF-2020M3A9I2081687). The SARS-CoV-2 resource (NCCP, No. 43326) for this study was provided by the National Culture Collection for Pathogens. The test compounds and their purity information were provided by the Korea Chemical Bank (KCB).

References

1. Maters PS, Perlman S. 2013. Coronaviridae, p 825-858. *In* Knife DM, Howley PM (ed), Fields Virology, vol 2. Lippincott Williams and Wilkins, Philadelphia, PA, USA.
2. Wu A, Peng Y, Huang B, Ding X, Wang X, Niu P, Meng J, Zhu Z, Zhang Z, Wang J, Sheng J, Quan L, Xia Z, Tan W, Cheng G, Jiang T. 2020. Genome Composition and Divergence of the Novel Coronavirus (2019-nCoV) Originating in China. Cell Host Microbe 27:325-328.
3. WHO. 12 June 2020. Coronavirus disease (COVID-19) pandemic. https://www.who.int/emergencies/diseases/novel-coronavirus-2019?gclid. Accessed 12 June 2020.
4. Gordon CJ, Tchesnokov EP, Woolner E, Perry JK, Feng JY, Porter DP, Gotte M. 2020. Remdesivir is a direct-acting antiviral that inhibits RNA-dependent RNA polymerase from severe acute respiratory syndrome coronavirus 2 with high potency. J Biol Chem 295:6785-6797.
5. Yan R, Zhang Y, Li Y, Xia L, Guo Y, Zhou Q. 2020. Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2. Science 367:1444-1448.
6. Sajib A. 2020. Repurposing of Approved Drugs with Potential to Block SARS-CoV-2 Surface Glycoprotein Interaction with Host Receptor. Preprints doi:doi: 10.20944/preprints202004.0369.v1.
7. Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, Shi Z, Hu Z, Zhong W, Xiao G. 2020. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. Cell Res 30:269-271.
8. Harford WV, Krejs GJ, Santa Ana CA, Fordtran JS. 1980. Acute effect of diphenoxylate

with atropine (Lomotil) in patients with chronic diarrhea and fecal incontinence. Gastroenterology 78:440-3.

9. Bao L, Deng W, Huang B, Gao H, Liu J, Ren L, Wei Q, Yu P, Xu Y, Qi F, Qu Y, Li F, Lv Q, Wang W, Xue J, Gong S, Liu M, Wang G, Wang S, Song Z, Zhao L, Liu P, Zhao L, Ye F, Wang H, Zhou W, Zhu N, Zhen W, Yu H, Zhang X, Guo L, Chen L, Wang C, Wang Y, Wang X, Xiao Y, Sun Q, Liu H, Zhu F, Ma C, Yan L, Yang M, Han J, Xu W, Tan W, Peng X, Jin Q, Wu G, Qin C. 2020. The pathogenicity of SARS-CoV-2 in hACE2 transgenic mice. Nature doi:10.1038/s41586-020-2312-y.

10. Sia SF, Yan LM, Chin AWH, Fung K, Choy KT, Wong AYL, Kaewpreedee P, Perera R, Poon LLM, Nicholls JM, Peiris M, Yen HL. 2020. Pathogenesis and transmission of SARS-CoV-2 in golden hamsters. Nature doi:10.1038/s41586-020-2342-5.

Figure legend

Figure 1. Antiviral activity of diphenoxylate against SARS-CoV-2 *in vitro*. **(A)** Use of a fluorescent image-based assay system to visualize the antiviral activity of diphenoxylate. Vero CCL-81 cells were infected with SARS-CoV-2 at an MOI of 0.1 in the presence of the compounds (0.1, 1.2, or 11.1 μM). Mock-infected or virus-infected cells treated with DMSO were used as controls. On day 2 post-infection (p.i.), the viral S protein was probed with a mouse anti-S antibody, followed by Alexa Fluor 488-conjugated goat anti-mouse IgG (green). Cell nuclei were counterstained with DAPI (blue). Magnification, ×20. **(B)** Dose-response curves of antiviral activity and cell viability on day 2 p.i. The number of green or blue spots per image was counted. Inhibitory activity (red line) and cell viability (black line) are expressed as percentages relative to the values obtained from SARS-CoV-2-infected cells in the presence of DMSO (defined as 100%). Data represent the mean ± standard deviation from triplicate samples. **(C)** Western blot showing reduced expression of the viral spike protein after exposure to diphenoxylate and remdesivir. Virus-infected cells were treated with DMSO delivery vehicle or each compound (10 or 100 μM). Two days later, cell lysates were harvested and loaded onto 10% SDS-PAGE gels (30 μg per well). The viral spike protein was detected using a specific antibody, followed by HRP-conjugated goat anti-mouse IgG. 'No virus' means the mock-infection control. Cellular β-actin was used as a loading control. Both proteins are indicated on the right side of the gels.