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Identification of non-covalent 3C-like protease inhibitors against severe acute respiratory syndrome coronavirus-2 via virtual screening of a Korean compound library

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ABSTRACT

The outbreak of coronavirus (CoV) disease 2019 (COVID-19) caused by the severe acute respiratory syndrome CoV-2 (SARS-CoV-2) has turned into a pandemic. The enzyme 3C-like protease ($3CL^{pro}$) is essential for the maturation of viral polyproteins in SARS-CoV-2 and is therefore regarded as a key drug target for treating the disease. To identify $3CL^{pro}$ inhibitors that can suppress SARS-CoV-2 replication, we performed a virtual screening of 500,282 compounds in a Korean compound bank. We then subjected the top computational hits to inhibitory assays against $3CL^{pro}$ in vitro, leading to the identification of a class of non-covalent inhibitors. Among these inhibitors, compound 7 showed an EC_{50} of 39.89 μM against SARS-CoV-2 and CC_{50} of 453.5 μM. This study provides candidates for the optimization of potent $3CL^{pro}$ inhibitors showing antiviral effects against SARS-CoV-2.

As of May 6, 2021, the coronavirus (CoV) disease 2019 (COVID-19)[1] has caused 155,813,360 confirmed cases and 3,254,882 deaths (https://covid19.who.int/). The first case was reported in December 2019.[1,2] The causative agent of COVID-19 is homologous to the severe acute respiratory syndrome (SARS)-associated CoV (SARS-CoV) that caused an outbreak in 2002–2003,[3,4] and it was thereby named SARS-CoV-2 by the World Health Organization. Another outbreak that occurred in the Middle East in 2012 and spread to South Korea in 2015 was also caused by a human CoV known as the Middle East respiratory syndrome CoV (MERS-CoV).[5,6] Targeting the enzymes essential for viral replication and the lifecycle of SARS-CoV-2 is a promising strategy for clinical therapy. At present, remdesivir treatment has shown a marginal (68%) benefit in clinical trials for patients with COVID-19,[7] and it was thereby approved for use by the USA-Food and Drug Administration (FDA). It was previously shown to inhibit SARS-CoV and MERS-CoV,[8] and it also antagonizes SARS-CoV-2 replication at a half-maximal effective concentration (EC_{50}) of 0.7 μM[9] by targeting the viral enzyme RNA-dependent RNA polymerase.[10]

A key protease called 3C-like protease ($3CL^{pro}$) undergoes auto-cleavage and then cleaves 11 sites on polyproteins generated by CoVs inside the host cells, which is essential for viral replication[11]; therefore, it has been used as a target for developing antivirals against SARS-CoV, MERS-CoV, and SARS-CoV-2.[12–14] $3CL^{pro}$ is a chymotrypsin-like enzyme; however, it utilizes the His-Cys catalytic dyad to cleave

Abbreviations: COVID-19, coronavirus (CoV) disease 2019; SARS-CoV-2, severe acute respiratory syndrome CoV-2; MERS-CoV, Middle East respiratory syndrome CoV; FDA, Food and Drug Administration; EC_{50}, half-maximal effective concentration; $3CL^{pro}$, 3C-like protease; IC_{50}, half-maximal inhibitory concentration; KCB, Korean compound bank; 3D, three-dimensional; PDB, Protein Data Bank; CC_{50}, half-maximal cytotoxic concentration; CPE, cytopathic effect; MOI, multiplicity of infection.

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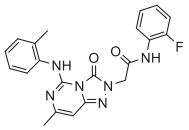
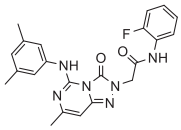
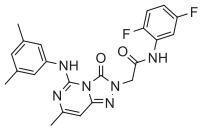
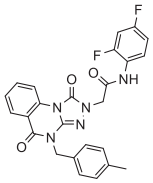
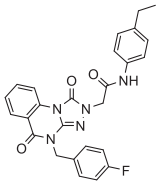
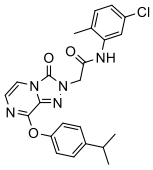
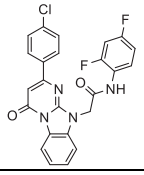
Table 1
IC_{50} and EC_{50} values of hit compounds in SARS-CoV-2 $3CL^{pro}$ and SARS-CoV-2.

Compound	Structure	IC_{50} (μM)	EC_{50} (μM)	Compound	Structure	IC_{50} (μM)	EC_{50} (μM)
1		8.7 ± 0.8	NA	**2**		10.9 ± 0.8	NA
3		8.0 ± 1.4	140.9 ± 3.80	**4**		9.5 ± 0.7	NA
5		12.1 ± 2.4	NA	**6**		8.9 ± 0.8	NA
7		5.7 ± 1.1	39.89 ± 0.17	**Remdesivir**		NA	11.1

conserved sequences of (Leu, Met, Phe)-Gln↓(Ser, Ala, Gly) on the polyproteins of SARS-CoV.[15] Due to the essential role of the protease, several rationally designed peptidomimetics based on its substrate specificity, as well as FDA-approved or experimental drugs such as disulfiram, ebselen, tideglusib, TDZD-8, carmofur, and PX12 have been investigated and shown to inhibit $3CL^{pro}$ activity and SARS-CoV-2 replication.[16–19] Moreover, boceprevir, GC-376, and calpain inhibitors II and XII have been demonstrated to inhibit SARS-CoV-2 viral replication by targeting $3CL^{pro}$.[20] As reported recently, the cysteine protease inhibitors MDL-28170 (calpain inhibitor III) and ONO 5334 (cathepsin K inhibitor) inhibit SARS-CoV-2 viral replication.[21] Furthermore, presently used pharmaceuticals and herbal medicines have been screened for activity against SARS-CoV-2; several compounds have been identified as inhibitors of $3CL^{pro}$ activity and SARS-CoV-2 replication.[22] Moreover, by screening collections of 1068 and 2701 FDA drugs for inhibition of the $3CL^{pro}$ and papain-like protease, respectively, we identified six drugs showing activity against SARS-CoV-2.[23]

Using an artificial intelligence approach, several drugs have been identified that inhibit SARS-CoV-2 replication in vitro. By performing quantitative high-throughput screening of 10,755 approved and investigational drugs and bioactive compounds, 23 small molecules have been found that inhibit SARS-CoV-2 $3CL^{pro}$ activity at a half-maximal inhibitory concentration (IC_{50}) ranging from 0.26 to 28.85 μM, among which seven had an anti-SARS-CoV-2 effect.[24] By analyzing the $3CL^{pro}$ pharmacophore clusters for application in virtual screening of 2122 FDA drugs, we identified nelfinavir and boceprevir for drug repurposing for treating COVID-19.[25] In this study, we performed virtual screening of the Korea Chemical Bank (KCB) library and identified a group of $3CL^{pro}$ inhibitors that showed IC_{50} values less than 10 μM. Among the inhibitors identified, the most potent candidate showed moderate antiviral activity at an EC_{50} of 39.89 μM. Our study provides candidates for inhibiting $3CL^{pro}$ activity and SARS-CoV-2 replication, which can be optimized further for developing a potential therapy for COVID-19.

Virtual screening of the KCB library to identify potential $3CL^{pro}$ inhibitors. To identify non-peptidomimetic chemical inhibitors of SARS-CoV-2 $3CL^{pro}$ from the KCB library, we performed ligand-based virtual screening using the three-dimensional (3D) shapes of the ligand-complexed SARS-CoV $3CL^{pro}$ structures, 3 MJ5, 3SN8, 3V3M, 4OVZ, 4OW0, and 4TWW, obtained from the Protein Data Bank (PDB) (http://www.rcsb.org). All ligand-based virtual screening was performed using the OpenEye Scientific software (OpenEye Scientific Software, Santa Fe, NM, USA). The database was prepared using OMEGA v3.0.1, which was used to generate a maximum number of 200 low-energy conformers for each molecule in the 500,282-membered KCB library. A 3D shape similarity search of ligands as a query was performed using ROCS v3.2.2, and the top 10,000 hits for each query molecule were selected based on the ROCS_TanimotoCombo score. We then performed a 3D electrostatic properties similarity search for the screened 10,000 compounds using EON v2.2.0, and the top 1,000 hits were selected based on the EON_ET_Combo score for each of the six query molecules. The sum of 3D shape- and electrostatically similar candidates comprised 6,000 compounds that were finally selected for structure-based virtual screening.

Structure-based virtual screening was performed using Schrodinger Suite 2020–1 (Schrödinger LLC, New York, NY, USA) and the X-ray crystal structure of SARS-CoV-2 $3CL^{pro}$ in complex with the peptide inhibitor obtained from PDB (ID 6LU7). The protein structure was modified using Protein Preparation Wizard in Maestro, and the receptor grid box was generated at a cubic size of 25 × 25 × 25 Å centered on a complexed ligand. The 6,000 compounds identified via ligand-based virtual screening and the 500,282 members of the KCB library were subjected to ligand preparation using the LigPrep module by applying an OPLS3e force field. During the process, tautomer and ionization states at pH 7.0 ± 2.0 were generated using the Epik module. Docking for each chemical library database was performed using the Glide v8.6 program in the Standard Precision mode. Based on visual inspection, 253 compounds were selected for performing $3CL^{pro}$ assays to determine the active hits.

Similarity-based virtual screening using two-dimensional fingerprints. To identify more analogues of the active hits, we performed a second round of virtual screening based on the fingerprint similarity method. The similarity between single pairs of compounds was calculated using Pipeline Pilot 2020 (Dassault Systèmes BIOVIA, San Diego, CA, USA). The KCB library database was screened using a Tanimoto

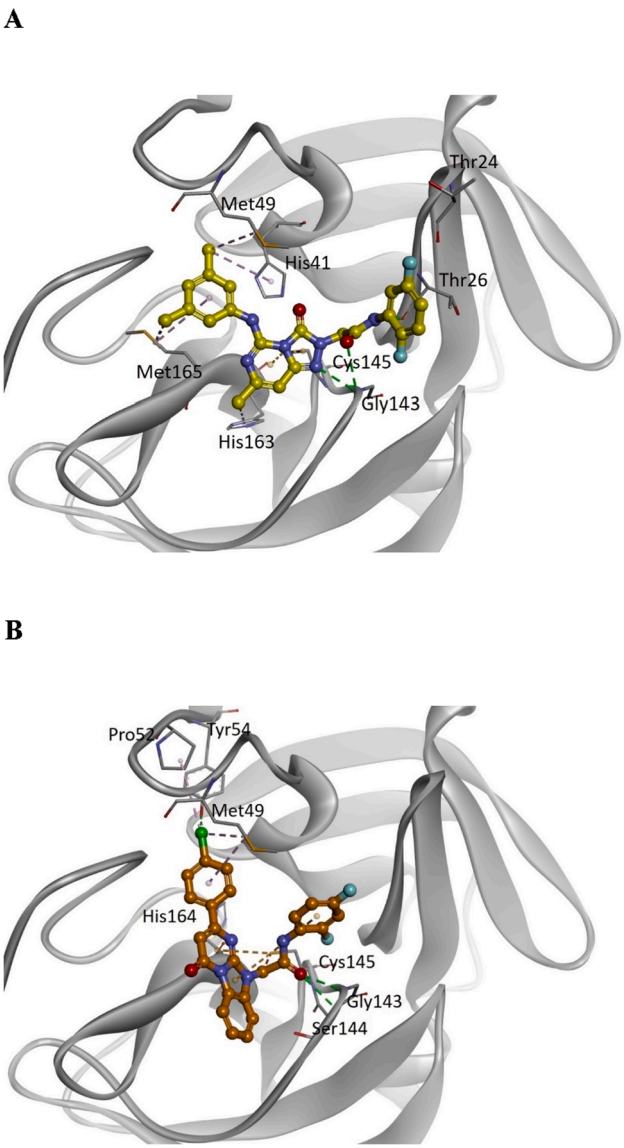


Fig. 1. Predicted binding models of compounds **3** and **7** with SARS-CoV-2 $3CL^{pro}$. (A) Binding of compound **3** (yellow) and (B) compound **7** (orange) to the SARS-CoV-2 $3CL^{pro}$ (grey). For clarity, key binding site residues are illustrated as sticks and labeled using the 3-letter amino acid codes. The hydrogen bonds are displayed as green lines with dashes, and hydrophobic interactions are shown as pink lines with dashes. The electrostatic interactions are indicated using orange lines with dashes.

coefficient ≥ 0.8 compared with the hits for selecting the compounds for IC_{50} measurements.

Determination of IC_{50} of the selected inhibitors. After identifying a collection of candidate compounds, we next tested their inhibitory activity against $3CL^{pro}$ using a fluorogenic peptide, Dabcyl-KTSAVLQSGFRKME-Edans, which contains a fluorescence quenching pair; the fluorescence increased when the peptide was cleaved by the protease.[22] Subsequently, the IC_{50} values of the active compounds were determined using reaction mixtures containing 2.5 nM $3CL^{pro}$ and 6 μM fluorogenic substrate in a buffer of 20 mM Bis-Tris (pH 7.0) in the absence and presence of various concentrations of the inhibitors. All experiments were performed in triplicate and the values are presented as the mean ± standard deviation (Table 1). The most active were compounds **3** and **7**, and their IC_{50} values were determined to be 8.0 ± 1.4 and 5.7 ± 1.1 μM, respectively.

Computer modeling of the binding modes of compounds 3 and 7. According to the in silico virtual screening and the $3CL^{pro}$ inhibition assay, compounds **3** and **7** were identified as the most potent inhibitors in the group. To determine their potential binding modes with $3CL^{pro}$, computer modeling was performed for these two compounds. In compound **3** (Fig. 1**A**), the triazolopyrimidineone ring was found to bind to the active site adjacent to Cys145 via electrostatic interactions, whereas the Gly143 backbone NH formed additional hydrogen bonds with the nitrogen of the ring. The amide bond formed two hydrogen bonds with the backbone NH groups of Thr26 and Gly143. The 1,4-difluoro substituted phenyl ring was located toward Thr24 and Thr26. The 3,5-dimethyl substituted phenyl group was bound to the hydrophobic pocket formed by His41, Met49, and Met165. In compound **7** (Fig. 1**B**), the benzoimidazopyrimidinone ring was found to bind to the active site adjacent to Cys145 via electrostatic interactions. The carbonyl group of the amide bond formed hydrogen bonds with the backbone NH groups of Gly143 and Ser144. The 1,3-difluoro substituted phenyl ring was located toward Leu27 and Met49 and was shorter than the 1,3-difluorophenyl ring in compound **3**. The chlorophenyl ring was found to bind to the same hydrophobic pocket that consisted of His41, Met49, and Met165 in compound **3**.

Antiviral activities of the $3CL^{pro}$ inhibitors 3 and 7 were determined against SARS-CoV-2 based on image assays. Vero E6 (ATCC CRL-1586) and Vero CCL-81 (ATCC CCL-81) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Cytiva, Marlborough, MA, USA) supplemented with 5% (v/v) fetal bovine serum (Cytiva) at 37 °C in a 5% CO_2 atmosphere.[26–27] The $3CL^{pro}$ inhibitor **3** and **7** showed antiviral activity against SARS-CoV-2 at an EC_{50} of 140.9 ± 3.8 μM and 39.89 ± 0.17 μM (Table 1). The CC_{50} values for inhibitors **3** and **7** were 468.7 ± 7.6 μM and 453.5 ± 19.9 μM, respectively, based on the cell viability inhibition percentages, represented by grey squares

As demonstrated here, we performed virtual screening of the Korean compound library containing 500,282 compounds to identify potential inhibitors of SARS-CoV $3CL^{pro}$. We then measured the IC_{50} values of the inhibitors using a peptide substrate with the fluorescence quenching pair Dabcyl/Edans at the N- and C-termini, respectively. The IC_{50} values of most inhibitors were less than 10 μM. Finally, the inhibitors were subjected to cell-based CPE assays to determine their anti-viral activity; compound 7 with more potent $3CL^{pro}$ inhibitory activity also displayed a lower EC_{50} of 39.89 ± 0.17 μM than that (140.9 ± 3.8 μM) of compound 3. Therefore, compound 7 with a selectivity index of more than 10 might serve as an initial hit for further optimization of this group of inhibitors. We thus provide here a candidate for further optimization for potential treatment of COVID-19.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Author contributions

J.Y.L. performed the in-silico virtual screening and molecular docking studies; C.J.K. conducted the $3CL^{pro}$ assay; J.S.S. and E.J. carried out the anti-viral EC_{50} measurements; Y.S.J and P.H.L. wrote the paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.128067.

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