

Malonic Acid-Type Fullerene Derivatives Strongly Inhibit The SARS-Cov-2 Main Protease

Daiki Katagishi

Keio University

Daisuke Yasuda

Keio University

Kyoko Takahashi

Nippon Medical School

Shigeo Nakamura

Nippon Medical School

Tadahiko Mashino

Keio University

Tomoyuki Ohe (✉ ohetm@pha.keio.ac.jp)

Keio University

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Abstract

COVID-19 is a disease that is causing a global pandemic. There is an urgent need to develop new drugs to treat it. In this study, we evaluated the inhibitory activities of a series of fullerene derivatives against the main protease of SARS-CoV-2, the virus that causes COVID-19. As a result, it was found that the malonic acid-type fullerene derivatives showed strong inhibitory activities.

Introduction

COVID-19 (coronavirus disease 2019) is an acute respiratory disease caused by SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2). It is generally believed that the first outbreak of COVID-19 occurred in Wuhan City, Hubei Province, People's Republic of China, in December 2019, [1] and the infection has spread all over the world. This pandemic is still ongoing, and at the time of writing this article (6:18 PM CET, 4 January 2022), the number of infected people worldwide exceeds 290,959,019, with 5,446,753 deaths. [2] Patients with COVID-19 have suffered a wide spectrum of symptoms ranging from asymptomatic/mild symptoms to severe pneumonia and death. [3]

SARS-CoV-2 is a positive-sense single-stranded RNA virus, and its genome length is 29.881 kb (GenBank No. MN908947), encoding 4 structural proteins, 16 nonstructural proteins (nsp1-nsp16), and accessory proteins. The structural proteins include the spike (S) protein, envelope (E) protein, membrane (M) protein, and nucleocapsid (N) protein. The N protein binds to viral genomic RNA and forms a nucleocapsid that is enclosed by a lipid membrane known as the viral envelope containing S, E, and M proteins. The S protein plays an important role in viral entry. It mediates cell infection by interacting with a receptor on the host cell surface, angiotensin-converting enzyme 2 (ACE2). [4] The E and M proteins are necessary for virus assembly and budding. Among the 16 nonstructural proteins, nsp3 (papain-like protease, PL^{Pro}), nsp5 (main protease, M^{Pro}, or 3-chymotrypsin-like protease, 3CL^{Pro}), nsp12 (RNA-dependent RNA polymerase), nsp13 (helicase), nsp14 (N7-methyltransferase), and nsp16 (2'-O-methyltransferase) act as enzymes. [5]

Thus, while there are many potential targets for COVID-19, SARS-CoV-2 M^{Pro} plays an important role in the cleavage of viral polyproteins into functional proteins and is recognized as an attractive target for antiviral drugs. SARS-CoV-2 M^{Pro} is a cysteine protease with a catalytic dyad comprised of Cys145 and His41. In the hydrolysis reaction of polyprotein, the imidazole group of His41 activates the SH group of Cys145, which acts as a nucleophile. [6]

Some inhibitors against SARS-CoV-2 have been reported, and most of them were originally drugs for other targets, [7-9] including anti-human immunodeficiency virus (HIV) drugs such as lopinavir, ritonavir, darunavir and cobicistat, [8] and anti-hepatitis C virus (HCV) drugs such as boceprevir and narlaprevir. [9] Drugs for the treatment of COVID-19 are being developed by pharmaceutical companies around the world. For example, molnupiravir, developed by Merck, was approved for use in some countries.

Fullerene is a third carbon allotrope represented by soccer-ball-shaped C₆₀ discovered by Kroto *et al.* [10] Its spherical and condensed aromatic rings with an extended p-conjugation system are so unique that C₆₀ has fascinated scientists all over the world. With the aim of pharmaceutical usage, its poor water solubility is a barrier. Therefore, many water-soluble fullerene derivatives have been synthesized by chemical derivatization, and their biological activities have been investigated. [11] We have previously reported inhibitory activities of fullerene derivatives against HIV protease, [12] HIV reverse transcriptase, [12-15] HCV protease [16] and HCV RNA-dependent RNA polymerase. [13, 16]

As mentioned above, there have been many reports that anti-HIV and HCV drugs deserve further attention as repurposed drugs for COVID-19. Therefore, fullerene derivatives exhibiting anti-HIV and anti-HCV activities could be drug candidates for the treatment of COVID-19. The aim of the current study was to comprehensively investigate the inhibitory activities of a variety of fullerene derivatives against SARS-CoV-2 M^{Pro}.

Materials And Methods

Reagents

The chemical structures of the test compounds used for the present study are shown in Figure 1. We have reported the synthesis of fullerene derivatives **1** to **4** and **8** to **13**. [12, 15-18] Our previous studies confirmed that **1**, **2**, and **8** are *trans* isomers and **3** is a *cis* isomer. Bis-adduct fullerene derivatives such as **7** and **12** are mixtures of several regioisomers. A polyhydroxylated fullerene, which is also known as fullerenol or fullerol, **5** (with 30-50 hydroxy groups) was obtained from SOLARIS CHEM (Vaudreuil-Dorion, Quebec, Canada). Compounds **6** and **7** were synthesized in the same manner as in the literature, [19] with minor modifications. Boceprevir and malonic acid were purchased from ChemScene (Monmouth Junction, NJ, USA) and Sigma–Aldrich (St. Louis, MO, USA), respectively.

SARS-CoV-2 M^{Pro} Inhibition Assay

The SARS-CoV-2 M^{Pro} inhibitory activities were examined in a similar manner to the HIV protease inhibition assay established by our laboratory, [12] although the incubation conditions were modified in accordance with the report by Jang *et al.*, [20] in which the inhibitory activities of polyphenols against M^{Pro} were evaluated. Boceprevir, which has already been reported to have M^{Pro} inhibitory activity, was used as a positive control.

Briefly, to each well of a 96-well plate, 8.0 mL of a 10% dimethyl sulfoxide (DMSO) solution of the test compounds, 8.0 mL of the SARS-CoV-2 M^{Pro} (BPS Bioscience, Inc., San Diego, CA, USA) solution and 50 mL of buffer were added and incubated at 37 °C for 1 h. Then, the enzymatic reaction was initiated by adding 14 mL of the 12-mer peptide TSAVLQSGFRKM (custom peptide synthesized by Sigma–Aldrich), which mimics the nsp4-5 junction. The final concentrations in the reaction mixture were 0.10-40 mM test compound, 20 mM tris(hydroxymethyl)aminomethane-HCl (pH = 7.5), 200 mM NaCl, 5 mM

ethylenediaminetetraacetic acid, 5 mM dithiothreitol, 1% DMSO, 50 mg/mL substrate peptide and 100 ng/mL M^{Pro}. After incubation at 37 °C for 1 h, 20 mL of 10% trifluoroacetic acid solution was added to stop the reaction. After centrifugation (1,000 rpm, 5 min), 60 mL of the supernatant was transferred to a 96-well plate, and the quantity of the cleaved peptides (SGFRKM) was measured by Agilent 1260 High Performance Liquid Chromatography (HPLC) (Agilent Technologies, Santa Clara, CA, USA) connected to an Agilent 6120 mass spectrometer (Agilent Technologies). Chromatographic separations were performed by an InertSustain®C18 3.0 mm (4.6 × 150 mm) column (GL Sciences, Tokyo, Japan) at a flow rate of 0.50 mL/min under isocratic 15% acetonitrile containing 0.1% formic acid for 10 min. The eluent was introduced directly into the mass spectrometer via electrospray ionization using the positive ion mode.

Results And Discussion

SARS-CoV-2 M^{Pro} inhibitory activities of various fullerene derivatives including proline-type derivatives (**1-3**), a derivative previously designed for HIV protease inhibition (**4**), fulleranol (**5**), malonic acid-type derivatives (**6, 7**), and cation-type derivatives (**8-13**) at a fixed concentration were determined by LC-MS. Figure 2 shows the residual activities of M^{Pro} after incubation with these compounds at 1.0 mM.

Among the assessed compounds, malonic acid-type derivatives, **6** and **7**, especially exhibited the most potent inhibitory activities. Proline-type derivatives (**1-3**) moderately inhibited M^{Pro}, but their inhibitory activities were obviously weaker than those of the malonic acid-type derivatives. Fulleranol (**5**) showed no inhibitory activity against M^{Pro} at 1.0 mM, indicating that a number of hydroxy groups attached to the fullerene surface lost the inhibitory activity. Compound **4**, which has a hydroxymethylcarbonyl moiety, is a transition-state mimic isostere of HIV protease substrate processing and showed potent efficacy against HIV protease in our previous report, [12] and it exhibited weak inhibition against M^{Pro}. Given the structural difference between the two proteases, this result might be reasonable. HIV protease is a homodimeric aspartyl protease composed of two identical subunits. The active site is located at the interface between the two monomers and contains catalytic Asp-Thr-Gly. In contrast, M^{Pro} is a cysteine protease that has a Cys-His catalytic dyad at its active site. Among the six cationic fullerene derivatives, only **8** and **12** showed moderate activities.

Further experiments with different concentrations confirmed that the half-maximal inhibitory concentrations (IC₅₀) for the proline-type derivative **2** and cationic fullerene derivative **8** in addition to the most potent derivatives **6** and **7** were 0.98, 1.3, 0.53 and 0.20 mM, respectively (Table 1). The IC₅₀ of boceprevir, an HCV NS3/4A protease inhibitor that has been reported to exhibit M^{Pro} inhibitory activity, was 28 mM in the current experiment, indicating that these fullerene derivatives have stronger activity than the existing drug that has been reported to be active. In particular, derivative **7** showed extremely strong activity with an IC₅₀ value lower than that of boceprevir by two orders of magnitude.

Table 1 IC₅₀ values of fullerene derivatives **2, 6, 7** and **8** for M^{Pro} inhibition

Compound	SARS-CoV-2 M ^{Pro} inhibitory activity (IC ₅₀ , mM)
2	0.98
6	0.53
7	0.20
8	1.3
Boceprevir	28

Furthermore, it was confirmed that malonic acid corresponding to the exo-substituent on the most potent derivatives (**6** and **7**) lost the activity (data not shown). From this result, it was shown that the inhibitory activity against SARS-CoV-2 M^{Pro} is dependent on the fullerene backbone, which is similar to our previous report that this is also the case for HCV protease and HIV protease.

Conclusions

In summary, it was found that malonic acid-type fullerene derivative **7** showed strong inhibitory activity against SARS-CoV-2 M^{Pro}. Since **7** has been previously reported to be nontoxic to HeLa cells in the range of 64 mM or less [21] and is expected to be applied to pharmaceuticals, **7** may be a potential drug candidate for the treatment of SARS-CoV-2. However, this compound is a bisadduct derivative of fullerene that contains multiple regioisomers, and it will be necessary to isolate each isomer and evaluate individual inhibitory activity to discover a more active compound. Additionally, it was found that there were remarkable differences in inhibitory activities among fullerene derivatives. To elucidate more detailed structure-activity relationships, further investigation will be required.

Abbreviations

COVID-19: coronavirus disease 2019; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; nsp: nonstructural proteins; S protein: spike protein; E protein: envelope protein; M protein: membrane protein; N protein: nucleocapsid protein; ACE2: angiotensin-converting enzyme 2; PL^{Pro}: papain-like protease; M^{Pro}: main protease; 3CL^{Pro}: 3-chymotrypsin-like protease; HIV: human immunodeficiency virus; HCV: hepatitis C virus; DMSO: dimethyl sulfoxide; HPLC: High Performance Liquid Chromatography; IC₅₀: the half-maximal inhibitory concentrations

Declarations

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conception and design of the research: Katagishi, Yasuda, Takahashi, Nakamura, Mashino and Ohe.

Acquisition and analysis of data: Katagishi.

Interpretation of data: Katagishi, Yasuda, Mashino, and Ohe

Drafted the manuscript: Katagishi.

Revised the manuscript: Yasuda, Takahashi, Nakamura, Mashino and Ohe.

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Author details

1. Department of Pharmaceutical Sciences, Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo, Japan.
2. Department of Chemistry, Nippon Medical School, 1-7-1 Kyonan-cho, Musashino, Tokyo, Japan

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Figures

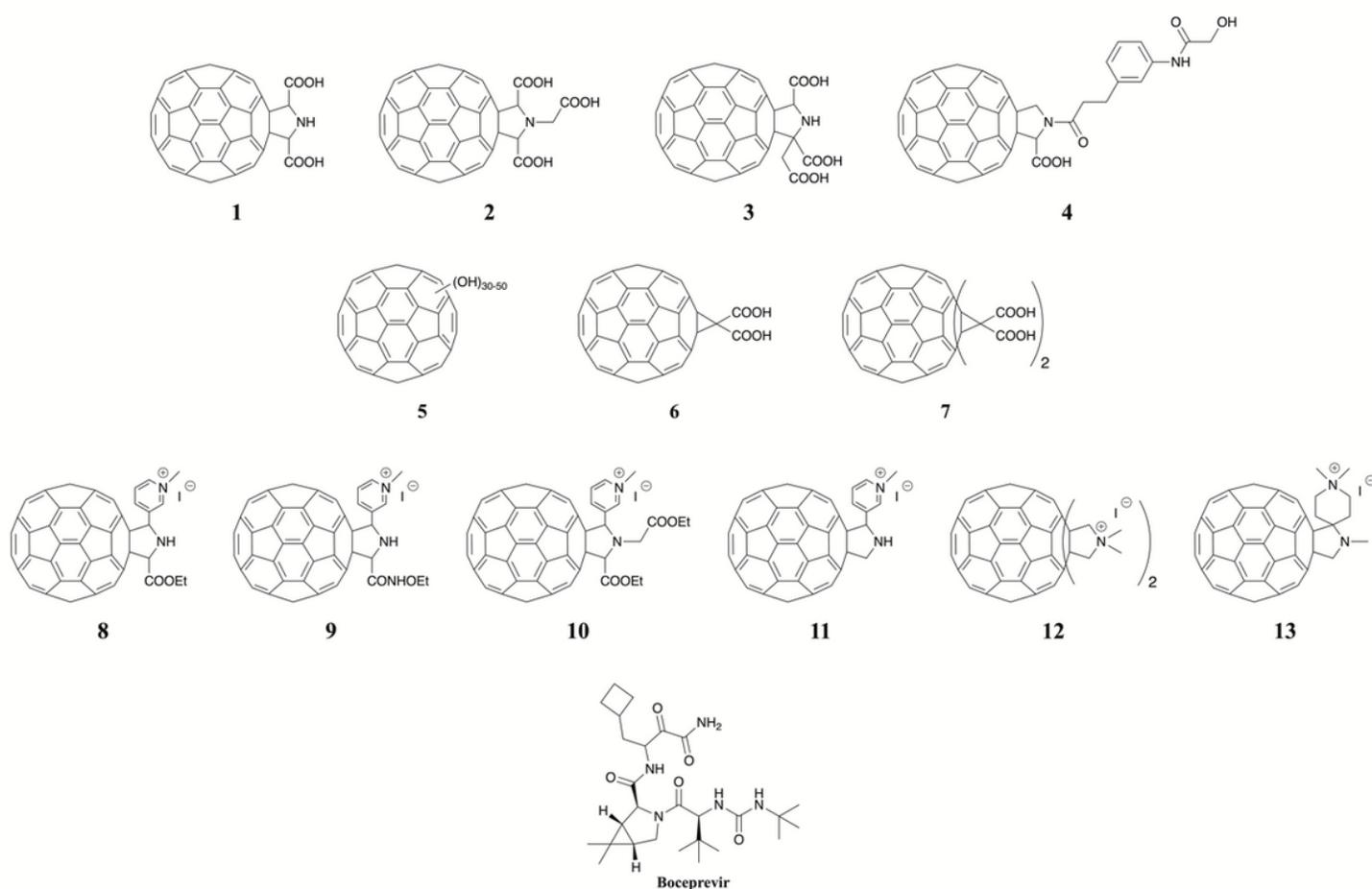


Figure 1

Test compounds

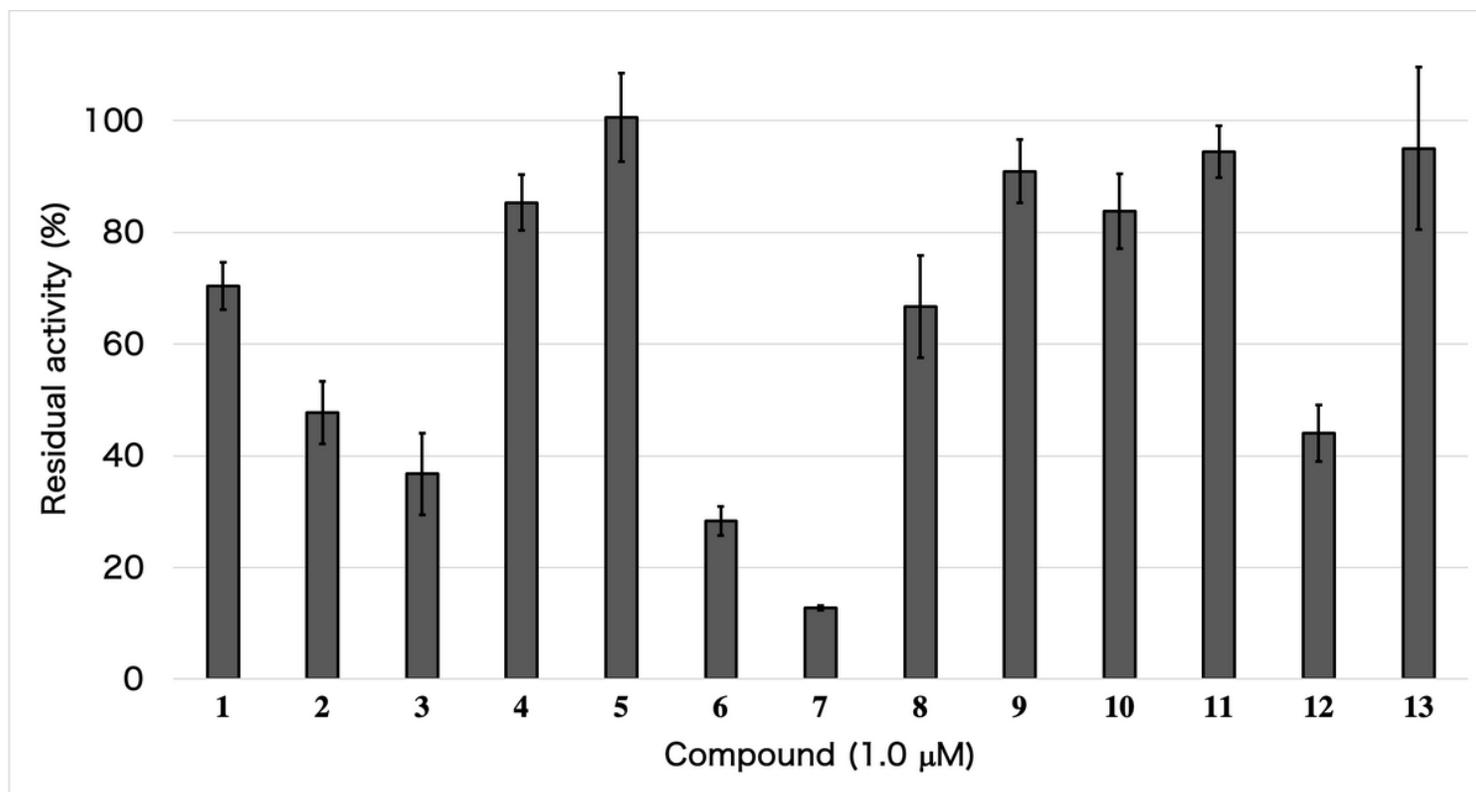


Figure 2

SARS-CoV-2 M^{Pro} inhibitory activities of fullerene derivatives at 1.0 mM