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Human immunodeficiency virus-reverse transcriptase inhibition and hepatitis C virus RNA-dependent RNA polymerase inhibition activities of fullerene derivatives

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Abstract—We examined the human immunodeficiency virus-reverse transcriptase and hepatitis C virus RNA-dependent RNA polymerase inhibition activities of cationic, anionic, and amino acid-type fullerene derivatives. Among the fullerene derivatives, the amino acid-type fullerene derivative was the most efficient in human immunodeficiency virus-reverse transcriptase inhibition.

Both the human immunodeficiency virus (HIV) and the hepatitis C virus (HCV) are RNA viruses and have similar enzymes. For example, HIV-reverse transcriptase and HCV-RNA-dependent RNA polymerase are RNA-dependent polymerases, which are essential for the replication of the virus. Indeed, HIV-RT is one of the major targets for anti-HIV agents.

HIV infection is one of the major causes of morbidity and mortality in the world. There are many anti-HIV agents, but their efficiency is not very high. Moreover, the emergence of drug-resistant mutant forms of HIV requires the development of effective and well-tolerated new remedies.

HCV is the major etiological virus of non-A and non-B hepatitis. An estimated 2–3% of the world population is chronically infected with HCV. HCV infection causes severe liver disease and can lead to the development of hepatocellular carcinoma. Now, interferon and repavirin are used for HCV therapy, but their therapeutic ratio is low. HCV RNA-dependent RNA polymerase, protease, and helicase, which are essential for the virus replication, are new targets for an anti-HCV drug.

These threats provide motivations to search for new types of lead compounds to be used as medicine against HIV and HCV infection.

The biological effects of fullerene and its derivatives are also of interest.[1,2] Some biological activities based on their unique physical properties and chemical reactivities have been reported. For example, DNA scissions[3,4] and the oxidation of biological materials[5] depend on photo-induced active oxygen production by the fullerene.[6] For other interesting enzymes, for example, glutathione transferase, glutathione reductase, and nitric oxide synthase, the inhibition activities of fullerene derivatives have also been reported.[7–10] We intend to develop fullerene derivatives as a new type of lead compound to be used as medicine and have reported that the anionic fullerene derivatives, carboxy fullerene, show interesting antioxidant activities,[11] and the cationic derivatives, C_{60}-bis(*N*,*N*-dimethylpyrrolidinium iodide) and its alkylated derivatives, have excellent antibacterial and antiproliferative activities.[12]

In this report, we studied the HIV-RT and HCV-RP inhibition activities of anionic (**1**), cationic (**2**–**6**), and amino acid types (**7**, **8**) fullerene derivatives (Fig. 1).

Keywords: Fullerene; Anti-HIV; Anti-HCV; Reverse transcriptase; RNA-dependent RNA polymerase.
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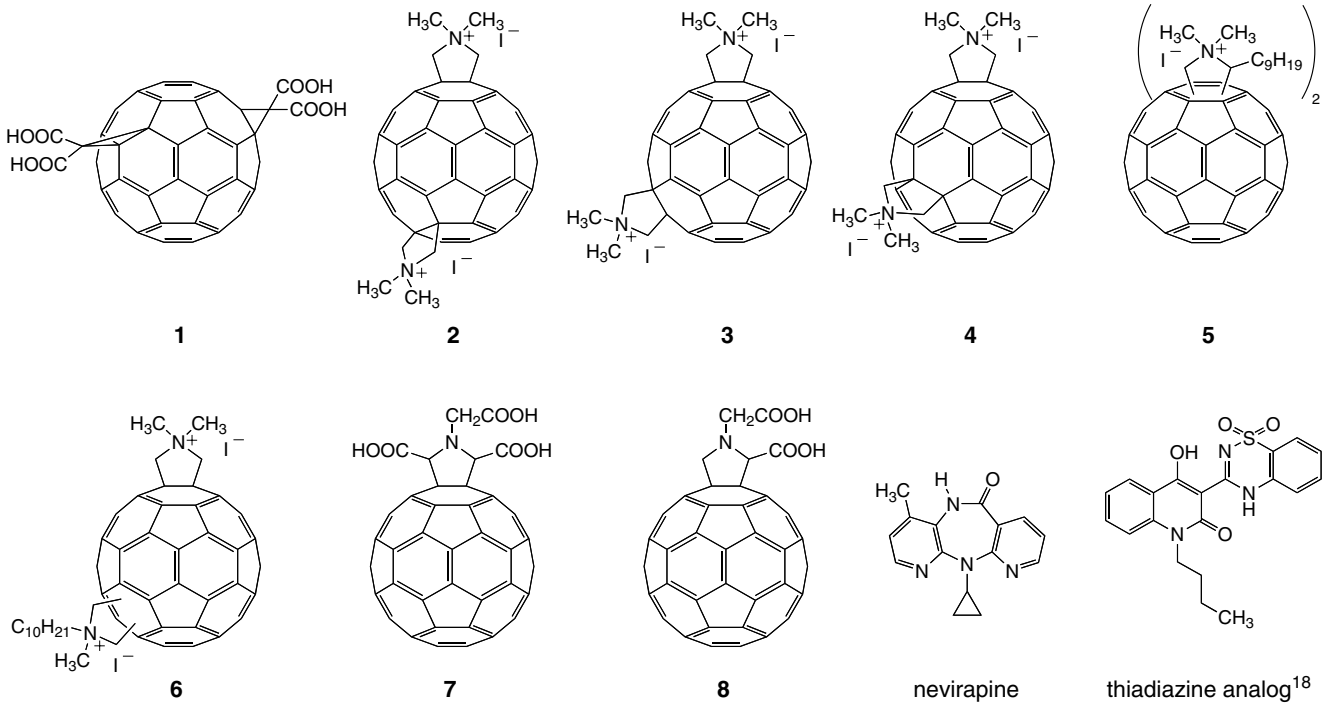

Fig. 1. Structures of fullerene derivatives **1**–**8**, nevirapine, and thiadiazine analog.

We previously reported the preparation of fullerene derivatives, **1** to **8**.[8,12] **5** and **6** were mixtures of regio-isomers, and **7** and **8** were mixtures of stereo-isomers. All fullerene derivatives were dissolved in DMSO to apply biological assay systems. Nevirapine, non-nucleoside analog of the HIV-RT inhibitor,[13] was also used as a DMSO solution.

The HIV-RT inhibition activities were examined according to Mizrahi et al.[14] DMSO sample solution (1 μL) and 1 μL of HIV-RT (0.01 U/mL) were added to a 18 μL reaction mixture containing 50 mM Tris–HCl (pH 8.3), 30 mM NaCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.125 mg/mL poly(rA)·oligo(dT),[12–18] and 2.5 μM dTTP including ^{32}P-dTTP. It was incubated for 1 h at 37 °C. Then, 10 μL of the reaction mixture was taken and placed on a Whatman® DE81 chromatography paper. After the chromatography paper was dried, it was washed three times with a 0.5 M NaH_2PO_4 buffer (pH 7.0), 70% ethanol, and ethanol. The radioactivity of the dried chromatography paper was counted with a liquid scintillator, and the HIV-RT activity was measured. IC_{50}s are means of three experiments.

The HIV-RT inhibition activity of the fullerene derivatives is listed in Table 1. All examined fullerene derivatives were more effective than the non-nucleoside analog of the HIV-RT inhibitor, nevirapine. Nevirapine, under the brand name Viramune®, is now used for HIV infection. Especially, the amino acid-type fullerene derivative, **7**, strongly inhibited HIV-RT. However, the other amino acid-type derivative, **8**, was less effective than **7**. Two carboxylic groups at the pyrrolidine ring appeared to be important for inhibition in the case of the amino acid derivatives. The HCV-RP inhibition activities of the three regio-isomers, **2**, **3**, and **4**, were not significantly different. In addition, the long alkyl group had a small effect.

Table 1. HIV-RT inhibition activity of fullerene derivatives

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
1	1.2	**6**	1.3
2	1.6	**7**	0.029
3	1.4	**8**	1.0
4	1.7	Nevirapine	3.0
5	0.50		

There are two major targets for anti-HIV agents, that is, HIV-protease and HIV-reverse transcriptase. Molecular modeling studies have revealed that the C_{60}-core could fit into the large and highly hydrophobic substrate-binding site of HIV protease. Indeed, some fullerene derivatives inhibited HIV protease.[15] However, this is the first report that the fullerene derivatives also have HIV-RT inhibition activity. Recently, Bosi et al. have reported the anti-HIV activity of fullerene derivatives.[16] They speculated that the mechanism of anti-HIV activity is HIV-protease inhibition but lacked experimental evidence. Our HIV-RT inhibition results demonstrate another possible mechanism of the anti-HIV activity of fullerene derivatives.

HCV-RP inhibition activities were examined according to Yamashita et al.[17] The assay was performed in a total volume of 40 μL containing 20 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 20 units of RNase inhibitor, 0.125 mg/mL poly(rA)·oligo(dU)$_{12-18}$, and 2.5 μM dUTP including ^{32}P-dUTP. It was incubated for 2 h at 30 °C. Then, 10 μL

Table 2. HCV-RP inhibition activity of the fullerene derivatives

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
1	3.2	**6**	1.4
2	0.24	**7**	2.0
3	0.31	**8**	>10
4	0.31	Thiadiazine analog[18]	0.10
5	1.4		

of the reaction mixture was taken and placed on a Whatman® DE81 chromatography paper. After the chromatography paper was dried, it was washed three times with a 0.5 M NaH_2PO_4 buffer (pH 7.0), 70% ethanol, and ethanol. The radioactivity of the dried chromatography paper was counted with a liquid scintillator, and the HCV-RP activity was measured. IC_{50}s are means of three experiments.

Among the examined fullerene derivatives, cationic derivatives were more effective than others (Table 2). Similarly to the HIV-RT inhibition results, the HCV-RP inhibition effects of the three regio-isomers, **2**, **3**, and **4**, were not significantly different. In the case of HIV-RT inhibition, the long alkyl group had a small effect, but the addition of the long alkyl chain into the fullerene derivative depressed the HCV-RP inhibition activity. The IC_{50} values of **2**–**4** were almost comparable to those of the benzo-1,2,4-thiadiazine analog (Fig. 1), a potent, specific inhibitor of the HCV-RP.[18] However, further optimization of the structure of the fullerene derivatives was required. **7** was not so effective to HCV-RP in comparison with the case of HIV-RP inhibition.

We have reported the antioxidant,[11] antibacterial, and antiproliferative activities of fullerene derivatives.[12] With regard to the antioxidant activity, carboxy fullerene derivatives such as **1** decreased the active oxygen toxicity. For the antibacterial and antiproliferative agents, cationic derivatives such as **2** to **6** were promising agents. The data in this report indicated that the amino acid-type fullerene derivative, **7**, was an excellent lead compound for an anti-HIV agent. Fullerene derivatives have many interesting biological activities, as we and other groups have already reported.[2–12] These activities depend on the properties of the fullerene core, while the substituents on the fullerene core control and modify the biological activities of fullerene derivatives.

The effects of three regio-isomers, **2**, **3**, and **4**, in HIV-RT inhibition, HCV-RP inhibition, antibacterial activity, and antiproliferative activity[12] were not significantly different. These findings indicate that it is not necessary to separate the regio-isomers to study the biological activities of fullerene derivatives.

We are now investigating the mechanisms of HIV-RT inhibition and HCV-RP inhibition.

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