Biological Activities of Water-soluble Fullerene Derivatives

S Nakamura and T Mashino

Department of Pharmaceutical Sciences, Faculty of Pharmacy, Keio University,
1-5-30 Shiba-koen, Minato-ku, Tokyo 105-8512, Japan

E-mail: mashino-td@pha.keio.ac.jp

Abstract. Three types of water-soluble fullerene derivatives were synthesized and their biological activities were investigated. C60-dimalonic acid, an anionic fullerene derivative, showed antioxidant activity such as quenching of superoxide and relief from growth inhibition of E. coli by paraquat. C60-bis(N,N-dimethylpyrrolidinium iodide), a cationic fullerene derivative, has antibacterial activity and antiproliferative effect on cancer cell lines. The mechanism is suggested to be respiratory chain inhibition by reactive oxygen species produced by the cationic fullerene derivative. Proline-type fullerene derivatives showed strong inhibition activities on HIV-reverse transcriptase. The IC50 values were remarkably lower than nevirapine, a clinically used anti-HIV drug. Fullerene derivatives have a big potential for a new type of lead compound to be used as medicine.

1. Introduction

Recently, fullerenes have gained attention in relation to toxicities of nano-materials. Suspension of fullerene was reported to increase oxidative stress in fish brain [1] or embryo [2]. On the other hand, problems of residual organic solvent for preparation of suspension are noted [3].

Fullerene itself is able to behave as a nano-material by aggregating in aqueous solution. However, the most different characteristic of fullerene from the other nano-materials, e.g. carbon nanotube and asbestos, is that fullerene is a molecule with the size of 7 to 8 Å in spherical shape. This means that fullerene is not a nano-material when it behaves as a molecule. Water-soluble fullerene derivatives should act differently as a molecule from unmodified fullerene.

Biological activities of water-soluble fullerene derivatives have attracted many researchers' attention. Generally, biological activities of a compound are related to its chemical properties. Some biological effects based on chemical reactivities of fullerenes have been reported. Fullerene derivatives with polyethylene glycol moiety were designed as an agent for photodynamic therapy [4] because fullerene is easily excited by light and produces singlet oxygen (1O2). Gadolinium-encapsulated fullerene is hopeful for use as an MRI-imaging agent [5]. The C60 core was reported to fit the hydrophobic substrate binding site of HIV protease, and the fullerene derivatives inhibit this enzyme [6].

The present problems of the medicinal chemistry are, first, that there is almost no medicine for intractable diseases, such as neurodegenerative diseases (Alzheimer and Parkinson's disease), cancer, and virus infection (AIDS, hepatitis C type, and influenza); and secondly, drug tolerance. To overcome these problems, the development of new ideal and epoch-making medicine is desired. The requirements of such medicine are novel chemical skeleton and novel chemical and physical...
properties. We believe that fullerene has these requirements, and we intend to develop fullerene derivatives as a new type of lead compound to be used as medicine.

We have synthesized some types of water-soluble fullerene derivatives and have reported their biological effects. This paper describes antioxidant, antibacterial, and antiproliferative activities of water-soluble fullerene derivatives. Inhibition activities on HIV reverse transcriptase (HIV-RT) of amino acid-type fullerene derivatives are also investigated.

2. Antioxidant activity of anionic fullerene derivatives

Reactive oxygen species (ROS) such as superoxide anion radical (O$_2^{-}$), hydroperoxide (ROOH), and hydroxyl radical (•OH) produced during oxidative stress cause various kinds of biological damage. Redox-active compounds often affect the production and/or decomposition of ROS in biological systems. Fullerene is a redox-active compound since it has a low LUMO level and a high HOMO level.

A polyhydroxylated C$_{60}$ derivative (fullerenol) was reported to have quenching activity for O$_2^{-}$ [7]. However, the properties of fullerenol are quite different from those of the parent C$_{60}$, since conjugated double bonds are widely broken. Therefore C$_{60}$-dimalonic acid (C$_{62}$(COOH)$_4$, 1, Fig. 1) was selected, because it has sufficient water solubility and is thought to have rather similar properties to C$_{60}$. C$_{62}$(COOEt)$_4$ was synthesized and its equatorial isomer was purified and then converted to 1. O$_2^{-}$ quenching activity of 1 was examined in the xanthine/xanthine oxidase system. The production of O$_2^{-}$ was measured in terms of cyt. c reduction determined from the increase in absorbance at 550 nm.

Figure 2 shows the effect of 1 on cyt. c reduction in the xanthine/xanthine oxidase system. When xanthine oxidase was added to the solution to generate O$_2^{-}$, cyt. c was reduced and the absorbance at 550 nm increased. The increase stopped at around 10 minutes after the addition of xanthine oxidase because cyt. c was completely reduced. When 1 was added to the complete system (B in Fig. 2), the initial rate of increase of the absorbance at 550 nm was slowed down. These results indicated that O$_2^{-}$ was quenched by 1.

Paraquat is a typical O$_2^{-}$-generator that is easily reduced by biological systems such as the respiratory chain and the one-electron-reduced form is able to pass an electron to O$_2$ to produce O$_2^{-}$.

Paraquat inhibited the growth of E. coli by the toxicity of O$_2^{-}$ (Fig. 3, b). The growth inhibition was relieved by the addition of 1 by quenching of O$_2^{-}$ (Fig. 3, c - f).
3. Antibacterial activities of cationic fullerene derivatives

In the previous section, we demonstrated that 1 decreased the toxicity of reactive oxygen generators in E. coli. During the same experiment with other fullerene derivatives, we found the bacteriostatic effect of C_{60}-bis(N,N-dimethylpyrrolidinium iodide) (Fig. 4, 2). Therefore, the inhibition of E. coli growth by cationic fullerene derivatives and the inhibition mechanism were investigated.

Cationic fullerene derivative 2 was used as a mixture of regioisomers. Figure 5 shows the effects of fullerene derivatives on E. coli growth. Anionic fullerene derivative 1 did not affect growth up to 50 µM and the growth was slightly suppressed at 100 µM (Fig. 5A). On the other hand, only 5 µM of 2 inhibited the growth completely (Fig. 5B).

![Figure 4. Structure of C_{60}-bis(N,N-dimethylpyrrolidinium iodide), 2.](image)

![Figure 5. Effect of fullerene derivatives on E. coli growth.](image)

To elucidate a mechanism of growth inhibition, O_{2} uptake by E. coli was examined in the presence of glucose. These fullerene derivatives seemed to inhibit energy metabolism in E. coli. Glucose was metabolized through a glycolytic pathway, TCA cycle, and then the respiratory chain, and finally O_{2} was consumed to produce H_{2}O.
As shown in Fig. 6A, the addition of an E. coli solution initiated O₂ consumption in the presence of glucose. Dioxygen uptake rates were measured and are shown as a dashed line in Fig. 6. In the presence of 50 µM of 2, the initial O₂ uptake rate was almost the same as that of the rate without 2 (46.6 µM/min), but after a short lag time (less than 30 seconds), consumption was inhibited (Fig. 6B, 4.1 µM/min). The addition of 50 µM of 1 did not affect consumption (42.2 µM/min).

The result of O₂ consumption inhibition was consistent with that of bacteriostatic effect. That is, 2 was effective in both the bacteriostatic effect and the O₂ uptake inhibition (inhibition of energy metabolism). Finally, 1 had almost no activity. These results strongly indicate that the mechanism of the bacteriostatic effect was energy metabolism inhibition in E. coli. As a result, cationic fullerene derivative was suggested to inhibit the respiratory chain from the effect of 2 on O₂ uptake caused by the inner membrane and NADH. These data mean that 2 was reduced by biological reductant system to anion radical of C₆₀ and this species reduced O₂ to produce O₂⁻, in contrast to the O₂⁻-quenching activity of 1 described in the previous section. The reduction potentials of 1 and 2 were –670 and –480 mV (vs. SCE), respectively. This means the reduction of cationic fullerene derivative 2 were easier than that of the anionic derivative 1. An electrochemical study revealed that reduced fullerene derivative 2 reacts with O₂ and produces H₂O₂ via O₂⁻ [8].

These results prompted us to investigate the antibacterial activities of the fullerene derivatives. Therefore, antibacterial activities of C₆₀-bis(N,N-dimethylpyrrolidinium iodide) regioisomers and alkylated C₆₀-bis(N,N-dimethylpyrrolidinium iodide) derivatives were investigated (Fig. 7).

The regioisomers of C₆₀-bis(N,N-dimethylpyrrolidinium iodide), 2-t₂, 2-t₃, and 2-t₄, had excellent antibacterial activity, which was comparable with that of vancomycin (VCM). The antibacterial effect of the three regioisomers was not significantly different. These findings indicate that it is not necessary to separate the regioisomers to study their biological activities. C₆₀-bis(2-alkyl-N,N-dimethylpyrrolidinium iodide) (3, 4) and C₆₀-bis(N-alkyl-N-methylpyrrolidinium iodide) (6, 7) also showed antibacterial activity, and 6 was the most effective. Moreover, these derivatives inhibited the growth of vancomycin-resistant E. faecalis.

In contrast to 2, 3, 4, 6, and 7, derivatives with a long alkyl chain, 5 and 8, had no antibacterial activity. In the respiratory chain inhibition, 5 and 8 had from none to slight activity. These results indicated that the mechanism of antimicrobial activity is a respiratory chain inhibition and that appropriate lipophilicity of the derivatives was suitable for the inhibition of the respiratory chain and for antibacterial activity. Tsao et al. [9] reported the antibacterial activity of carboxy fullerenes. They have shown that its action is achieved by insertion into the cell walls and disruption of the cell structure. This mechanism is different from the respiratory chain inhibition. The bacteriostatic effect of fullerene derivatives has been reported by Bosi et al. [10], but the mechanism was not investigated.
Figure 7. Structure of C60-bis(N,N-dimethylpyrrolidinium iodide) regioisomers (2-t-2, 2-t-3, 2-t-4) and alkylated C60-bis(N,N-dimethylpyrrolidinium iodide) derivatives (3 - 6).

Table 1. Antibacterial activity of fullerene derivatives.

|     | MIC (µg/mL) |     |     |     |     |     |     |     |     |
|-----|-------------|-----|-----|-----|-----|-----|-----|-----|-----|
|     | 2-t-2       | 2-t-3 | 2-t-4 | 3   | 4   | 5   | 6   | 7   | 8   | VCM |
| S.aureus 209P JC-1 | 1.56 | 0.78 | 3.12 | 6.25 | 3.12 | >100 | 0.36 | 6.25 | >100 | 1.56 |
| S.aureus M133 (MRSA) | 0.78 | 1.56 | 3.12 | 6.25 | 6.25 | >100 | 0.36 | 6.25 | >100 | 1.56 |
| S.aureus M126 (MRSA) | 3.12 | 1.56 | 3.12 | 6.25 | 3.12 | >100 | 0.78 | 6.25 | >100 | 1.56 |
| S.eidermidis ATCC 14990 | 3.12 | 1.56 | 3.12 | 6.25 | 3.12 | >100 | 0.78 | 6.25 | >100 | 1.56 |
| E.hirae ATCC 8043 | 12.5 | 6.25 | 6.25 | 6.25 | 25.0 | >100 | 1.56 | 6.25 | >100 | 3.12 |
| E.faecalis W-73 | 12.5 | 6.25 | 6.25 | 6.25 | 12.5 | >100 | 1.56 | 6.25 | >100 | 3.12 |
| E.faecium vanA (VRE) | 12.5 | 6.25 | 6.25 | 12.5 | 6.25 | >100 | 1.56 | 6.25 | >100 | >100 |
| E.faecalis NCTC 12201 (VRE) | 12.5 | 3.12 | 6.25 | 6.25 | 6.25 | >100 | 1.56 | 6.25 | >100 | >100 |

4. Antiproliferative activity of cationic fullerene derivatives

The antiproliferative activities of fullerene derivatives were evaluated and compared with well-known anticancer agents by using a panel of 36 human cancer cell lines. The antiproliferative activity was measured by the MTT assay, giving the fullerene derivative concentration required for 50 % growth inhibition (G150). The average of the logarithm of G150 (MID-G150) is presented in Table 2.

Fullerene derivatives 2 – 5 were effective in the antiproliferative activity, which was comparable to that of cisplatin (MID-G150 = –5.30). In case of antibacterial activity, 5 was ineffective. It seems that the mechanism of antiproliferative activity is not the same as that of antibacterial activity and/or that the distribution of these derivatives in a mammalian cell is different from that in bacteria.

Tabata et al. have reported the anticancer activity of a fullerene derivative with laser photo-irradiation [4]. A laser photo-irradiated fullerene derivative produces active oxygen to kill cancer cells. The action was not replicated in the present experiment because photo-irradiation was not applied.
Table 2. Antiproliferative activity of fullerene derivatives.

| compound | MID-GI$_{50}$ |
|----------|---------------|
| 2-t-2    | -5.44         |
| 2-t-3    | -5.18         |
| 2-t-4    | -5.18         |
| 3        | -5.42         |
| 4        | -5.48         |
| 5        | -5.61         |
| cisplatin| -5.30         |

5. Human immunodeficiency virus-reverse transcriptase inhibition activities of proline-type fullerene derivatives

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. These threats provide motivation to search for new types of lead compounds to be used as medicine against HIV infection. We therefore examined the HIV-RT inhibition activities of anionic (1), cationic (2–8), and proline-type (Fig. 8, 9–12) fullerene derivatives.

The HIV-RT inhibition activity of the fullerene derivatives is listed in Table 3. 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 proline-type fullerene derivatives, 10 and 12, strongly inhibited HIV-RT.

Figure 8. Structure of proline-type fullerene derivatives and nevirapine

The X-ray crystal structure of the complex of HIV-RT and nevirapine has already been available (PDB ID: 3HVT). A docking simulation study of HIV-RT and fullerene derivative 10 was investigated.

Figure 9. Superimposition of nevirapine and 10 at nevirapine-binding site of HIV-RT.

Docking simulation was performed by DS Molding 1.2-SBD.
and 10 was able to fit the nevirapine-binding site of HIV-RT. Figure 9 shows a superimposition of the binding states of nevirapine and 10. The pyrrolidine ring of 10 is located on the B-ring of nevirapine, and the position of C₆₀ core is on the side of the cyclopropane ring of nevirapine.

**Table 3. HIV-RT inhibition activity of fullerene derivatives.**

| compound | IC₅₀ (µM) | compound | IC₅₀ (µM) |
|----------|----------|----------|----------|
| 1        | 1.2      | 6        | 2.5      |
| 2-t-2    | 1.8      | 7        | 1.9      |
| 2-t-3    | 1.7      | 8        | 8.9      |
| 2-t-4    | 1.9      | 9        | 0.15     |
| 3        | 1.1      | 10       | 0.022    |
| 4        | 0.84     | 11       | 0.073    |
| 5        | 0.59     | 12       | 0.029    |
| nevirapine| 3.0      |          |          |

The HIV-RT inhibition activities were examined according to Mizrahi et al. [11]. One µL of a DMSO sample solution 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 MgCl₂, 5 mM dithiothreitol, 0.125 mg/mL poly(rA)•oligo(dT)₁₂-₁₈, and 2.5 µM dTTP including ³²P-dTTP. It was incubated for 1 h at 37 ºC. 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 NaH₂PO₄ 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. IC₅₀S are the means of three experiments.

There are two major targets for anti-HIV agents, i.e., HIV-protease and HIV-reverse transcriptase. Molecular modeling studies have revealed that the C₆₀-core could fit into the large and highly hydrophobic substrate-binding site of HIV-protease. Indeed, some fullerene derivatives inhibited HIV-protease [12]. However, we propose that the fullerene derivatives also have HIV-RT inhibition activity. Bosi et al. have reported the anti-HIV activity of fullerene derivatives [13]. 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.

6. Conclusion

We have reported the antioxidant, antibacterial, and antiproliferative activities of fullerene derivatives. With regard to the antioxidant activity, anionic fullerene derivatives such as 1 decreased the active oxygen toxicity by O₂-• quenching. Anionic fullerene derivative 1 also relieved E. coli from the growth inhibition by O₂-• generator. For the antibacterial and antiproliferative agents, cationic derivatives such as 2 to 8 were promising agents. Cationic fullerene derivatives were suggested to produce reactive oxygen species, and the respiratory chain was inhibited. The proline-type fullerene derivative, 11, was an excellent lead compound for the anti-HIV agent. Fullerene derivatives have many interesting biological activities, as we and other groups have already reported. 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.
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