

# Water-soluble Fullerene Derivatives for Drug Discovery

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## Abstract

Fullerenes (represented by buckminsterfullerene, C<sub>60</sub>) are a new kind of organic compound with a cage-like structure. A great deal of attention has been focused on their unique properties. From the viewpoint of drug discovery, fullerenes could be novel lead compounds for drug discovery. However, fullerenes are poorly soluble in aqueous media. Incorporation of water-soluble groups into the fullerene core enables investigation of its biological activities. Certain fullerene derivatives show inhibitory activity against human immunodeficiency virus reverse transcriptase. Hepatitis C virus RNA polymerase is also inhibited by fullerene derivatives. Therefore, fullerene derivatives are candidate antiviral agents. In addition, fullerene derivatives exhibit antiproliferative activity by inducing apoptosis related to the generation of reactive oxygen species. Fullerene derivatives also have the potential to be anticancer drugs.

(J Nippon Med Sch 2012; 79: 248-254)

**Key words:** fullerenes, human immunodeficiency virus reverse transcriptase, antitumor agents, apoptosis, reactive oxygen species

## Introduction

The development of drugs to treat intractable diseases and overcome drug resistance is an important research subject. A lead compound is needed to develop drugs with a new structure, because groundbreaking new drugs cannot expect to be developed by improving existing drugs. Furthermore, such new drugs should have chemical properties different from those of conventional organic compounds.

Fullerenes (represented by buckminsterfullerene, C<sub>60</sub>) were discovered by Kroto et al. in 1985 as the third allotropic form of carbon after diamond and

graphite<sup>1</sup>. Fullerenes are spherical molecules 0.7 nm in diameter. They are condensed aromatic ring compounds with an extended  $\pi$ -conjugated system. Fullerenes are a new kind of organic compound with a cage-like structure, and a great deal of attention has been focused on their unique properties. We believe that fullerenes could be novel lead compounds for drug development. However, fullerenes are poorly soluble in aqueous media. Recently, water-soluble groups have been incorporated into the fullerene core<sup>2,3</sup>. The biological activities of water-soluble derivatives of fullerene are described in this review.

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### Inhibition of HIV-protease by Fullerene Derivatives

Acquired immunodeficiency syndrome (AIDS) was first reported in 1981, and the human immunodeficiency virus (HIV) was discovered in 1983<sup>4</sup>. Three essential enzymes for the replication of HIV are reverse transcriptase, integrase, and protease. Therefore, these enzymes are targets for major anti-HIV drugs.

HIV protease has a cylindrical substrate-binding site composed of lipophilic amino-acid residues. Molecular modeling studies suggest that fullerenes could be adapted to the binding site because of their hydrophobicity and size. Friedman et al. have synthesized a fullerene derivative **1** (Fig. 1). They showed that the fullerene derivative inhibits HIV protease and also has activity against HIV-infected

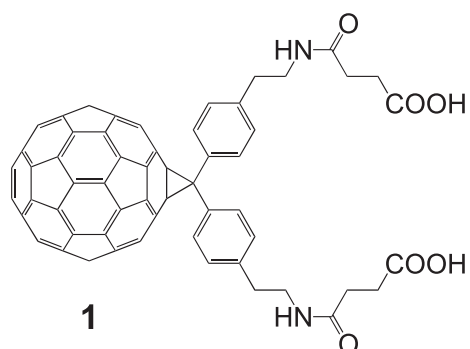


Fig. 1 Fullerene derivative with inhibitory activity against HIV protease

cells *in vitro*<sup>5,6</sup>. This fullerene derivative was reported to have no cytotoxicity, and therefore, could be an excellent choice as an anti-HIV agent.

### Inhibition of HIV Reverse Transcriptase by Fullerene Derivatives

Two kinds of HIV reverse transcriptase inhibitors, i.e., nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), are used. NRTIs (e.g., azidothymidine) are analogs of nucleic acids. Their inhibitory action is due to their incorporation as substrates in place of nucleic acids. Conversely, NNRTIs (e.g., nevirapine) bind at different sites on the substrate-binding site. The binding of NNRTIs changes the protein structure of the substrate-binding site and results in the inhibition of the reverse transcription reaction<sup>7,8</sup>. The HIV reverse transcriptase inhibitory activities of 3 kinds of fullerene derivative, anionic fullerene derivative **2**, cationic fullerene derivative **3**, and zwitterion-type fullerene derivative **4**, have been investigated (Fig. 2)<sup>9,10</sup>. The half-maximal inhibitory concentrations ( $IC_{50}$ ) of fullerene derivatives **2**, **3**, and **4** are 1.2  $\mu$ M, 1.1  $\mu$ M, and 0.15  $\mu$ M, respectively. These fullerene derivatives are more effective than nevirapine (3.0  $\mu$ M). Marchesan et al. have examined the anti-HIV effect of fullerene derivative **3** using HIV-infected CEM cells<sup>11</sup>. They hypothesized that the activity is derived from the inhibition of HIV protease.

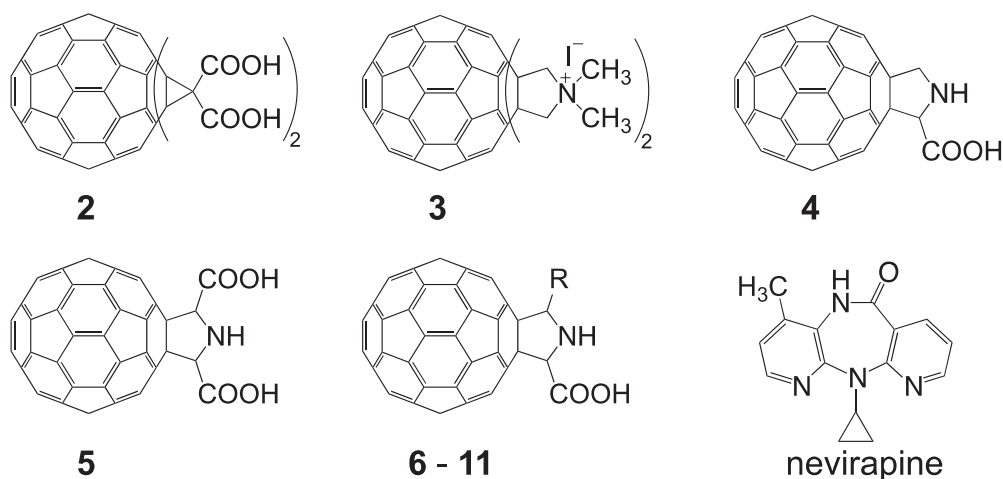


Fig. 2 Fullerene derivatives with inhibitory activity against HIV reverse transcriptase

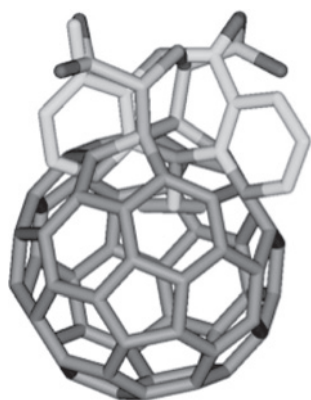


Fig. 3 Superimposition of nevirapine and fullerene derivative **5** at the binding site of nevirapine in HIV reverse transcriptase

Incorporation of an additional carboxyl group to fullerene derivative **4** increases inhibitory activity against HIV reverse transcriptase. The  $IC_{50}$  of fullerene derivative **5** is  $0.032 \mu\text{M}$  and is less than that of **4**. Analysis of the inhibition kinetics of HIV reverse transcriptase by fullerene derivative **5** has revealed that the inhibition is a mixed-type noncompetitive mode that has also been reported in the case of nevirapine<sup>12</sup>. A docking simulation of HIV reverse transcriptase and fullerene derivative **5** suggests that fullerene derivative **5** can adapt to the binding site of nevirapine<sup>13</sup>. Superimposition of nevirapine and fullerene derivative **5** at the binding site of nevirapine reveals that the pyrrolidine ring of **5** is located on the B-ring of nevirapine and that the nitrogen and oxygen atoms are at the same position as in nevirapine (Fig. 3). In addition, it was suggested that a carboxyl group could be replaced by a hydrophobic group because it overlaps with the methyl group of nevirapine. Therefore, fullerene derivatives **6–11** have been synthesized, and HIV reverse transcription inhibitory activities have been investigated. Almost all other fullerene derivatives show activities equal to or greater than that of fullerene derivative **5** (Table 1). These fullerene derivatives can be readily synthesized from  $C_{60}$  with glycine ester and the corresponding aldehyde by 1,3-dipolar cycloaddition. These fullerene derivatives should be lead compounds for the development of anti-HIV drugs.

Highly active antiretroviral therapy is widely used for HIV-infected patients<sup>14</sup>. This therapy aims at

Table 1 Inhibitory activities of amino acid-type fullerene derivatives on HIV reverse transcriptase

Fullerene derivative	R	$IC_{50}$ ( $\mu\text{M}$ )
<b>4</b>	none	0.15
<b>5</b>	carboxyl	0.032
<b>6</b>	isopropyl	0.018
<b>7</b>	isopropenyl	0.019
<b>8</b>	<i>t</i> -butyl	0.011
<b>9</b>	cyclohexyl	0.042
<b>10</b>	phenyl	0.018
<b>11</b>	<i>p</i> -nitrophenyl	0.21
nevirapine	—	3.0

suppressing HIV reproduction by a combination of several kinds of anti-HIV drugs. However, the most serious problem of anti-HIV drugs is the emergence of drug-resistant forms. Bulky amino acids, such as tyrosine, tryptophan, and valine, are present at the NNRTI-binding site. These amino acids are often replaced by smaller ones in NNRTI-resistant mutants<sup>15,16</sup>. If NNRTI binds to wild-type HIV reverse transcriptase, the structure of the substrate-binding site is changed, and the binding of nucleic acids is inhibited. However, if the mutation occurs at the NNRTI-binding site, the NNRTI has no effect on the structural change of the substrate-binding site. Because of their large size, fullerene derivatives may affect substrate binding even in the case of drug-resistant mutants (Fig. 4).

#### Inhibition of Hepatitis C Virus RNA Polymerase by Fullerene Derivatives

In 1989, hepatitis C virus (HCV) was proven to be the virus that causes non-A non-B hepatitis<sup>17</sup>. The standard therapy is polyethylene glycol-interferon and ribavirin; however, it is not sufficiently effective against genotype 1<sup>18,19</sup>. Therefore, novel anti-HCV drugs effective against all virus types are desired. NS5B RNA polymerase, a major enzyme for the replication of HCV, is a target of anti-HCV drugs.

Fullerene derivatives **2**, **3**, and **5** inhibit NS5B RNA polymerase with  $IC_{50}$  values of  $3.2 \mu\text{M}$ ,  $0.30 \mu\text{M}$ , and  $2.0 \mu\text{M}$ , respectively<sup>9,10</sup>. Interestingly, the inhibitory activity of fullerene derivative **5** against NS5B RNA polymerase is not as great as that

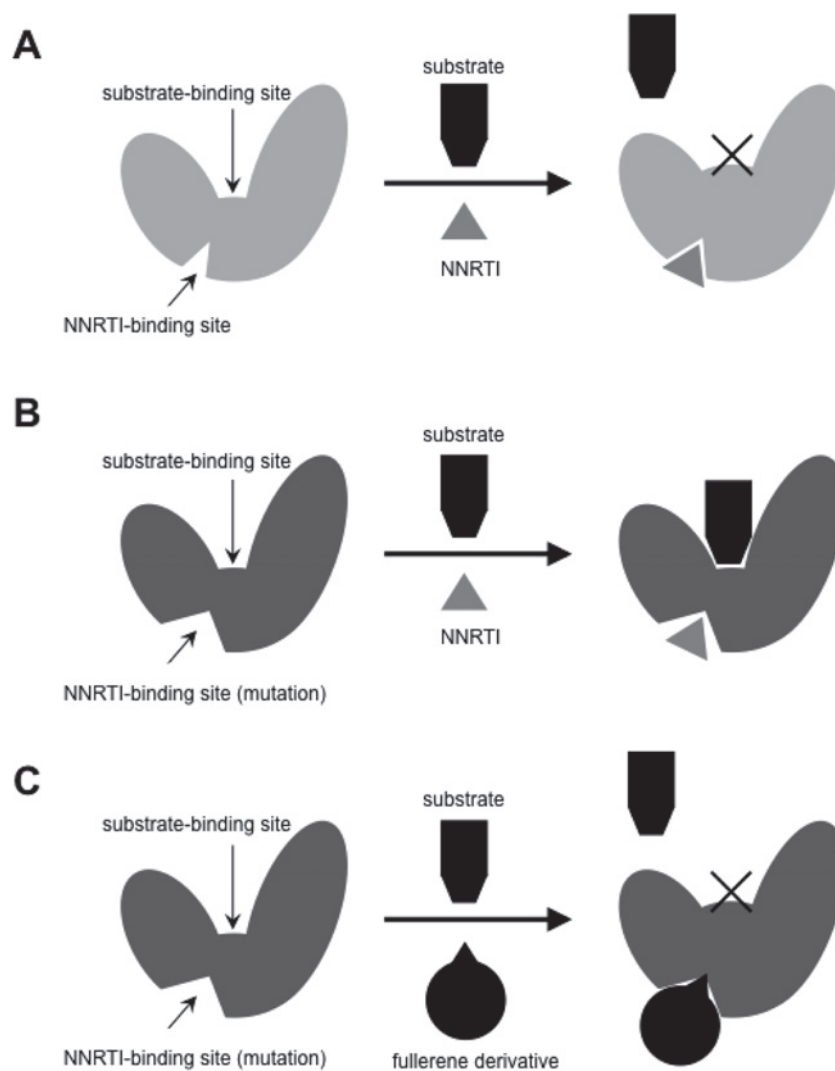


Fig. 4 Schematic illustration of wild-type and NNRTI-resistant mutant of HIV reverse transcriptase

**A.** Substrate binding is inhibited by the structural change of the binding site caused by NNRTI in wild-type HIV reverse transcriptase. **B.** Mutation enables substrate binding because NNRTI does not affect the structure of the binding site in the NNRTI-resistant mutant. **C.** Because of their large size, fullerene derivatives may be effective against NNRTI-resistant mutants.

against HIV reverse transcriptase. However, fullerene derivative **3**, the most effective derivative, might not be a good anti-HCV drug because it is cytotoxic<sup>20</sup>. Further structural conversion of fullerene derivatives is necessary for them to be lead compounds for the development of anti-HCV drugs.

### Antiproliferative Activities of Fullerene Derivatives

Panels of human cancer cell lines are used as screening systems for anticancer agents<sup>21</sup>. These systems enable evaluation of the antiproliferative activities of a compound using 39 human cancer cell lines (e.g., lung cancer, colorectal cancer, gastric cancer, ovarian cancer, breast cancer, renal cancer, melanoma, glioma, and prostate cancer). The

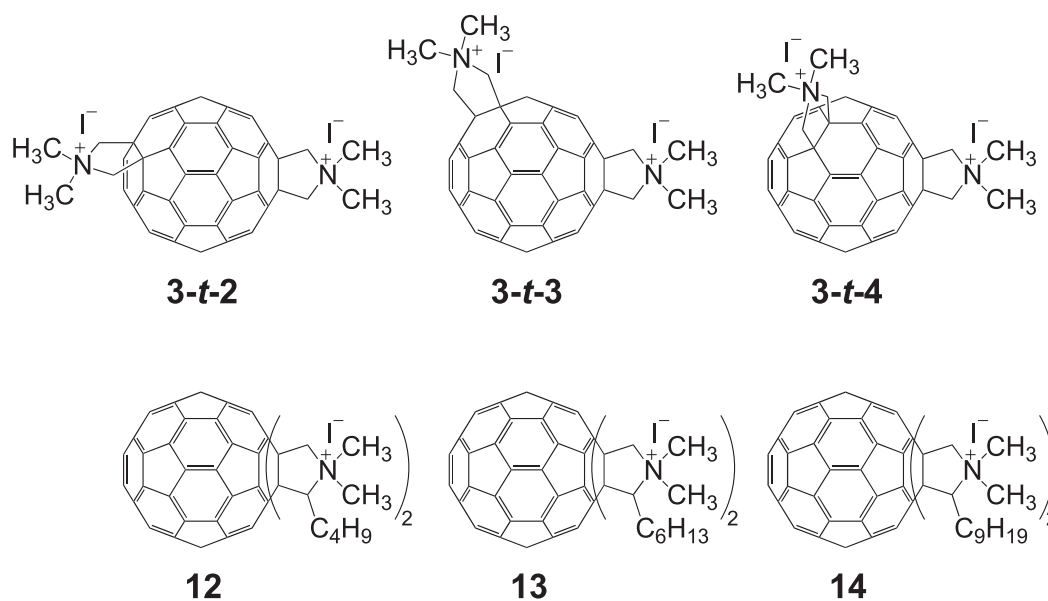


Fig. 5 Cationic fullerene derivatives with antiproliferative activity

Table 2 Antiproliferative activities of cationic fullerene derivatives

Fullerene derivative	MID-GI <sub>50</sub>	Highest correlation coefficient ( $r_{\max}$ )	Anticancer agent
3-t-2	-5.44	0.49	bleomycin
3-t-3	-5.18	0.52	taxol
3-t-4	-5.18	0.54	actinomycin-D
12	-5.42	0.70	actinomycin-D
13	-5.48	0.71	vincristine
14	-5.61	0.64	taxol
cisplatin	-5.30	—	—

spectrum of activities shows a characteristic pattern for each drug because the drug concentration required for 50% growth inhibition (GI<sub>50</sub>) is different for each cell line. Therefore, the spectrum of antiproliferative activity can be used to predict the mechanism of action of the drug.

Various water-soluble fullerene derivatives have been evaluated using panels of human cancer cell lines<sup>22</sup>. The cationic pyrrolidinium fullerene derivatives shown in **Figure 5** have antiproliferative activity. The mean log GI<sub>50</sub> (MID-GI<sub>50</sub>) value of fullerene derivatives are comparable to those of cisplatin and are less than -5, an index of an active anticancer drug (**Table 2**). The correlation coefficient values ( $r$ ) of standard drugs have also been evaluated. When the spectrum of antiproliferative

activity of a compound is not similar to that of standard drugs, the compound is assumed to possess a unique mechanism. Fullerene derivatives are expected to be unique anticancer agents with a novel mechanism of action because the highest correlation coefficient values ( $r_{\max}$ ) of fullerene derivatives range from 0.5 to 0.75.

#### Induction of Apoptosis by Fullerene Derivatives

The antiproliferative mechanism of fullerene derivatives has been investigated in human promyeloleukemia (HL-60) cells<sup>20</sup>. Exposure of HL-60 cells to various concentrations of fullerene derivative **3** results in a dose-dependent decrease in cell viability. The IC<sub>50</sub> value of fullerene derivative **3** is approximately 10  $\mu$ M, and all cells die after exposure to 50  $\mu$ M of fullerene derivative **3**. From the analysis of the cell cycle, fullerene derivative **3** lead to apoptosis, as indicated by the appearance of the sub-G<sub>1</sub> phase. Appearance of DNA fragmentation and condensation of nuclear chromatin is also observed on exposure to fullerene derivative **3**. Activation of the caspase cascade and release of cytochrome *c* suggest that fullerene derivative **3** induces apoptosis in HL-60 cells.

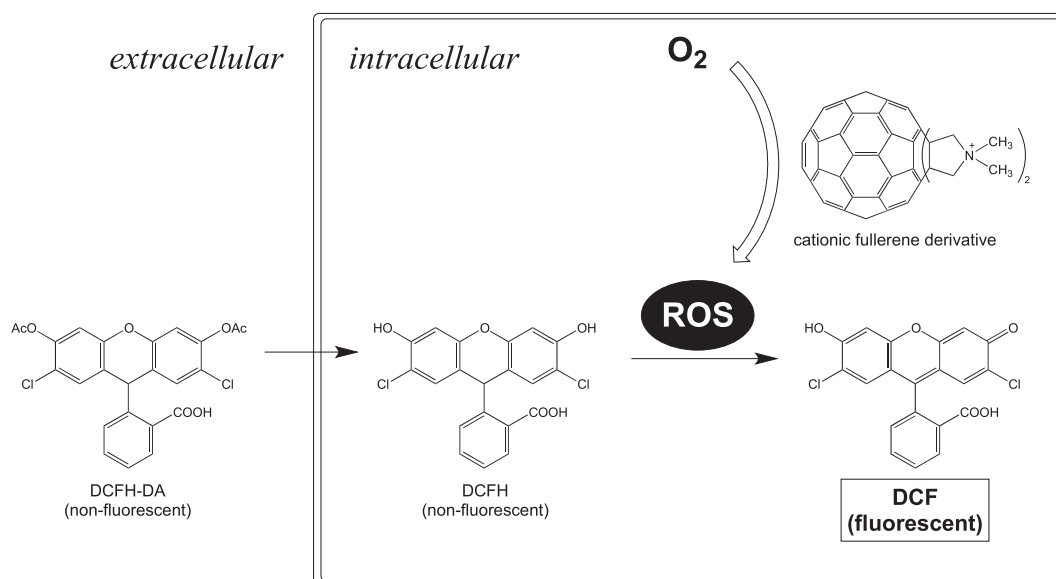


Fig. 6 Evaluation of intracellular generation of ROS by a cationic fullerene derivative using DCFH-DA

### Intracellular Generation of Reactive Oxygen Species by Fullerene Derivatives

Intracellular oxidative stress can be measured using the reactive oxygen species (ROS)-sensitive fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA)<sup>23</sup>. DCFH-DA is taken up by cells and is then deacetylated by esterases to DCFH. When ROS are generated in cells, DCFH is oxidized to fluorescent 2',7'-dichlorofluorescein (DCF) (**Fig. 6**). Generation of ROS by fullerene derivative **3** in HL-60 cells is suggested by enhancement of the fluorescence of DCF. Pretreatment with the typical antioxidant  $\alpha$ -tocopherol (vitamin E) suppresses the intracellular generation of ROS in a dose-dependent manner.  $\alpha$ -Tocopherol also reduces the amount of cell death by fullerene derivative **3**. These results suggest that the antiproliferative activity of fullerene derivative **3** is related to the intracellular generation of ROS.

### Conclusions

Fullerene derivatives have inhibitory activity against HIV reverse transcriptase and HCV RNA polymerase. The increase in the number of HIV patients is a serious social problem, and new kinds of anti-HIV drugs are continuously desired. A variety

of anti-HIV drugs are required for the treatment of drug-resistant mutants. Fullerene derivatives that have novel and unique molecular skeletons could be lead compounds in the development of antiviral agents. In addition, the bulkiness of the fullerene core is expected to be an advantageous feature against resistant viruses.

Pyrrolidinium fullerene derivatives show antiproliferative activities related to apoptosis by the intracellular generation of ROS. Several clinically used anticancer agents are known to induce apoptosis in cancer cells, which are affected by ROS generation because the ROS level in cancer cells is originally higher than that in normal cells. Pyrrolidinium fullerene derivatives have the potential to be a new kind of anticancer agent.

### References

1. Kroto HW, Heath JR, O'Brien SC, Curl RF, Smalley RE: C<sub>60</sub>: Buckminsterfullerene. *Nature* 1985; 318: 162-163.
2. Bingel C: Cyclopropanierung von Fullerenen. *Chem Ber* 1993; 126: 1957-1959.
3. Prato M: [60] Fullerene chemistry for materials science applications. *J Mater Chem* 1997; 7: 1097-1109.
4. Barre-Sinoussi F, Chermann JC, Rey F, et al: Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* 1983; 220: 868-871.
5. Friedman SH, DeCamp DL, Sijbesma RP, Srdanov G, Wudl F, Kenyon GL: Inhibition of the HIV-1 protease

- by fullerene derivatives: model building studies and experimental verification. *J Am Chem Soc* 1993; 115: 6506–6509.
6. Friedman SH, Ganapathi PS, Rubin Y, Kenyon GL: Optimizing the binding of fullerene inhibitors of the HIV-1 protease through predicted increases in hydrophobic desolvation. *J Med Chem* 1998; 41: 2424–2429.
  7. Cohen KA, Hopkins J, Ingraham RH, et al.: Characterization of the binding site for nevirapine (BI-RG-587), a nonnucleoside inhibitor of human immunodeficiency virus type-1 reverse transcriptase. *J Biol Chem* 1991; 266: 14670–14674.
  8. Smerdon SJ, Jäger J, Wang J, et al.: Structure of the binding site for nonnucleoside inhibitors of the reverse transcriptase of human immunodeficiency virus type 1. *Proc Natl Acad Sci USA* 1995; 91: 3911–3915.
  9. Mashino T, Shimotohno K, Ikegami N, et al.: Human immunodeficiency virus reverse transcriptase inhibition and hepatitis C virus RNA-dependent RNA polymerase inhibition activities of fullerene derivatives. *Bioorg Med Chem Lett* 2005; 15: 1107–1109.
  10. Nakamura S, Ikegami N, Harada M, Shimotohno K, Takahashi K, Mashino T: Synthesis of novel amino acid-type fullerenes and their inhibition activity of HIV reverse transcriptase and HCV RNA polymerase. *J Kyoritsu Univ Pharm* 2006; 1: 77–84.
  11. Marchesan S, Da Ros T, Spalluto G, Balzarini J, Prato M: Anti-HIV properties of cationic fullerene derivatives. *Bioorg Med Chem Lett* 2005; 15: 3615–3618.
  12. Enzo T, Yung-chi C: HIV-1 reverse transcriptase inhibition by a dipyrrodoiazepinone derivative: BI-RG-587. *Biochem Pharmacol* 1992; 43: 1371–1376.
  13. Nakamura S, Mashino T: Biological activities of water-soluble fullerene derivatives. *J Phys Conf Ser* 2009; 159: 012003.
  14. Bartlett JA, Fath MJ, Demasi R, et al.: An updated systematic overview of triple combination therapy in antiretroviral-naïve HIV-infected adults. *AIDS* 2006; 20: 2051–2064.
  15. Kelly TA, Proudfoot JR, McNeil DW, et al.: Novel non-nucleoside inhibitors of human immunodeficiency virus type 1 reverse transcriptase. 5. 4-Substituted and 2,4-disubstituted analogs of nevirapine. *J Med Chem* 1995; 38: 4839–4847.
  16. Das K, Clark AD Jr, Lewi PJ, et al.: Roles of conformational and positional adaptability in structure-based design of TMC125-R165335 (etravirine) and related non-nucleoside reverse transcriptase inhibitors that are highly potent and effective against wild-type and drug-resistant HIV-1 variants. *J Med Chem* 2004; 47: 2550–2560.
  17. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M: Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989; 244: 359–362.
  18. Feld JJ, Hoofnagle JH: Mechanism of action of interferon and ribavirin in treatment of hepatitis C. *Nature* 2005; 436: 967–972.
  19. Iwasaki Y, Ikeda H, Araki Y, et al.: Limitation of combination therapy of interferon and ribavirin for older patients with chronic hepatitis C. *Hepatology* 2005; 43: 54–63.
  20. Nishizawa C, Hashimoto N, Yokoo S, et al.: Pyrrolidinium-type fullerene derivative-induced apoptosis by the generation of reactive oxygen species in HL-60 cells. *Free Radical Res* 2009; 43: 1240–1247.
  21. Dan S, Tsunoda T, Kitahara O, et al.: An integrated database of chemosensitivity to 55 anticancer drugs and gene expression profiles of 39 human cancer cell lines. *Cancer Res* 2002; 62: 1139–1147.
  22. Mashino T, Nishikawa D, Takahashi K, et al.: Antibacterial and antiproliferative activity of cationic fullerene derivatives. *Bioorg Med Chem Lett* 2003; 13: 4395–4397.
  23. Negre-Salvayre A, Augé N, Duval C, et al.: Detection of intracellular reactive oxygen species in cultured cells using fluorescent probes. *Methods Enzymol* 2002; 352: 62–71.

(Received, February 6, 2012)

(Accepted, February 24, 2012)