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Photodynamic Activity of Liposomal Photosensitizers via Energy Transfer from Antenna Molecules to [60]Fullerene

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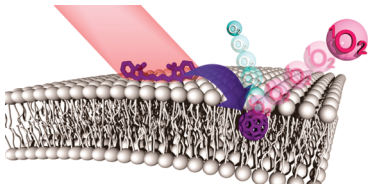
ABSTRACT Photodynamic therapy (PDT) is an emerging approach for the treatment of tumor diseases that has received growing interest in the past few years. In this study, we constructed liposomal photosensitizers (PS) for PDT by shoehorning as light-harvesting "antenna" molecules and dense [60]fullerene (C_{60}) into lipid membrane bilayers. The liposomal PS showed improved photodynamic activity toward human cancer cells via the photoenergy transfer from photoactivated antenna molecules to C_{60}.

KEYWORDS Energy transfer, fullerenes, photodynamic therapy, photosensitizers, liposomes



Photodynamic therapy (PDT) is a next-generation cancer treatment based on cytotoxic reactive oxygen species (ROS) producing photochemical reactions between photoexcited photosensitizers (PS) and molecular oxygen.[1,2] Due to the formation of a long-lived triplet state and the high photoproduction ability of ROS, fullerenes have attracted significant attention as PS of PDT.[3−13] We have previously reported that liposomal lipid membrane can be used for solubilization of unmodified [60]fullerenes (C_{60}) to overcome their poor water solubility. The produced cationic lipid membrane incorporating C_{60} ($LMIC_{60}$) showed high cytotoxicity under light irradiation between 350−500 nm.[10−13] However, from a practical application standpoint, the poor absorption of C_{60} between 600 and 700 nm, which is the optimal wavelength range for PDT, remains problematic.

In photosynthesis, solar energy is efficiently converted into chemical potential energy by highly organized multiprotein assemblies in the thylakoid membrane architecture of chloroplasts. In this process, photon energy is absorbed by the pigment antenna molecules and transferred to the reaction centers. The energy transfer model which occurs between pigment antenna molecules and the reaction centers represents an attractive approach to overcome the poor absorption of C_{60} at long wavelengths. In this study, thylakoid membrane architecture mimetic liposomal PS of PDT were constructed by shoehorning light-harvesting "antenna" molecules and dense C_{60} into lipid membrane bilayers (Figure 1). In this PS, photoenergy was absorbed by antenna molecules and transferred to C_{60} to generate the ROS. To construct the liposomal PS, $LMIC_{60}$ is a convenient platform for placing antenna molecules and C_{60} in close proximity because the antenna molecule-introduced liposome can be easily prepared through liposome staining using a lipid membrane probe. By employing liposomes, a direct covalent link between the antenna molecules and C_{60} can be avoided, resulting in the efficient generation of ROS because unmodified fullerenes generate ROS more efficiently than other chemically modified C_{60} derivatives.[14]

1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine (DiD) molecules, dialkylated carbocyanine lipid membrane probes, were used as light-harvesting antenna molecules because dialkylated carbocyanine lipid membrane probes have no appreciable cytotoxicity,[15,16] and DiD molecules have an absorption maximum (λ_{max}) of 648 nm in liposomes, which matches the optimal wavelength range for PDT. Light-harvesting liposomes of $LMIC_{60}$-DiD were prepared via fullerene exchange from the γ-cyclodextrin (γ-CDx) cavity to DiD contained liposomes (liposome-DiD) following a previously reported method.[10−13] Due to the efficient intracellular uptake of liposomes by tumor cells in our previously reports, liposomes with cationic surfaces were used for the preparation of $LMIC_{60}$-DiD.[10,11] The structures of DiD and lipids are shown in Figure S1 in the Supporting Information. $LMIC_{60}$-DiD, composed of DiD, lipids and C_{60}, were produced in a molar ratio of 1:40:4. The size distributions of the $LMIC_{60}$-DiD were measured by a dynamic light scattering (DLS) method. Unfortunately size distributions of $LMIC_{60}$-DiD could not be measured because the absorption of DiD interferes with the laser equipped in the measuring instrument. Therefore, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI), which is structurally similar to DiD, was employed for measurements (Figure S1 in the Supporting Information). The average diameter of $LMIC_{60}$-DiI was approximately 90 nm and did not change when irradiated with light at a power of 17 mW cm^{-2} and wavelengths longer than 510 nm (510−740 nm) for 30 min. To confirm that the

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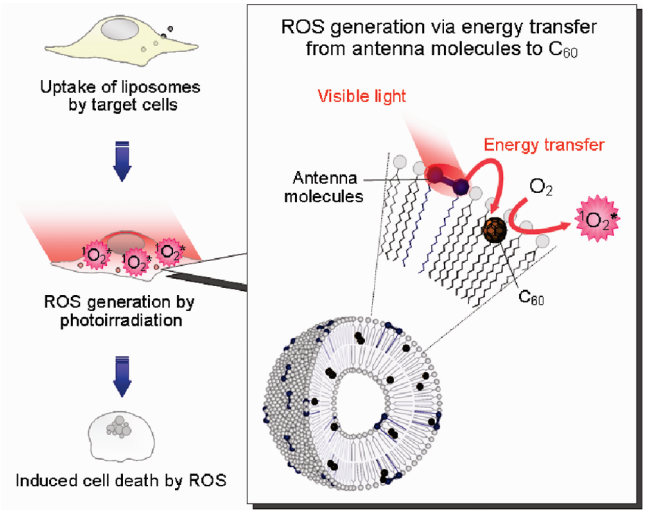

Figure 1. Light-harvesting liposomal PS of $LMIC_{60}$-DiD. Light-harvesting liposomal PS was prepared as a lipid membrane incorporating C_{60} and DiD antenna molecules. The energy of light absorbed by antenna molecules and transferred to C_{60} to generate ROS (singlet oxygen; $^1O_2$).

photon energy absorbed by DiD antenna molecules is transferred to C_{60} in $LMIC_{60}$-DiD, the C_{60}-dependent fluorescence quenching of DiD was first analyzed. Although the UV−vis absorption spectra of DiD for liposome-DiD and $LMIC_{60}$-DiD (Figure S2 in the Supporting Information) were similar, the fluorescence of DiD was reduced in $LMIC_{60}$-DiD compared with the fluorescence of DiD in liposome-DiD (Figure S3 in the Supporting Information). This indicated that the light energy absorbed by DiD may be transferred to C_{60}.

The photodynamic activity of $LMIC_{60}$-DiD using human cervical cancer HeLa cells was subsequently evaluated. To verify that photodynamic activity of $LMIC_{60}$-DiD is dependent on the antenna molecules, DiI was employed as a reference antenna molecule. DiI has a λ_{max} at 551 nm and barely absorbs light over 600 nm (Figures S1 and S2 in the Supporting Information). Following incubation with $LMIC_{60}$-DiI and $LMIC_{60}$-DiD, the cells were exposed to light with wavelengths longer than 610 nm (610−740 nm), at which the light is absorbed by DiD. Using the WST-8 assay, the cell viability was measured in light irradiated and unirradiated cells as a ratio (%) compared with untreated cells. The results showed that no samples had dark toxicity, even at the highest concentrations used (Figure 2a). Moreover, only $LMIC_{60}$-DiD reduced the viability of HeLa cells dependent on photoirradiation. All other samples were found to have no photodynamic activity. This photodynamic activity of $LMIC_{60}$-DiD was drug dose-dependent, and the medium inhibitory concentration (IC_{50} value) was estimated to be ca. 2 μM of DiD (Figure 2b). The IC_{50} value of $LMIC_{60}$-DiD was approximately equal to that of Photofrin, the most widely used clinical photosensitizer[17,18] (the IC_{50} value of Photofrin under the same conditions is ca. 2 μM when the number of moles is converted to porphyrin units because Photofrin comprises porphyrin oligomers containing 2−8 units; Figures S1 and S4 in the Supporting Information). Furthermore, antenna molecule-dependent photodynamic activity

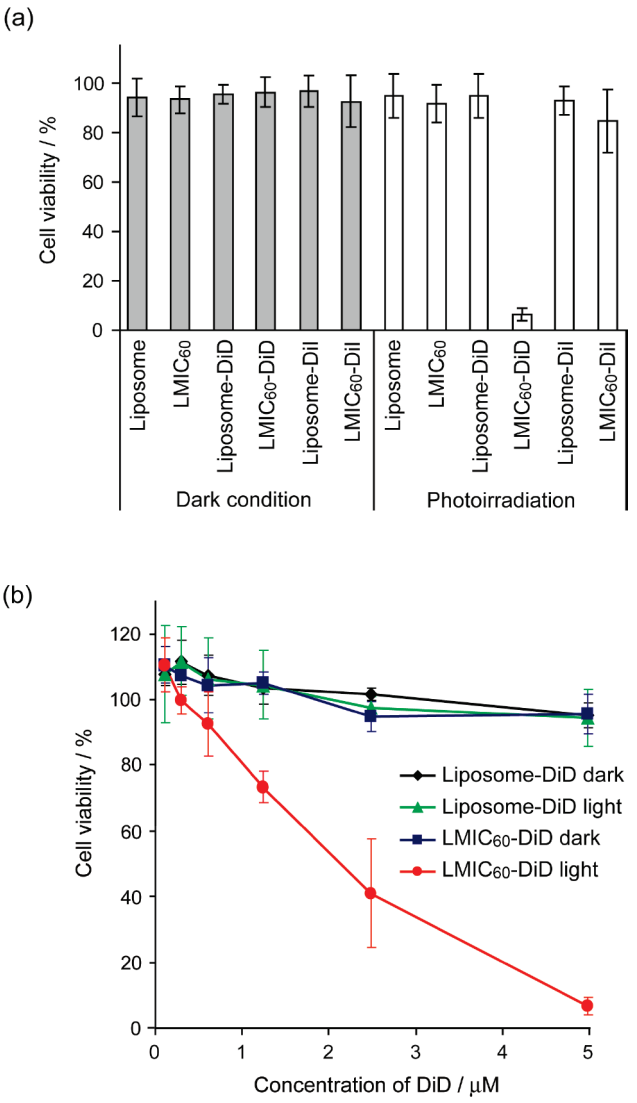

Figure 2. Photodynamic activity of $LMIC_{60}$-DiD. (a) Photodynamic activity was determined using the WST-8 assay at a fluorochrome concentration of 5 μM. Native liposomes and $LMIC_{60}$ were added at the same lipid concentrations as $LMIC_{60}$-DiD. (b) Dose-dependent photodynamic activity of $LMIC_{60}$-DiD. Each value represents the mean ± SD of three experiments.

was monitored not only for $LMIC_{60}$-DiD but also for $LMIC_{60}$-DiI treated cells when the photoirradiation wavelength was changed to longer than 510 nm (Figure S5 in the Supporting Information). These data clearly indicate that photodynamic activity of light-harvesting liposomal PS is related to the absorption wavelength of the antenna molecules and that the energy transfer from photoactivated antenna molecules to C_{60} can occur within the liposomes.

To identify the importance of placing antenna molecules and C_{60} in close proximity, HeLa cells were simultaneously treated with $LMIC_{60}$ and liposome-DiD. Although the cells were treated with the same concentrations of C_{60} in $LMIC_{60}$ and DiD in liposome-DiD compared with that of $LMIC_{60}$-DiD ($[C_{60}]$ = 10 μM, [DiD] = 2.5 μM), the photodynamic activity on simultaneously treated cells was significantly reduced

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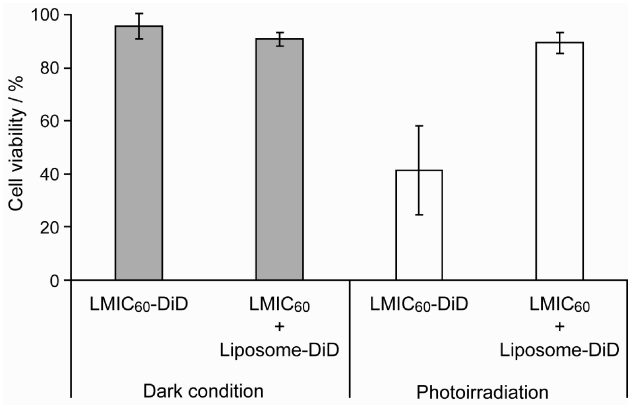

Figure 3. The importance of placing antenna molecules and C_{60} in close proximity. Photodynamic activity was measured at $LMIC_{60}$ and liposome-DiD simultaneously treated HeLa cells. HeLa cells were treated with 10 μM of C_{60} and 2.5 μM of DiD. Each value represents the mean ± SD of three experiments.

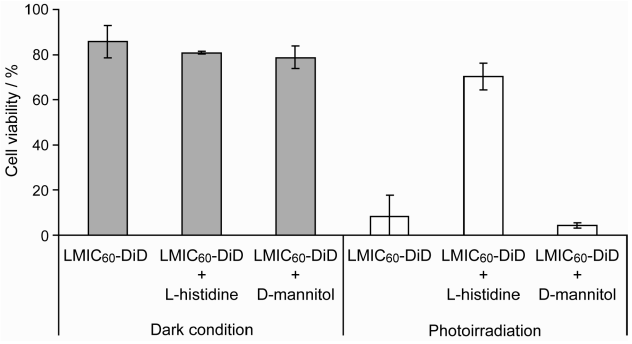

Figure 4. Effect of ROS scavengers on photodynamic activity of $LMIC_{60}$-DiD. Photodynamic activity of $LMIC_{60}$-DiD (5 μM of DiD) was measured in the presence of 50 mM L-histidine or 50 mM D-mannitol. Each value represents the mean ± SD of three experiments.

(Figure 3). These data indicate that the neighboring of antenna molecules and C_{60} in liposomes is necessary to induce the efficient photodynamic activity of $LMIC_{60}$-DiD.

ROS are well-known to be generated from excited C_{60} via an electron transfer type I reaction which generates superoxide anions ($O_2^{\bullet-}$) yielding hydroxyl radicals, and/or an energy transfer type II reaction which generates singlet oxygen molecules ($^1O_2$).[19] To identify the species of reactive oxygen generated from the $LMIC_{60}$-DiD, the effect of D-mannitol and L-histidine, the scavengers of hydroxyl radicals and $^1O_2$, respectively,[20,21] were analyzed. As shown in Figure 4, L-histidine blocked the photocytotoxicity of $LMIC_{60}$-DiD effectively, whereas D-mannitol was ineffective. The results suggest that $^1O_2$ generated via the energy transfer type II reaction plays a major role in the photodynamic activity of $LMIC_{60}$-DiD. Furthermore, the results also suggest that photon energy absorbed by DiD molecules is transferred to C_{60} via the energy transfer mechanism. If the radical anion of C_{60} ($C_{60}^{\bullet-}$) were generated via electron transfer from photoactivated DiD to C_{60}, only $O_2^{\bullet-}$ could have been produced by the type I reaction between $C_{60}^{\bullet-}$ and oxygen (Figure S6 in the Supporting Information).

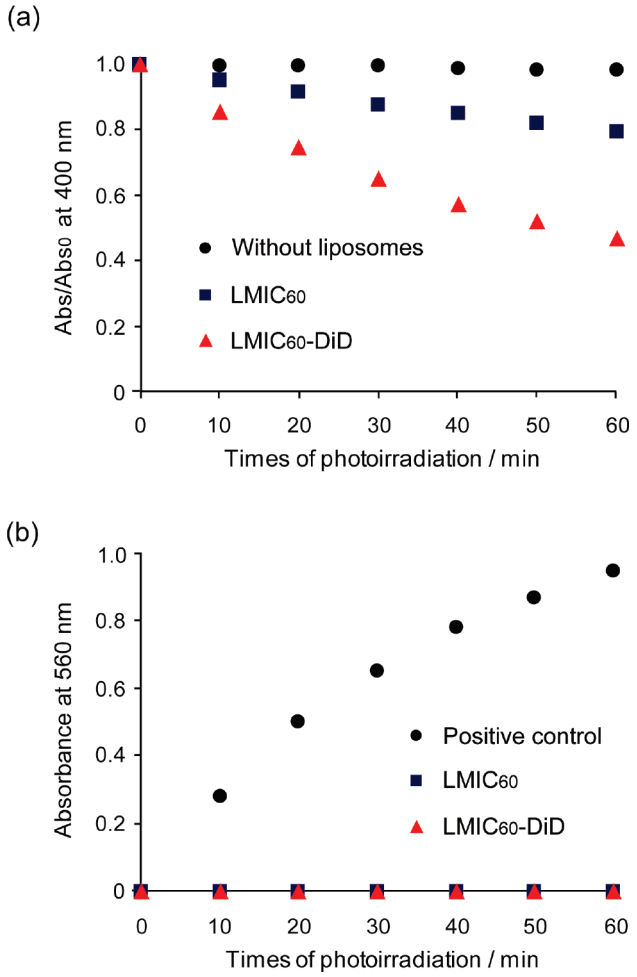

Figure 5. Detection of ROS generation by chemical methods (a) $^1O_2$ generation was detected by the APA bleaching method. The bleaching of APA was monitored as a reduction in the absorbance at 400 nm. (b) $O_2^{\bullet-}$ generation was detected by the NBT method. Formazan generation by a reduction of NBT in the presence of $O_2^{\bullet-}$ is detected as an increase in the absorption at 560 nm.

To further confirm these results, $^1O_2$ and $O_2^{\bullet-}$ generation was detected by chemical methods using 9,10-anthracenedipropionic acid (APA) and nitroblue tetrazolium (NBT) as detectors, respectively.[13,19] APA is converted to endoperoxide form by the reaction with $^1O_2$, which in turn leads to a reduction in absorbance at 400 nm (Figure S7a in the Supporting Information). Formazan generation by reduction of NBT in the presence of $O_2^{\bullet-}$ is detected as an increase of the absorption at 560 nm (Figure S7b in the Supporting Information). The decrease of APA absorption was monitored as a function of time after irradiating samples. As shown in Figure 5a, photoirradiation time-dependent bleaching of APA was severe for the $LMIC_{60}$-DiD samples compared with the $LMIC_{60}$ samples. Reduction of NBT by $O_2^{\bullet-}$, on the other hand, could not be detected in the photoirradiated $LMIC_{60}$-DiD samples, although formazan was readily detected in the C_{60}·γ-CDx complex positive control samples in the presence of NADH (Figure 5b).[22] These data clearly indicate that photon energy absorbed

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by DiD molecules is transferred to C_{60} via an energy transfer mechanism and $^1O_2$ was generated from C_{60} (Figure S6 in the Supporting Information).

In this study, we have demonstrated that light-harvesting liposomal PS of LMIC$_{60}$-DiD showed photodynamic activity when exposed to light with wavelengths longer than 610 nm (610−740 nm). This is the first demonstration of photodynamic activity of C_{60} under irradiation matched with the optimal wavelength for PDT. This photodynamic activity is dependent on the photon energy absorbed by antenna molecules, and the excitation of C_{60} occurs via the energy transfer from the antenna molecules to C_{60} resulting in the generation of $^1O_2$. Liposomal PS of LMIC$_{60}$-DiD were simply prepared by shoehorning antenna molecules and a dense C_{60} into liposomes. Degradation of liposomes *in vivo* can easily occur by metabolism. This high degradation rate of the liposomes results in the antenna molecules and C_{60} separating from each other. Therefore, this event leads to the inhibition of photoenergy transfer and this light-harvesting liposomal PS system may help to relieve photosensitive dermatitis, a known side effect of PDT.[23−25] Although additional improvements of the light-harvesting liposomal PS are necessary to enhance the photodynamic activity (actually the molar ratio of C_{60} and DiD in liposomes can be reduced; Figures S8 and S9 in the Supporting Information), these findings imply that the concept of light-harvesting liposomal PS of LMIC$_{60}$ shows significant promise in the field of medicinal chemistry. Applications of light-harvesting liposomal PS are currently under study in our laboratories.

SUPPORTING INFORMATION AVAILABLE

Experimental procedures and Figures S1−S9. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions: A.I. conceived and designed the experiment. M.A. performed the experiments. A.I. and M.A. prepared the manuscript. All authors discussed the results and commented on the manuscript.

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REFERENCES

(1) Dougherty, T. J.; Gomer, C. J.; Henderson, B. W.; Jori, G.; Kessel, D.; Korbelik, M.; Moan, J.; Peng, Q. Photodynamic therapy. *J. Natl. Cancer Inst.* **1998,** *90*, 889–905.

(2) Kato, H. Photodynamic therapy for lung cancer: a review of 19 years experience. *J. Photochem. Photobiol. B* **1998,** *42*, 96–99.

(3) Tabata, Y.; Ikada, Y. Biological functions of fullerene. *Pure Appl. Chem.* **1999,** *71*, 2047–2053.

(4) Bosi, S.; Da Ros, T.; Spalluto, G.; Prato, M. Fullerene derivatives: an attractive tool for biological applications. *Eur. J. Med. Chem.* **2003,** *38*, 913–923.

(5) Nakamura, E.; Isobe, H. Functionalized fullerenes in water. The first 10 years of their chemistry, biology, and nanoscience. *Acc. Chem. Res.* **2003,** *36*, 807–815.

(6) Guldi, D. M.; Prato, M. Excited-State Properties of C_{60} Fullerene Derivatives. *Acc. Chem. Res.* **2000,** *33*, 695–703.

(7) Akiyama, M.; Ikeda, A.; Shintani, T.; Doi, Y.; Kikuchi, J.; Ogawa, T.; Yogo, K.; Takeya, T.; Yamamoto, N. Solubilisation of [60]fullerenes using block copolymers and evaluation of their photodynamic activities. *Org. Biomol. Chem.* **2008,** *6*, 1015–1019.

(8) Ikeda, A.; Nagano, M.; Akiyama, M.; Matsumoto, M.; Ito, S.; Mukai, M.; Hashizume, M.; Kikuchi, J.; Katagiri, K.; Ogawa, T.; Takeya, T. Photodynamic Activity of C_{70} Caged within Surface-Crosslinked Liposome. *Chem.-Asian J.* **2009,** *4*, 199–205.

(9) Ikeda, A.; Matsumoto, M.; Akiyama, M.; Kikuchi, J.; Ogawa, T.; Takeya, T. Direct and short-time uptake of [70]fullerene into the cell membrane using an exchange reaction from a [70]fullerene−γ-cyclodextrin complex and the resulting photodynamic activity. *Chem. Commun.* **2009,** 1547–1549.

(10) Ikeda, A.; Sato, T.; Kitamura, K.; Nishiguchi, K.; Sasaki, Y.; Kikuchi, J.; Ogawa, T.; Yogo, K.; Takeya, T. Efficient photocleavage of DNA utilising water-soluble lipid membrane-incorporated [60]fullerenes prepared using a [60]fullerene exchange method. *Org. Biomol. Chem.* **2006,** *3*, 2907–2909.

(11) Ikeda, A.; Doi, Y.; Nishiguchi, K.; Kitamura, K.; Hashizume, M.; Kikuchi, J.; Yogo, K.; Ogawa, T.; Takeya, T. Induction of cell death by photodynamic therapy with water-soluble lipid-membrane-incorporated [60]fullerene. *Org. Biomol. Chem.* **2007,** *5*, 1158–1160.

(12) Ikeda, A.; Doi, Y.; Hashizume, M.; Kikuchi, J.; Konishi, T. An Extremely Effective DNA Photocleavage Utilizing Functionalized Liposomes with Fullerene-Enriched Lipid Bilayer. *J. Am. Chem. Soc.* **2007,** *129*, 4140–4141.

(13) Doi, Y.; Ikeda, A.; Akiyama, M.; Nagano, M.; Shigematsu, T.; Ogawa, T.; Takeya, T.; Nagasaki, T. Intracellular Uptake and Photodynamic Activity of Water-Soluble [60]- and [70]Fullerenes Incorporated in Liposomes. *Chem.—Eur. J.* **2007,** *14*, 8892–8897.

(14) Hamano, T.; Okuda, K.; Mashino, T.; Hirobe, M.; Arakane, K.; Ryu, A.; Mashikoc, S.; Nagano, T. Singlet oxygen production from fullerene derivatives: effect of sequential functionalization of the fullerene core. *Chem. Commun.* **1997,** 21–22.

(15) Honig, M. G.; Hume, R. I. Fluorescent carbocyanine dyes allow living neurons of identified origin to be studied in long-term cultures. *J. Cell Biol.* **1986,** *103*, 171–187.

(16) Honiga, M. G.; Hume, R. I. Dil and DiO: versatile fluorescent dyes for neuronal labelling and pathway tracing. *Trends Neurosci.* **1989,** *12*, 333–341.

(17) Kessel, D. Photosensitization with derivatives of haematoporphyrin. *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med.* **1986,** *49*, 901–907.

(18) Kessel, D. Proposed structure of the tumor-localizing fraction of HPD (hematoporphyrin derivative). *Photochem. Photobiol.* **1986,** *44*, 193–196.

(19) Yamakoshi, Y.; Umezawa, N.; Ryu, A.; Arakane, K.; Miyata, N.; Goda, Y.; Masumizu, T.; Nagano, T. Active Oxygen Species

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Generated from Photoexcited Fullerene (C_{60}) as Potential Medicines: $O_2^{-\bullet}$ versus $^1O_2$. *J. Am. Chem. Soc.* **2003,** *125*, 12803–12809.

(20) Goldstein, S.; Czapski, G. Mannitol as an $OH^\bullet$ scavenger in aqueous solutions and in biological systems. *Int. J. Radiol.* **1984,** *46*, 725–729.

(21) Lindig, B. A.; Rodgers, M. A. J. Rate parameters for the quenching of singlet oxygen by water-soluble and lipid-soluble substrates in aqueous and micellar systems. *Photochem. Photobiol.* **1981,** *33*, 627–634.

(22) Nakanishi, I.; Fukuzumi, S.; Konishi, T.; Ohkubo, K.; Fujitsuka, M.; Ito, O.; Miyata, N. DNA Cleavage via Superoxide Anion Formed in Photoinduced Electron Transfer from NADH to γ-Cyclodextrin-Bicapped C_{60} in an Oxygen-Saturated Aqueous Solution. *J. Phys. Chem. B* **2002,** *106*, 2372–2380.

(23) Wooten, R. S.; Smith, K. C.; Ahlquist, D. A.; Muller, S. A.; Balm, R. K. Prospective study of cutaneous phototoxicity after systemic hematoporphyrin derivative. *Lasers Surg. Med.* **1988,** *8*, 294–300.

(24) Dougherty, T. J.; Cooper, M. T.; Mang, T. S. Cutaneous phototoxic occurrences in patients receiving Photofrin. *Lasers Surg. Med.* **1990,** *10*, 485–488.

(25) Moriwaki, S. I.; Misawa, J.; Yoshinari, Y.; Yamada, I.; Takigawa, M.; Tokura, Y. Analysis of photosensitivity in Japanese cancer-bearing patients receiving photodynamic therapy with porfimer sodium (Photofrin). *Photodermatol., Photoimmunol. Photomed.* **2001,** *17*, 241–243.

DOI: 10.1021/ml100021x | *ACS Med. Chem. Lett.* **2010,** *1*, 115–119