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Photoinduced Electron-Transfer Mechanisms for Radical-Enhanced Photodynamic Therapy Mediated by Water-Soluble Decacationic C₇₀ and C₈₄O₂ Fullerene Derivatives

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Abstract

Fullerenes are promising candidates for photodynamic therapy (PDT). Thus, C₇₀ and novel C₈₄O₂ fullerenes were functionalized with and without an additional deca-tertiary ethyleneamino-chain as an electron source, giving rise to two distinct pairs of photosensitizers, the monoadducts **LC-17**, **LC-19** and the bisadducts **LC18** and **LC-20** to perform PDT in HeLa cells with UVA, blue, green, white and red light. Shorter wavelengths gave more phototoxicity with **LC-20** while **LC-19** was better at longer wavelengths; the ratio between killing obtained with **LC-19** and **LC-20** showed an almost perfect linear correlation ($R = 0.975$) with wavelength. The incorporation of a deca-tertiary amine chain in the C₈₄O₂ fullerene gave more PDT killing when excited with shorter wavelengths or in presence of low ascorbate concentration through higher generation of hydroxyl radicals. Photoactivated C₈₄O₂ fullerenes induced apoptosis of HeLa cancer cells, together with mitochondrial and lysosomal damage demonstrated by acridine orange and rhodamine 123 fluorescent probes.

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Supplementary material Available. Additional characterization and spectroscopic data for all compounds utilized.

Keywords

photodynamic therapy; decacationic [70]fullerene derivatives; decacationic [84]fullerene-dioxide derivatives; electron transfer; ascorbic acid

BACKGROUND

Photodynamic therapy (PDT) is an alternative treatment for several diseases including cancer that uses a non-thermal photochemical reaction among non-toxic photosensitizers (PS), harmless visible light and ambient oxygen. PDT offers several advantages over traditional therapies, such as reduced side effects and improved tumor selectivity, since only the lesion that is exposed to the light suffers damage, while other tissues are not affected. The PS may be delivered topically, systemically or locally and the light may be delivered inside the body via endoscopic or interstitial fiber optics.

The PS molecule needs to be excited by the appropriate wavelengths and can then further transfer the photon energy to surrounding oxygen molecules in order to generate reactive oxygen species (ROS) and kill cancer cells. A wide range of chemical structures are able to act as PS and fullerenes have been a notable addition to the armamentarium. Fullerenes may be good PS because they are excellent absorbers of light, good electron acceptors and have high quantum yields of the triplet state. They are good models for electron transfer research and may be able to generate ROS through both type-I and type-II photochemical mechanisms. Moreover fullerenes are much more photo-stable and demonstrate less photo-bleaching when compared with tetrapyrroles and synthetic dyes. However, fullerenes need to be derivatized by chemists to increase solubility in water and biological systems.

Different cage sizes of fullerenes have been employed to perform PDT. The most well known and the first to be recognized for its photodynamic effect on tumors was C₆₀ fullerene, which has a well-known unique arrangement of 60 carbon atoms arranged in a soccer ball structure. Besides C₆₀, C₇₀ fullerenes are established as electron acceptors in photo-induced electron-transfer processes with electron donors and C₇₀ derivatives have been used to carry out PDT. When PDT is fullerene mediated with a long incubation time it can kill multiple types of cancer cells including head and neck, breast and esophageal cancer that respond poorly to alternative cancer therapies. The present study however is the first to our knowledge to demonstrate the effectiveness of C₈₄O₂-based fullerenes for PDT.

Fullerenes can accept up to six electrons, while the excited states of the fullerene, particularly the triplet, may be even better electron acceptors. We devised a synthetic scheme in which a decacationic chain could be attached to the fullerene in order to provide water solubility and at the same time increase the binding affinity to the negatively charged cancer cells and microbial cells that are the target of PDT. This strategy produced **LC-17** when C₇₀ fullerene was employed and **LC-19** when the C₈₄O₂ fullerene oxide was used. We also wanted to test the hypothesis that electron transfer mechanisms are important for PDT so we attached an additional deca-tertiary amine chain that, alone or with an external electron donor could provide additional electrons for the photochemical reactions. The analogous structures were **LC-18** for C₇₀ fullerene with both a deca-cationic chain and a deca-tertiary amine chain attached, and **LC20** the C₈₄O₂ fullerene oxide with both a deca-cationic chain and a deca-tertiary amine chain attached.

Changes in the electronic and geometrical structures of molecules, as well as their interaction with other molecules in the surrounding environment can be induced by the photo-excitation of these molecules. The electron transfer can take place in both an inter and intra-molecular fashion. When C₆₀ fullerene cages were covalently bound to photoactive

chromophores, there was enhanced photo-induced intramolecular energy transfer or electron-transfer events. Furthermore, relative small variations in the irradiation wavelengths may cause changes in the photophysical behavior of dyes.

We first tested this synthetic approach using the more available C₇₀ fullerene as a starting material and then moved on to examine the less accessible C₈₄O₂ compounds. We compared the effect of the additional tertiary amine chain in the in vitro PDT killing of HeLa cells using different light sources that ranged through the spectrum from UVA to red light. We tested the hypothesis that more electron transfer, higher generation of hydroxyl radicals and enhanced cell killing would be encouraged by a fullerene with an attached tertiary amine chain along with the use of shorter excitation wavelengths and low concentrations of ascorbic acid as an external electron donor.

METHODS

Materials

Reagents consisting of γ -butyrolactone, BF₃·Et₂O, triethylamine, pyridine, iodomethane, 1,8-diazabicyclo[5,4,0]-undec-7-ene (DBU), tetrabromomethane (CBr₄), iodine, trifluoroacetic acid, and *L*-ascorbate were purchased from Aldrich Chemicals and used without further purification. Malonyl chloride was purchased from TCI America. A C₇₀ sample with a purity of 98.0% was purchased from Term USA, Inc. and a C₈₄O₂ sample was provided by Nano-C, Inc. Sodium sulfate was employed as a drying agent. Solvents were routinely distilled before use. 3'-*p*-(Hydroxyphenyl)fluorescein (HPF) were obtained from Invitrogen, Ltd. as a solution in dimethyl formamide.

Synthesis and characterization

This is fully described in supporting information section.

Determination of the Absorption Spectra of LC-19, LC-19-I₃⁻, and LC-20

The PS were dissolved in DMA to form 50 μ M solutions. 2.0 mL of each solution was added to quartz cuvettes and then the absorption spectrum of each dye was obtained with a spectrophotometer (Evolution 300 UV-Vis Spectrophotometer - Thermo Fisher Scientific Inc., Waltham, MA, USA).

Calculation of Wavelength Absorption by LC-19-I₃⁻ and LC-20

To calculate how much of the wavelengths used each dye would absorb, the emission spectra of all light sources (UVA, blue, green, white and red) were plotted against the absorption spectrum of both dyes. The overlapping regions between each light source emission band and LC-19-I₃⁻ or LC-20 were obtained by calculating the overlapped areas under the curves with the results shown in Table 1.

Photodynamic Therapy and Cell Viability

HeLa cells were cultured in RPMI-1640 Medium (R 8758, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma-Aldrich) at 37°C under 5% CO₂. For cell viability assay, cells were cultured in 96-well clear tissue-culture treated microplates (353072, BD Falcon, San Jose, CA, USA) at 1×10^4 /well for 24 h and then incubated for 24h with LC-17 or LC-18 or LC-19-I₃⁻ or LC-20 all compounds at 4.0 μ M to form different experimental groups: control 1 (no dye and no light); control 2 (only light); control 3 (only dye incubation); PDT group with LC-19-I₃⁻; and PDT group with LC-20. After washing the cells twice with PBS, light irradiation was performed with the wavelengths UVA (350-380 nm/ Ultraviolet Examination Light - UV

501, Burton Medical, Chatsworth, CA, USA), blue (390–420 nm/ OMNILUX PN EL 1600, Phototherapeutics Inc., CA, USA), green (525–555 nm/ LC122, LumaCare Medical Group, MBG Technologies Inc., CA, USA), white (400–700 nm/ LC122, LumaCare Medical Group) and red light (615–645 nm/ OMNILUX PN EL 1600). The irradiances used were 4, 50, 80, 160 and 100 mW/cm², respectively. The energy densities were 1, 2, 4 and 8 J/cm² for UVA and 10, 20, 40 and 80 J/cm² for every other wavelength used. After illumination the cells were again incubated for 24 h and the cells viability was determined with a 4-h MTT assay.

Killing Ratios between LC-19-I₃⁻ and LC-20

To easily compare the efficacy of both dyes in killing HeLa cells the areas under the curves obtained with the MTT assay for every dye and wavelength were calculated to give an absolute number. The values obtained for **LC-19-I₃⁻** were divided by the values obtained for **LC-20** for each wavelength comprising a wavelength dependence behavior of the ratios between both dyes.

Detection of Hydroxyl Radicals

The fluorescent probe hydroxyphenyl fluorescein (HPF) (H36004, Molecular Probes, Life Technologies, NY, USA) was used to selectively detect the HO• formation upon the illumination of **LC-19-I₃⁻** and **LC-20** dyes (4.0 μM) in aqueous environment and with or without the presence of 10 μM of *L*-ascorbic acid (AA) (Sigma-Aldrich). For this purpose 7 distinct groups (n = 5) were configured in a 96-well clear microplate (353072, BD Falcon) using PBS and acetonitrile in the ratio of 50/50% as solvent: Group 1 contained only solvent; group 2 was HPF probe (5.0 μM); group 3 consisted of AA (10 μM); group 4 was HPF probe (5.0 μM) + AA (10 μM); group 5 contained **LC-19-I₃⁻** (4.0 μM); group 6 was HPF probe (5.0 μM), **LC-19-I₃⁻** (4.0 μM); finally, group 7 consisted HPF probe (5.0 μM), AA (10 μM), **LC-19-I₃⁻** (4.0 μM). Groups 5–7 were repeated with **LC-20** (4.0 μM). The total volume of each well added up to 100 μL and the plates were then irradiated with the UVA or red light sources as described above. The fluorescence emission was measured (SpectraMax M5, Molecular Devices, Sunnyvale, CA) using excitation/emission wavelengths of 490/515 nm, as the fluences increased up to 38 J/cm² for the UVA group and up to 313 J/cm² for the red light group. The generation of hydroxyl radicals was also checked with different concentrations of ascorbic acid after photo-stimulation of both dyes. For that, 5.0 μM of HPF was added in wells containing PBS solutions with 0, 2.5, 5, 10, 20, 40, 50, 80, 100, 200 and 400 μM of *L*-ascorbic acid. The plates were then illuminated with 313 J/cm² of red light or 38 J/cm² of UVA light and the fluorescence emission was measured as described above.

Confocal Fluorescence Imaging

HeLa cells were cultured (1 × 10⁴/well) in the same conditions described for the cell viability assay in black 96-well tissue culture treated with clear bottom plates (353219, BD Falcon, San Jose, CA, USA) for 24 h and then incubated for 24h with both **LC-19-I₃⁻** and **LC-20** compounds at 4μM. To determine the intracellular generation of hydroxyl radicals, after the 24h incubation with the dyes, HeLa cells were incubated with 5.0 μM of HPF (H36004, Molecular Probes) in complete medium for 30 minutes at 37 °C. All groups were washed twice with PBS and the experimental groups were irradiated with UVA light (4 J/cm²). Immediately after PDT the cells were observed by confocal microscopy (Fluoview FV1000, Olympus America Inc., PA, USA). To determine the location of intracellular damage caused by PDT, the cells were incubated immediately after PDT (24 hour incubation with **LC-19-I₃⁻** or **LC-20** and 4.0 J/cm² of UVA light) with acridine orange (0.5μM/ Sigma-Aldrich) or rhodamine 123 (0.5μM/ Sigma-Aldrich) and with 4μg/mL

Hoechst (Hoechst 33342, Invitrogen) for 5 min in complete medium at 37 °C. Next the cells were washed and the intracellular localization of the dye was observed by confocal microscopy. Both probes were excited with Ar-laser 488-nm and emission wavelengths were AO (green fluorescence 525 nm $\pm$ 10 nm, or red fluorescence 580-nm longpass) and Rho 123 (525 nm $\pm$ 10 nm). As controls, HeLa cells were incubated with either AO or Rho 123 but did not receive PDT.

RESULTS

Synthesis of C₇₀- and C₈₄O₂-Malonate Quaternary Ammonium Iodide Salts

The unmodified fullerenes are not soluble in polar solvents, which demands their chemical modification so they can become suited for biological purposes, such as PDT. Accordingly, functional moieties of C₇₀ and C₈₄O₂ fullerenes were designed to increase both the water-solubility and provide surface binding interactions with *-D-Ala-D-Ala* residues of the bacteria cell wall by incorporating multiple H-bondings and positive quaternary ammonium charges. We first synthesized a malonate precursor arm M(C₃N₆⁺C₃)₂, shown in the structure of C₇₀[>M(C₃N₆⁺C₃)₂] **1 (LC-17)** and C₈₄O₂[>M(C₃N₆⁺C₃)₂] **2 (LC-19)** in Figure 1. It included two esters and two amide moieties to give a sufficient number of carbonyl and -NH groups in a short length of ~20 Å to provide effective multi-binding sites with the use of a well-defined water-soluble pentacationic moiety C₃N₆⁺C₃-OH at each side of the arm. (See supplementary material).

ROS-Generation Mechanism and Wavelength-Dependent PDT Efficacy of Decacationic Fullerene Derivatives

The activity of photoinduced excitation of PS can be correlated to its relative optical absorption extinction coefficients at different wavelengths of PDT application. Chemical functionalization of fullerene cages to their monoadduct and bisadduct derivatives changes the π -conjugation length at different regions of the cage surface and, thus, varies the optical absorption profile over the UV-vis range. Figure 2 summarized UV-vis spectra (solid lines) of **LC-17**, **LC-18**, **LC-19**, and **LC-20** with the plot against the spectra (dashed lines) of five light sources employed. In the visible wavelength region, absorption extinction coefficients follow the order of **LC-17** > **LC-19-I⁻** > **LC-20** > **LC-18**. This order changes in the UVA wavelength region (315–400 nm) giving the slightly higher coefficient for decacationic C₈₄O₂ bisadduct **LC-20** followed by decacationic monoadducts **LC-17** and **LC-19-I⁻** with a lower value for decacationic C₇₀ bisadduct **LC-18**. When all the iodide counter anions (I⁻) of **LC-19-I⁻** were exchanged to triiodides (I₃⁻) by the treatment with iodine (I₂) giving the corresponding **LC-19-I₃⁻**. The optical absorption of I₃⁻ at 294 and 366 nm enhances the overall value of extinction coefficient of this compound in the UVA region. The area under each overlap between the absorption spectra of decacationic fullerene derivatives and light spectral curves were integrated and calculated in order to give an indication of relative numbers of photons absorbed at different wavelengths ranging from UVA, blue, green to red and white, as summarized in Table 1.

We initially tested the effect of varying the excitation wavelength using either UVA or red light with C₇₀[>M(N₆⁺C₃C₁)₂] (**LC-17**) and C₇₀[>M(C₃N₆⁺C₃)₂][>M(C₃N₆C₃)₂] (**LC-18**) on HeLa cell PDT killing. As can be seen in Figure 3A using UVA light, **LC-18** that possessed the additional deca-tertiary ethyleneamine chain gave significantly more cell killing than that found with **LC-17** that lacked the deca-tertiary amine chain. When red light was used the situation was reversed and the decacationic **LC-17** gave significantly more killing than that found with **LC-18** with both chains (Figure 3B). This interesting and unexpected finding prompted us to repeat the HeLa cell killing studies with the novel compounds C₈₄O_x[>M(C₃N₆⁺C₃)₂] (**LC-19-I₃⁻**) and C₈₄O₂[>M(C₃N₆⁺C₃)₂]

[$>M(C_3N_6C_3)_2$] (**LC-20**) derivatives using a wider range of different wavelengths of light (Figure 4, (A) UVA, (B) blue, (C) green, (D) white and (E) red). There was more killing with **LC-20** than with **LC-19-I₃⁻** when UVA was used (Figure 4A) and when blue light was used (Figure 4B). These differences are presumably due to the greater likelihood of electron transfer processes taking place with shorter wavelengths and the presence of electron donating tertiary-ethyleneamine chain. When white light was used (Figure 4C) the difference between **LC-20** and **LC-19-I₃⁻** was smaller but **LC-20** still gave more killing, while green light (Figure 4D) gave equal killing for the two fullerenes. Interestingly when red light was used (Figure 4E) the situation was reversed and **LC-19-I₃⁻** gave significantly more killing than **LC-20**. All the utilized compounds induced a very low dark toxicity at these concentrations (Figures 3A,B and 4A-E). When the areas under the curve (AUC) for the killing were calculated and the mean ratios of AUC for **LC-20** and **LC-19-I₃⁻** were plotted against wavelength (Figure 4F) an excellent linear correlation was obtained ($R = 0.975$).

We used a fluorescent caspase-3 assay to measure apoptosis 24 h after PDT. The results are shown in Figure S8. **LC-19-I₃⁻** with both UVA and red light and **LC-19-I₃⁻** with UVA light all gave comparable amounts of caspase-3 activation, but **LC-19-I₃⁻** with red light gave significantly more caspase-3 activation.

Since the hypothesis was that the presence of the deca-tertiary amine chain and the use of shorter wavelengths both encouraged electron transfer that is considered to lead to type-I photochemistry, we used the fluorescence probe of 3'-*p*-(hydroxyphenyl)-fluorescein (HPF) that has been reported to be (reasonably) selective for hydroxyl radicals (HO[•]) that are the most cytotoxic species generated during type-I PDT. By this method, the measured probe fluorescence intensity can be correlated roughly to the yield of hydroxyl radicals produced. Besides, superoxide radical (O₂^{-•}) is the precursor species to the formation of HO[•], directly monitoring the presence of HO[•] will also serve to confirm superoxide radical formation. Moreover since it has been reported that adding an external source of electrons such as ascorbate to the PDT reaction can further potentiate type I photochemistry by facilitating electron transfer, provided the ascorbate is at a sufficiently low concentration, we tested the addition of 10 μM ascorbate. In addition, ascorbate can also efficiently reduce each I₃⁻ anion to 3I⁻ anions *in situ* in PBS solution. Figure 5 shows the generation of HPF fluorescence by **LC-19-I₃⁻** and **LC-20** by both UVA light and red light in the presence and absence of ascorbate. Figure 5A shows that **LC-19-I₃⁻** alone activated HPF significantly more than **LC-20** when UVA was used consistent with the larger absorption band of **LC-19-I₃⁻** in the UVA region of the spectrum (Figure 2 and Table 1).

Actually, in aqueous media the generation of hydroxyl radical was found to be always significantly higher with the **LC-19-I₃⁻** (Figure 5), regardless of the photo-stimulation being with red or UVA light. However, when low concentration of ascorbate was added to the reaction mixture an interesting phenomenon was observed. The rate of activation of HPF by **LC-19-I₃⁻** and UVA was significantly decreased by ascorbate, while the rate of activation of **LC-20** by UVA light was increased in the presence of ascorbate. This observation suggests that the electron donating ability of ascorbate was effective in the presence of the deca-tertiary ethyleneamine chain (**LC-20**) allowing increased electron transfer and higher generation of HO[•], while in the absence of the tertiary hexa(ethyleneamine) chain (**LC-19**) the ROS quenching effect of the ascorbate predominated and HO[•] concentration was reduced. Figure 5B shows the same experiments repeated with red light. Again the overall rate of activation of HPF was higher for **LC-19-I₃⁻** compared to **LC-20**, despite the absorption of red light being apparently higher for **LC-20** compared to **LC-19-I₃⁻**. When the effect of ascorbate was examined it can be seen that for **LC-19-I₃⁻** there was a significant reduction in HPF activation, while for **LC-20** there was no significant effect. These data

suggest that the electron transfer reaction is more pronounced in the case of UVA excitation than it is for red light and offers some explanation for the differences observed in the cell killing studies.

As the potentiation of HPF activation by low concentrations of ascorbate was somewhat surprising we decided to employ a wide range of ascorbate concentrations ranging from 1.0 to 400 μM to explore the biphasic effects of ascorbate. For UVA excitation (Figure 5C), there was a pronounced biphasic effect of ascorbate concentration on HPF activation in the case of **LC-20**, and to a much lesser and non-significant extent in the case of **LC-19-I₃⁻**. The peak in potentiation was seen with 5.0 and 10 μM ascorbate and as the concentrations were steadily increased the potentiation disappeared and quenching was apparent from 50 μM to 400 μM . Statistical analysis showed that ascorbate concentrations of 5 and/or 10 μM gave more HPF fluorescence than 1 and/or 2.5 μM , and furthermore that most concentrations higher than 2 and/or 40 μM gave less HPF than 5 and/or 10 μM thus demonstrating a biphasic relationship. In the case of red light excitation (Figure 5D) there was no potentiation apparent, and quenching occurred throughout the whole range of ascorbate concentrations.

The fullerenes were not intrinsically fluorescent, so it was not possible to establish cellular uptake and subcellular localization (mitochondria, lysosomes or endoplasmic reticulum) by the traditional methods of confocal microscopy. The uptake of the fullerenes into the cells was established by using the HPF probe intracellularly. The HPF was added to the cells shortly before light exposure and as can be seen in Figure 6A green fluorescence increased in both **LC-19-I₃⁻** (Figure 6C) and **LC-20** (Figure 6E) after light exposure.

Intracellular Localization of Decacationic C₈₄O₂-Malonate Quaternary Ammonium iodide/triiodide Salts

The intracellular localization was investigated by using probes that report damage provoked by PDT to different intracellular organelles. This was done using rhodamine 123 (Rh 123) and acridine orange (AO) fluorescent dyes (Figures 6G-O) added immediately after light delivery. Due to the mitochondrial transmembrane potential, Rh123 accumulates in the mitochondria of living cells and not in their nuclei. As a consequence of PDT damage to mitochondria, the mitochondrial transmembrane potential is lost, resulting in Rh123 being taken up as diffuse fluorescence throughout the cancer cells, including the nucleus (Figure 6G-I). Hydroxyl radical-induced tumor cell death is indeed associated with the reduction of the mitochondrial transmembrane potential.

On the other hand, AO accumulates in lysosomes in intact cells (punctate fluorescent organelles) where it is in an aggregated state and displays both red and green fluorescence. When the lysosomes suffer PDT-mediated damage the AO is taken up into cells in a more diffuse pattern including to the nucleus where it binds to the double stranded helical DNA in a monomeric molecular form and causes much stronger green fluorescence and in some cases (such as the present) binds to damaged DNA that has suffered decreased structural organization, leading to a higher emission of red fluorescence. Figure 6 (J-O) showed that the PDT treated cells (both **LC-19-I₃⁻** and **LC-20**) showed distinct changes from intact cells giving more green fluorescence that spread over the whole cell including the nucleus, and also more red fluorescence. The data therefore suggest that these fullerenes accumulate in both mitochondria and in lysosomes. This is not unusual in PS that have both hydrophobic regions and cationic charges in the same molecule.

DISCUSSION

The phenomenon of wavelength-dependent alteration of PDT mechanisms has not been much reported (if at all). When PDT is performed with **LC-20** and shorter wavelengths, higher cell killing is achieved, but **LC-19-I₃⁻** gives higher killing when used along with longer wavelengths, such as red light. The presence of tertiary amine electron donors predisposes towards better killing after short wavelength excitation. Knowing that high amounts of hydroxyl radical induce the apoptosis of cells and that the generation of hydroxyl radical was always significantly higher with the **LC-19-I₃⁻**, regardless of the photo-stimulation being with red or UVA light, the intracellular environment seems to have influenced the results.

Accordingly, when an external electron donor (ascorbate) was added to the experiment, the situation significantly changed, leading to a reduction of fluorescence generation in the case of both **LC-19-I₃⁻** and **LC-20** when red light was used, and a remarkable increase in formation of HPF fluorescence only for **LC-20/UVA** light. Ascorbate is a well-known reducing agent and antioxidant, but it is also well-known to have both antioxidant and prooxidant effects depending on the concentration and other parameters. Ascorbate can act as a quencher of singlet oxygen and hydroxyl radical, especially in aqueous systems at higher concentrations, while at lower concentrations ascorbate may act as an electron donor.

Among the several intracellular electron donors suggested, ascorbate is one of the most important, and acts as a major electron donor to tPMET, the trans-plasma membrane electron transport system that oxidizes intracellular electron donors to reduce extracellular oxidants. Primary cultures of pulmonary endothelial cells may benefit from a restricted intracellular pool of ascorbic acid to reduce the extracellular mildly oxidizing dye FIC and thiazine dyes. Martirosyan et al found a biphasic-dose response with antioxidants (ascorbate and quercetin) in the hypericin-mediated photodynamic hemolysis of isolated erythrocytes. Concentrations of ascorbate at about 3.5 μM potentiated hemolysis and this was attributed to increased electron transferring.

Although it has not been proved from which side of the plasma membrane the AA employs its stimulatory effect, the presence of this electron donor in the intracellular environment may significantly change the phototoxicity of the two C_{84}O_2 -fullerenes that only differ by the incorporation of deca-tertiary amines. This was confirmed by adding AA to the aqueous system, what mimicked the intracellular environment and changed the relation of hydroxyl radical generation between **LC-19-I₃⁻** and **LC-20**. In addition, the cells probably have other sources of electron donors in their natural state, such as the flavonoids quercetin and myricetin in erythrocytes.

There are some literature references suggesting that relatively small variations in the irradiation wavelengths may cause changes in the photophysical behavior of dyes. It was reported that there was a change from an intramolecular electron transfer mechanism to triplet energy transfer in a ruthenium porphyrin/perylene bisimide compound when it was excited by 585 or 530 nm light, respectively.

The intramolecular electron transfer of crystal violet lactone is also an absorption-wavelength-dependent process. In addition, the thermodynamics and kinetics of the intramolecular electron transfer following photoexcitation of crystal violet lactone in $[\text{Pr}_{31}^+][\text{Tf}_2\text{N}^-]$ is dependent on the solvent and on the environment. Moreover, the pathway of charge separation may be excitation wavelength dependent in reaction centers of the purple nonsulfur photosynthetic bacterium *Rhodobacter sphaeroides*, once the amount of stimulated

emission is not the same if shorter wavelength excitation is used instead of 860 nm excitation.

It is known that covalently combining electron donors like porphyrin and phthalocyanine with efficient electron acceptors, such as fullerenes, strongly affects the electron transfer mechanisms. The photoinduced electron transfer involves the interaction between donors and acceptors in a way to form transient exciplex states, barrierless charge separation and recombination reactions, independently of the temperature.

Standard PDT that generates singlet oxygen causes caspase-dependent apoptosis and it has been reported that high amounts of exogenous hydroxyl radical cause caspase-independent cell death, thus it could be theorized that type-II photochemical mechanism could cause more caspase-dependent apoptosis than type-I mechanisms. This is in fact consonant to what found herein, but this hypothesis will require more experimental testing.

One of main reasons why fullerenes are particularly efficient in mediating PDT is their extended π system, which absorbs UV or visible light and generates ROS through energy and electron-transfer processes from the triplet and singlet states. The efficiency of photo-induced cytotoxicity depends on both the degree of modification of the cage and also on the cage size of the fullerenes. The more extended is the π system, the higher is the PDT efficiency expected to be. The photodynamic activity of C70 fullerenes is higher than that of C60, due to the former's relatively larger cage. Thus, LC-19 and LC-20 utilized herein are highly water-soluble polycationically charged C₈₄O₂ fullerene derivatives with even more extended π -conjugate systems which have, for the first time, been shown to be effective PDT agents.

We found a wavelength-dependent relationship between the cellular phototoxicity and the presence of an electron donating deca-tertiary amine chain, as a covalent addend moiety to C₈₄O₂, which predisposed towards photoinduced radical ROS. We also found that the generation of hydroxyl radicals can be maximized by the application of short wavelength excitation in the presence of a combination of electron donating substituents and low concentrations of ascorbic acid. The latter is regarded as an effective reducing agent to the cationic tertiary-amine radical, produced upon the photoinduced intramolecular electron-transfer from the bis-hexa(aminoethyl)amidated malonate donor arm moiety to the fullerene cage.

The electron reduction of ascorbate allows the regeneration of neutral deca-tertiary ethyleneamine moieties and, thus, the continuation of photoinduced redox cycles that ultimately led to higher generation of hydroxyl radicals. C₈₄O₂ fullerene derivatives were demonstrated to be efficient photosensitizers that induced, at low concentrations, minimal dark toxicity and high generation of hydroxyl radicals when light stimulated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The structures of the PS

Decacationic C_{70} monoadduct **1 (LC-17)**, $C_{84}O_2$ monoadduct **2-I⁻/I₃⁻ (LC-19-I⁻/I₃⁻)**, C_{70} bisadduct **3 (LC-18)**, and $C_{84}O_2$ bisadduct **4 (LC-20)**.

Figure 2.

UV-vis absorption spectra of: decacationic C_{70} monoadduct **1** (**LC-17**) (A), $C_{84}O_2$ monoadduct **2-I⁻** (**LC-19-I⁻**) (B), **2-I₃⁻** (**LC-19-I₃⁻**) (C), C_{70} bisadduct **3** (**LC-18**) (D), and $C_{84}O_2$ bisadduct **4** (**LC-20**) (E), all solution in DMF with a concentration of 2.0×10^{-5} M, except 1.2×10^{-5} M for (C). The emission wavelengths of five light sources are shown for the correlation of fullerene absorptions.

Figure 3. Fluence-dependent killing curves of LC17 and LC18
Photokilling of HeLa cells by LC-17 and LC-18 (incubated at $4\ \mu\text{M}$ for 24 h) after irradiation with stated fluences of UVA (A) and red (B) wavelengths.

Figure 4. Fluence-dependent killing curves of LC-19- I_3^- and LC-20 and Caspase-3 activity
 Photokilling of HeLa cells by novel $C_{84}O_x[>M(C_3N_3^+C_3)_2]$ (LC-19- I_3^-) and $C_{84}O_2[>M(C_3N_6^+C_3)_2][>M(C_3N_6C_3)_2]$ (LC-20) derivatives (incubated at 4 μ M for 24 h) using a wider range of different wavelengths of light ((A) UVA, (B) blue, (C) green, (D) white, and (E) red). (F) shows linear correlation between the ratio of areas under the killing curves of LC20/ LC-19- I_3^- and the wavelength determined from Figs 4 A-E. (G) HeLa cells were incubated with LC-19- I_3^- or LC-20 for 24 h and treated with doses of UVA or red light to produce 50% cell killing. * $P < 0.05$, *** $P < 0.01$ LC19 vs LC20 (Student's t-test).

Figure 5. Fluence-dependent HPF fluorescence and Ascorbic acid concentration variation
 HPF fluorescence dependent on fluence with both LC-19-I_3^- and LC-20 under irradiation of either UVA (A) or red light (B) with or without ascorbic acid. HPF fluorescence dependent on ascorbic acid concentration with both LC-19-I_3^- and LC-20 and the illumination at wavelengths of UVA (C) and red light (D). In Fig 5C * $p < 0.05$ for ascorbate of 5 and/or 10 μM vs 1 and/or 2 μM ; # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ for ascorbate of 5 and/or 10 μM vs concentrations greater than 20 and/or 40 μM . This applies to both LC19 and LC20 .

Figure 6. Photomicrographs of HeLa cells

In each case blue is Hoechst 33342 to mark nuclei. (A–F) Studies with HPF (green) added for 30 min before light. HeLa cells were incubated with HPF alone (A & B), **LC-19-I₃⁻** for 24 h + HPF (C & D), **LC-20** for 24 h + HPF (E & F), and exposed to 4.0 J/cm² of UVA light. Scale bar = 400 μm. (G–I) Studies with Rho123 (green) showing blue/green overlap. HeLa cells were incubated with nothing (G), **LC-19-I₃⁻** for 24 h (H), or **LC-20** for 24 h (I), irradiated with 4.0 J/cm² of UVA light and Rho123 and Hoechst added immediately after. Scale bar = 100 μm. (J–O) Studies with AO (green and red), no Hoechst used. HeLa cells were incubated with nothing (J & K), **LC-19-I₃⁻** for 24 h (L & M), **LC-20** for 24 h (N & O), irradiated with 4.0 J/cm² of UVA light, and AO added immediately after. Scale bar = 100 μm.

Table 1

Relative numbers of photons absorbed from each light source by each compound (decacationic fullerene monoadducts and bisadducts).

	UVA	Blue	Green	White	Red
LC-17, 1	12.8	10.4	7.17	50.4	2.91
LC-19-I⁻, 2-I⁻	13.2	9.96	5.16	42.3	2.93
LC-19-I₃⁻, 2-I₃⁻	35.1	6.36	0.91	10.4	0.38
LC-18, 3	8.76	5.84	2.51	18.2	1.00
LC-20, 4	14.7	9.91	4.33	32.0	1.76

Values were based on data in Figure 2.