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Photoinduced Nitric Oxide Release from a Nitrobenzene Derivative in Mitochondria

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Abstract: We report a novel NO donor (RpNO), containing a 2,6-dimethylnitrobenzene moiety for photocontrollable NO release and a rhodamine moiety for targeting to mitochondria. Photorelease of NO from RpNO in aqueous solution was confirmed by means of ESR analysis. Cellular release of NO from RpNO was confirmed with the aid of DAF-FM DA, an NO-specific fluorescence probe. RpNO was colocalized with MitoTracker Green FM, a mitochondrial stain, in HCT116 colon cancer cells and exhibited photodependent cytotoxicity. Our results indicate that RpNO is an effective NO donor for time-controlled, mitochondria-specific NO treatment.

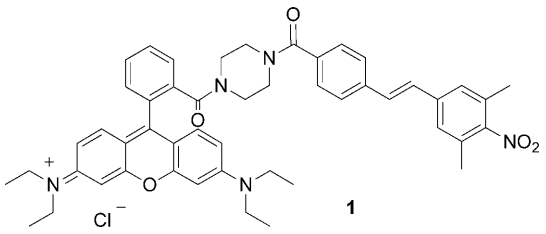
Keywords: caged compounds · cytotoxicity · mitochondria · NO donor · photolysis

Introduction

Nitric oxide (NO), although it exists as an unstable free radical under ambient conditions, is involved in the maintenance and regulation of many vital functions,[1] serving as, for example, a pleiotropic bioregulator of blood pressure, a neuromodulator, and a biodefense agent. Consequently, many NO donors have been developed and employed for biological studies.[2] However, most NO donors currently used, such as NOCs (secondary amine-derived diazenium diolates)[3] and NORs (α,β-unsaturated oxime derivatives),[4] release NO by spontaneous autolysis. Photocontrollable NO donors are far more attractive tools, allowing both spatial and temporal control of NO release.[5] Although sodium nitroprusside (SNP) is available as a photoinducible NO donor, it has the drawback of potential toxicity due to the cyanide ligands.[6]

We have previously reported 4-substituted 2,6-dimethylnitrobenzenes as a new type of NO donor.[7] Irradiation of 2,6-dimethylnitrobenzenes with extended π-electron systems at their 4-positions with ultraviolet A light results in efficient NO release as a result of rearrangement to arylnitrites. We have also demonstrated that our 2,6-dimethylnitrobenzene derivatives work as photocontrollable NO donors in HCT116 human colon cancer cells. Because concentrations and the biological influence of NO differ in different organelles, organelle-specific photocontrollable NO donors would be extremely useful.[9] Because the mitochondrion is a key organelle for apoptotic signal transduction, and because NO induces cellular apoptosis through mitochondrion-dependent pathways, mitochondria are a potential target for NO donors to initiate apoptotic cancer cell death.[10] Furthermore, after recent reports of mitochondrial nitric oxide synthase activity,[11] mitochondria-specific NO donors would also be useful for elucidation of NO functions in mitochondria.

With the aim of developing functional photoactivatable NO donors, we designed and synthesized RpNO (**1**), containing both a 2,6-dimethylnitrobenzene moiety for photocontrollable NO release and a rhodamine moiety for targeting to mitochondria. The rhodamine moiety effectively targets mitochondria as a result of its hydrophobic cationic character.[12]



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Here we show that our novel NO donor RpNO is distributed exclusively to the mitochondria, where it releases NO in response to UV-A irradiation. Its irradiation-dependent cytotoxicity towards HCT116 cells was also evaluated.

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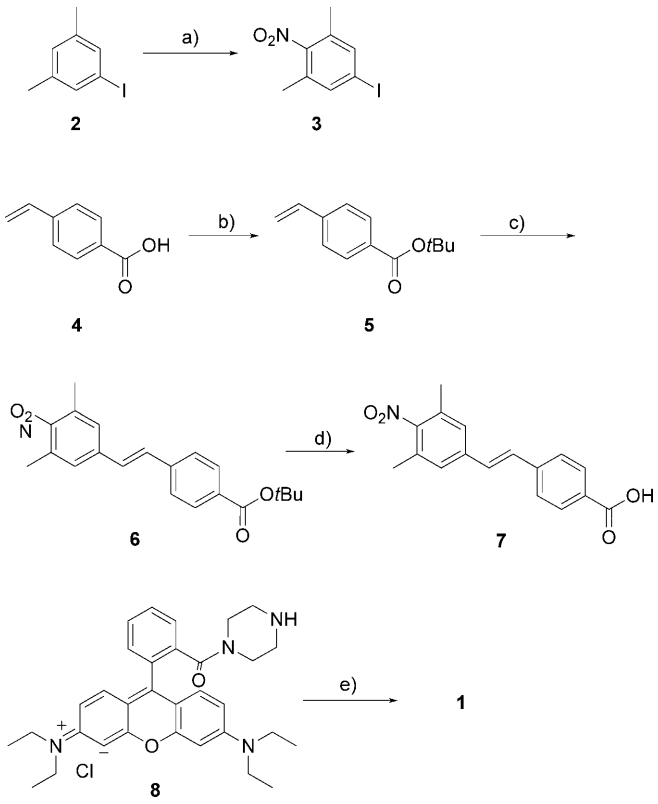
Results and Discussion

Molecular design and synthesis: We focused on use of the rhodamine component to achieve mitochondrial localization and of the 2,6-dimethylnitrobenzene unit as the NO-releasing moiety, and aimed to couple commercially available rhodamine B to the 2,6-dimethylnitrobenzene moiety at the 2′-carbonyl group (Scheme 1).[13] Formation of a tertiary amide bond with piperazine as a linker moiety prevents cyclization of RpNO into a nonfluorescent lactam form.[14] The NO donor moiety was thus connected to a conjugated π-electron system known to be photoresponsive in biological applications. RpNO was synthesized as shown in Scheme 1 and characterized by ^{1}H and ^{13}C NMR spectroscopy, HPLC, melting point measurement, and mass spectroscopy.



Scheme 1. Synthesis of RpNO (**1**). Reagents and conditions; a) nitric acid (70%), 100°C, 1 h, 49%; b) $(Boc)_2O$, DMAP, *t*BuOH, RT, 3 h, 75%; c) **3**, $Pd(OAc)_2$, PPh_3, Et_3N, $AgNO_3$, MeCN, 100°C, 3.5 h, 67%; d) TFA, $CHCl_3$, RT, 93%; e) **7**, $(COCl)_2$, DMF, CH_2Cl_2, RT, 5 h, 45%. Boc = *tert*-butoxycarbonyl, DMAP = 4-dimethylaminopyridine.

Release of NO from RpNO in vitro: NO release from RpNO was determined by an ESR spin-trapping method with an Fe-*N*-methyl-D-glucamine dithiocarbamate (MGD) complex, which is known to react with NO to give the stable paramagnetic complex [(MGD)-Fe^{2+}-NO]. In its ESR spectrum, the [(MGD)-Fe^{2+}-NO] complex gives a broadened three-line signal with $a^N = 1.25$ mT and $g^{iso} = 2.04$. When RpNO was photoirradiated in MilliQ water containing DMSO (7.5%) for 15 min in the presence of Fe-MGD, a three-line spectrum typical of [(MGD)-Fe^{2+}-NO] was observed by ESR spectroscopy, indicating that NO had been released from RpNO upon photoirradiation (Figure 1).

Figure 1. A representative ESR spectrum of a solution containing Fe-MGD and RpNO after photoirradiation (330–380 nm) with a 6% ND filter. Samples contained RpNO (750 μM), MGD (60 mM), and $FeSO_4$ (15 mM) in MilliQ water containing DMSO (7.5%). ESR spectra were recorded after photoirradiation for 15 min with a modulation width of 1.25 G and a microwave power of 10 mW.

From a calibration curve prepared with [(MGD)-Fe^{2+}-NO], the rate of NO release from RpNO was 6% upon 15 min photoirradiation with the light source (100 W mercury lamp) of a fluorescence microscope fitted with a WU filter (330–380 nm band-pass filter) and a 6% ND filter (see Figure S4 in the Supporting Information).

Confocal microscopy: For confocal microscopy, HCT116 cells were cultured in McCoy's 5 A culture medium. For the experiments, cells were incubated for 2 days and then washed with D-PBS (Dulbecco's PBS) and treated with RpNO (10 nM) for 5 min in the dark, followed by washing once with D-PBS. The cells were then stained for 15 min with MitoTracker Green FM, a well-established mitochondrial dye, and subjected to confocal fluorescence microscopy. RpNO was found to be localized in the mitochondria (Figure 2, and Figure S5 in the Supporting Information), as would be expected in view of the presence of the membrane-permeable and cationic rhodamine moiety, because of

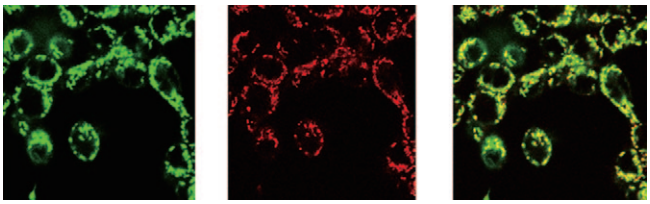


Figure 2. HCT116 human colon cancer cells were stained with MitoTracker Green FM and RpNO and observed under a confocal fluorescence microscope. The distribution of MitoTracker Green FM (left, green), the distribution of RpNO (center, red), and the merged image (right) of the same field are shown.

the highly inside-negative membrane potential across the inner mitochondrial membrane.

Intracellular release of NO from RpNO: Photorelease of NO from RpNO in HCT116 cells was confirmed with the aid of DAF-FM DA,[15] a cell-membrane-permeable NO-specific fluorescence probe. DAF-FM DA itself is a nonfluorescent compound, but is known to be hydrolyzed and to react with NO to form triazole DAF-FM T, which has strong green fluorescence. For confocal microscopy, the cells were plated and cultured for 2 days and were then treated with RpNO (100 μM) in the same manner as in the colocalization experiment. DAF-FM DA was loaded into the cells for 10 min and the cells then were irradiated with UV-A light for 5 min. Confocal microscopic observation revealed that the cells showed green fluorescence derived from DAF-FM T only when exposed to both RpNO and UV light. This suggested that RpNO released NO in the cells upon photoirradiation (Figure 3), or put another way, that RpNO acts as a photocontrollable NO donor (see also Figure S6 in the Supporting Information).

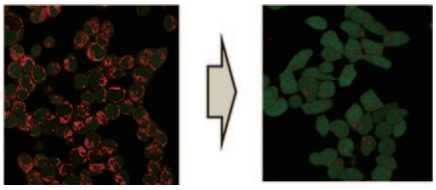


Figure 3. Fluorescence images of HCT116 human colon cancer cells loaded with RpNO and DAF-FM DA, left) before, and right) after photoirradiation (330–380 nm) with a 1.5% ND filter. Samples contained RpNO (100 μM) and DAF-FM DA (10 μM).

As shown in Figure 3, the cells were consistently stained with green fluorescence, even though it had been confirmed that RpNO was localized specifically in the mitochondria. This is probably because the diffusion rates of DAF-FM T or of NO itself are very fast, so that it is hard to identify the location of NO production in this experiment, even though RpNO is localized in mitochondria and so should release NO there.

Cytotoxicity towards cancer cells: It has been reported that NO mediates the cytotoxic action of macrophages towards tumor cells through inhibition of mitochondrial enzyme activity.[16] We had previously reported that nonlocalizing 2,6-dimethylnitrobenzene derivatives induced cytotoxicity in HCT116 cells through the release of NO under photoirradiation conditions.[7] In the presence of RpNO (10 μM), light in the wavelength range of 325–375 nm was applied for 5 min on a fixed area of a culture plate containing human colon cancer HCT116 cells. The cells were observed after incubation for 24 h at 37 °C in the dark. As expected, the cells within the irradiated area were detached and appeared dead (Figure 4 A). Treatment either with RpNO alone or with photoirradiation alone had no effect. This photoirradiation-dependent cytotoxicity was effectively diminished in the presence of β-NADH and P450 nor,[17] an NO reductase (Figure 4 B). This indicates that the cytotoxicity is due to NO release from RpNO under photoirradiation conditions (see also Figure S7 in the Supporting Information).

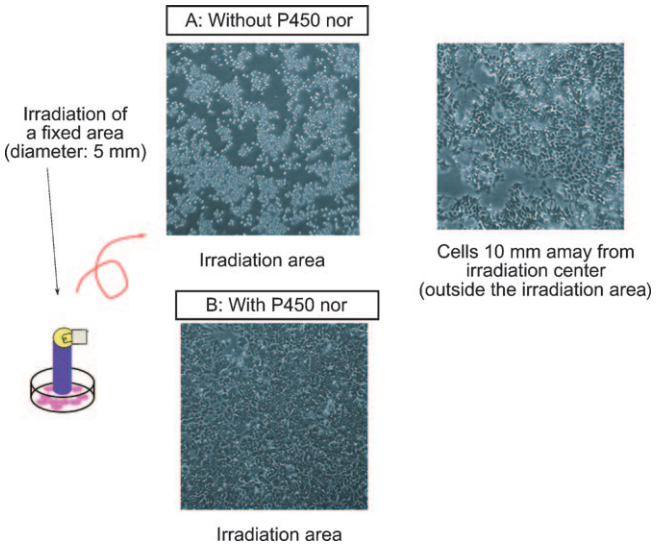


Figure 4. Cytotoxicity of NO released from RpNO under photoirradiation conditions. Representative phase-contrast images of cells within the irradiation area A) in the absence of P450 nor, or B) in the presence of P450 nor are shown.

Conclusion

We have demonstrated photoinduced NO generation in the mitochondria of living cells through the use of RpNO, a 2,6-dimethylnitrobenzene derivative linked to a rhodamine dye, which serves to drive localization of RpNO into mitochondria. This is the first example of a photocontrollable mitochondria-localizing NO donor. RpNO is expected be a useful tool for studies of the biological roles of NO.

Experimental Section

General methods: Melting points were determined with a Yanagimoto micromelting point apparatus. ^{1}H and ^{13}C NMR spectra were recorded with a JEOL JNM-LA500 or a JEOL JNM-A500 spectrometer in the indicated solvent. Chemical shifts (δ) are reported in parts per million relative to tetramethylsilane as internal standard. Elemental analyses were performed with a Yanaco CHN CORDER NT-5 analyzer, and all values were within ±0.4% of the calculated values. Fast atom bombardment (FAB) mass spectra were recorded with a JEOL JMS-SX102 A mass spectrometer. UV/Vis spectra were recorded with an Agilent 8453 spectrophotometer. MGD was obtained from Dojindo. All other reagents and solvents were purchased from Aldrich, Tokyo Kasei Kogyo, Wako Pure Chemical Industries, Nacalai Tesque, and Kanto Kagaku, and were used without purification. Flash column chromatography was performed with silica gel 60 (particle size 0.046–0.063 mm, Merck). Photoirradiation was performed with use either of the light source of an Olympus BX-60 fluorescence microscope or of an Asahi Spectra MAX-302 irradiating apparatus.

Synthesis

Compound 3: 1-Iodo-3,5-dimethylbenzene (**2**, 8.04 g, 34.65 mmol) was added at 0 °C to a solution of HNO_3 (70%, 25 mL). The mixture was stirred for 1 hour at 100 °C and was then poured onto ice, neutralized with saturated $NaHCO_3$ solution, and extracted with Et_2O. The Et_2O layer was washed with brine and dried over Na_2SO_4. Filtration and concentration afforded a residue, which was subjected to silica gel flash column chromatography (toluene/*n*-hexane 1:20) to give **3** (4.60 g, 16.66 mmol, 48.1%) as a pale yellow solid: ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 7.51 (s, 2H), 2.27 ppm (s, 6H).

Compound 5: $(Boc)_2O$ (33.15 g, 152.08 mmol, 1.5 equiv) and DMAP (24.78 g, 202.77 mmol, 2.0 equiv) were added to a solution of 4-ethenylbenzoic acid (**4**, 15.01 g, 101.39 mmol) in *tert*-butanol (500 mL). The mixture was stirred for 3 h at room temperature and was then poured into water and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with brine and dried over Na_2SO_4. Filtration and evaporation afforded a residue, which was subjected to silica gel flash column chromatography (AcOEt/*n*-hexane 1:10) to give **5** (15.68 g, 76.85 mmol, 75.8%) as a colorless oil: ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 7.94 (d, J = 8.2 Hz, 2H), 7.44 (d, J = 8.2 Hz, 2H), 6.75 (dd, J = 20.5 Hz, 11.0 Hz, 1H), 5.85 (d, J = 17.7 Hz, 1H), 5.36 (d, J = 10.7 Hz, 1H), 1.59 ppm (s, 9H).

Compound 6: A mixture of $Pd(OAc)_2$ (248.19 mg, 1.11 mmol, 0.10 equiv), PPh_3 (608.36 mg, 2.32 mmol, 0.21 equiv), Et_3N (1.54 mL, 11.12 mmol, 1.00 equiv), $AgNO_3$ (2190.00 mg, 13.04 mmol, 1.17 equiv), compound **3** (3067.9 mg, 11.12 mmol), and compound **5** (2712.0 mg, 13.29 mmol, 1.20 equiv) in acetonitrile (150 mL) was stirred under Ar at 100 °C for 3.5 h. The mixture was filtered and the filtrate was poured into water. After extraction with $CHCl_3$, the $CHCl_3$ layer was washed with brine and dried over Na_2SO_4. Filtration and evaporation afforded a residue, which was subjected to silica gel flash column chromatography (toluene/*n*-hexane 1:1→toluene/*n*-hexane/AcOEt 10/10/1) to give **6** (2.60 g, 7.37 mmol, 66.3%) as a yellow solid: ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 7.99 (d, J = 8.5 Hz, 2H), 7.54 (d, J = 8.2 Hz, 2H), 7.27 (s, 2H), 7.16 (d, J = 16.4 Hz, 1H), 7.10 (d, J = 16.1 Hz, 1H), 2.35 (s, 6H), 1.61 ppm (s, 9H).

Compound 7: TFA (5 mL) was added to a suspension of **6** (1355.4 mg, 3.84 mmol) in $CHCl_3$ (5 mL). The mixture was stirred for 6.5 h at room temperature and then concentrated in vacuo, and the residue was suspended in *n*-hexane. Filtration gave **7** (1033.3 mg, 3.48 mmol, 90.6%) as a yellow powder: ^{1}H NMR (DMSO, 500 MHz): δ = 7.96 (d, J = 8.2 Hz, 2H), 7.74 (d, J = 8.2 Hz, 2H), 7.57 (s, 2H), 7.47 (d, J = 16.4 Hz, 1H), 7.39 (d, J = 16.4 Hz, 1H), 2.30 ppm (s, 6H).

Compound 1 (RpNO): DMF (catalytic amount) was added to a solution of **7** (50 mg, 0.17 mmol) and ethanedioyl dichloride (40 μL, 0.47 mmol, 2.8 equiv) in CH_2Cl_2 (4 mL). The mixture was stirred at room temperature for 1.5 h. Evaporation of the solvent in vacuo gave a yellow solid. A solution of the yellow solid in CH_2Cl_2 (3 mL) was added at 0 °C to a solution of the rhodamine derivative **8**[13] (137 mg, 0.25 mmol, 1.5 equiv) in CH_2Cl_2 (3 mL) and Et_3N (1.5 mL). The mixture was stirred at room temperature for 5 h, and solvents were removed in vacuo. Purification of the residue by silica gel flash chromatography (MeOH/$CHCl_3$ 1:15 to 1:10) and recrystallization from MeOH/Et_2O gave **1** (RpNO, 62.3 mg, 45.3%) as a dark red solid: m.p. 185–187 °C; ^{1}H NMR (CD_3OD, 500 MHz): δ = 7.79–7.30 (m, 3H), 7.65 (d, J = 5.0 Hz, 2H), 7.52 (m, 1H), 7.42 (s, 2H), 7.38 (d, J = 8.5 Hz, 2H), 7.26 (m, 4H), 7.18 (d, J = 5.0 Hz, 2H), 6.97 (s, 2H), 3.69 (q, J = 7.1 Hz, 8H,), 3.44–3.07 (m, 8H), 2.30 (s, 6H), 1.31 ppm (t, J = 7.5 Hz, 12H); ^{13}C NMR (DMSO, 500 MHz): δ = 162.7, 160.1, 149.8, 147.7, 147.4, 130.9, 130.8, 126.9, 123.7, 122.7, 121.8, 121.7, 120.1, 119.5, 118.6, 118.5, 105.9, 105.4, 87.9, 37.4, 30.6, 16.9 ppm; ESI-MS 790 $[M+H-Cl]^+$; HPLC purity; 97.004%.

Confocal microscopy: HCT116 human colon cancer cells were purchased from American Type Culture Collection (ATCC) and cultured in McCoy's 5 A culture medium containing penicillin and streptomycin and supplemented with fetal bovine serum according to the manufacturer's instructions. The cells were maintained at 37 °C in a humidified incubator (CO_2, 5%, v/v) under subconfluent conditions. For the experiments, the cells were plated on 3 cm glass dishes at 1.0×10^5 cells per dish with the culture medium (2 mL). The cells were incubated at 37 °C in a humidified incubator (CO_2, 5%, v/v) for 2 days. The dishes were then washed with Dulbecco's PBS (D-PBS) and the medium was replaced with D-PBS (1 mL). The cells were treated with RpNO (10 nM) for 5 min in the dark, washed once with D-PBS, stained with MitoTracker Green FM (100 nM) according to the manufacturer's instructions, and subjected to confocal fluorescence microscopy without washing.

Photoirradiation: For the ESR experiment, a quartz flat cuvette containing RpNO and a NO trap was irradiated by use of a conventional fluorescence microscope (Olympus BX-60, 100 W mercury lamp) with a WU filter (330–380 nm band-pass filter, 3.84 mW cm^{-2}). For detection of NO with DAF-FM DA, cells were irradiated with same microscope light source as in the ESR experiment. For photoirradiation experiments, the cells were placed at a distance of 10 cm from a lens attached to a MAX-302 light source (Asahi Spectra, Tokyo) with a xenon lamp (300 W) and irradiated with UV-A light (325–375 nm) through a 50% ND filter for the time indicated (15.1 mW cm^{-2}).

ESR analysis: The Fe^{2+} complex of MGD (Fe-MGD) was used to trap NO. A fresh solution of Fe-MGD (1:4) was prepared by addition of ferrous ammonium sulfate to a MilliQ water solution of MGD. A sample containing RpNO (750 μM) and Fe-MGD (15 mM) in MilliQ water (7.5% DMSO) was introduced into a quartz flat cuvette. Photoirradiation was carried out for 15 min with the light source (100 W mercury lamp) of a fluorescence microscope fitted with a WU filter (330–380 nm band-pass filter) and a 6% ND filter, and ESR spectra were recorded with a JES-RE 2X spectrometer (JEOL Co. Ltd., Tokyo, Japan). For the calibration curve, standard solutions containing designated amounts of Fe-MGD (12.5 μM to 400 μM) were treated with NOC7 (excess) in MilliQ water (7.5% DMSO). The ESR signals of standard solutions were recorded with an ESR spectrometer in the same manner, and the peak areas of the NO complex were plotted against the concentration of [(MGD)-Fe^{2+}-NO] (see the Supporting Information). The spectrometer settings were as follows: modulation frequency 100 kHz, modulation amplitude 2.0 G, scan time 4 min, microwave power 16 mW, and microwave frequency 9.42 GHz.

Detection of NO with DAF-FM DA: HCT116 human colon cancer cells were treated in the same manner as in the confocal microscopy experiments. Briefly, the cells were plated on 3 cm glass-bottomed dishes at 1.0×10^5 cells per dish with McCoy's 5 A culture medium (2 mL) and were then incubated at 37 °C in a humidified incubator (CO_2, 5%, v/v) for 2 days. The dishes were washed with D-PBS and the culture medium was replaced with D-PBS (1 mL). The cells were treated with DAF-FM DA (10 μM) for 15 min in the dark and were then washed twice with D-PBS. The cells were subsequently treated with RpNO (100 μM) for 15 min in the dark, washed twice with D-PBS, and subjected to photoirradiation for 5 min with the light source (100 W mercury lamp) of a fluorescence microscope fitted with a WU filter (330–380 nm band-pass filter) and a 1.5% ND filter. After irradiation, the cells were subjected to confocal fluorescence microscopy (LSM 510, Carl Zeiss Japan Co. Ltd., Tokyo, Japan).

Cytotoxicity to HCT116 cells: HCT116 human colon cancer cells were treated in the same manner as in the confocal microscopy experiments, with slight modifications. In brief, the cells were plated on 6 cm culture dishes at 2.5×10^5 cells per dish with McCoy's 5 A culture medium (5 mL) and incubated at 37 °C in a humidified incubator (CO_2, 5%, v/v) for 1 day. On the day of the experiment, the culture medium was replaced and its volume was reduced to 2 mL per 6 cm culture dish. The cells were treated with RpNO in DMSO (20 μL) to give a final concentration of 10 μM, which resulted in a final DMSO concentration of less than 1% (v/v) in the culture media; this concentration of DMSO alone had no apparent effect on the appearance of the cells. The cells were also treated with RpNO in the same manner as described above, but in the presence of P450 nor (200 nM, Wako Pure Chemical Industry Co. Ltd, Osaka, Japan) and β-NADH (10 μM) for scavenging. The treated cells were photoirradiated for 5 min with the light source (xenon lamp) of an irradiating apparatus (Asahi Spectra MAX-302) fitted with a UV filter (325–375 nm band-pass filter). The irradiation was limited to a circular area of 5 mm diameter. After 24 h incubation, the cells were observed under an invert-

ed-phase contrast microscope and images were taken with a digital camera and processed on a personal computer.

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