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A novel mitochondria-localizing nitrobenzene derivative as a donor for photo-uncaging of nitric oxide

Taeko Horinouchi [a], Hidehiko Nakagawa [a,c,*], Takayoshi Suzuki [a], Kiyoshi Fukuhara [b], Naoki Miyata [a,b,*]

[a] Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467-8603, Japan
[b] Organic Chemistry National Institute of Health Sciences, Setagaya, Tokyo 158-8501, Japan
[c] PRESTO, Japan Science and Technology Agency (JST), 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan

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ABSTRACT

We report a novel green-fluorescent NO donor, NBDNO, bearing a 2,6-dimethylnitrobenzene moiety for photocontrollable NO release and a triphenylphosphonium moiety for targeting to mitochondria. Photorelease of NO from NBDNO was confirmed by means of ESR analysis in aqueous solution. Intracellular release of NO from NBDNO was confirmed by using DAR-4M AM, an NO-specific fluorescence probe. NBDNO was colocalized with MitoRed, a mitochondrial stain, in HCT116 colon cancer cells. Our results indicate that NBDNO is an effective NO donor for time-controlled, mitochondria-specific NO treatment.

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] For example, it serves as a pleiotropic bioregulator of blood pressure, a neuromodulator, and a biodefence agent. Consequently, many NO donors have been developed and employed for biological studies.[2] Most NO donors currently used, such as NOCs[3] and NORs,[4] release NO by spontaneous autolysis. NO donors that permit spatially and temporally controllable NO release are expected to be useful for a variety of biological studies.

Photoirradiation on/off systems for temporal control of NO release are easy to use and serve to minimize the effect of irradiation on living cells.[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] Ultraviolet A (UV-A) irradiation of 2,6-dimethylnitrobenzenes having an extended π-electron system at the 4-position results in efficient NO release as a result of rearrangement to arylnitrite.

We have also demonstrated that our 2,6-dimethylnitrobenzene derivatives work as photocontrollable NO donors in HCT116 human colon cancer cells. Because the concentration and biological influence of NO differ among organelles, organelle-specific photocontrollable NO donors would be extremely useful.[8] Since the mitochondrion is a key organelle for apoptotic signal transduction, and NO induces cellular apoptosis via mitochondria-dependent pathways, mitochondria are a potential target for NO donors to initiate apoptotic cancer cell death.[9] Furthermore, it was recently reported that mitochondrial fission is induced by S-nitrosylated Drp1 protein,[10] and this mechanism may be involved in Altzheimer disease.[11] Mitochondria are also known to have nitric oxide synthase activity.[12] Therefore, mitochondria-specific NO donors would be useful to elucidate NO functions in mitochondria.

With the aim of developing functional photoactivatable NO donors, we designed and synthesized **NBDNO** (Fig. 1), bearing a 2,6-dimethylnitrobenzene moiety for photocontrollable NO release, a triphenylphosphonium[13] moiety for targeting to mitochondria and a fluorescent 7-nitrobenz-2-oxa-1,3-diazole[14] moiety for visu-

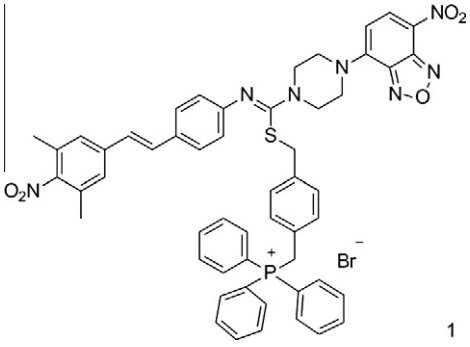

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Figure 1. Chemical structure of **NBDNO** (**1**)

* Corresponding authors. Tel./fax: +81 52 836 3407 (H.N.).
E-mail address: deco@phar.nagoya-cu.ac.jp (H. Nakagawa).

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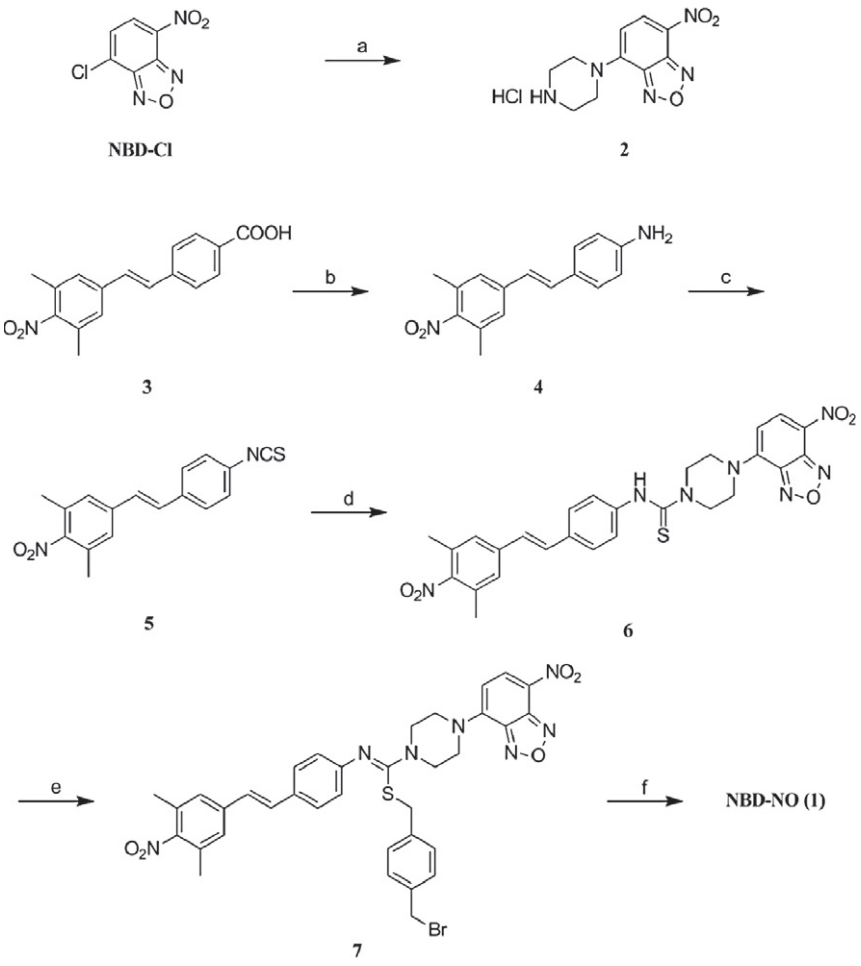
alizing the compound by means of microscopy. The triphenylphosphonium moiety is expected to target mitochondria effectively due to its hydrophobic cationic character. Furthermore, an NO donor bearing a NBD green fluorescent moiety is expected to be useful in multi-color labeling experiments, because many mitochondria-localizing compounds have red fluorescence. Here, we show that our novel NO donor, **NBDNO**, is distributed exclusively to mitochondria, where it releases NO in response to UV-A irradiation.

Molecular Design and Synthesis: We focused on the triphenylphosphonium moiety to achieve mitochondrial localization and the 2,6-dimethylnitrobenzene group as the NO-releasing moiety. With the use of piperazine as a linker moiety, **NBDNO** is structurally fixed in an extended form, which reduces of the possibility of intramolecular interaction between the NBD moiety and NO donor moiety. The NO donor moiety was thus connected to a conjugated π-electron system, which is known to be photoresponsive in biological applications. **NBDNO** was synthesized as shown in Scheme 1 and characterized by ^{1}H NMR, ^{13}C NMR, HPLC, melting point measurement, and mass spectroscopy.

*Release of NO from **NBDNO** in vitro*: NO release from **NBDNO** was determined by means of an ESR spin-trapping method with Fe–*N*-methyl-D-glucamine dithiocarbamate (Fe–MGD) complex, which is known to react with NO to give a stable paramagnetic complex [(MGD)–Fe^{2+}–NO]. On ESR spectroscopy, the [(MGD)–Fe^{2+}–NO] complex gives a broadened three-line spectrum with a^{N} = 1.25 mT and g^{iso} = 2.04. When **NBDNO** was photoirradiated in MilliQ water containing 7.5% DMSO for 15 min in the presence of Fe–MGD, a typical three-line spectrum of [(MGD)–Fe^{2+}–NO] was observed in ESR spectroscopy, indicating that NO was released from **NBDNO** upon photoirradiation (Fig. 2). From a calibration curve obtained by using separately prepared [(MGD)–Fe^{2+}–NO], the rate of NO release from **NBDNO** was found to be 10.8% for 15 min photoirradiation with the light-source (xenon lamp) of an irradiating apparatus equipped with a UV filter (325–385 nm band-pass filter) through a 50% ND filter (see Fig. S2 in Supplementary data).

Confocal microscopy: For confocal microscopy, HCT116 cells were cultured in McCoy's 5A culture medium. For the experiments, cells were incubated for 2 days and then washed with D-PBS and treated with 10 μM **NBDNO** for 30 min in the dark, followed by washing once with D-PBS. The cells were then stained for 10 min with MitoRed®, a well-established mitochondrial dye, and subjected to confocal fluorescence microscopy. **NBDNO** was found to be localized to mitochondria (Fig. 3), as expected from the presence of the membrane-permeable and cationic triphenylphosphonium moiety, because of the highly inside-negative membrane potential across the inner mitochondrial membrane.

*Intracellular release of NO from **NBDNO**:* Photorelease of NO from **NBDNO** in HCT116 cells was confirmed by using DAR-4M AM[15], a cell-membrane-permeable NO-specific fluorescence probe. Rhodamine-based DAR-4M AM, which itself is nonfluorescent, is hydrolyzed intracellularly and the hydrolysis product reacts with NO to form triazole DAR-4M T, which has strong red fluorescence in



Scheme 1. Synthesis of **NBDNO**. Reagents and conditions: (a) Boc-piperazine, Cs_2CO_3, $CHCl_3$, CH_3CN; 4 N HCl/dioxane, $CHCl_3$, quant; (b) DPPA, Et_3N, *t*-BuOH, reflux 52.5%; 4 N HCl/dioxane, $CHCl_3$; $NaHCO_3$ aq 64.0%; (c) $SCCl_2$, Et_3N, CH_2Cl_2, 77.3%; (d) Compound **2**, Et_3N, THF, DMF, 91.7%; (e) α,α′-dibromoxylene, $NaHCO_3$, DMF, 57.5% (f) PPh_3, acetone, CH_2Cl_2, 70.5%.

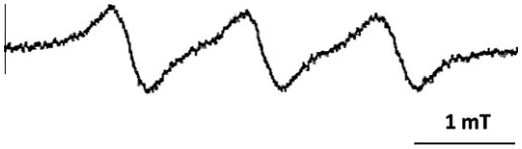


Figure 2. A representative ESR spectrum of a solution containing Fe–MGD and **NBDNO** after photoirradiation (325–385 nm) via a 50% ND filter. Samples contained 750 μM **NBDNO**, 60 mM MGD, and 15 mM $FeSO_4$ in MilliQ water containing 7.5% DMSO. ESR spectra were recorded after photoirradiation for 15 min with a modulation width of 1.25 G and a microwave power of 10 mW.

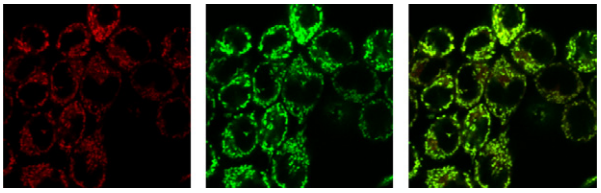


Figure 3. HCT116 human colon cancer cells were stained with MitoRed® and **NBDNO**, and observed with a confocal fluorescence microscope. The distribution of MitoRed® (left, red), the distribution of **NBDNO** (center, green), and the merged image (right) of the same field are shown.

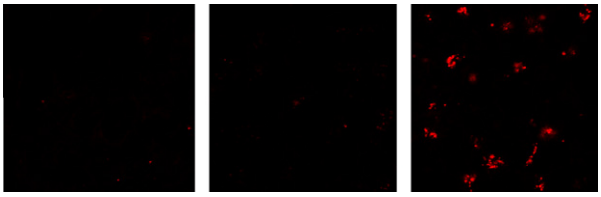


Figure 4. HCT116 human colon cancer cells were treated with 10 μM DAR-4M AM and then photoirradiated (left), treated with 10 μM **NBDNO** without irradiation (center) or treated with 10 μM DAR-4M AM and 10 μM **NBDNO** with photoirradiation (right), then subjected to confocal fluorescence microscopy.

mitochondria. For confocal microscopy, the cells were plated and cultured for 2 days, and then treated with 10 μM **NBDNO** in the same manner as in the colocalization experiment. DAR-4M AM was loaded into the cells for 10 min and then the cells were irradiated with UV-A light for 5 min with the same irradiating apparatus and conditions as used for the ESR experiment. Confocal microscopic observation revealed that the cells showed red fluorescence derived from DAR-4M T only when exposed to both **NBDNO** and UV light. This suggested that **NBDNO** released NO in the cells upon photoirradiation (Fig. 4), that is, it indeed serves as a photocontrollable NO donor.

We have demonstrated specific photoinduced NO generation in mitochondria of living cells from **NBDNO**, a 2,6-dimethylnitrobenzene derivative bearing both a triphenylphosphonium moiety, which serves to drive the localization of **NBDNO** to mitochondria, and green-fluorescent NBD dye. **NBDNO** is expected be a useful tool for studies on the biological roles of NO.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.02.027.

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