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Novel mitochondria-localizing TEMPO derivative for measurement of cellular oxidative stress in mitochondria

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Abstract—Neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, and apoptosis, are thought to be associated with oxidative stress affecting mitochondria. In this study, we designed and synthesized a fluorescein-tagged TEMPO derivative, compound **1**, with triphenylphosphino moiety. Synthesized **1** localized in mitochondria and detected oxidative stress in an endotoxic model of a mouse macrophage-like cell line. Compound **1** is therefore a potentially useful probe for evaluating oxidative stress in mitochondria.

Oxygen taken into our bodies is used to produce energy, and about 1% of oxygen is transformed into reactive oxygen species (ROS). ROS are considered to play important roles; for instance, they serve as protective factors in inflammation, and they work as neuromodulators.[1] It is known that a variety of functions of cellular components, such as lipids, proteins, sugars, and DNA, suffer from oxidative stress when ROS are produced in excess.[2] Superoxide can be produced by electron transfer in mitochondria, and thus oxidative damage may accumulate more rapidly in mitochondria.[3] Oxidative stress affecting mitochondria is considered to be closely related to neurodegenerative disorders,[4] such as Alzheimer's[5] and Parkinson's diseases,[6] and apoptosis.[7]

However, there have been only a few attempts to measure the oxidative stress induced by ROS in specific cellular regions.[8] ROS can be measured indirectly by means of their reaction with stable radical compounds in the cell, through which radical probes are readily reduced to non-radical species.[9]

Among these radical species, 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO) can be reduced to 2,2,6,6-tetramethylpiperidin-1-ol under physiological conditions, that is, TEMPO converts to the non-radical form by reduction.[10] When ROS are upregulated and cells are in a relatively oxidative environment, cellular reduction will be downregulated. Electron spin resonance (ESR) measurement is a useful approach to detect radical species in biological systems. Using TEMPOL (4-hydroxyl-2,2,6,6-tetramethylpiperidin-1-oxyl), a useful TEMPO derivative, oxidative stress can be measured by ESR spectrometry. TEMPOL is easily introduced into cells, but, due to its amphiphilic nature, can easily exit cells as well.[11] TEMPO derivatives, which localize to a particular cellular region, would be useful for measuring regional oxidative stress, such as stress at mitochondria. A TEMPO derivative localizing to mitochondria would be advantageous.

For this purpose, the TEMPO derivative requires a radical moiety for ESR detection, a fluorescent group for identifying its cellular distribution, and a functional group for localizing to a particular region. We have already developed such probes localizing to the cell membrane and succeeded in evaluating oxidative stress at the cell membrane.[12]

In this study, we designed a TEMPO derivative (Fig. 1), that has a cationic triphenylphosphonium moiety for localization to the mitochondria,[13] nitroxyl radical moiety for measuring ESR, and fluorescein moiety for confirming distribution in cells. Compound **1** was synthesized as shown in Scheme 1, and we demonstrated that this radical probe was able to detect oxidative stress in mitochondria in an endotoxic model of a mouse macrophage-like cell line.

Keywords: Fluorescein; Redox; Electron spin resonance; Reactive oxygen species; Superoxide; Inflammation.
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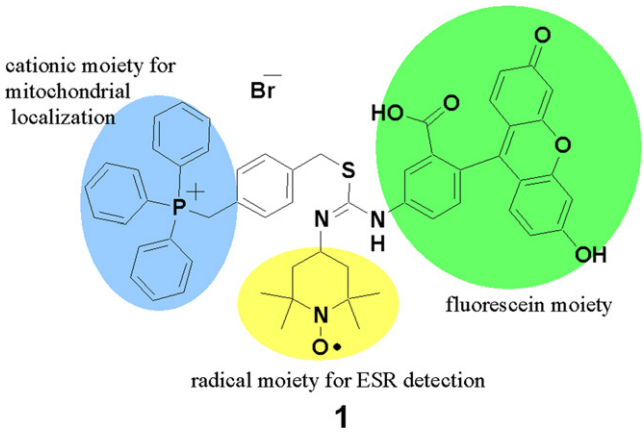


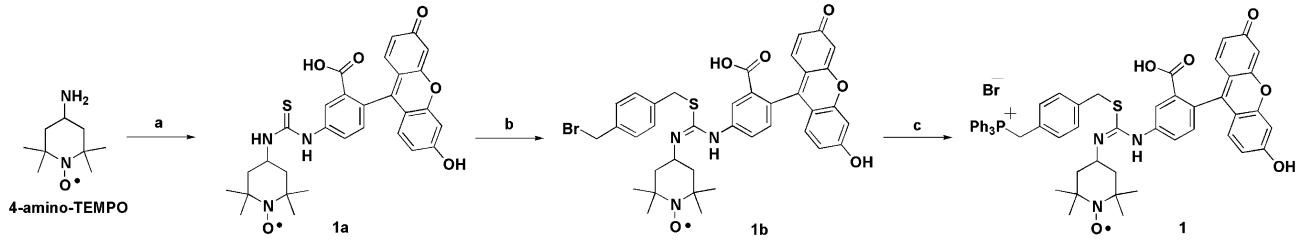
Figure 1. Structure of a TEMPO derivative (1) designed to localize to mitochondria.

Mouse RAW264.7 cells were cultured in DMEM culture medium containing penicillin and streptomycin, supplemented with fetal bovine serum. For the experiments, the cells were plated onto 10-cm culture dishes at 1.5×10^7 cells/dish with 15 mL of DMEM culture medium. The cells were incubated at 37 °C in a humidified 5% (v/v) CO_2 incubator for 2 days. Then, the culture medium was replaced with 5 mL of serum-free DMEM, and the cells were treated with LPS (*Escherichia coli*, 0.5 μg/mL) and IFN-γ (human recombinant, 150 U/mL). The treated cells were subsequently cultured for 5 h, scraped into 2 mL of Dulbecco's PBS (D-PBS), and washed with D-PBS. Then the cells were treated with 50 μM of **1** for 15 min under dark conditions, followed by washing 3 times with D-PBS. The cell suspension (1 mL) was used for the ESR experiments. There was no significant difference in cell number between non-treated- and LPS/IFN-γ-treated cell suspension (supporting information).

Each suspension of the treated cells was placed in a flat quartz cuvette. ESR measurements were started 10 min after treatment with **1**. The ESR signal was recorded at 5-min intervals. The signal intensity of TEMPO derivative (I) was calculated from the second integral of the center peak of the signal trace and expressed as a ratio (I/I_0) by comparing it to the intensity of the standard Mn^{2+} signal (I_0). The signal decay rate ($-k$) was calculated as the pseudo first order rate of the decrease in the ratio (I/I_0). For confocal microscopy, the cells were plated on a 3-cm glass-bottomed culture dish at 1.5×10^5 cells/dish with 1.5 mL of DMEM culture medium and incubated at 37 °C in a humidified 5% (v/v) CO_2 incubator for 2 days. The cells were treated with **1** in the same manner as in the ESR experiments without detachment. The cells were subsequently stained with MitoRed, which is known as a rhodamine-based well-established mitochondria dye, for 10 min and subjected to confocal fluorescence microscopy. The confocal microscopic study of the RAW264.7 cells treated with compound **1** indicated that **1** was localized to mitochondria as expected (Fig. 2). Its membrane-permeable cationic moiety probably contributed to the localization to mitochondria because mitochondria are negatively charged compared with cytosol. The ESR signal of **1** was measured in RAW264.7 cells to evaluate oxidative stress in mitochondria (Figs. 3 and 4). In the control cells, the signal intensity of **1** was gradually decreased at $0.0080 \pm 0.0004\ min^{-1}$ under our conditions. The upregulation of oxidative stress was evaluated after endotoxic stimulation (Fig. 4a). ESR spectra of **1** were measured in RAW264.7 cells, which had been treated with 500 ng/mL LPS and 150 U/mL IFN-γ for 5 h. The rate of signal decay observed in cells treated with LPS/IFN-γ was decreased to $0.0063 \pm 0.0009\ min^{-1}$. The decreased rate as a result of the LPS/IFN-γ treatment was restored to $0.0077 \pm 0.0007\ min^{-1}$ in the presence of 100 U/mL SOD and 10 U/mL catalase during measurement (Fig. 4b).

In the absence of the RAW264.7 cells, the signal failed to decay (data not shown). Since cells generally exist in a reductive environment, compound **1** was found to be gradually reduced to the non-radical species in the presence of the control cells.

Treatment with LPS/IFN-γ is known to activate the cells and to increase the production of reactive oxygen and nitrogen species (ROS/RNS). In this study, LPS/IFN-γ treatment decreased the decay rate of nitroxyl radical. The decrease in the rate was recovered in the presence of 100 U/mL SOD and 10 U/mL catalase. Although ROS were still upregulated by LPS/IFN-γ treatment, they were considered to be at least partially scavenged by SOD and catalase. These two enzymes seem to contribute to reduce ROS around the mitochondria by scavenging around cells, although these enzymes may be incapable of approach to mitochondria. LPS/IFN-γ treatment is known to increase ROS production by activating NADPH oxidase on endosomes. One possibility is that SOD and catalase may scavenge ROS around the cell membrane and reduce total ROS, so that the population of ROS diffusing to mitochondria may be



Scheme 1. Synthesis of compound **1**. Reagents and conditions: (a) fluorescein-5-isothiocyanate, THF, 87%; (b) α,α′-dibromo-*p*-xylene, $NaHCO_3$, DMF, 44%; (c) PPh_3, AcOEt, 94%.

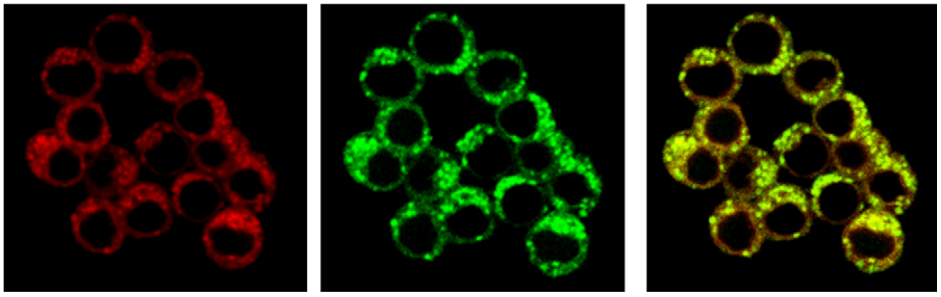


Figure 2. RAW264.7 cells were stained simultaneously with MitoRed and compound **1**, and observed by confocal fluorescence microscopy. Distribution of MitoRed (left, red), distribution of compound **1** (center, green), and merged image (right) at same field are shown.

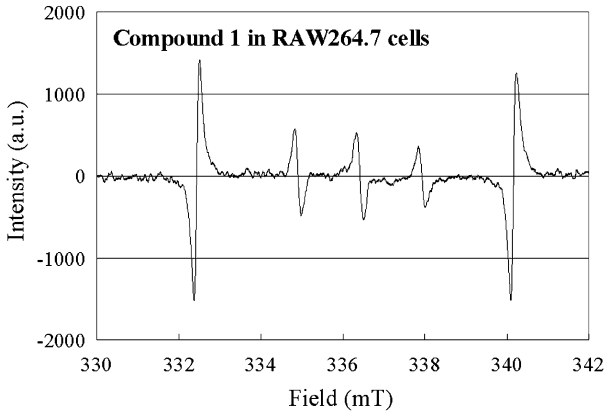


Figure 3. ESR spectra were recorded with a JES-RE 2X spectrometer (JEOL Co. Ltd., Tokyo, Japan). The settings used were as follows: microwave power, 10 mW; frequency, 9.42 GHz; field, 336.5 mT; sweep width, 7.5 mT; sweep time, 1 min; modulation width, 0.063 mT; gain, 2500; and time constant, 0.03. The signal intensity of TEMPO derivative (I) was calculated from the second integral of the signal trace and expressed as a ratio (I/I_0) by comparing it to the intensity of the standard Mn^{2+} signal (I_0).

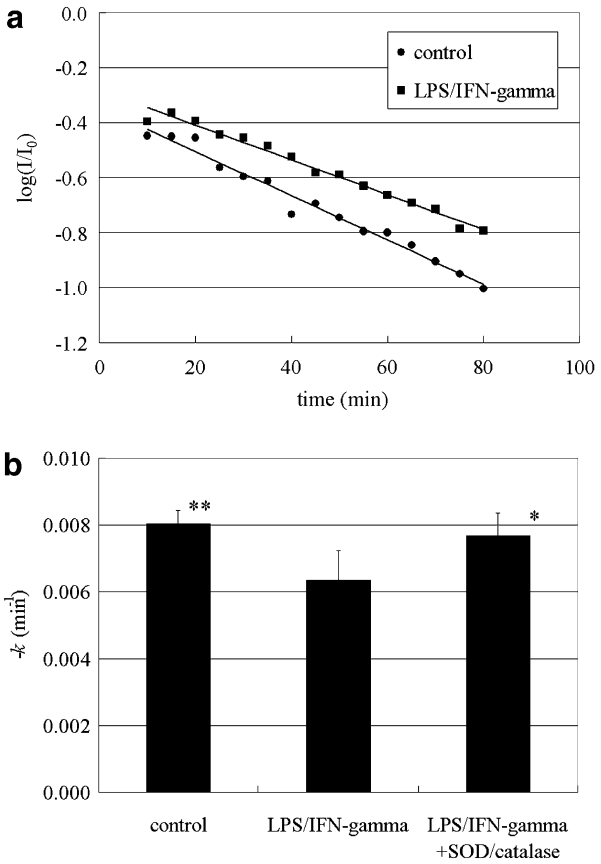


Figure 4. Signal decay rate of compound **1** in RAW264.7 cells. (a) The time course of the relative signal intensity measured at 5-min intervals, control cells (•), LPS/IFN-γ-treated cells (■). I, compound **1** peak area; I_0, Mn^{2+} external standard peak area. (b) Signal decay rate of **1** in RAW264.7 cells. The signal decay rates of **1** with RAW264.7 cells were calculated from ESR signal intensities of **1** in RAW264.7 cells treated with vehicle, or with LPS/IFN-γ, in the presence or absence of SOD/catalase. Values are presented as means ± SD of 4–7 experiments. ANOVA and Bonferroni-type multiple t test indicated significant differences between LPS/IFN-γ and the control ($^{**}P < 0.01$), and LPS/IFN-γ + SOD/catalase ($^{*}P < 0.05$).

also decreased. The change in this rate after LPS/IFN-γ-treatment was assumed to be due to either a decrease in the cellular reductants by ROS upregulation or an increase in the oxidation of hydroxylamine, a reduced form of **1**.[14] The TEMPO moiety can be oxidized to the ESR-silent oxonium cation formed by superoxide.[15] However, this cation was known to be rapidly reduced back to TEMPO by superoxide itself.[15] Compound **1** might be considered to be repeatedly oxidized and reduced in mitochondria. The direct oxidation of TEMPO itself by superoxide probably does not affect the signal decay rate under the conditions used for these measurements.

Cells generally exist in a reductive environment, and radical compounds are reduced to non-radical species by intracellular reductants such as glutathione. Reductant concentration is considered to be kept at a constant value ($[Red]_{const}$) in a living cell (Eq. 1) by homeostasis.

When treated with LPS/IFN-γ, ROS/RNS are upregulated, and reductants are consumed to reduce ROS/RNS in a cell, so that the intracellular reductant concentration is considered to be shifted to a smaller constant value ($[Red]'_{const}$), at which reductant consumption and regeneration are balanced. Therefore, the reducing rate of a TEMPO derivative would be decreased as shown in Eqs. 2 and 3. Here, [T·] is for TEMPO radical concentration, [Red] is for reductant concentration, k and k_{obs} are for rate constant and observed pseudo first order rate constant, respectively.

In untreated cells

$$-\mathrm{d}[\mathrm{T}\cdot]/\mathrm{d}t = k[\mathrm{Red}]_{\mathrm{const}}[\mathrm{T}\cdot] = k_{\mathrm{obs}}[\mathrm{T}\cdot]. \quad (1)$$

In treated cells

$$-\mathrm{d}[\mathrm{T}\cdot]/\mathrm{d}t = k[\mathrm{Red}]'_{\mathrm{const}}[\mathrm{T}\cdot] = k'_{\mathrm{obs}}[\mathrm{T}\cdot] \quad (2)$$

$$k_{\mathrm{obs}} > k'_{\mathrm{obs}} (\text{when } [\mathrm{Red}]_{\mathrm{const}} > [\mathrm{Red}]'_{\mathrm{const}}). \quad (3)$$

The difference in reducing rate of [T·] reflects the difference between k_{obs} and k'_{obs}. The difference between k_{obs} and k'_{obs} comes from the difference between $[\mathrm{Red}]_{\mathrm{const}}$ and $[\mathrm{Red}]'_{\mathrm{const}}$. Since the change of $[\mathrm{Red}]_{\mathrm{const}}$ (to $[\mathrm{Red}]'_{\mathrm{const}}$) is dependent on the generation of ROS/RNS, it is assumed to reflect the concentration of ROS/RNS. Both k_{obs} and k'_{obs} do not include the term [T·], so that the rates are able to be compared to each other without consideration of the difference in initial concentration of the radicals. Direct oxidation of the hydroxylamine, the reduced form of **1**, by ROS may also contribute to the decrease of the decay rate of **1** in LPS/IFN-γ-treated cells.[16] This reaction is also slowed by the reduction of ROS/RNS in the presence of SOD and catalase, indicating a recovery of the decay rate.

In comparison with the decay rate measured at cell membrane using a membrane-localizing probe reported previously,[12] the change of the decay rate of the probe in mitochondria is smaller than that in the cell membrane by LPS/IFN-γ treatment. It is assumed that mitochondria have intense reducing ability or produced lesser amounts of ROS/RNS than cell membranes with LPS/IFN-γ treatment. These results suggest that oxidative damage in mitochondria is smaller than in the cell membrane in this endotoxic model. Since NADPH oxidase is upregulated near the cell membrane in this model, it is consistent that the decay rate in cell membrane is significantly larger than in mitochondria.

In conclusion, compound **1** was found to be a useful probe for evaluating oxidative stress in mitochondria.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.01.011.

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