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Bioorganic & Medicinal Chemistry Letters 17 (2007) 1451–1454
Bioorganic & Medicinal Chemistry Letters

Novel membrane-localizing TEMPO derivatives for measurement of cellular oxidative stress at the cell membrane

Shizuka Ban, Hidehiko Nakagawa,* Takayoshi Suzuki and Naoki Miyata*

Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467-8603, Japan

Received 22 August 2006; revised 30 October 2006; accepted 13 November 2006
Available online 16 November 2006

Abstract—Oxidative stress affecting lipid membranes is considered to be closely related to cardiovascular disease and brain ischemia. In this study, we designed and synthesized membrane-localizing TEMPO derivatives and demonstrated that one of these synthesized probes, compound **1**, localized and detected oxidative stress in the cell membrane in an endotoxic model of a mouse macrophage-like cell line. Compound **1** is therefore a potentially useful probe for evaluating oxidative stress at the cell membrane.

It is known that about 1% of the oxygen taken into our bodies by breathing is metabolized to reactive oxygen species (ROS). ROS are considered to play important roles, not only in inflammation as protective factors, but also in signal transduction.[1] On the other hand, it is also known that ROS react with lipids, proteins, sugars, and DNA, causing oxidative stress when produced in excess.[2] These reactions are suggested to be a major cause of a variety of diseases and oxidative stress caused by their reaction with lipids is considered to be closely related to cardiovascular disease[3] and brain ischemia.[4] It appears important to evaluate oxidative stress with respect to lipids in order to understand the pathological conditions underlying these kinds of diseases. However, there have been only a few attempts to measure the oxidative stress induced by ROS in specific cellular regions.[5] ROS can be measured indirectly by means of their reaction with stable radical compounds in the cell, through which the radical probes are readily reduced to non-radical species.[6]

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.[7] 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-tetramethyl-piperidin-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 from cells as well.[8] TEMPO derivatives which localize to a particular cellular region would be useful for measuring oxidative stress, including stress on the cell membrane, and TEMPO derivatives localizing to the lipid membrane would be advantageous.

For this purpose, the TEMPO derivatives would require a radical moiety for ESR detection, a fluorescent group for identifying its cellular distribution, and a functional group for localizing to the lipid membrane in a cell.

In this study, we designed and synthesized TEMPO derivatives with an alkyl chain for localization to the cell membrane (Fig. 1), and demonstrated that these radical probes were able to detect oxidative stress in lipid bilayers in an endotoxic model of a mouse macrophage-like cell line.

Mouse RAW264.7 cells were cultured in DMEM 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 DMEM. The cells were incubated at 37 °C

Keywords: Fluorescein; Redox; Electron spin resonance; Reactive oxygen species; Superoxide; Inflammation.
* Corresponding authors. Tel.:/fax: +81 52 836 3407; e-mail addresses: deco@phar.nagoya-cu.ac.jp; miyata-n@phar.nagoya-cu.ac.jp

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doi:10.1016/j.bmcl.2006.11.040

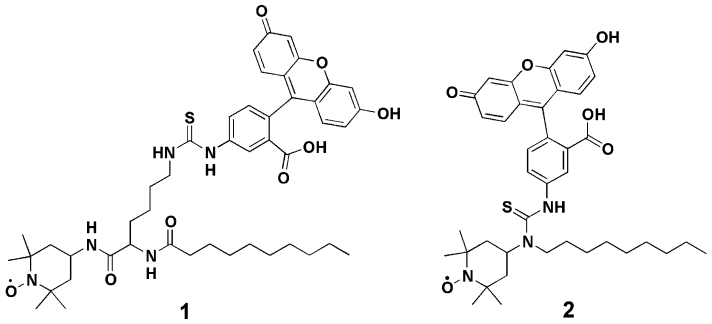


Figure 1. Structures of TEMPO derivatives designed to localize to cell membranes.

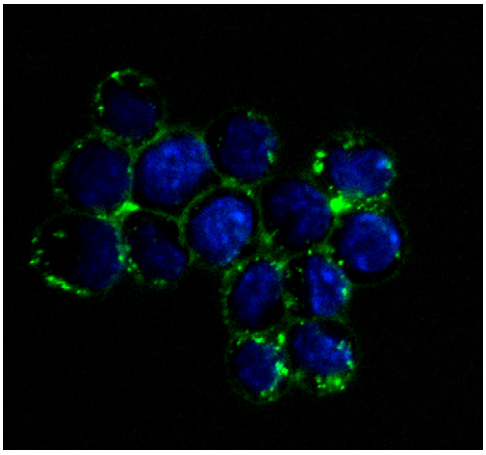
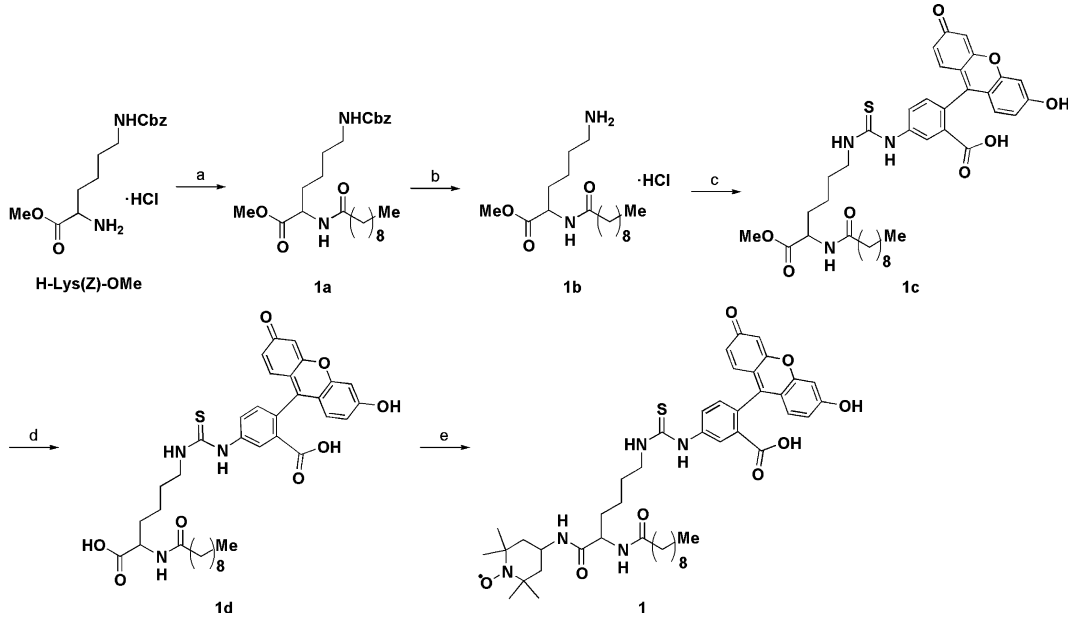


Figure 2. Distribution of **1** in RAW264.7 cells. The cells were stained with **1** (green) and Hoechst 33342 (blue), and then observed by confocal fluorescence microscopy.

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 (*E. coli*, 0.5 μg/mL) and IFN-γ (human recombinant, 150 U/mL). The treated cells were subsequently cultured for 5 h, washed 2 times with Dulbecco's PBS (D-PBS), and then treated with 100 μM of **1** for 10 min in dark. Following this, they were washed 3 times with D-PBS. The cells were then scraped into 2 mL D-PBS, and 1 mL of the cell suspension was used for ESR experiments.

Each suspension of treated cells was placed in a flat quartz cuvette. ESR measurements were started 15 min after the treatment with **1**. The ESR signal was recorded at 5-min intervals. The signal intensity (I) was calculated from the 2nd 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). The signal decay rate 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 1.5×10^5cells/dish with 1.5 mL DMEM, and incubated at 37 °C in a humidified 5% (v/v) CO_2 incubator for 2 days. The cells were treated with **1** or **2** in the same manner as for the ESR experi-

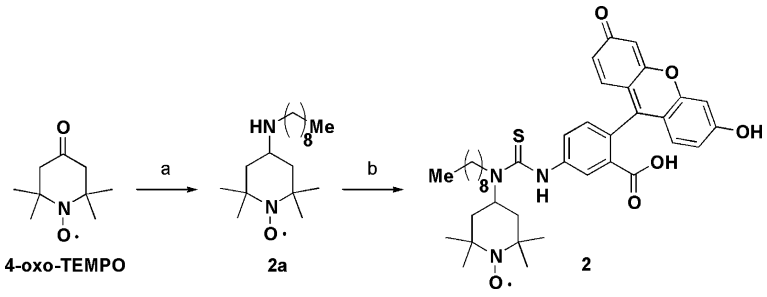


Scheme 1. Synthesis of compound **1**. Reagents and conditions: (a) *n*-decanonyl chloride, Et_3N, CH_2Cl_2, 99%; (b) Pd–C, H_2, MeOH, then 4 N HCl/AcOEt, 88%; (c) FITC, Et_3N, EtOH, 51%; (d) LiOH aq, THF, EtOH, 98%; (e) 4-amino-TEMPO, EDCI, HOBt, DMF, 55%.

ments. The cells were subsequently stained with Hoechst 33342 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 the cell membrane as expected (Fig. 2), due to the presence of its alkyl chain. The TEMPO moiety was assumed to be close to the surface in the lipid bilayer because the signal shape was not broad, as compared with the signal of TEMPOL in solution (Supporting information). This probe is considered to be able to penetrate into the lipid bilayer for its adequate lipid solubility. Compounds **1** and **2** are assumed to be distributed at the both leaflets of the cell membrane. Thus, the probe can measure the oxidative stress at the cell membrane (Scheme 1 and Scheme 2).

There are no differences in localization and sensitivity between compounds **1** and **2** in the observation by confocal fluorescence microscopy. Compound **1** was used for ESR analysis because it was more stable than compound **2** in solution. The ESR signal of **1** was measured in RAW264.7 cells to evaluate oxidative stress in the cell



Scheme 2. Synthesis of compound **2**. Reagents and conditions: (a) *n*-nonylamine, THF, then $NaBH(OAc)_3$, 84%; (b) FITC, THF, 41%.

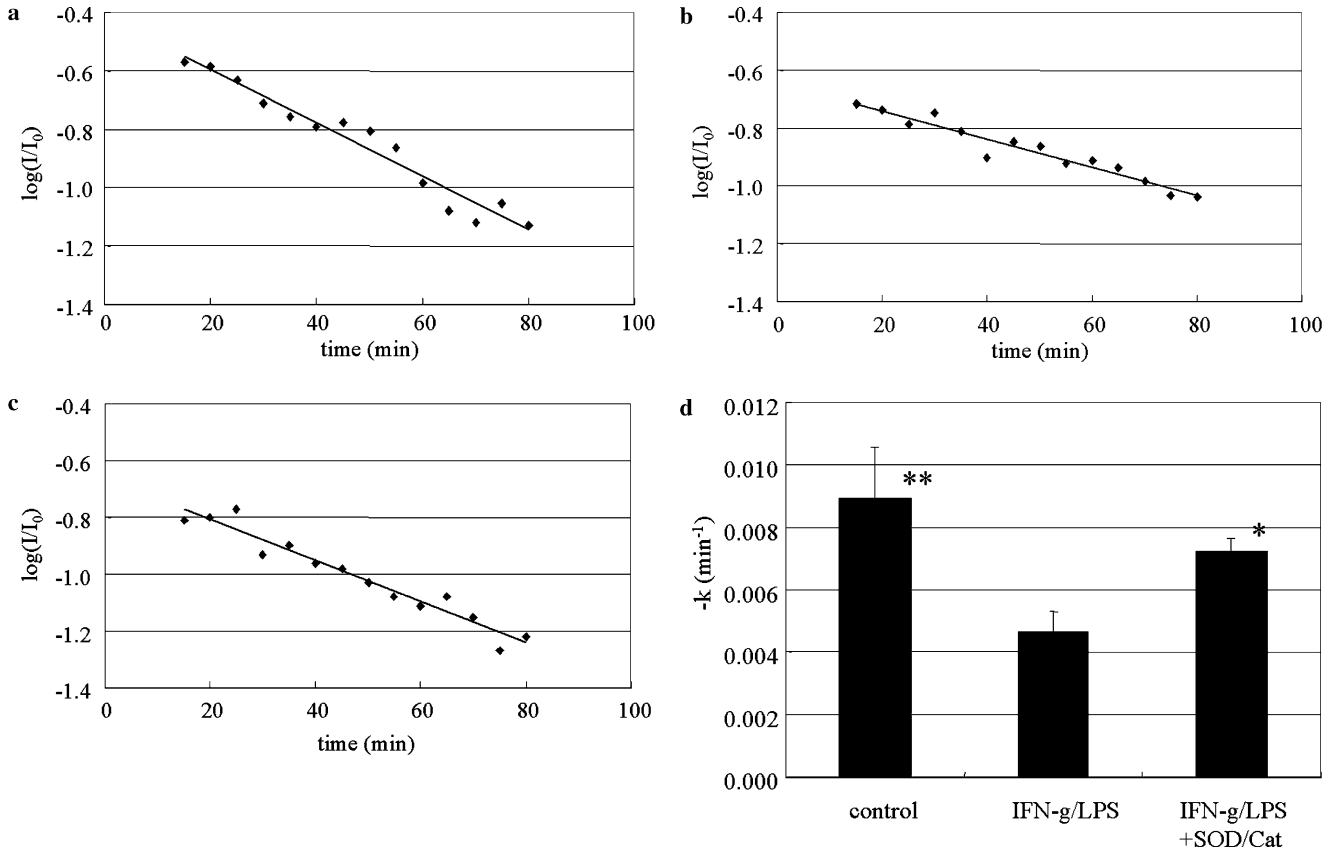


Figure 3. The time course of the relative signal intensity measured at 5-min intervals, (a) control cells, (b) LPS/IFN-γ-treated cells, and (c) LPS/IFN-γ-treated cells in the presence of SOD and catalase. *I*, compound **1** peak area; I_0, Mn^{2+} external standard peak area. (d) Signal decay rate of **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 3 or 4 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 ($^{*}P < 0.05$).

membrane. In the control cells, the signal intensity of **1** was gradually decreased at $0.0091 \pm 0.002\ min^{-1}$ under our conditions. The upregulation of oxidative stress was evaluated after endotoxic stimulation. ESR spectra of **1** were measured in RAW264.7 cells 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 $0.0049 \pm 0.0007\ min^{-1}$. The decreased rate as a result of the LPS/IFN-γ treatment was restored to $0.0072 \pm 0.0004\ min^{-1}$ in the presence of 100 U/mL SOD and 10 U/mL catalase during measurement (Fig. 3d). 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 activated the cells and increased the production of reactive oxygen and nitrogen species (ROS/RNS). LPS/IFN-γ treatment also decreased the decay rate of nitroxyl radical. The decrease in this 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. The probe was reduced under any conditions in our experiment, and the decay rate was considered to reflect the cellular local reducing ability for the probe, which would correlate with the oxidative stress in the cell membrane. Thus, the decay rate of **1** was not assumed to reflect specific ROS production, but the local oxidant upregulation, although the production of superoxide was upregulated under this experimental condition, and superoxide, hydrogen peroxide, and hydroxyl radical were considered to play an important role in this result. It was suggested that the cell membrane was exposed to an oxidative environment due to the increase in ROS by LPS/IFN-γ-pretreatment. Although SOD and catalase were thought not to be distributed inside the cells, they were able to get close to the cell membrane, where compound **1** was localized. These two enzymes may contribute to the scavenging of ROS around the cell membrane, and this is consistent with the fact that ESR signal decay rate was restored under these conditions. The change in this rate 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**.[9] The TEMPO moiety can be oxidized to the ESR-silent oxonium cation form by superoxide.[10] However, this cation was found to be rapidly reduced back to TEMPO by the superoxide itself.[10] Compound **1** was considered to be repeatedly oxidized and reduced in the cell membrane. The direct oxidation of TEMPO itself by superoxide probably does not affect the signal decay rate under the condition used for these measurements.

In conclusion, although there remain some detailed characteristics to be clarified, compound **1** was found to be a useful probe for evaluating oxidative stress at the cell membrane.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.11.040.

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