Radioiodinated Nitroxide Derivative for the Detection of Lipid Radicals

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S Supporting Information

ABSTRACT: Thus far, no accurate measurement technology has been developed to detect lipid alkyl radicals (lipid radicals), which cause lipid peroxidation. Therefore, we aimed to develop a nuclear medical imaging probe that can be taken up in the lipophilic site in cells such as biological membranes, by reacting specifically with the lipid radicals generated there. We designed and synthesized 4-(4-[¹²⁵I]iodobenzamido)-2,2,6,6-tetramethylpiperidine-1-oxyl, which shows high reactivity to lipid radicals with a high radiochemical yield and purity. Intracellular retention was found to increase significantly when lipid radicals were produced.

KEYWORDS: Radical, radioisotope, lipid, nitroxide

Oxidative degradation of lipids is known as lipid peroxidation.^{1,2} The reaction is initiated by the extraction of a hydrogen atom at the position between the allyl moiety of unsaturated fatty acids to generate lipid carbon-centered radicals.^{3,4} Thereafter, the lipid carbon-centered radicals react with molecular oxygen to form peroxyl radicals, which extract hydrogen atoms from allyl moieties of other nearby lipid molecules and get converted into lipid peroxides. Hydrogen atom-extracted fatty acids get converted to lipid carbon-centered radicals, and this reaction occurs in chain.

Recently, it has been shown that the onset and progress of liver cancer can be markedly suppressed by preadministration of a compound that captures lipid carbon-centered radicals in a liver cancer animal model, established by using a nitrosamine carcinogen.⁵ Similarly, with regard to age-related macular degeneration (AMD), it has been reported that pretreatment with a lipid carbon radical-scavenging compound could suppress retinal damage by light exposure, reduce lipid peroxides, and delay disease progression.⁶ These studies suggest that lipid carbon-centered radicals contribute to the onset and progress of various diseases. If these carbon-centered radicals can be measured *in vivo*, diagnosis and assessment of the degree of disease progression will become possible.

The above-mentioned candidates for liver cancer and AMD are nitroxide-based compounds, which are highly reactive to carbon-centered radicals. These compounds are also developed as fluorescent probes that can detect lipid carbon radicals in tissue sections.⁷ However, the fluorescence has low biopermeability and is not adequate for external body measurements.

Therefore, we focused on nuclear medical imaging, which enables noninvasive and quantitative diagnosis. On the other hand, it is necessary to label compounds with radioisotopes (RI). So far, only one tritium-labeled nitroxide derivative has been reported as a nuclear battery,⁸ but tritium is not suitable for *in vivo* imaging. Therefore, the purpose of this study was to label the nitroxide compound with RI, in order to use it as an imaging probe. We selected radioiodine, as it enables diagnosis using single photon emission computed tomography (SPECT).⁹ Generally ¹²³I is used for SPECT; here we used ¹²⁵I, which decays by electron capture and has a long half-life (59.49 d) to be easy to handle, to develop a radiolabeled nitroxide derivative for the detection of lipid radicals. In addition, the developed radiolabeled probes were tested in a cultured cell system to assess whether the probes were retained inside the cell when the lipid carbon radicals were generated.

Nitroxide, which is also called aminoxyl or nitroxyl radical, is a molecular species containing a >NO• radical structure.¹⁰ There are several types of basic structures such as cyclic and chained nitroxides. In this study, we chose the same type of nitroxide, i.e. TEMPO (2,2,6,6-tetramethylpiperidin-N-oxyl), as the one used for the treatment of liver cancer and AMD in a previous study.^{5,6} TEMPO has a chemically modifiable substituent at the 4-position of the piperidine ring. Some of these derivatives are commercially available.

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The tin-halogen exchange reaction is widely known as a radioiodination method.¹¹ In general, aliphatic halogens often cause elimination reactions, which raises concerns about reduced purity and potential adverse effects on the living body. Therefore, we planned to synthesize 2,2,6,6-tetramethyl-4-(4-(tributylstannyl)benzamido)piperidine-1-oxyl (**3**), in which an aromatic tin substituent was introduced into the 4-position of the TEMPO derivative; thereafter it converts to the desired radioactive 4-(4-[¹²⁵I]iodobenzamido)-2,2,6,6-tetramethylpiperidine-1-oxyl ([¹²⁵I]**2**) (Scheme 1).

Scheme 1. Synthetic Scheme for Radioiodinated Nitroxide [¹²⁵I]2**^a**

^a(i) *p*-Iodobenzoic acid, DMT-MM, THF, 79%; (ii) (SnBu₃)₂, Pd(PPh₃)₄, toluene, 51%; (iii) Na¹²⁵I, NCS, MeOH/AcOH (100:1).

Moreover, 4-(4-iodobenzamido)-2,2,6,6-tetramethylpiperidine-1-oxyl (**2**), which is a nonradioactive iodine analog, is required to identify radiolabeled **2**. Therefore, we first synthesized **2** by condensation of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (**1**) with *p*-iodobenzoic acid. In fact, when the reaction was carried out using DMT-MM, which is a dehydration condensation agent, **2** could be obtained in a yield of 79%. Next, the synthesis of **3** was attempted by a tin-halogen exchange reaction. The stannylation was performed using a palladium catalyst and bis(tributyltin) to afford **3** in a 51% yield. **3** was identified by mass spectrometry (MS) measurement, whose spectrum showed a typical isotopic peak pattern derived from tin atom.

Next, the reactivity of **2** toward lipid radicals was determined by electron spin resonance spectroscopy (ESR). It showed a marked ESR signal decay over time in the arachidonic acid/lipoxygenase reaction system. However, for reactive oxygen species such as superoxide, hydroxyl radical, hydrogen peroxide, and hypochlorous acid, the ESR signal did not change (Figure 1). These results are in agreement with the previous report that nitroxide formed adducts with lipid carbon radicals in a reaction system of fatty acid and lipoxygenase.¹²

Figure 1. Reactivity of **2** with lipid alkyl radicals and reactive oxygen species. ●: lipid alkyl radical generated from 0.8 mM arachidonic acid + 0.05 mg/mL lipoxygenase; □: hydroxyl radical generated from 1% H₂O₂ + 0.2 mM FeSO₄; ◇: superoxide generated from 1.25 mM hypoxanthine + 78 mU/mL xanthine oxidase; △: 0.1% NaClO; ×: 1% H₂O₂.

And it is also consistent with the reported paper that the reactivity of TEMPO nitroxide with hydroxyl radical and superoxide is varied with the substituents at the 4-position of the piperidine ring; oxo- and amino-TEMPO are reported less reactive to hydroxyl radical and no-substituted TEMPO are relatively reactive, and almost all nitroxides are nonreactive to superoxide itself though it can react only when the two electron reductants such as NADH exist.¹³ And it is also reported that these reactivities are well correlated with the redox potential of oxoammonium cation and the nitroxide redox couple. The present compound **2** has the amide group at the 4-position of the piperidine ring which raises the redox potential to be less reactive to hydroxyl radical and superoxide, though we could not measure the redox potential of **2** due to the poor water solubility. From the above, **2** exhibits high selectivity to lipid carbon radicals.

Subsequently, ¹²⁵I-labeling of the tin precursor **3** was examined. *N*-Chlorosuccinimide (NCS) was used as the oxidizing agent for iodine. Compound **3** was mixed with Na¹²⁵I under acidic condition (MeOH/AcOH = 100:1) at room temperature for 15 min and then HPLC purification was conducted. As a result, the chromatogram of radioactivity showed a main peak at about 9 min. Then, this peak was collected, and HPLC analysis was carried out by co-injection with the nonradiolabeled compound **2**. Then both UV and RI peaks were detected at almost the same time at about 9 min (Figure 2). The radiochemical yield was 82%, and the

Figure 2. HPLC chromatogram of [¹²⁵I]**2** and co-injected **2**. (a) UV chromatogram; (b) RI detected chromatogram.

radiochemical purity was >95%. As mentioned above, we succeeded at obtaining the ¹²⁵I-labeled nitroxide. In addition, the radiochemical yield was similar even when the reaction was carried out under the same conditions, while changing the reaction time to 5, 10, and 30 min. In addition, when the reaction was carried out using chloramine T as an oxidizing agent instead of NCS, labeling was possible with a similar radiochemical yield and purity.

We next examined whether compound **2** was trapped in cells during lipid radical production. It has been reported that the lipid carbon radicals can be generated during metabolic processes by adding arachidonic acid to cultured cells.^{14,15} On the other hand, arachidic acid was used for the control experiment which is the saturated analog of arachidonic acid possessing the similar lipophilicity and is not converted to lipid alkyl radicals due to the lack of active methylene. Therefore, the amount of radioactivity in the cells was measured using a γ-counter with or without arachidonic acid or arachidic acid

added together with [^{125}I]2. The amount taken up over time in each group increased up to 15 min (Figure 3). In the condition

Figure 3. Accumulation of [^{125}I]2 in HepG2 cells with arachidonic acid (■), with arachidic acid (▲), and without additives (●). * $p < 0.01$ vs without additives, # $p < 0.01$ vs arachidic acid.

of no additives, the uptake amount was minimal among these three conditions. With arachidonic acid, the uptake was slightly increased compared with no additives. This difference may be derived from the lipophilicity of arachidonic acid which promoted slightly the uptake of [^{125}I]2.¹⁶ Instead, the uptake amount was higher in the addition of arachidonic acid much more than arachidic acid. This result indicated that the developed compound 2 was trapped in the cells by reacting with lipid carbon radicals derived from the stimuli of arachidonic acid though there was a little effect of the lipophilicity of coadded arachidonic acid. On the other hand, the TEMPO-type nitroxide which has four methyl groups at the α -position of the N–O moiety is known to be susceptible to bioreduction.¹⁷ This is assumed to cause the loss of the reactivity toward lipid radicals. To improve the stability to bioreduction, we are now developing new probes.

In this study, we designed and synthesized a new radioiodinated nitroxide derivative for the development of SPECT probes for lipid carbon radicals. We were the first to obtain a radioiodinated nitroxide derivative with high radiochemical yield and high radiochemical purity. In addition, the developed compound exhibited high reaction selectivity to lipid carbon radicals and the cellular uptake was found to increase over time in cells in which lipid carbon radical production was induced as compared to that in nonstimulated cells. This indicates that it is possible to use this compound as a detection probe for lipid carbon radicals.

EXPERIMENTAL PROCEDURES

Detailed experimental procedures are found in the [Supporting Information](#).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmchemlett.9b00416>.

Experimental methods and compound characterization data (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

RI, radioisotope; SPECT, single photon emission computed tomography; TEMPO, 2,2,6,6-tetramethylpiperidin-*N*-oxyl; DMT-MM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; THF, tetrahydrofuran; Na¹²⁵I, [^{125}I] sodium iodide; NCS, *N*-chlorosuccinimide; MS, mass spectrometry; ESR, electron spin resonance.

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