Synthesis and Evaluation as a Blood–Brain Barrier-Permeable Probe of 7-N-(PROXYL-3-yl-methyl)theophylline

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It is known that damage due to reactive oxygen species (ROS) and nitric oxide (NO) underlies diseases including cancer and Alzheimer’s disease.1–3 Much attention has been devoted to ROS activity with the goal of preventing these serious diseases. Although in vivo measurement of free radical activity is needed to improve disease prevention methods, in vivo measurement of free radicals such as ROS and NO is difficult due to their short half-lives and low concentrations.4,5) Using a nitroxide-spin probe reactive with free radicals, and an anti-oxidant such as ascorbic acid (AsA), it has been reported that some nitroxide-radical spin probes such as tetramethylpyrrolidine-1-yloxy (TETEPONE) can pass through the blood–brain barrier (BBB) and allow noninvasive EPR imaging of redox status in living mice.6–11) It is expected that these nitroxide radicals can be used as a probe for monitoring the redox status of the brain. However, the development of new hybrid probes that can simultaneously measure ROS activity and the BBB permeability has not yet been achieved.

Recently, we synthesized several hybrid compounds that can conjugate a nitroxide radical with a drug that shows pharmacological action in the mouse brain, and explored their BBB permeability in mice. These studies failed to identify a hybrid compound that satisfied both lipophilicity and molecular size requirements for BBB permeability, and hydrophilicity requirements for vein injection. Many synthesized hybrid probes were almost too lipophilic to prepare suitable emulsions with a surfactant that could be injected into the mouse via the tail vein. Here, we describe the synthesis and in vitro and in vivo properties of a novel BBB-permeable hybrid (Fig. 1).

We synthesized the nitroxide-based hybrid probe by choosing theophylline as the drug component. The 7-N-substituted theophylline is part of the xanthine family and pharmacologically similar to caffeine (7-N-methylxanthine). 7-N-Alkylxanthines show specific chemotherapeutic activity with lower toxicity and side effects.15) Thus theophylline is expected to be relatively non-toxic. 7-N-(PROXYL-3-yl-methyl)-theophylline (3) was successfully prepared by connecting theophylline to HMP, Compound 3 has an N–C linkage that connects theophylline to HMP, which is different from the previously reported compounds 1 and 2, in which the drug and nitroxide were connected by an ester bond, which was hydrolyzed in the brain prior to its anti-inflammatory activity. Without hydrolysis, 3 has to show pharmacological activity unmodified. Partition coefficients (P ow) of nitroxides 3, 3-carboxy-PROXYL (COP), and HMP between n-octanol and phosphate buffered saline (PBS) (0.1 M, pH 7.4) were measured by EPR spectroscopy and compared.16–18) In vivo stability and distribution of 3 was examined in mice.

Results and Discussion

Synthesis To begin with, the synthesis of 3 was practiced by a substitution reaction using 3-bromomethyl-PROXYL (4), which was synthesized via seven steps from 4-piperidone as per the established method19–21) (Chart S1). A substitution reaction of 4 to theophylline in the presence of NaH (1.2 eq) in anhydrous N,N-dimethylformamide (DMF) at 50°C for 1 d af-
forded \(3\) in a maximum yield of 28%. Use of other bases such as NaOEt and Et\(_3\)N did not yield \(3\). The use of CsCO\(_3\) gave a 10% yield of \(3\). The use of sulfonate in place of bromide as a nitroxide donor also failed to yield \(3\). On the other hand, synthetic method established by Mohammad Navid Soltani Rad’s group\(^{22}\) in which reflux reaction of 7-N-tosyltheophylline (TsTh) and 3-hydroxymethyl-PROXYL in acetonitrile in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) proceeded smoothly and the maximum yield of \(3\) was 58% (Chart 1).

The purity of \(3\) was determined by \(^1\)H-NMR and EPR spectroscopy (Figs. S1 and S2, and Fig. 2). In \(^1\)H-NMR spectroscopy, the assignment was achieved as a hydroxylamine by adding an excess of hydrazobenzene. The EPR spectrum of \(3\) in 0.1 m PBS (pH 7.4) showed triplet patterns due to \(^{14}\)N hyperfine splitting (\(a_N=1.85\) mT, \(g=2.007\)), similar to that of HMP. The assignment of \(3\) was confirmed by heteronuclear multiple bond connectivity (HMBC) correlation. Since in the HMBC correlation of \(3\), C5 and C8 were correlated to 3-CH\(_2\) of the PROXYL and C4 to the 3-N-CH\(_3\), it was demonstrated that the 3-methyl-PROXYL moiety is bonded at the 7-N position of the theophylline, not at the 9-N position (Fig. 3).

![Fig. 3. HMBC Correlation of 3](chart)

**Table 1. Partition Coefficients of Nitroxides between \(n\)-Octanol and PBS**

| Nitroxides | \(P_{o/w}\) |
|------------|-------------|
| 3          | 1.86        |
| COP        | 0.012       |
| HMP        | 1.41        |

**Table 2. Second-Order Rate Constants, \(k\ (M^{-1}\ min^{-1})\), for Initial Rates of Reduction of 3 and HMP (20\(\mu\)M) with 100-Fold Excess AsA, or 100-Fold Excess of AsA Plus 125-Fold Excess of GSH in 0.1 m PBS (pH 7.4) at 20°C for 30 min Based on Fig. 4**

|                  | \(k\ (M^{-1}\ min^{-1})\) | \(R^2\)  |
|------------------|-----------------------------|----------|
| 3+AsA            | 11.9±5.3                    | 0.9972   |
| 3+AsA+GSH        | 22.3±6.8                    | 0.9896   |
| HMP+AsA          | 6.1±5.2                     | 0.9956   |
| HMP+AsA+GSH      | 9.1±2.4                     | 0.9968   |

**Partition Coefficients of Nitroxides between \(n\)-Octanol and PBS (0.1 m, pH 7.4)**
were measured by EPR spectroscopy. The $P_{ow}$ of the BBB-permeable HMP was 1.41, and that of the impermeable COP was 0.012. Since the $P_{ow}$ of 3 was 1.86 and indicated more lipophilicity than HMP did, 3 is expected to be BBB permeable. Despite this lipophilicity, it could be emulsified with polyoxyethylene sorbitan trioleate (Tween 85). The $P_{ow}$ values of ibuprofen- and ketoprofen-PROXYLs ($1\text{ }\text{ and }\text{ }2$) indicated no radical in the PBS layer due to greater lipophilicity (Table 1).

Reduction Rate Constants, $k$ (m$^{-1}$ min$^{-1}$) of 3 and HMP in the Presence of 100-Fold Excess of AsA, or 100-Fold Excess of AsA Plus 125-Fold Excess of Glutathione (GSH)$^{23-27}$ The rates of reduction for 3 and HMP were stable, with high $R^2$ of 0.995 and 0.998, respectively. Their rate constants were approximately equal, at $11.9\pm5.3$ and $6.1\pm5.2\text{m}^{-1}\text{min}^{-1}$, respectively. In the presence of 125-fold excess GSH, the rate constants were $22.3\pm6.8$ and $9.1\pm2.4\text{m}^{-1}\text{min}^{-1}$, respectively, slightly increased compared to those achieved without GSH, as reported by Andrzej Rajca’s group.$^{20}$

Since 3 showed similar reactivity to HMP in antioxidants such as AsA and GSH, it is expected that 3 will function similarly to HMP in the mouse (Fig. 4, Table 2).

Evaluation of in Vivo Distribution of 3 in the Mouse Head by EPR Imaging Three-dimensional EPR images of the mouse head were captured after injection of probe 3 into the tail vein, and used to produce slice-selected two-dimensional (2D) EPR images. A 2D EPR image of the mouse head obtained after injection of 3 is shown in Fig. 5G, along with 2D EPR images obtained after injection of BBB-permeable HMP (Fig. 5C) and BBB-impermeable 3-carboxyl-PROXYL (COP, Fig. 5E). According to comparison of Fig. 5G with Figs. 5C and E, probe 3 appears to enter the mouse brain by passing through the BBB. To confirm this, a 2D EPR image was co-registered to the anatomical image of the mouse head obtained by MRI for 3, HMP, and COP. Co-registered images (Figs. 5H, D, F) indicate that probe 3 can enter the brain by passing through the BBB, though the distribution and concentration of 3 were reduced in comparison to those of HMP.

Theophylline is known to increase blood flow. After injection of 3 into the mouse tail vein, we confirmed by naked eye that the skin of the mouse leg visibly reddened (Fig. S3). This symptom was observed for at least 5 min after injection of 3 (1.5 mmol/g body weight) into the tail vein and may indicate enhanced blood flow in the mouse. For future studies, we will consider methods for measuring changes in the blood flow in mice.

Conclusion

Compound 3 was successfully synthesized by coupling TsTh with HMP. The stability of 3 in the presence of AsA with or without GSH was determined to be similar to that of HMP. The partition coefficient and molecular weight of 3 made it suitable for passing through the BBB. in vivo EPR imaging of the mouse head clearly revealed the distribution of 3 in the mouse brain and its BBB permeability. The reddening of the skin on the mice’ legs after injection of 3 led us to expect a pharmacological effect, because theophylline and caffeine are known to enhance blood flow.$^{28-30}$

Experimental

Synthesis

Materials and Apparatus
All starting materials and reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Sigma-Aldrich Corporation (U.S.A). Reactions were monitored by TLC on 0.25-mm silica gel F254 plates (E. Merck, Germany). UV radiation and a heated 7% solution of phosphomolybdic acid in ethanol were used for coloration. Flash column chromatography was performed on a silica gel (silica-gel 60, 40–50 μm, Kanto Chemical Co., Inc., Tokyo, Japan) to separate and purify the reaction products. Melting point

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**Fig. 5. Distribution of Nitroxide Probe in the Mouse Head**

(A) Photograph of the mouse head. (B) MRI of examine mouse head before injection of nitroxide probe. 2D EPR images of the mouse head taken 0.5 min after injection of BBB-permeable HMP (C), BBB-impermeable COP (E), and 3 (G). (D, F, H) Co-registration of the EPR images (C, E, G) and the anatomical image obtained by MRI of the same mouse before administration of each probe. (Color figure can be accessed in the online version.)
was determined using an ASONE micro-melting point apparatus. The uncorrected value is reported in “7-N-(PROXYL-3-yl-methyl)theophylline (3).” IR spectra were recorded on a Horiba FT-720 IR spectrometer using KBr disk. NMR spectra were recorded using a JEOL ECX-500 spectrometer with Me$_4$Si as the internal standard. Proton NMR spectra of hybrid compounds were measured using the corresponding hydroxylamines, which were prepared by reduction of nitrooxide radicals in the presence of excess hydrazobenzene. Mass spectral data were obtained by FAB-MS using 3-nitrobenzyl alcohol (NBA) as the matrix on a JEOL JMS-AX505HA instrument. Elemental analyses were performed on a Perkin-Elmer PE 2400 II instrument. After being dried at 80°C under reduced pressure for more than 2 h, each product was subjected to elemental analysis.

7-N-(PROXYL-3-yl-methyl)theophylline (3)

TsTh (233 mg, 0.698 mmol), HMP (100 mg, 0.581 mmol), and DBU (174 mL, 1.162 mmol) were added in anhydrous CH$_2$CN (4 mL) and the resulting mixture was refluxing for 9 h. After confirmed no change of the products on the silica-gel TLC, the reaction mixture was evaporated to remove the organic solvent. The residue was dissolved in CHCl$_3$ (10 mL) added washed with water (10 mL) and brine (3 mL), and then dried over anhydrous Na$_2$SO$_4$. After removing of CHCl$_3$, the residue was separated by column chromatography (CHCl$_3$–MeOH=100:0 ca. 20:1) to give 3 (113.7, 58.6 mg) as a colorless solid.

Pale-yellow crystal. mp 188–189°C. IR ν (KBr) 3521, 3457, 3356, 3249, 3095, 2973, 2876, 1705, 1666, 1602, 1550, 1477, 1461, 1436 cm$^{-1}$. $^1$H-NMR (CDCl$_3$+hydrazobenzene) δ: (PROXYL moiety) 1.064, 1.078, 1.180, and 1.193 (each 3H, s, CH$_3$), 1.50 (2H, m, 4-CH$_2$), 2.33 (1H, m, H3), 3.93 (1H, dd, J=13.3, 10.4Hz, 3-CH$_3$), 4.43 (1H, dd, J=13.3, 5.0Hz, 3-CH$_3$), (theophylline moiety) 3.43 and 3.62 (each 3H, s, CH$_3$), 7.43 (1H, s, H8). $^{13}$C-NMR (CDCl$_3$+hydrazobenzene) δ: (PROXYL moiety) 17.36, 26.28, 26.43, and 28.46 (CH$_3$), 40.1 (C4), 43.5 (C5), 47.8 (CH$_3$), 60.96 and 64.88 (C2, 6), (theophylline moiety) 113.7, 58.6 mg) as a colorless solid.

Calcd for C$_{16}$H$_{24}$N$_5$O$_3$: C, 57.47; H, 7.23; N, 20.94. Found: C, 57.48; H, 7.44; N, 20.65. g = 2.007, aN=1.85.

Analysis

Apparatus

EPR spectra were obtained using a JEOL JES-FR30 EPR spectrometer. Samples were drawn into quartz capillaries. The bottoms of the capillaries were sealed, and capillaries were placed in standard 2-mm (i.d.) quartz EPR tubes. The EPR spectrometer settings were as follows: microwave power, 4.0mW; frequency, 9.5 GHz; and, modulation amplitude, 1.25 G. Quick mixing of the sample was conducted using VOLTEx®

Reagents

The reagents used in this study were commercial products (Wako Pure Chemical Industries, Ltd.): n-octanol, l-ascorbic acid and GSH, and dimethyl sulfoxide.

Partition Coefficients of Nitroxides between n-Octanol and PBS

To each 1 mL of 33 mM solution of compound 3, HMP, and COP in 0.1 M PBS (pH 7.4), 1 mL of n-octanol was added. The resultant solution was vortexed for 1 h, and then the mixture was centrifuged at 3000 rpm for 5 min. Both octanol and PBS were subjected to EPR measurement.

Conditions of EPR measurement: field: 336.5 mT, power: 4.00 mW; gain: 10.0, sweep width: 5.0 mT, modulation width: 0.1 mT, sweep time: 0.5 min, time constant: 0.1 s, date points: 0, accumulation: 1, accumulation method: no, frequency: 9.2 GHz.

EPR intensity was calculated by the following equation:

$$
\text{EPR intensity} = \frac{\text{lowest peak height of the three line}}{\text{peak height of Mn marker}}
$$

The partition coefficient between n-octanol and PBS ($P_{o/w}$) was calculated by the ratio of the EPR intensities of octanol and PBS.

EPR Intensity Measurement of Time-Dependent Reduction of 3 and HMP by Addition of 100-Fold Excess AsA and 125-Fold Excess of GSH

Compound 3, HMP, and AsA were dissolved in 0.1 M PBS (pH 7.4) to prepare 2 mM stock solutions, respectively. Twenty microliters of 2 mM 3 or HMP, and 1.98 mL of a mixture of 2.02 mM AsA and 2.52 mM GSH in 0.1 M PBS (pH 7.4) were mixed by VOLTEX and the mixture was subjected to EPR measurement after 2 min and at intervals from 5 to 180 min. PBS (0.1 M, pH 7.4) was deoxygenized before use.

EPR measurements and calculations of EPR intensity were performed as in 5.3.

Twenty microliters of 2 mM 3 or HMP was diluted with 1.98 mL of 0.1 M PBS (pH 7.4) to prepare 20 µM sample solutions. The EPR intensity of each 20 µM sample solution was measured, and the relative value of these EPR intensities was set to 1.0.

Animals

The Sapporo Medical University Animal Care Committee approved the protocols for all animal experiments according to the National institutes of Health Animal Care and Use Protocol (NIH, Bethesda, MD, U.S.A.). One male C57BL/6 mouse aged 5 weeks with a body weight of 22 g was used. The animal was housed in a temperature- and circadian rhythm-controlled room with unrestricted access to food and water.

In Vivo EPR Spectrometer and Imager

EPR images were obtained using a custom-made 750-MHz CW-EPR imager. A main static magnetic field of 27 mT was generated with permanent magnets (Hitachi Metals, Ltd., Tokyo, Japan). Three sets of coils for magnetic field gradients and field ramp changes, and the minimum scanning time was 50 ms. Data acquisition was controlled with a custom-written program in LabVIEW software (National Instruments Inc., Texas, U.S.A.). Image reconstruction from the EPR spectra was based on the filtered back-projection method, and 3D EPR images were obtained by the single-stage filtered back-projection (FBP) algorithm. The imaging matrix was 128×128×128, and the field of view (FOV) was 50×50×50 mm.

In Vivo EPR Imaging of Mouse Brain

A mouse was anesthetized with isoflurane (1.5%) in air at 250 mL/min. The tail vein was cannulated with a 27 gauge needle for injection of nitroxides. A solution of 3 (1.5 µmol/g weight of body) in PBS was injected for about 15 s. The body temperature of the mouse was maintained at 37±0.5°C during EPR imaging experiments.
Conflict of Interest  The authors declare no conflict of interest.

Supplementary Materials  The online version of this article contains supplementary materials.

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