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Rapid Communication

Highly amphiphilic manganese porphyrin for the mitochondrial targeting antioxidant

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Takayuki Haruyama, Shoichiro Asayama and Hiroyoshi Kawakami*

Department of Applied Chemistry, Tokyo Metropolitan University, and 1-1 Minami-Osawa, Hachioji, Tokyo 192-0397, Japan

*Hiroyoshi Kawakami, Department of Applied Chemistry, Tokyo Metropolitan University, 1-1 Minami-Osawa, Hachioji, Tokyo 192-0397, Japan, Tel: +81-42-677-1111 Ext. 4972, Fax: +81-42-677-2821, E-mail: kawakami-hiroyoshi@c.metro-u.ac.jp

Here, we communicate that the design of the anti-oxidant consisting of amphiphilic manganese porphyrin with one dimethylimidazolium group and three phenyl groups for mitochondrion targeting. The resulting Mn-porphyrin MnMImP$_3$P exhibited high partition coefficient ($\log P_{ow} = +4.78$) as well as significant superoxide dismutase and peroxynitrite decomposition activities. Accordingly, the MnMImP$_3$P exhibited a little increase in fluorescence intensity attributed to 3,3′-dipropyl-2,2′-thiadicarbocyanine iodide [diS-C$_3$-(5)], a tracer dye to assess the mitochondrial membrane potential, which suggested the interaction of the MnMImP$_3$P, leading to the release of the fluorescence dye from the mitochondrial membrane, with the mitochondria. As a result, the MnMImP$_3$P rescued the cell death under oxidative stress concerned with mitochondrial damage.

Keywords: Manganese porphyrin/Mitochodrial targeting/Antioxidant/Amphiphilicity.

Abbreviations: SOD, superoxide dismutase; PD, Parkinson's disease; 6-OHDA, 6-hydroxydopamine.

Here, we have reported the design of the anti-oxidant consisting of amphiphilic manganese (Mn) porphyrin for mitochondrial targeting. Mitochondria are the major source of superoxide, and are responsible for oxidative damage, *e.g.* neurodegenerative disorders like Parkinson's and Alzheimer's diseases (*1*). In mitochondria, manganese superoxide dismutase (Mn–SOD) catalyzing the reduction from superoxide radical anion ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) is the key enzyme for the protection of oxidative stress (*2*). We have already reported that water-soluble Mn-porphyrins with SOD activity works as anti-oxidant in various biochemical fields (*3,4*). If the availability of anti-oxidant drugs at the mitochondrial level within cells increase, the radical approach to therapy would be promising. Therefore, we have already synthesized the Mn-porphyrin modified with signal peptide for mitochondrion targeting (*5*). Although the resulting Mn-porphyrin–oligopeptide conjugate exhibits anti-oxidative activity for cells with mitochondrial damage, the drug carrier to deliver into cytoplasm is necessary for rescue of the cells. In this study, we have synthesized the amphiphilic Mn-porphyrin with one dimethylimidazolium group and three phenyl groups for mitochondrion targeting (Scheme 1). This article describes the synthesis and characterization of the resulting amphiphilic Mn-porphyrin in the context of its catalytic activity, 1-octanol/water partition coefficients ($\log P_{ow}$), mitochondrion membrane potential, and protective effect of human neuroblastoma cells against experimental Parkinson's disease (PD) model; detailed studies on the mechanism of anti-oxidative activity are outside the scope of the present study.

To synthesize the amphiphilic Mn-porphyrin with SOD activity, as shown in Scheme 1, we first synthesized 5-(1-methylimidazole-2-yl)-10,15,20-triphenyl-21H,23H-porphine (**4**) according to literature methods (*6,7*) modified by us. 1-Methylimidazole-2-carboxaldehyde (**1**), pyrrole (**2**) and benzaldehyde (**3**) were refluxed in propionic acid. The six possible porphyrin isomers were separated by using column chromatography with basic active alumina, followed by that with silica gel and a chloroform solvent system. Then, the methylation of the resulting porphyrin (**4**) with methyl iodide was carried out, producing 5-(1,3-dimethylimidazolium-2-yl)-10,15,20-triphenyl-21H,23H-porphine (**5**): ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm 9.10 (2H, s, 4,5-dimethylimidazolium), 8.90 (4H, m, 2,3,7,8-pyrrole β), 8.76 (4H, m, 12,13,17,18-pyrrole β), 8.21 (6H, m, *o*-phenyl), 7.81 (9H, m, *m*- and *p*-phenyl), −2.63 (2H, s, internal pyrrole). Finally, the insertion of manganese into the resulting porphyrin (**5**) with manganese (II) acetate gave 5-(1,3-dimethylimidazolium-2-yl)-10,15,20-triphenyl-porphinatomanganese (**6**): λ_{max} (CH_3OH) 374, 396, 463 and 558 nm.

Accordingly, we examined whether the resulting amphiphilic Mn-porphyrin worked as an anti-oxidant. The results of the catalytic activity measured by stopped-flow kinetic analysis (*8*) are summarized in Table 1. The catalytic rate constant of SOD activity (k_{dis}) for the MnMImP$_3$P (**6**) was $8.3 \pm 5.0 \times 10^6$ M^{-1}s^{-1}. Although $O_2^{\bullet-}$ is known to react with nitric oxide (NO) at rate of at least 3.7×10^7 M^{-1}s^{-1} to form the powerful oxidant peroxynitrite ($ONOO^-$) (*9*), furthermore, the MnMImP$_3$P was capable of decomposing $ONOO^-$. The rate constant of $ONOO^-$ decomposition (k_{dec}) for the MnMImP$_3$P was $1.0 \pm 0.1 \times 10^6$ M^{-1}s^{-1}.

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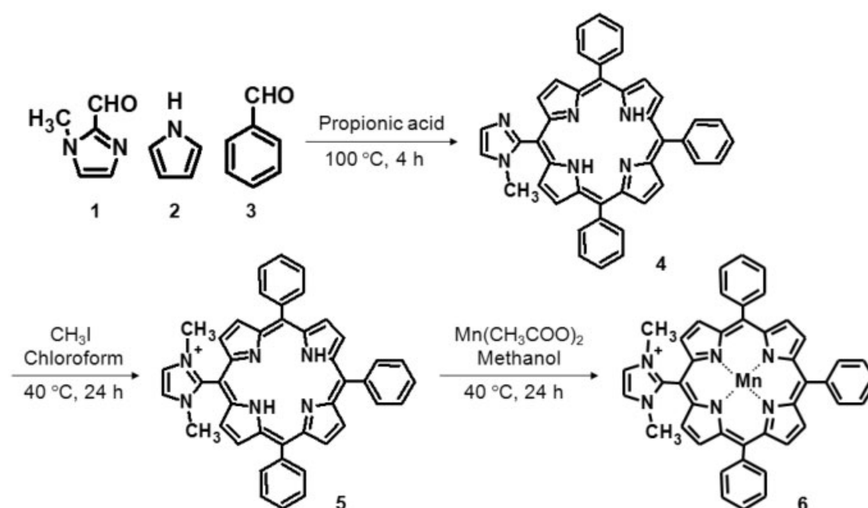

Scheme 1 Synthesis of 5-(1,3-dimethylimidazlium-2-yl)-10,15,20-triphenylporphinatomanganese ($MnMImP_3P$).

Table 1. SOD (k_{dis}), peroxynitrite decomposition (k_{dec}) activity and partition coefficient of Mn-porphyrins[a].

Mn-porphyrin	k_{dis} ($10^6\,M^{-1}s^{-1}$)	k_{dec} ($10^6\,M^{-1}s^{-1}$)	log P_{ow}
$MnMPy_4P$	20 ± 3.5	2.0 ± 0.6	N.D.
$MnMIm_4P$	57 ± 2.6	4.8 ± 0.2	N.D.
$MnMIm_2P_2P$	12 ± 0.9	1.6 ± 0.2	−1.11
$MnMImP_3P$	8.3 ± 5.0	1.0 ± 0.1	4.78

[a]The decay of O_2^- was spectrophotometrically monitored at 245 nm. The absorbance data were processed to obtain an observed rate constant ($k_{dis,obs}$) which was calculated from the slope of the plot of ln(absorbance at 245 nm) versus time. The k_{dis} was determined from the slope of a plot of $k_{dis,obs}$ versus the concentration of the Mn-porphyrin. The decay of $ONOO^-$ was spectophotometrically monitored at 302 nm. The k_{dec} was determined in the presence of 2 mM ascorbic acid as above. Partition coefficients ($logP_{ow}$) were determined using equal volumes of water and 1-octanol. N.D.: Not detectable.

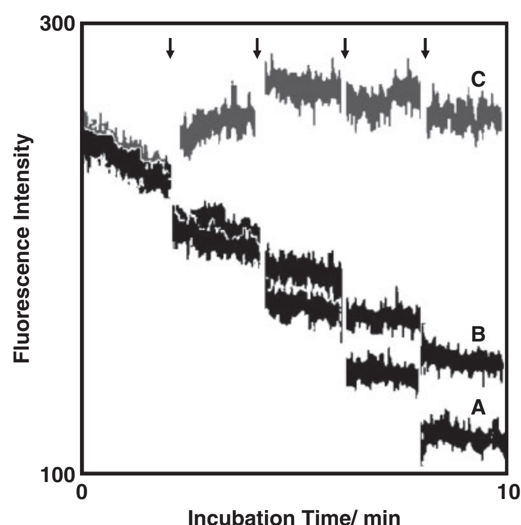

Fig. 1 Effect of Mn-porphyrins on the mitochondrial membrane potential measured by a tracer fluorescence dye, diS-C_3-(5): **(A)** $MnMIm_4P$, **(B)** $MnMPy_4P$, **(C)** $MnMImP_3P$. Mn-porphyrins (100 μM) were added to the mitochondrion solution in the presence of 20mM succinate and 0.1 μg/ml diS-C_3-(5) per 2 min (↓). The resulting fluorescence (ex. 622 nm, em. 670 nm) was chased for 10 min. The membrane potential was generated by adding succinate. Data are typical traces of the experiments repeated at least 2–3 times.

Control Mn-porphyrins, which were 5,10,15,20--tetrakis(1,3-dimethylimidazolium-2-yl)-porphinatomanganese ($MnMIm_4P$), 5,10,15,20-tetrakis(*N*-methyl-4-pyridyl)porphinatomanganese ($MnMPy_4P$) and di(1,3-dimethylimidazolium-2-yl)di-phenylporphinatomanganese ($MnMIm_2P_2P$) exhibited higher SOD and $ONOO^-$ decomposition activity than the $MnMImP_3P$ with one cationic groups to exhibit enzyme activity. It can be said that the $MnMImP_3P$ showed significant SOD and $ONOO^-$ decomposition activity despite the substitution of non-ionic phenyl groups for cationic dimethylimidazolium. However, the $MnMImP_3P$ was shown to have the highest $logP_{ow}$ value among the Mn-porphyrins in this study. The $logP_{ow}$ value of the $MnMImP_3P$ was +4.78, which was almost same hydrophobicity of a mitochondrion-targeted anti-oxidant, $MitoQ_{15}$ ($logP_{ow} = +4.30$) (*10*). Therefore, if the $MnMImP_3P$ are concentrated at an intracellular site, mitochondrion, the apparent activity of the enzyme is expected to increase.

To confirm the recognition ability of the $MnMImP_3P$ for mitochondria, we examined the effect of the $MnMImP_3P$ on the membrane potential of the isolated mitochondria from human neuroblastoma SH-SY5Y cells. Figure 1 shows the effect of the Mn-porphyrins on the mitochondrial membrane potential estimated by fluorescence intensity attributed to a tracer dye, 3,3′-dipropyl-2,2′-thiadicarbocyanine iodide [diS-C_3-(5)] (Cosmo Bio Co., Ltd., Tokyo, Japan) (*11*). An increase in fluorescence intensity is considered to be a decrease in membrane potential, due to the release of diS-C_3-(5) from mitochondria. The $MnMImP_3P$ exhibited a little increase in fluorescence intensity, which suggested a little decrease in mitochondrial membrane potential. The control Mn-porphyrins, $MnMIm_4P$ and $MnMPy_4P$, on the

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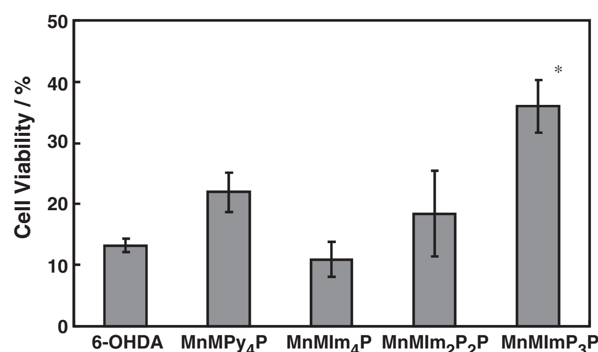

Fig. 2 Effect of the Mn-porphyrins on the viability of human neuroblastoma SH-SY5Y cells. After 4 h preincubation with each Mn-porphyrin (1 μM), 150 M 6-OHDA was added to the cells (2.3×10^4 cells/well) in the Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and incubated for 20 h. By further incubation for 4 h, the cell viability was measured by Alamar Blue assay. Symbols and error bars represent the mean and standard deviation of the measurements made in paired samples ($n = 5$). Asterisk indicates statistical significance ($P < 0.005$) when compared with each control value.

other hand, exhibited a decrease in fluorescence intensity, presumably owing to the dilution by adding the Mn-porphyrin solution. The $MnMImP_3P$ is, therefore, considered to be interacted with the mitochondrial membrane, leading to the release of the fluorescence dye from the mitochondrial membrane. However, there seem to be some possibilities of the mechanisms for mitochondrial potential down, such as direct or indirect inhibition of electron transfer.

To prove the recognition ability of the $MnMImP_3P$ for mitochondria, we finally examined whether the $MnMImP_3P$ protected the death of human neuroblastoma cells against experimental PD model. Cell death mechanisms could be studied through the use of mitochondrial complex I neurotoxins, such as 6-hydroxydopamine (6-OHDA) (*12*). 6-OHDA is one of the most commonly used neurotoxins in *in vitro* and *in vivo* experimental PD models, which induces apoptosis in the catecholaminergic cells specifically. 6-OHDA can produce reactive oxygen species such as $O_2^{\bullet-}$ *in vivo* as well as *in vitro*. Figure 2 shows the effect of the Mn-porphyrins on the viability of the human neuroblastoma SH-SY5Y cells incubated with 6-OHDA. Although this simple *in vitro* experiment is considered to be a cause of parkinsonian conditions, because PD is much complex pathological conditions, it is worth noting that the $MnMImP_3P$ increased cell viability from 13% to 36% [by Alamar Blue assay (*13*)], where the $MnMImP_3P$ itself exhibited no cytotoxicity. On the other hand, the control $MnMIm_4P$ exhibited no increase in the cell viability, and another control $MnMIm_2P_2P$ did not significantly ($P > 0.1$) increase the cell viability. Although the control $MnMPy_4P$ increased the cell viability, furthermore, the increase by the $MnMPy_4P$ was less than that by the $MnMImP_3P$. These results suggest that the $MnMImP_3P$ passed through cell membrane and accumulated in mitochondria to produce $O_2^{\bullet-}$. If the $MnMImP_3P$ is accumulated and membrane potential is decreased, the cells would be affected by the $MnMImP_3P$ because the cellular energy production would be down-regulated. However, the $MnMImP_3P$ was non-toxic, presumably due to a little degree of the membrane potential decrease, which affected no significant cell viability.

To support the above hypothesis, we examined the amount of lipid hydroperoxide generated by oxidative stress in mitochondria and the activity of aconitase, existing mitochondria and cytoplasm, inactivated by $O_2^{\bullet-}$. The amount of lipid hydroperoxide in mitochondria in this PD model was decreased by the administration of the $MnMImP_3P$ (Supplementary Fig. S1). The $MnMImP_3P$ also recovered the aconitase activity (Supplementary Fig. S2). Both anti-oxidative effects of the $MnMImP_3P$ was higher than that of the control $MnMPy_4P$, in spite of the lower SOD activity of the $MnMImP_3P$ ($k_{dis} = 8.3 \times 10^6\,M^{-1}s^{-1}$; Table 1), which supported that the $MnMImP_3P$ accumulated in mitochondria by adequate hydrophobicity ($\log P_{ow} = +4.78$; Table 1). Moreover, 6-OHDA exposure is reported to induce up-regulation of neuronal nitric oxide synthase (nNOS) and inducible NOS (iNOS), resulting in NO generation (*14*). If $O_2^{\bullet-}$ and NO form $ONOO^-$, the $MnMImP_3P$ ($k_{dec} = 1.0 \times 10^6\,M^{-1}s^{-1}$; Table 1) is considered to decompose the resulting $ONOO^-$. Although there are other possibilities for the protection mechanism, such as the suppression of the auto-oxidation of 6-OHDA generating H_2O_2, which cause cytotoxicity (*15*), Mn-porphyrin is known to exhibit no significant catalase activity, which catalyses the reduction from H_2O_2 to H_2O (*16*).

Taking these results into account, the amphiphilic Mn-porphyrin $MnMImP_3P$ is considered to be the promising anti-oxidant for mitochondrial targeting. Although a detailed analysis concerning the direct evidence that the $MnMImP_3P$ exerts protective effects as the anti-oxidant is now in progress, the chemical versatility of Mn-porphyrins and their structure–activity relationship are generally expected to be potential approach to control the intracellular distribution of antioxidants.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

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