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Design of Manganese Porphyrin Modified with Mitochondrial Signal Peptide for a New Antioxidant

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Abstract: A new design of antioxidant, consisting of manganese (Mn) porphyrin and signal peptide for mitochondrial targeting, is reported. The resulting Mn-porphyrin−oligopeptide conjugate exhibited significant superoxide dismutase (SOD) activity and decomposed peroxynitrite ($ONOO^-$). The new antioxidant caused the swelling of isolated mitochondria. By using the pH-sensitive drug carrier for intracellular delivery, furthermore, the new conjugate recovered the viability of lipopolysaccharide (LPS)-stimulated macrophage RAW 264.7 cells. These results suggest that the Mn-porphyrin modified with signal peptide for mitochondrial targeting is promising for a new class of antioxidants.

Keywords: Water-soluble manganese porphyrin; superoxide dismutase activity; signal peptide; mitochondrion; organella targeting

Here we have reported a new design of antioxidant consisting of manganese (Mn) porphyrin and signal peptide for mitochondrial targeting. Mitochondria are the major source of superoxide and are responsible for oxidative damage, e.g., neurodegenerative disorders such as Alzheimer's and Parkinson's diseases.[1] In mitochondria, manganese superoxide dismutase (Mn-SOD) is the key enzyme for the protection of oxidative stress.[2] We have already reported that water-soluble Mn-porphyrins with SOD activity work as antioxidants in various biochemical fields.[3,4] If the availability of antioxidant drugs at the mitochondrial level within cells were to increase, the radical approach to therapy would be promising. In this study, we have designed the Mn-porphyrin modified with signal peptide for mitochondrion targeting (Figure 1). This paper describes the design and characterization of the resulting Mn-porphyrin−oligopeptide conjugate in the context of its catalytic activity, mitochondrial swelling, and protection of cells from oxidative damage; detailed studies on the structure of the Mn-porphyrin−oligopeptide conjugate and the mechanism of antioxidative activity are outside the scope of the present study.

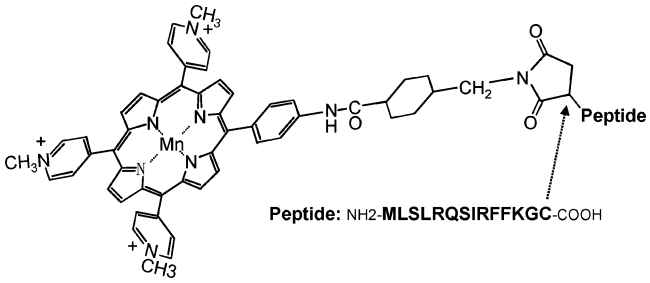

Figure 1. Chemical structure of Mn-porphyrin modified with signal peptide.

To modify Mn-porphyrin with signal peptide, we first synthesized 5-(4-aminophenyl)-10,15,20-tris(*N*-methyl-4-pyridyl)porphine [(APh)MPy_3P] according to the literature.[5] Then, an amino group of (APh)MPy_3P was reacted with succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) as cross-linking reagent between amino and thiol groups, followed by Mn introduction.[6] The maleimidomethyl group of SMCC was subsequently reacted[7] with

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(1) Dessolin, J.; Schuler, M.; Quinart, A.; De Giorge, F.; Ghosez, L.; Ichas, F. Selective targeting of synthetic antioxidants to mitochondria: towards a mitochondrial medicine for neurodegenerative diseases? *Eur. J. Pharmacol.* **2002**, *447*, 155−161.

(2) Quijano, C.; Hernandez-Saavedra, D.; Castro, L.; McCord, J. M.; Freeman, B. A.; Radi, R. Reaction of peroxynitrite with Mn-superoxide dismutase. Role of the metal center in decomposition kinetics and nitration. *J. Biol. Chem.* **2001**, *276*, 11631−11638.

(3) Asayama, S.; Mizushima, K.; Nagaoka, S.; Kawakami, H. Design of Metalloporphyrin-carbohydrate conjugates for a new superoxide dismutase mimic with cellular recognition. *Bioconjugate Chem.* **2004**, *15*, 1360−1363.

(4) Asayama, S.; Mori, T.; Nagaoka, S.; Kawakami, H. Chemical modification of manganese porphyrins with biomolecules for new functional antioxidants. *J. Biomater. Sci., Polymer Ed.* **2003**, *14*, 1169−1179.

(5) Kawakami, H.; Ohse, T.; Kawano, M.; Nagaoka, S. Superoxide dismutase activity of a novel macromolecular manganese porphyrin. *Polym. Adv. Technol.* **1999**, *10*, 270−274.

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Table 1. SOD (k_{dis}) and Peroxynitrite Decomposition (k_{dec}) Activity of Mn-Porphyrins

Mn-porphyrin	k_{dis} [10^6 M^{-1} s^{-1}]	k_{dec} [10^4 M^{-1} s^{-1}]
$MnMPy_4P$	11 ± 2.5	5.2 ± 1.3
$MnMPy_3P$−peptide[a]	1.8 ± 0.8	1.8 ± 0.7

[a] The decay of $O_2^{\bullet-}$ was spectrophotometrically monitored at 245 nm. The absorbance data (0.132−0.039; 0−20 ms) 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 (2−6 μM) of the Mn-porphyrin, dissolved in phosphate buffer containing a slight amount of trifluoroacetic acid. Furthermore, the decay of $ONOO^-$ was spectrophotometrically monitored at 302 nm. The absorbance data (0.249−0.111; 0−0.3 s) were processed to obtain an observed rate constant ($k_{dec,obs}$) which was calculated from the slope of the plot of ln(absorbance at 302 nm) versus time. The k_{dec} was determined from the slope of $k_{dec,obs}$ versus the concentration (0−5 μM) of the Mn-porphyrin. The rate constants were determined from the mean at least 3 experiments.

the thiol group of C-terminal cysteine of mitochondrial leader sequence[8] with glycine spacer (Met-Leu-Ser-Leu-Arg-Gln-Ser-Ile-Arg-Phe-Phe-Lys-Gly-Cys). The conjugation and the absence of free signal peptide were confirmed by gel permeation chromatography using *N*,*N*-dimethylformamide as a mobile phase and sulfhydryl determination using 4,4′-bis-dimethylaminodiphenylcarbinol.[9]

Accordingly, we examined whether the resulting Mn-porphyrin−oligopeptide conjugate ($MnMPy_3P$−peptide) worked as an antioxidant. The results of the catalytic activity measured by stopped-flow kinetic analysis are summarized in Table 1. The catalytic rate constant of SOD (k_{dis}) for the conjugate was $1.8 \pm 0.8 \times 10^6$ M^{-1} s^{-1}, while that for a control Mn-porphyrin, 5,10,15,20-tetrakis(*N*-methyl-4-pyridyl)porphinatomanganese ($MnMPy_4P$), was $11 \pm 2.5 \times 10^6$ M^{-1} s^{-1} under these conditions. Although superoxide ($O_2^{\bullet-}$) 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 ($ONOO^-$),[10] furthermore, the conjugate was capable of decomposing $ONOO^-$. The rate constant of $ONOO^-$ decomposition (k_{dec}) for the conjugate was $1.8 \pm 0.7 \times 10^4$ M^{-1} s^{-1}, while that for $MnMPy_4P$ was $5.2 \pm 1.3 \times 10^4$ M^{-1} s^{-1} under these conditions. It can be said that the Mn-porphyrin−oligopeptide conjugate showed significant SOD and $ONOO^-$ decomposition activity despite the chemical modification of the metalloporphyrin. If the new antioxidants are concentrated at an intracellular site, mitochondrion, the apparent activity of the enzyme is expected to increase.

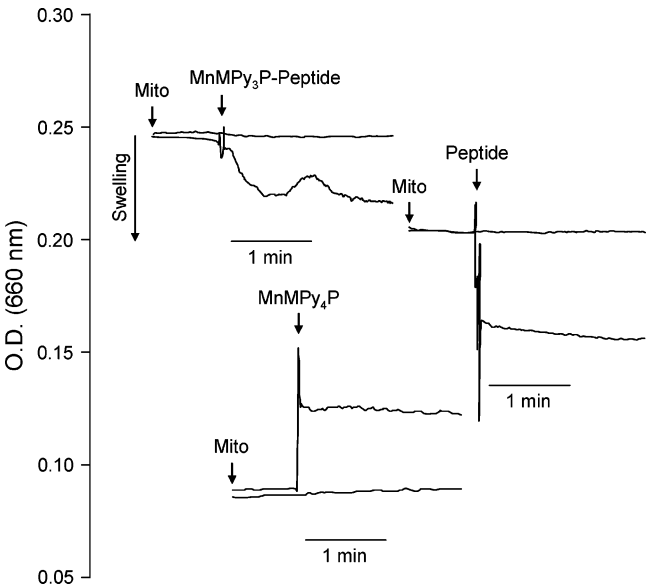

Figure 2. The Mn-porphyrin modified with signal peptide induced swelling in liver mitochondria suspended in KCl assay medium supplemented with ATP and Mg^{2+}. Arrows indicate timing of addition of mitochondria (Mito), signal peptide (Peptide), unmodified Mn-porphyrin ($MnMPy_4P$), and the Mn-porphyrin modified with signal peptide ($MnMPy_3P$−Peptide).

To confirm the recognition ability of the resulting Mn-porphyrin−oligopeptide conjugate for mitochondria, we examined the effect of the conjugate on the permeability of rat liver mitochondria by mitochondrial swelling assay.[11] Figure 2 shows the mitochondrial swelling in KCl media supplemented with ATP and Mg^{2+} in the presence of the conjugate. A decrease in light scattering is considered to be an increase in mitochondrial volume, so that the Mn-porphyrin−oligopeptide caused mitochondrial swelling. The signal peptide alone also exhibited a decrease in the light

(6) A typical procedure is as follows: (APh)MPy_3P (5 mg) and SMCC (10 mg) were dissolved in 1.1 mL of dimethyl sulfoxide containing 2.7 μL of triethylamine. The reaction mixture was incubated at 38 °C for 20 h, followed by dialysis against distilled water using a Spectra/Por cellulose ester membrane (molecular weight cutoff = 500) to remove unreacted SMCC. The resulting porphyrin obtained by freeze−drying was then incubated with 3 equiv of $Mn(OAc)_2 \cdot 4H_2O$ in H_2O at 38 °C for 48 h. The reaction was monitored by UV−visible spectroscopy and was stopped when the Soret band shift was completed (from 422 to 464 nm). The reaction mixture was dialyzed again against distilled water using a Spectra/Por cellulose ester membrane (molecular weight cutoff = 500) for 3 days to remove unreacted $Mn(OAc)_2$. The resulting Mn-porphyrin was obtained by freeze−drying.

(7) A typical procedure is as follows: The resulting (APh)MPy_3P−SMCC (19 mg) and the mitochondrial leader sequence (8.7 mg) were dissolved in 1.1 mL of dimethyl formamide. The reaction mixture was incubated at 37 °C for 3 days, followed by dialysis against distilled water using a Spectra/Por 7 membrane (molecular weight cutoff = 10^3) to remove unreacted (APh)MPy_3P−SMCC. The resulting Mn-porphyrin was obtained by freeze−drying.

(8) Lu, Y.; Beavis, A. D. Effect of leader peptides on the permeability of mitochondria. *J. Biol. Chem.* **1997**, *272*, 13555−13561.

(9) Rohrbach, M. S.; Humphries, B. A.; Yost, F. J., Jr.; Rhodes, W. G.; Boatman, S.; Hiskey, R. G.; Harrison, J. H. The reaction of 4,4′-*bis*-dimethylaminodiphenylcarbinol with the sulfhydryl group. A new reagent for sulfhydryl analysis. *Anal. Biochem.* **1973**, *52*, 127−142.

(10) Saran, M.; Michel, C.; Bors, W. Reaction of NO with $O_2^{\bullet-}$ implications for the action of endothelium-derived relaxing factor (EDRF). *Free Radical Res. Commun.* **1990**, *10*, 221−226.

(11) Kushnareva, Y. E.; Polster, B. M.; Sokolove, P. M.; Kinnally, K. W.; Fiskum, G. Mitochondrial precursor signal peptide induces a unique permeability transition and release of cytochrome c from liver and brain mitochondria. *Arch. Biochem. Biophys.* **2001**, *386*, 251−260.

scattering. Therefore, the signal peptide alone opened the mitochondrial inner membrane pore, leading to permeability transition and thereby swelling. The control Mn-porphyrin $MnMPy_4P$ alone, on the other hand, exhibited an increase in light scattering, presumably owing to nonspecific formation of aggregation between mitochondria. These results suggest that the Mn-porphyrin−oligopeptide conjugate had potential ability in targeting mitochondria.

Finally, we examined whether the Mn-porphyrin−oligopeptide conjugate worked as antioxidant in lipopolysaccharide (LPS)-stimulated macrophage RAW 264.7 cells. LPS treatment produced NO synthesis and NO-mediated apoptosis.[12] NO-mediated apoptosis is generally considered to be mediated by DNA or mitochondrial damage.[13] It is likely that the reaction of NO with $O_2^{\bullet-}$ generated from mitochondria produced $ONOO^-$, accelerating cell damage. Figure 3 shows the effect of the Mn-porphyrin−oligopeptide on the cell viability of RAW 264.7 stimulated with LPS. The Mn-porphyrin−oligopeptide $MnMPy_3P$−peptide as well as the control $MnMPy_4P$ increased cell viability from 20% to 40% (by Alamar Blue assay[14]), where these Mn-porphyrins themselves exhibited no cytotoxicity. Under these experimental conditions (in the absence of drug carrier), the effect of mitochondrial signal peptide is unclear, presumably owing to endosomal/lysosomal degradation. Therefore, we used aminated poly(L-histidine),[15,16] $PLH\text{-}NH_2$, as drug carrier for endosomal/lysosomal escape. It should be noted that the administration after complex formation of $MnMPy_3P$−peptide with $PLH\text{-}NH_2$ enhanced cell viability up to 80%. The control $MnMPy_4P$, on the other hand, did not significantly enhance the viability in spite of the complex formation of $PLH\text{-}NH_2$. These results suggest that the $MnMPy_3P$−peptide exhibited antioxidative activity against LPS-stimulated macrophages by intracellular and mitochondrial delivery.

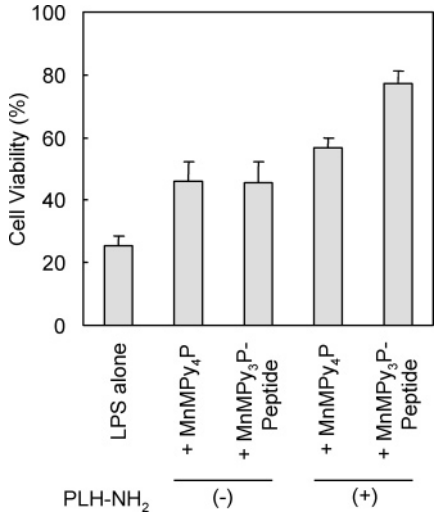

Figure 3. Effect of the Mn-porphyrins on the viability of RAW 264.7 cells (2×10^4 cells/well) stimulated with LPS (20 μg/mL) for 48 h in EMEM supplemented with 10% heat-inactivated fetal bovine serum. By further incubation for 5 h, the cell viability was measured by Alamar Blue assay. Before stimulation, each Mn-porphyrin (10 μM) was incubated for 24 h in the presence (+) or absence (−) of aminated poly(L-histidine) ($PLH\text{-}NH_2$) (1 mg/mL) as drug carrier. Symbols and error bars represent the mean and standard deviation of the measurements made in quadruplicate wells.

In conclusion, we have designed the Mn-porphyrin modified with mitochondrial signal peptide for a new class of antioxidant. The antioxidative activity of the resulting Mn-porphyrin−oligopeptide conjugate suggests that the Mn-porphyrin could protect mitochondria from oxidative damage, which is the topic of our future investigation. The new antioxidant designed in this study is expected to be the first artificial enzyme targeting mitochondria by using the signal peptide.

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(12) Yoo, Y. H.; Lim, Y. J.; Park, S. E.; Kim, J. M.; Park, Y. C. Overexpression of redox factor-1 negatively regulates NO synthesis and apoptosis in LPS-stimulated RAW 264.7 macrophages. *FEBS Lett.* **2004**, *556*, 39−42.

(13) Brüne, B.; von Knethen, A.; Sandau, K. B. Nitric oxide and its role in apoptosis. *Eur. J. Pharmacol.* **1998**, *351*, 261−272.

(14) Unsworth, J. M.; Rose, F. R. A. J.; Wright, E.; Scotchford, C. A.; Shakesheff, K. M. Seeding cells into needled felt scaffolds for tissue engineering applications. *J. Biomed. Mater. Res.* **2003**, *53*, 617−620.

(15) Asayama, S.; Kawakami, H.; Nagaoka, S. Facile chemical modification of the poly(L-histidine) for a new pH-sensitive polypeptide. *Chem. Lett.* **2003**, *32*, 1152−1153.

(16) Asayama, S.; Sekine, T.; Hamaya, A.; Kawakami, H.; Nagaoka, S. Poly(L-histidine) with several aminoethyl groups for a new pH-sensitive DNA carrier. *Polym. Adv. Technol.* **2005**, *16*, 567−570.