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NOVEL IRON COMPLEXES BEHAVE LIKE SUPEROXIDE DISMUTASE *IN VIVO*

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Novel iron and copper complexes having *tris*[N-(5-methyl-2-pyridylmethyl)-2-aminoethyl]amine (5MeTPAA), *tris*[N-(3-methyl-2-pyridylmethyl)-2-aminoethyl]amine (3MeTPAA), *tris*[N-(5-methoxycarbonyl-2-pyridylmethyl)-2-aminoethyl]amine (TNAA), *tris*[(2-thienylmethyl)-2-aminoethyl]amine (TTAA), *tris*[(2-furylmethyl)-2-aminoethyl]amine (TFAA) or *tris*[(2-imidazolyl)-2-aminoethyl]amine (TIAA) as ligand, were synthesized to examine the superoxide dismutase (SOD) activity. The concentrations of Fe-3MeTPAA and Fe-TIAA equivalent to 1 unit of SOD (IC_{50}) were 0.5 μM and 1.0 μM, respectively. Fe-3MeTPAA and Fe-TIAA had higher SOD activity than other Fe and Cu complexes and protected *Escherichia coli* cells from paraquat toxicity. In case of using *tris*[N-(6-methyl-2-pyridylmethyl)-2-aminoethyl]amine (6MeTPAA) as ligand, the Fe complex could not be obtained, which may be due to the steric hindrance of 6-methyl substituent. Generally, Cu complexes had low SOD activity, compared with Fe complexes, and could not suppress paraquat toxicity.

KEY WORDS: Superoxide, superoxide dismutase (SOD), SOD mimic, paraquat, *Escherichia coli*, iron complex.

INTRODUCTION

Superoxide dismutase (SOD) mimics may be useful as pharmaceuticals, because SOD provides a defense system against several diseases in which superoxide ($O_2^{\cdot -}$) appears to play an important role. Copper-amino acids or peptides,[1-3] copper-salicylates,[4-6] copper-penicillamine,[7,8] copper-polyamine,[9,10] copper-*o*-phenanthroline,[11] copper-cimetidine[12] and manganese-desferrioxamine[13-15] have been known as potent SOD mimics. Copper-diisopropylsalicylate has been reported to have anti-tumor effects.[6] SQD activities of copper complexes, however, are generally *in vivo* lowered by chelating agent such as proteins, which are ordinarily found in living cells. There may be some problems concerning SOD actitivies of these copper complexes *in vivo*.

There is little research about SOD mimics containing iron and manganese, though Mn-desferrioxamine has been reported to be effective *in vivo* by Fridovich and other investigators.[13,15]

We recently reported that two iron complexes, Fe(II)-tetrakis-N,N,N′,N′-(2-pyridylmethyl)ethylenediamine (Fe-TPEN) and Fe(III)-*tris*[N-(2-pyridylmethyl)-2-aminoethyl]amine (Fe-TPAA), have high SOD activity and can protect *Escherichia coli* cells from paraquat toxicity.[16] These complexes behave like SOD both *in vitro* and *in vivo*, because the complexes can inhibit ferricytochrome c reduction by xanthine–xanthine oxidase system and suppress the toxicity of paraquat. Paraquat has been

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reported to enhance extremely the generation of O_2^- in *E. coli* cells and do damage to the cells.[17]

Now we synthesized several, novel metal complexes containing TPAA analogues as ligand. This paper reports SOD activities of these complexes *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Tris-2-aminoethylamine, methyl-6-methyl nicotinate, 2-imidazole carboxy aldehyde, 2-furaldehyde, 6-methyl-2-pyridine carboxyaldehyde and 2-thiophene carboxyaldehyde were purchased from Aldrich. $FeSO_4 \cdot 7H_2O$ and 2,5-lutidine were from Wako Pure Chemical Industry Ltd. $CuSO_4 \cdot 5H_2O$ was from Kanto Chemical Co, Ltd. 2,3-Lutidine was from Tokyo Kasei Kogyo Ltd. Agar and bacto-tryptone were from Difco Laboratories. Cytochrome c (Type III), bovine serum albumin, xanthine sodium salt, methyl viologen (paraquat), cyanocobalamin (vitamin B_{12}) and glucose were from Sigma. Xanthine oxidase was from Boehringer Mannheim GmbH.

Lethality of E. coli

E. coli B B_{12} (ATCC29682) was kindly provided by Dr. I. Fridovich. Loss of viability was determined as follows. The incubation system (10.0 ml) contained 2×10^7 *E. coli* cells/ml and glucose (0.5 w/v) in 10 mM phosphate buffer (pH 7.4). Viable cells were enumerated by spreading appropriate dilutions, in triplicate, on agar plates containing 2% agar, 1% bacto-tryptone and 0.5% sodium chloride. The survival ratios were evaluated from colony counts after overnight incubation at 37°C.

Assay of SOD Activity

SOD activities were measured according to the method of Ref 18 except omitting EDTA.

Syntheses of Chelators and their Metal Complexes

Tris[N-(3-methyl-2-pyridylmethyl)-2-aminoethyl]amine (3-Me-TPAA) 2,3-Lutidine (500 mg) reacted with I_2 (820 mg) to make lutidyliodide. The iodide dissolved in a small amount of dimethylsulfoxide (DMSO) was added to 10 ml of DMSO heated to 120–130°C. After neutralized with aqueous Na_2CO_3 solution and extracted with ether, 3-methylpyridine-2-aldehyde (440 mg) was obtained in 80%. Full details of the procedure can be found.[19] The aldehyde (330 mg) and *tris*(2-aminoethyl)amine (97 mg) were dissolved in anhydrous methanol, and the reaction mixture was refluxed under an argon atmosphere. After 3 h, $NaBH_4$ was added to the solution in order to reduce the schiff base. After evaporation of methanol, aqueous Na_2CO_3 solution was added to the residue and the product was extracted with CH_2Cl_2. The CH_2Cl_2 extract was dried over anhydrous Na_2CO_3 and evaporated to form a yellow oil (840 mg). ^1H NMR ($CDCl_3$/TMS):

δ2.1 (s, 9H) δ2.5 (br, 12H) δ2.7 (br, 3H, D_2O exchangeable)
δ3.7 (s, 6H) δ7.0 (m, 6H) δ8.2 (br, 3H)

Tris[N-(6-methyl-2-pyridylmethyl)-2-aminoethyl]amine (6MeTPAA), tris[N-(5-methyl-2-pyridylmethyl)-2-aminoethyl]amine (5MeTPAA), tris[N-(5-methoxycarbonyl-2-pyridylmethyl)-2-aminoethyl]amine (TNAA), tris[(2-thienylmethyl)-2 aminoethyl]amine (TTAA), tris[(2-furylmethyl)-2-aminoethyl]-amine (TFAA) or tris[(2-imidazolyl)-2-aminoethyl]amine (TIAA) The corresponding aldehydes were allowed to react with *tris*(2-aminoethyl)amine in a similar manner as above, and then the schiff bases were reduced by $NaBH_4$ to form the desired amines (Scheme I).

6MeTPAA:^{1}H NMR ($CDCl_3$/TMS),
δ2.5 (s, 9H) δ2.5 (br, 3H, D_2O exchangeable) δ2.7 (br, 12H)
δ3.9 (s, 6H) δ7.3 (m, 9H)

5MeTPAA:^{1}H NMR ($CDCl_3$/TMS),
δ2.3 (s, 9H) δ2.3 (br, 3H, D_2O exchangeable) δ2.7 (br, 12H)
δ3.9 (s, 6H) δ7.3 (m, 6H) δ8.3 (s, 3H)

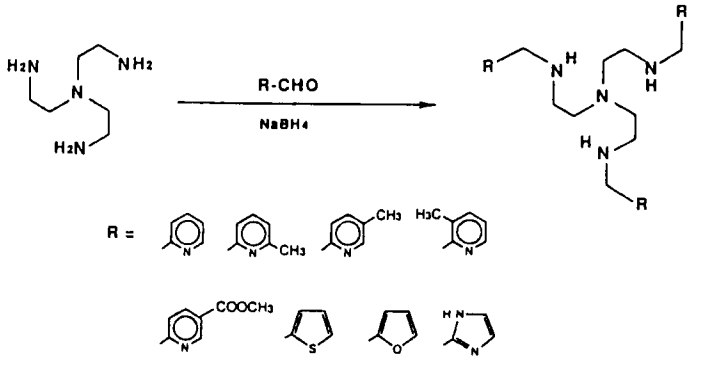
TNAA:^{1}H NMR ($CDCl_3$/TMS):
δ2.6 (br, 12H) δ2.6 (br, 3H, D_2O exchangeable) δ3.8 (s, 9H)
δ3.8 (s, 9H) δ3.8 (s, 6H) δ7.3 (d, 3H, J = 12 Hz)
δ8.2 (dd, 3H, J = 12 Hz, 4 Hz) δ8.9 (d, 3H, J = 4 Hz)

TTAA:^{1}H NMR ($CDCl_3$/TMS):
δ1.8 (s, 3H, D_2O exchangeable) δ2.6 (br, 12H) δ3.9 (s, 6H)
δ6.8 (m, 6H) 7.1 (m, 3H)

TFAA:^{1}H NMR ($CDCl_3$/TMS):
δ1.7 (s, 3H, D_2O exchangeable) δ2.5 (br, 12H) δ3.5 (s, 6H)
δ6.0 (m, 3H) δ6.2 (m, 3H) δ7.2 (m, 3H)

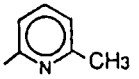
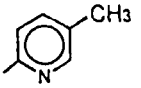
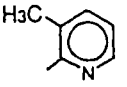
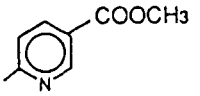
TIAA:^{1}H NMR ($CDCl_3$/TMS)
δ2.5 (br, 12H) δ3.9 (s, 6H) δ6.9 (br, 6H, D_2O exchangeable)
δ7.3 (s, 6H)

Syntheses of Chelators



SCHEME I

TABLE I
Absorption Maximum of Iron and Copper Complexes

	R	abbreviation	Fe Complex		Cu Complex	
			λ_{max} (nm)	ε	λ_{max} (nm)	ε
1		TPAA	590	2,300	661	150
2		6-Me TPAA	—	—	783	180
3		5-Me TPAA	593	2,000	624	140
4		3-Me TPAA	589	2,800	644	150
5		TNAA	634	3,530	621	140
6		TTAA	300(s)	2,200	833	170
7		TFAA	304	4,000	822	190
8		TIAA	530(s)	4,000	643	120

The spectrum was measured in 5 min after forming complex in 50 mM phosphate buffer pH 7.4. [chelator]/[metal] > 1.5 in all cases.

Formation of the Metal Complexes The reaction of these chelators except 6MeTPAA with ferric ion in methanol formed corresponding complexes, whose absorption maxima in water are shown in Table I. In case of using 6MeTPAA as ligand, the Fe complex could not be obtained, which may be due to the steric hindrance of 6-methyl substituent. Copper complexes were prepared in a similar manner, whose absorption maxima in water are also shown in Table I. All of the isolated complexes are readily dissolved both in water and in polar organic solvents such as methanol, methylene chloride etc.

RESULTS

Table II shows the superoxide dismuting activities of iron and copper complexes determined by xanthine–xanthine oxidase-cytochrome c method. The IC_{50} in Table II means the concentration of the complex which exerts the SOD activity equivalent to 1 unit of native SOD. In the iron complexes, Fe(III)-3MeTPAA (IC_{50}: 0.5 μM) and

TABLE II
IC_{50} Values of Iron and Copper Complexes

	Fe Complex		Cu Complex	
abbreviation	IC_{50} (μM)	BSA inhibition	IC_{50} (μM)	BSA inhibition
TPAA	4.0	–	> 400	n.d.
6-Me TPAA	—	—	100	+ + +
5-Me TPAA	4.0	–	> 400	n.d.
3-Me TPAA	0.5	–	100	+
TNAA	6.0	–	40	+ +
TTAA	20	–	20	–
TFAA	10	–	10	–
TIAA	1.0	–	> 200	n.d.

n.d. = not determined

Xanthine oxidase (4.0 milliunits/ml) was added to 50 mM phosphate buffer (pH 7.4) solution including 10 μM cytochrome c, 50 μM xanthine and catalase (2,500 units/ml) in the presence of metal complex. [chelator]/[metal] > 1.5 in all cases.

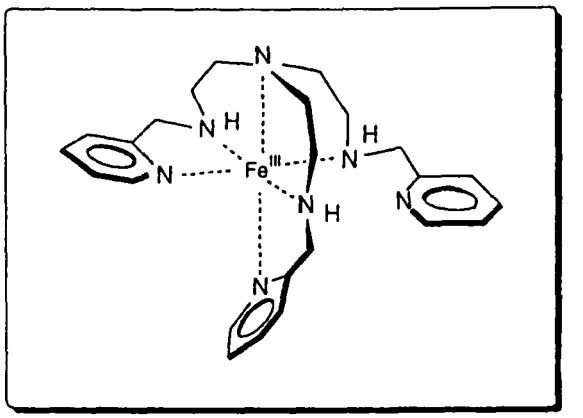
Fe(III)-TIAA (1.0 μM) exhibited relatively high SOD activities. Their activities were not inhibited by bovine serum albumin (BSA). The fact that Fe-TTAA and Fe-TFAA had low SOD activities indicates that nitrogen is better than sulfur and oxygen as ligand atom. Cu-TFAA was the best SOD mimic in the copper complexes. The IC_{50} values of Cu-TFAA and Cu-TTAA were 10 μM and 20 μM, respectively and exceptionally their activities were not inhibited by BSA, while other Cu complexes were inhibited. Cu-TPAA and Cu-5MeTPAA was inactive in the dismutation of superoxide. In case of using 6MeTPAA as ligand, Cu-6MeTPAA was easily formed, but decomposed by addition of BSA.

Table III demontrates the effects of these iron and copper complexes against *E. coli* toxicity by paraquat. All of iron complexes protected *E. coli* cells from paraquat toxicity. The activities increased with methyl or methyl carboxyl substituents on pyridine ring. Only Fe-TFAA and Fe-TTAA exacerbated paraquat toxicity at the high concentration (1.0 mM) (data not shown), though they suppressed the toxicity at the low concentration (0.1 mM). Copper complexes except Cu-TIAA had no protective effect against paraquat toxicity.

TABLE III
Effect of Iron and Copper Complexes on the Lethality of *E. coli* by Paraquat

	SURVIVAL (%)			
	Fe Complex		Cu Complex	
additives	30 min	60 min	30 min	60 min
Paraquat	28.7	14.8	31.2	12.8
TPAA	49.6	44.4	2.8	0.4
6-Me TPAA	—	—	4.5	0
5-Me TPAA	68.0	70.0	2.2	1.7
3-Me TPAA	73.8	60.5	9.1	0
TNAA	50.7	44.0	0	0
TTAA	68.0	45.2	2.2	0
TFAA	47.8	41.9	4.6	0
TIAA	70.9	61.7	41.5	37.5

Paraquat (1.0 mM) was added to the incubation system (10 mM phosphate buffer pH 7.4) including glucose (0.5% w/v) and 0.1 mM metal complex.



SCHEME II

DISCUSSION

The previous study showed that Fe-TPAA exhibits higher SOD activity than Fe-TPEN *in vivo*, although the former has lower SOD activity than the latter *in vitro*.[16] This study also demonstrates that the activities *in vitro* do not necessarily correspond to those *in vivo*. The location in the cell and/or the penetration into the membrane of the complexes may be the important factor(s) as much as their SOD activity. Generally, iron complexes containing TPAA derivatives exhibited high SOD activities, but Fe-TTAA and Fe-TFAA showed relatively low activities. This result demonstrates that the nitrogen atom is suitable as the iron ligands for the activities. The result that Fe-TTAA and Fe-TFAA exacerbated the paraquat toxicity at 1.0 mM may be due to the decomposition of these complexes in the living cells, though addition of BSA did not have any effect on their SOD activities. This result seems to confirm the hypothesis which the ligand containing nitrogen atom is essential for the formation of the stable complex.

Native SODs have three or four imidazole rings at the active center[20-23] and Fe-TIAA complex seems to resemble active sites of native SODs. But the activity of Fe-TIAA was nearly equal to that of Fe-TPAA, which shows that imidazole is not necessary for the activity. 6-Methyl substituted TPAA did not react with ferric ion. 6-Methyl functional group is probably too bulky to form the Fe complex. This assists the proposed hexacoordinated structure of Fe-TPAA shown in Scheme II.

Cu-TTAA and Cu-TFAA had relatively good activities in the copper complexes *in vitro*, and oxygen or sulfur atom appears to be more suitable for the SOD activity of Cu complex than nitrogen atom. But these copper complexes also could not decrease the paraquat toxicity. Copper complexes appear to be unstable in the living system.

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