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Mangansese-containing SOD Mimetics

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Abbreviations

$O_2^{\bullet -}$	superoxide
$ONOO^-$	peroxynitrite
NBT	nitroblue tetrazolium
SOD	superoxide dismutase
MnSOD	manganous-superoxide dismutase
ROS	reactive oxygen species
RNS	reactive nitrogen species

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1. Abstract

Superoxide dismutase (SOD) represents an essential defense system against oxygen-derived free radicals, specifically superoxide ($O_2^{\bullet -}$). Superoxide can initiate a series of free radical reactions that yield other oxygen radicals, which together are thought to act as inflammatory mediators and induce cellular damage. A vast number of diseases are believed to be caused by superoxide-mediated damage, and the addition of native SODs in these settings has had mixed success. This variability may in part be due to the limitations of native SOD enzyme. Thus, the development of SOD mimetics that overcome these limitations would serve beneficial in large number of clinical settings. This review will discuss the chemistry, biochemistry, and clinical applications of native SOD, highlighting its limitations. This review will further discuss an attractive class of SOD mimetics, the manganese-containing complexes, which have conquered some of the limitations posed by native SOD. Lastly, the methods of detecting SOD activity will be discussed highlighting the key differences and pitfalls of each approach.

2. Introduction

Superoxide ($O_2^{\bullet -}$) is generated during normal cellular respiration and many other cellular pathways. Once formed, $O_2^{\bullet -}$ can initiate a chain of free radical reactions, potentially yielding the highly reactive hydroxyl radical *via* the iron-catalyzed Fenton reaction (reactions 1-2) or peroxynitrite ($ONOO^-$) (reaction 3) [1].

$$Fe^{3+} + O_2^{\bullet -} \rightarrow O_2 + Fe^{2+} \quad \{1\}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet} \quad \{2\}$$

$$O_2^{\bullet -} + NO^{\bullet} \rightarrow ONOO^- \quad \{3\}$$

Superoxide and other oxygen radicals can react with biological targets (lipid, nucleic acids, catecholamines, receptors, *etc.*) causing inflammation and subsequent tissue damage (**Figure 1**) [1, 2]. Fortunately, aerobes have engineered overlapping systems to defend against the deleterious effects of reactive oxygen species (ROS). There exists a multitude of catalytic entities such as superoxide dismutase (SOD), glutathione peroxidase, and catalase; as well as non-catalytic antioxidants namely glutathione, ascorbate, α-tocopherol, uric acid, and others [3]. The SODs are enzymes that rapidly dismutate superoxide to yield the nonradical products dioxygen (O_2) and hydrogen peroxide (H_2O_2), thereby shunting superoxide away from deleterious reactions with potential biological targets. However, these defenses can be overwhelmed in many disease settings whereby ROS-induced cellular damage ensues [1].

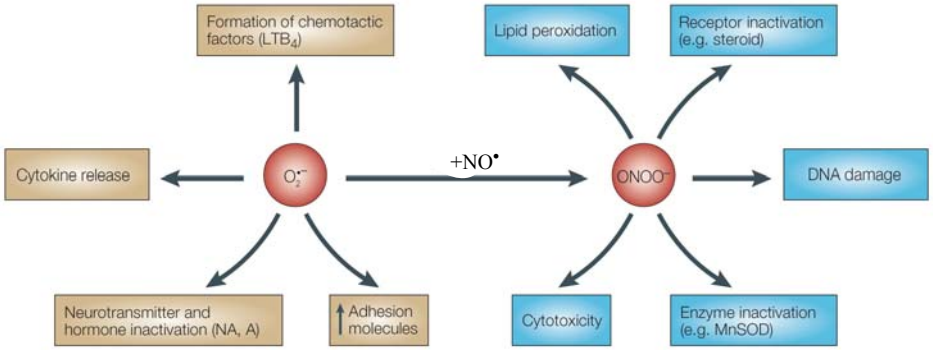

Fig. 1 Effects of superoxide on biological targets and inflammatory processes. A, adrenalin; LTB_4, leukotriene B_4; NA, noradrenalin; $ONOO^-$, peroxynitrite. Adapted from [11].

Thus, agents that possess SOD activity would prove to have valuable clinical applications. This review will focus on Mn-containing SOD mimetics for three main reasons: (i) Mn-containing SOD mimetics are highly stable, (ii) free Mn^{2+} is the least toxic (compared to $Fe^{2+/3+}$ and Cu^{2+}) and does not participate in Fenton chemistry, and (iii) mice deficient in the Mn-containing SOD succumbed to death (compared to mice lacking the Cu/Zn-SODs) [1]. The chemical and biochemical properties that are deemed important for SOD mimetic activity will be discussed. Further, this review will cover the assays available to detect SOD activity highlighting the key differences and pitfalls of each approach.

3. Properties of Native Superoxide Dismutase

3.1 Chemistry and Biochemistry

Superoxide dismutases are a class of oxidoreductases that contain either Mn (SOD2 or MnSOD), Cu/Zn (SOD1, SOD3), or Fe at the active site [4]. Human MnSOD is expressed in the mitochondria, whereas SOD1 is found in the cytosol and SOD3 in the extracellular compartment. These metalloproteins rapidly catalyze the dismutation of superoxide to yield O_2 and H_2O_2 (reaction 6). The proposed mechanism involves alternate reduction and oxidation of the metal (*e.g.*, Mn^{2+}/Mn^{3+}) by $O_2^{\bullet-}$ in a 'ping-pong' type fashion (reactions 4-6).

$$M^{n+} + O_2^{\bullet-} \rightarrow M^{(n-1)+} + O_2 \quad \{4\}$$

$$M^{(n-1)+} + O_2^{\bullet-} + 2H^+ \rightarrow M^{n+} + H_2O_2 \quad \{5\}$$

$$\text{Net: } 2\ O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2 \quad \{6\}$$

$$O_2^{\bullet-} \leftrightarrow O_2 + e^- \qquad E_m = -330 \text{ mV } (-160 \text{ mV}) \quad \{7\}$$

$$H_2O_2 \leftrightarrow O_2^{\bullet-} + 2H^+ + e^- \qquad E_m = +890 \text{ mV} \quad \{8\}$$

This 'ping-pong' mechanism is contingent on Mn-SOD (and the other SODs) having midpoint potentials that lie between the two redox couples involved in $O_2^{\bullet-}$ dismutation (reactions 7 and 8) [5]. Human Mn-SOD has a redox potential of 393 ± 29 mV, which allows efficient catalysis of both the oxidative

(reaction 4) and reductive (reaction 5) reactions. The k_{cat} for human MnSOD is approximately 2 x 10^8 M^{-1} s^{-1} under biological conditions [2]. These efficient reactions do not require reducing agents, and thus do not require cell energy to proceed.

3.2 Preclinical studies

Preclinical studies have shown that MnSOD has a protective role in animal models of ischemia-reperfusion injury, inflammation, tissue injury, Parkinson's disease, cancer, AIDS, and radiation therapy induced tissue damage [6]. These studies were performed using either exogenous native SOD or transgenic mice overexpressing MnSOD. Differences in efficacy between the two models were evident for diseases that necessitated the enzyme to cross the blood-brain barrier, i.e., Parkinson's disease and stroke-induced ischemia-reperfusion injury. In these settings, the exogenous native MnSOD showed no protective effects presumably due to its large size, ~88 kDa. Recombinant human MnSOD was also shown to exhibit a bell-shaped response curve. At low doses, MnSOD displayed anti-inflammatory effects whereas high doses exhibited pro-inflammatory effects [7]. At high doses, MnSOD is thought to react with its dismutation product H_2O_2 to generate hydroxyl radicals via Fenton chemistry [8].

3.3 Clinical Studies

Human clinical studies with Orgotein® (bovine CuZnSOD) revealed positive anti-inflammatory effects in patients suffering from rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, and radiation therapy-induced damage (e.g., pulmonary fibrosis) [11]. However, Orgotein® was eventually pulled from the market due to several major side effects, primarily arising from its immunogenicity. These studies revealed the major limitations of using native SOD therapeutically (**Table 1**). Thus, synthetic SOD mimetics that overcome these limitations should have clinical applications.

Table 1. Limitations of Native SOD [11]

- Immunogenicity of non-human enzymes
- Inability to cross membranes to gain intracellular access where $O_2^{\bullet -}$ is produced or cross the blood-brain barrier
- Short biological half-life (6hr)
- Bell-shaped dose response curve
- Cost

4. Properties of Mn-containing SOD Mimetics

An ideal SOD mimetic should have a redox metal center similar to native SODs, high stability, high specificity for $O_2^{\bullet-}$, high rate constant, non-antigenic, non-toxic, and high membrane permeability [3]. Further, an SOD mimetic should possess a redox potential similar to native MnSOD (-330 mV < E_0 < +889 mV) for optimal catalytic activity [5]. There are several classes of putative Mn-containing SOD mimetics: (i) Mn(III)-salen complexes, (ii) Mn(III) metalloporphyrins, and (iii) Mn(II)-pentaazamacrocyclic ligand-based.

4.1 Mn(III)-salen Complexes

Mn(III)-salen (salicylaldehyde-ethylenediamine Schiff's base adduct) complexes are reported to exhibit both $O_2^{\bullet-}$ scavenging and catalase activity [10]. The proposed mechanism for $O_2^{\bullet-}$ dismutation is similar to MnSOD, in which $O_2^{\bullet-}$ reduces Mn(III) to Mn(II) (reaction 5). The Mn(II) is subsequently oxidized back to Mn(III) by a second molecule of $O_2^{\bullet-}$ (reaction 6).

$$Mn(III) + O_2^{\bullet-} \rightarrow Mn(II) + O_2 \quad \{5\}$$

$$2H^+ + Mn(II) + O_2^{\bullet-} \rightarrow Mn(III) + H_2O_2 \quad \{6\}$$

The prototypes of this class are EUK-8 and EUK-134 (**Figure 2**) [11]. However, Czapski *et al.* reported that Mn-salen complexes do not exhibit SOD activity when measured using stopped-flow kinetics, and they act as $O_2^{\bullet-}$ scavengers [9].

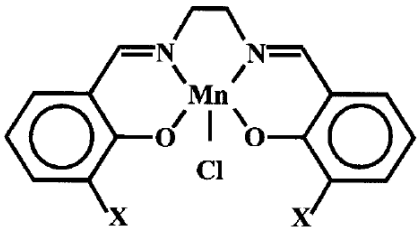

Fig. 2 The structure of Mn-salens: In EUK-8, X is H; in EUK-134, X is OCH_3 [10].

Mn(III)-salen complexes have shown efficacy in a variety of *in vivo* disease models. Melov *et al.* showed that MnSOD-deficient mice treated with EUK-8 or EUK-134 were rescued from spongiform encephalopathy and lifespan was extended to >20 days (versus ~8 days in untreated mice) [14]. Thus, the Mn-salen complexes can cross the blood-brain barrier and effectively scavenge $O_2^{\bullet-}$ and other ROS.

4.2 Mn(III) Metalloporphyrins

Mn(III)-metalloporphyrins have been well-characterized and have been shown to possess four distinct antioxidant properties: scavenging (i) $O_2^{\bullet-}$, (ii) H_2O_2, (iii) $ONOO^-$, and (iv) lipid peroxyl radicals [3]. The $O_2^{\bullet-}$ dismutation activity is attributed to the Mn moiety, which changes its valence between Mn(III) and Mn(II) in a similar fashion to native MnSOD. The prototypes of this class are MnTBAP, MnTM-4-PyP, MnTM-2-PyP, and MnOBTM-4-PyP (**Figure 3**). These compounds are stable toward EDTA, which is known to chelate the active metal ion of other SOD mimetics rendering it inactive [11]. The antioxidant properties and redox potentials of the prototypical Mn-metalloporphyrins are shown in **Table 2**.

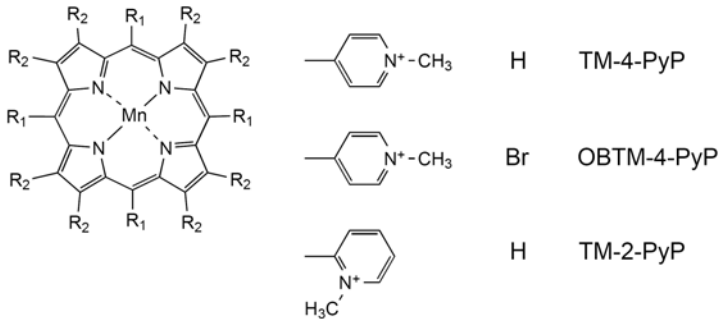

Fig. 3 The structures of Mn-metalloporphyrins [3].

Antioxidants	SOD (U mg^{-1})[a]	Catalase (% Act)[b]	Redox potential ($E_{1/2}$, V)[c]	Lipid peroxidation IC_{50} [μM][d]
CuZnSOD	5100[e]	–	+0.35[f]	15[g]
MnTBAP	179[h]	0.2[i]	−0.19[j]	29[g]
MnTM-4-PyP	550[e]	0.3[i]	+0.06[e]	16[g]
MnTM-2-PyP	8500[e]	0.9[k]	+0.22[e]	1[g]
MnOBTM-4-PyP	18 460[j]	0.5[k]	+0.48[j]	1[g]

Table 2. Comparison of the antioxidant properties of metalloporphyrins. SOD activity measured via cytochrome c assay, catalase activity via O_2 electrode,and lipid peroxidation via thiobarbituric acid reactive substances (TBARS). Redox potentials measured at pH 7.8. Adapted from [3].

Mn-metalloporphyrins have been shown to be efficacious in both *in vitro* and *in vivo* disease models, *e.g.*, protection from paraquat-induced cell damage and lung injury (mice), sequelae of endotoxemia (tissue damage, shock), and carrageenan-induced paw edema [1, 2, 11]. However, whether these effects are mediated through $O_2^{\bullet-}$ dismutation or scavenging of other ROS/RNS is difficult to assess. Further, the

potency and efficacy of Mn-metalloporphyrins vary considerably depending on the oxidative stress model. This in part is due to its large size, ~31 kDa [3].

4.3 Mn(II)-pentaazamacrocyclic Ligand-based Complexes

Mn(II)-pentaazamacrocyclic ligand-based SOD mimetics represent the most $O_2^{\bullet-}$-selective class, largely generated *via* computer-aided design. Mn(II) complexes are inherently kinetically stable to dissociation (log K > 17, 90% intact *in vivo* 30 min after injection), a major concern with other metallic SOD mimetics [12, 15]. The prototypes of this class are M40403 and M40401, which are derivatives of the 15-membered pentaazacyclopentadecane that contain added bis(cyclohexylpyridine) groups (**Figure 4**). They are low molecular weight, 483 Da and 501 Da, respectively (vs. 88 kDa and >30 kDa for MnSOD and other mimetics, respectively). The catalytic activity is equal or superior to native MnSOD, with rate constants of k_{cat} of 2 x 10^9 M^{-1} s^{-1} for M40401 and k_{cat} of 2 x 10^7 M^{-1} s^{-1} for M40403 [12]. In addition, they are non-immunogenic, membrane permeable, and highly active. These agents do not show the bell-shaped dose curve as observed for native MnSOD and other mimetics, which can be attributed to the high selectivity for $O_2^{\bullet-}$ [1]. The high $O_2^{\bullet-}$ selectivity is due to the state of the manganese(II) center. Since the resting state is the reduced Mn(II), the complex is unreactive towards reducing agents until it is oxidized to Mn(III) by protonated superoxide. However, this oxidized state is rapidly reduced back to the Mn(II) state. Further, these complexes are difficult to oxidize (E_m = + 780 mV). Lastly, two-elecron non-radical potent oxidants (*e.g.*, peroxynitrite) cannot kinetically capable to oxidize the complex because they operate *via* one-electron oxidations [8]. M40403 has been shown to be protective in various models of acute and chronic inflammation, ischemia-reperfusion injury, and shock [1, 6, 12].

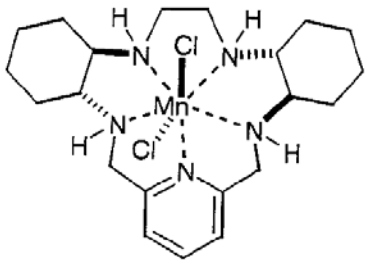

Fig. 4 Structure of M40403 [12]

5. Detection of SOD Activity

Traditionally, SOD activity is measured indirectly using either the cytochrome c or nitroblue tetrazolium (NBT) assay. Employing the xanthine/xanthine oxidase system to generate $O_2^{\bullet-}$, ferricytochrome c or NBT is reduced by $O_2^{\bullet-}$ to yield the reduced form of cytochrome c or formazan, respectively, which can be detected *via* a spectrophometric change. Thus, SOD activity correlates to a decrease in cytochrome c or formazan production. Measuring the production of uric acid rules out interference of the agent (SOD mimetic) on $O_2^{\bullet-}$ production [4].

Interpreting data collected from these indirect assays warrants caution. SOD mimetics may oxidize the reduced cytochrome (false positive for SOD activity), reduce ferricytochrome c (false negative for SOD activity), or react stiochiometrically versus catalytically (or acts as a scavenger) [4].

A direct method of detecting SOD activity is stopped-flow kinetic analysis [13]. This assay monitors the decay of $O_2^{\bullet-}$ spectrophometrically which enables one to decipher catalyzed decay from uncatalyzed decay (indicative of a scavenger).

SOD activity can also be determined by the aerobic growth of *E. coli* strain JI 132 which lacks SOD activity (SOD-null) [4]. In the absence of SOD, the bacteria fail to replicate. Bacterial growth determined with a turbidity measurement at 700 nm.

Aside from SOD activity, it is important to determine the stability of SOD mimetics *in vitro* or *in vitro*. Electron spin resonance yields distinct spectra for metal-complexes (e.g., Mn(II)-complex) and free metal ions (e.g., Mn^{2+}). This assay can be performed on biological fluids with little manipulation [4].

6. Summary and Conclusions

SODs play a vital role in defense against $O_2^{\bullet-}$, and a multitude of disease have implicated the role for superoxide-mediated damage. Treatment with native SOD, *e.g.,* MnSOD, has had limited success in part due to the limitations of the enzyme. Thus, the need for SOD mimetics that overcome these limitations would have great clinical significance. An attractive class of mimetics is the manganese-

containing complexes, which are highly stable. The Mn(II)-pentaazamacrocyclic ligand-based SOD mimetics (*e.g.,* M40403) are unique because they are highly specific for $O_2^{\bullet-}$ and catalytically efficient, which makes them valuable tools for teasing out the role of $O_2^{\bullet-}$ in disease settings, as well as an useful therapeutic agent.

7. References

1. Cuzzocrea S, Riley DP, Achille PC, Salvemini D. (2001) Antioxidant therapy: A new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. *Pharmcol Rev* **53:** 135-159.
2. Salvemine D, Riley DP, Cuzzocrea S. (2002) SOD mimetics are coming of age. *Nature Reviews*. **1:** 367-374.
3. Patel M, Day BJ. (1999) Metalloporphyrin class of therapeutic catalytic antioxidants. *Trends Pharmacol Sci.* **20(9):**359-64.
4. Weiss R, Riley DP. (1996) Manganese(II)-based superoxide dismutase mimetics: Rational drug design of artificial enzymes. *Drugs of the Future*. **21(4):** 383-389.
5. Leveque V, Vance CK, Nick HS, Silverman DN. (2001) Redox properties of human manganese superoxide dismutase and active-site mutants. *Biochemistry*. **40:**10586-10591
6. Salvemini D, Muscoli C, Riley DP, Cuzzocrea S. (2002) Superoxide dismutase mimetics. *Pulm Pharmacol Ther.* **15(5):** 439-47.
7. Dowling EJ, Chandler CL, Claxson AW, Lillie C, Blake DR. (1993) Assessment of human recombinant manganese superoxide dismutases in models of inflammation. *Free Radic Res Commun*. **18(5):** 291-298.
8. Mao GD, Thomas PD, Lopaschuk GD, Poznansky MJ. (1993) Superoxide dismutase (SOD)-catalase conjugates. Role of hydrogen peroxide and the Fenton reaction in SOD toxicity. J *Biol Chem.* **268(1):** 416-20.
9. Goldstein S, Czapski G. (1991) Comparison between different assays for superoxide dismutase-like activity. *Free Rad Res Comms.* **12-13:** 5-10
10. Sharpe MA, Ollosson R, Stewart VC, Clark JB. (2002) Oxidation of nitric oxide by oxomanganese-salen complexes: A new mechanism for cellular protection by superoxide dismutase/catalase mimetics. *Biochem J*. **366:** 97-107.
11. Faulknew KM, Liochev SL, Fridovich I. (1994) Stable Mn(III) porphyrins mimic superoxide dismutase *in vitro* and substitute for it *in vivo*. *J Biol Chem.* **269(38):** 23471-23476.
12. Aston K, Rath N, Slomczynska U, Schall O, Riley DP. (2001) Computer-aided design (CAD) of Mn(II) complexes: Superoxide dismutase mimetics with catalytic activity exceeding the native enzyme. *Inorg Chem.* **40:** 1779-1789.
13. Riley DP, Rivers WJ, Weiss RH. Stopped-flow kinetic analysis for monitoring superoxide decay in aqueous systems. *Anal Biochem*. **196:** 344.
14. Melov J, Dostrow SR, Coskun PE, Huffman K, Wallace DC, Malfoy B. (2001) Lifespan extension and rescue f spongiform encephalopathy in SOD-2 nullizygous mice treated with SOD-catalse mimetics. *J Neurosci.* **21:** 8348-53.
15. Salvemini D, Wang ZQ, Zweier JL, Samouilov A, Macarthur H, Misko TP, Currie MG, Cuzzocrea S, Sikorski JA, Riley DP. (1999) A nonpeptidyl mimic of superoxide dismutase with therapeutic activity in rats. *Science*. **286:** 304-306.