Functional Mimics of Superoxide Dismutase Enzymes as Therapeutic Agents

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Contents

Dennis P. Riley joined MetaPhore Pharmaceuticals, Inc. as vice-president of research in 1999. The emphasis for MetaPhore is both chelation therapy and the use of metal complexes as pharmaceuticals. He joined MetaPhore following his career at Monsanto as a Senior Science Fellow and manager of the Metal-Mediated Chemistry group in Monsanto's Corporate Research Division. He completed his undergraduate education with majors in chemistry and mathematics (*cum laude*) at Heidelberg College and earned his Ph.D. at Ohio State University in inorganic chemistry in 1975. Following post-doctoral training at the University of Chicago and several years at Procter & Gamble (Section Head), he joined Monsanto's Corporate Research center in St. Louis as Senior Research Group leader in Homogeneous Catalysis. He is an Adjunct Professor of Chemistry at Washington University, has been elected a Fellow of the American Association for the Advancement of Science, elected Chairperson and organizer of the 1998 Gordon Research Conference on Inorganic Chemistry, and was the recipient of the ACS St. Louis Section's Chemist of the Year Award for 1999. His primary research interests include activation of small molecules (i.e., O_2 and CO_2), catalytic oxidations, asymmetric catalytic conversions, and bioinorganic chemistry (*Synzymes*–small molecule functional mimics of enzymes). He has been granted over 30 U.S. patents and over 200 foreign patents, as well as published over 80 papers and authored several review articles and book chapters.

1. Introduction

Superoxide dismutase (SOD) enzymes are ubiquitous in living systems.[1] These enzymes serve a vital role in defending oxygen-utilizing life forms from oxidative damage.[1] The naturally occurring superoxide dismutase (SOD) enzymes appear in many forms in both animals and plants. Cu/Zn-containing forms are found in the extracellular spaces of mammals, as well as in the cytosols of eukaryotic cells, the periplasms of gram-negative bacteria, and the plastids of plants. Mn-containing forms of SOD are found in the mitochondria of all mammalian cells and in *E. coli*; while all anaerobic prokaryotes, if they possess SOD activity, will contain Fe SOD exclusively.[1] All obligate aerobes possess Mn SOD enzymes exclusively, and facultative anaerobes contain both. Additionally, a Ni SOD has recently been discovered in *Streptomyces griseus*.[2]

Many disease states afflicting mankind can be broadly characterized as ones in which the body fails to adequately contain the overproduction of an undesired chemical byproduct. One such example of this inability to control and limit the concentration of a potentially harmful agent occurs as a result of our need to metabolize energy stores. All mammalian life consumes oxygen as the ultimate oxidant supporting cellular respiration, but a considerable portion of the oxygen is metabolized through its one-electron reduction product, superoxide anion. Under normal circumstances in healthy individuals, the radical burden is contained by the complement of SOD enzymes present in the mitochondria of cells (Mn based), in the plasma (Cu/Zn based), and in the extracellular spaces (Cu/Zn based). This control of the free radical flux derived from oxygen is jeopardized in many circumstances in which superoxide (SO) anion production is excessive. This overproduction of SO can overwhelm the body's ability to catalytically dismute superoxide and reduce or eliminate the radical bur-

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den. This deleterious oxygen-derived free radical has been demonstrated to be a mediator of reperfusion diseases, such as those following acute myocardial infarct or stroke, shown to be associated with development and continuation of inflammatory processes, involved in diseases such as arthritis, and play a major role in the initiation of neurological disorders such as Parkinson's disease.

At the molecular level recent reports by Dix et al. have demonstrated that perhydoxyl radical ($HO_2^{\bullet}$)—protonated superoxide—is the most competent and selective initiator of the autoxidation of lipid membrane components with its site of attack being *only* at the allylic positions in unsaturated fatty acids.[3,4] Some of the products from the resultant highly selective chain autoxidations are the leukotrienes (e.g., LTB_4) and prostanoids, which are responsible for the upregulation of TNF_α and other cytokines responsible for mediating inflammation. Further, it has been shown that $HO_2^{\bullet}$ is a very selective and active agent for the site-selective DNA nicking via 5′-hydrogen abstraction of the deoxyribose,[5] leading to an extremely mutagenic DNA nick.

The rational design and synthesis of low molecular weight catalysts which mimic a natural enzyme function (a *synzyme*[6]) has potential for use as a human pharmaceutical in the treatment or prevention of such diseases. Synthetic enzymes (*synzymes*) have as a potential utility the ability to address the many diseases in which the overproduction of an undesired, toxic metabolic byproduct is a factor in the initiation or continuation of the disease. Thus, the application of such *synzyme* technology for removal/elimination of superoxide (a product of cellular respiration, activated polymorphonuclear leukocytes, endothelial cells, and mitochondrial electron flux) would seem to be well-suited.

Many research groups, including our own, have been pursuing the possibility of developing such *synzymes* as an approach to managing the types of diseases outlined in the previous discussion. Tremendous progress has been made in this area in recent years both in defining a role for such a synthetic enzyme as a human pharmacological agent utilizing a number of animal models for disease and in progressing toward development of actual drug candidates. The following review briefly introduces the chemistry of the SOD enzymes, surveys recent advances in the synthesis of low molecular weight SOD mimics, and attempts to introduce some of the issues involved with the testing for SOD activity and the chemical design constraints one must satisfy in order to synthesize a highly active enzyme mimic which can function as a human pharmaceutical agent. This latter discussion involves considerations of toxicity and stability—critical design elements for the development of a human pharmaceutical. In particular, emphasis will be made in this review on the considerations of biological studies for the metal complexes reported to possess SOD *synzyme* activity.

A. Native Enzymes

From the initial seminal discovery by Fridovich and McCord that a class of serum copper- and zinc-containing proteins catalyze the dismutation of superoxide to oxygen and hydrogen peroxide,[1b,7] there has been a tremendous amount of research which has continued to elaborate the role that the SOD enzymes play in maintaining the health of organisms. The SOD enzymes are a class of oxidoreductases which contain either Cu/Zn, Fe, or Mn at the active site and catalyze this dismutation of the free radical superoxide, the one-electron reduction product of molecular oxygen (eq 1) (eqs 1 and 2, where M^n is the metalloenzyme in the reduced state and M^{n+1} is the enzyme in the oxidized state) to the nonradical products. These enzymes perform this catalytic cycle of dismutation with incredible efficiency, i.e., at rates approaching diffusion-controlled! For example, the mammalian Cu/Zn SOD's have been shown to possess catalytic rates in excess of $2 \times 10^9\ M^{-1}\ s^{-1}$, while the Mn and Fe SOD enzymes have been shown to function at rates that are somewhat slower, approximately an order of magnitude slower depending on the source of the enzyme. The SOD enzymes have been shown to have efficacy in animal models of disease states proposed to be, in part, mediated by superoxide, such as myocardial ischemia-reperfusion injury,[8−11] inflammation,[12−15] and cerebral ischemia-reperfusion injury.[16−18] Further evidence for superoxide as a mediator of disease states continues to accrue, such as in ALS and neuronal apoptosis,[19−23] cancer,[24−28] and AIDS.[25,26]

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

$$HO_2^{\bullet} + M^n \rightarrow H_2O_2 + M^{n+1}$$

In light of the clinical data surrounding the use of the SOD enzymes,[31−33] low molecular weight mimics of the enzyme SOD have been proposed for the treatment of a great variety of diseases. SOD mimics could have distinct advantages over the natural enzymes as a pharmaceutical agents, including the ability to access intercellular space, cellular permeability, the lack of immunogenicity, a longer half-life in the blood (the human enzymes are stable in vivo for only short periods, i.e., $t_{1/2} \approx$ minutes), potential for oral delivery, and a lower cost of goods. There are a wide range of therapeutic applications for SOD mimics considering the multitude of deleterious effects known to derive from oxidative stress induced by an excess of superoxide (oxidative stress is defined as a disease process or tissue damage which results in increased endogenous superoxide radical generation with its attendant radical daughter reactive oxygen intermediates (ROI) combined with insufficient radical elimination defenses).[31]

2. Considerations for Drug Design

A. Measuring SOD Activity

For any effort to develop true catalytically active SOD mimics, it is critical that one be able to rapidly and quantitatively assay any putative mimic for such catalytic SOD activity. This can be an issue since the indirect assays most often employed possess several inherent problems which limit their ability to yield

quantitative rate data and often affect the interpretation of results.[34] The discovery and activity of the SOD enzymes were first reported by Fridovich and McCord using a cytochrome *c* assay. In this assay, a reporter molecule (e.g., ferricytochrome *c*) is reduced by superoxide to the reduced form of the reporter affording a spectrophotometric change or a fluorescence. Inhibition of this reduction of cytochrome *c* by scavenging or reducing the superoxide concentration is taken as a measure of SOD activity. An SOD mimic will quench the superoxide and diminish the response of indicator. Cytochrome *c* or nitro blue tetrazolium (NBT) are the most often used indicators in a system using xanthine/xanthine oxidase to generate steady-state low levels of superoxide. The indirect methods are limited in that true catalytic rate constants cannot be directly determined. However, this method may indicate whether a compound is an SOD mimic at lower levels of activity than the direct methods. It is important to verify, however, that the compound being tested is not participating in a redox short circuit, i.e., simply reducing the indicator or inhibiting the xanthine/xanthine oxidase production of superoxide or reacting with the hydrogen peroxide (catalase has been added to scavenge H_2O_2, but this adds another potential source of cross reaction and hence interpretive error) or oxygen products of the self-dismutation of superoxide, a reaction which at pH = 7.4 has a second-order rate constant of $\sim 1.5 \times 10^5$ M^{-1} s^{-1} at 21 °C.[35] It is also important to note that the indirect assay is carried out under conditions in which there is a steady-state (continuous) generation of superoxide from the xanthine enzyme; thus, the assay is often not carried out with a large initial excess of substrate over putative catalyst. In fact, there can be an excess of "catalyst" over the initial concentration of superoxide. This can lead to a false interpretation of catalytic activity, when there is a fast stoichiometric reaction with superoxide. Although not perfect, the indirect method has value for testing lower activity SOD mimics provided the proper consideration is given to the factors which affect the accuracy of the assay.[34]

The direct methods for measuring SOD activity fall into two categories: stopped-flow kinetic analysis and pulse radiolysis. Both of these methods allow precise measurement of the rate of dismutation of superoxide by visualizing directly the spectrophotometric decay of the superoxide anion in buffer solution. As with the indirect methods, the radiolysis methods often rely on a steady-state generation of superoxide, where the initial concentration of dissolved oxygen in the water (which is about 100 mM under 1 atm of air at 25 °C) is the limiting factor for superoxide flux. Kinetic data from both methods can also be readily obtained as a function of other variables, including pH.[35] With pulse radiolysis, as with the xanthine generated superoxide, the superoxide is generated by pulse irradiation of oxygen-saturated aqueous solutions in the presence of formate. The reaction with the putative SOD mimic can then be measured by observation of the spectrum of superoxide.[35] With the stopped-flow method, the putative catalyst in buffer solution is rapidly mixed with potassium superoxide dissolved in a small amount of dry dimethyl sulfoxide (aqueous buffer:DMSO, ~20:1). Unlike the other methods, the initial concentration of superoxide can be very high compared to the putative catalysts being evaluated (>1000:1). With this stopped-flow method, if a pure first-order decay of superoxide is obtained (as monitored by the disappearance of superoxide on the millisecond time scale) over more than 3 half-lives, then a true catalytic effect is validated. Both the stopped-flow and pulse radiolysis methods provide rates for the catalytic dismutation of superoxide in aqueous solution at physiological pH or higher. The stopped-flow procedure is limited in that rates can only be determined for compounds which possess catalytic activities greater than $k_{cat} > 10^{5.5}$ M^{-1} s^{-1} (at pH = 7.4) due to the competing background second-order self-dismutation of superoxide.[35] From the analysis of stopped-flow derived data, an uncatalyzed decay of superoxide (second-order kinetics) can readily be distinguished from a catalyzed decay of superoxide (first-order kinetics) in the presence of a large excess of superoxide over the complex being screened. A second-order catalytic rate constant (k_{cat}) can be obtained for an agent with true catalytic SOD activity by assessing the first-order decay of superoxide at various concentrations of metal complex being evaluated. This direct determination of an actual k_{cat} can be utilized to directly compare and quantitate the SOD activities of enzymes and/or mimetics under a given set of conditions (e.g., defined pH and temperature).[34,35]

As noted in the preceding discussion, it is important to determine whether a compound is in fact acting as a true catalytic SOD mimic, as opposed to a stoichiometric superoxide scavenger. Many metal complexes are known to react with and be reduced by superoxide or oxidized by its conjugate acid, the strong oxidant, $HO_2^{\bullet}$, but may nevertheless not function as a catalyst. Thus, the determination of true catalytic superoxide reactivity is critical to the claim that a complex is an SOD mimic. It must be emphasized that a necessary criterion for a complex to be an actual SOD mimic is that it must function as a true catalyst, i.e., it will perform many turnovers of the catalytic cycle rather than a single stoichiometric turnover and will consequently provide a first-order decay of superoxide over several half-lives of decay with no deviation to second-order behavior, indicative of self-dismutation.

Another consideration regarding the actual catalytic activity of a putative SOD mimic is that it may be possible for a compound to act as an oxidoreductase both in an assay and in vivo. As oxidoreductases, putative SOD mimic complexes may be either reduced or oxidized at a faster rate with molecules other than superoxide or its conjugate acid, $HO_2^{\bullet}$. An example of this potential interpretation problem is the fact that all of the methods used to screen or assay SOD activity will of necessity make hydrogen peroxide, the product of the dismutation. Since many redox-active complexes will react with H_2O_2, it is very possible that an apparent catalytic cycle may be observed but it may in fact be due to hydrogen peroxide contributing as either a reductant or an

oxidant to the redox cycle. Thus, separate studies which assess a putative catalyst's reactivity with hydrogen peroxide are important so that an unambiguous determination of SOD catalytic activity can be made. Also, it is important to keep in mind that none of the assays currently employed can exactly replicate the environment encountered by these SOD mimics in the body. One must understand the assumptions made and use the information from these assays as valuable physical data for designing or screening SOD mimics.

B. Selection of Metal

As noted above, there are but a few metals known (e.g., Cu, Mn, Fe, and perhaps Ni) whose complexes will catalyze the dismutation of superoxide to hydrogen peroxide and oxygen. The focus of this review will be limited to those complexes of manganese and iron which have been reported to function as low molecular weight SOD mimics. This decision to pursue Fe and Mn complexes and exclude those of copper from the scope of this review is based on a number of reasons. First, throughout the 1970s and 1980s, most papers published on metal complexes as mimics of SOD enzymes focused on the use of copper complexes, and this area has been the subject of a number of reviews,[36] including a comprehensive review by Sorenson in 1989.[37] Second, the study of copper mimics is complicated by the fact that the aquo Cu(II) ion is itself a very excellent SOD catalyst with nearly the activity of the Cu/Zn enzymes.[35,38] This presents an issue with interpretation of SOD mimic activity reported for a Cu complex, i.e., is it really due to the complex or a trace amount of free metal due to dissociation of the complex or to free Cu(II) impurity. A third major issue confronting the use of copper complexes involves considerations of toxicity when designing a metal-based pharmaceutical agent. Most of the SOD mimics currently being developed as drugs are based on manganese rather than copper or iron. One reason for favoring manganese is that decomposition of the catalyst could produce the free metal in vivo. Since both copper[39,40] and iron[41−43] ions are very toxic in vivo and manganese ion is not (manganese is the least toxic to mammalian systems as the free aquated metal ion and is also the least likely of the three aquo M^{2+} ions to react with hydrogen peroxide to generate hydroxyl radicals (Fenton chemistry)), manganese has been selected most often for use in these synthetic catalysts designed for pharmaceutical use. Since the aquated ions of copper and iron are quite toxic as the free ions in mammalian systems and promote the "Fenton" reaction with hydrogen peroxide producing hydroxyl radicals, it becomes extremely important that a metal-based drug is very stable in its complexed state in vivo and does not liberate such redox-active, toxic metal ions in an inappropriate biological site. This stability issue raises the question of whether ligands can be devised for copper which can provide a platform for facilitating the redox cycle of Cu(II)/Cu(I) and at the same time provide a stable complex for the reduced Cu(I) ion—an ion which is extremely kinetically labile and which is characterized by rapid ligand exchange rates. Thus, for example, one approach to contain Cu ions would be via macrocyclic ligands. The Cu complexes with simple macrocyclic ligands (e.g., cyclam) are indeed quite stable but are difficult to reduce and generally possess little or no catalytic SOD activity.[44]

C. Metal Complex Stability

The stability of a metal complex is critical for its use as a pharmaceutical. As noted above, the possibility that a potentially harmful redox-active metal ion could be liberated from a complex in an inappropriate biological compartment is clearly a possibility to be avoided. There are several aspects to stability which should be considered when addressing a pharmaceutical application, including those factors which contribute to the overall chemical stability and metabolic stability. Metal complexes are invariably susceptible to proton (acid)-assisted dissociation, and most complexes are potentially susceptible to oxidative ligand degradation. In addition, many complexes can be labilized for ligand dissociation via reduction of the metal so that a more labile metal center is generated. All three pathways are conceivable in a biological in vivo milieu. For example, in the acid pH of the stomach or the lowered pH of ischemic tissue (oxygen-deprived tissue becomes acidic with the pH approaching 5) or in the strong oxidizing environment of the liver, oxidative complex degradation could be expected to be a major source of liberation of free and potentially toxic metal ions. Furthermore, in the strongly reducing environment of most cells, reduction of a metal center in a complex could well play an important role for decomposition of a complex. Thus, when considering the stability of a metal complex SOD mimic, not only are the inherent thermodynamic and kinetic stability in water key parameters, but the oxidative and reductive stability of the complex should be considered. Obviously, there is no better measure of stability than the actual stability of a complex as monitored in vivo. The most desirable attribute would be that the kinetics of excretion are far faster than the kinetics of dissociation. In any event, assessments of the in vivo kinetic stability and clearance rate for an intact complex are critical aspects of the drug design studies.

While the thermodynamic stability or binding constant (log K) is an important aspect of the stability question and can be measured in a relatively straightforward manner for most metal complexes via a number of means, including potentiometric titrations,[45] the kinetic stability or rate of dissociation (k_{diss}) is probably the most important parameter. An important criteria is the stability of the complex to acid dissociation and the inherent stability in water. This can be measured in a straightforward manner as a function of pH by observing the formation of free ligand and aquated metal ion as a function of time at various pH's so as to assess the $[H^+]$ dependence on the kinetics of dissociation. This can be conveniently done via many approaches including competition methods involving free ligand complexation with a competing metal ion or by monitoring the dissociation of the metal complex via HPLC (high-pressure liquid chromatography) separation and analysis.[46]

D. Toxicity Considerations

In addition to the obvious problems discussed in the previous section regarding the stability of a complex and the toxicity of free redox-active metal ions, there are considerations of side effects due to the mechanism-based activity of the synzyme itself, namely, due to the SOD activity of the complex. The possibility of mechanism-based toxicity is certainly conceivable and may potentially limit therapeutic utility. Clearly, it is an aspect of this approach to human pharamaceutical development which must be monitored and, if necessary, be addressed. For example, SOD mimics could possess the ability to potentiate levels of nitric oxide, a vasorelaxant and anti-thrombotic,[47,48] by reducing or eliminating the known diffusion-controlled reaction between superoxide anion and nitric oxide, which yields peroxynitrite, another toxic metabolite derived from oxygen.[49] Even though the reaction of nitric oxide with superoxide has been measured to proceed with a nearly diffusion-controlled rate,[50] reduction of the superoxide levels by administration of an SOD mimic could be expected to reduce this pathway for nitric oxide loss/metabolism. This would, of course, depend on the local concentration of mimic and its catalytic effectiveness. Since NO is a vasorelaxant and will lower blood pressure, the apparent beneficial effect of an SOD mimic in blocking production of a toxic metabolite like peroxynitrite could also possibly be deleterious if NO levels become potentiated, thus affording too high a circulating [NO], i.e., a serious blood pressure lowering could be produced. Clearly, such an effect would depend on the biodistribution of an SOD mimic drug following administration and its catalytic effectiveness. While this discussion is aimed at identifying a potential problem and is not meant to document an actual known problem, it does point out that altering the normal metabolic levels of free radical products, such as superoxide, is a possible area of concern.

Another area which could conceivably be an area of potential toxicity would be the lack of specificity in reactivity of an SOD mimic. By this it is meant that a complex should not itself react with such small molecules as hydrogen peroxide or peroxynitrite to produce even more noxious agents such as hydroxyl radicals or nitrogen dioxide ($NO_2^{\bullet}$). Additionally, a reaction with nitric oxide forming a stable complex with a metal complex could exert deleterious side effects in elevating blood pressure by reducing the concentration of free nitric oxide. A less direct but nevertheless real possibility for a deleterious side effect could arise if a hypothetical SOD mimic complex catalyzed the decomposition of hydrogen peroxide to the less noxious molecules oxygen and water (as a catalase mimic); this could conceivably weaken the immune system by deactivating one of the major pathways for bacterial killing via reducing the H_2O_2 levels produced by neutrophils.[51] Again, the point of this discussion is not to suggest that such side effects are actually known problems, but to point out that they are potential realities that should be considered when designing and testing agents as human pharmaceuticals. Consequently, studies to assess the reactivity of an SOD mimic with such biologically relevant small molecules may be quite relevant to the design of such agents, and when such data exists, it will be cited in the following discussion of specific SOD mimics.

Finally, due to the catalytic nature of the *synzyme* drug (i.e., an SOD mimic, with optimal biodistribution and a high catalytic rate), it could be efficacious at very low concentrations, making this a potential therapeutic regimen with low inherent side effect probability. Thus, the challenge is to design SOD mimics with high catalytic rate (i.e., diffusion-controlled), optimal biodistribution, and high kinetic stability (i.e., both chemical and metabolic).

E. Biological Considerations

A significant aspect of classical drug design is the optimization of partitioning of a drug to the desired tissue. This is classically assessed by measuring and comparing the partition coefficients (so-called log P, where $P = [\text{drug}]_{n-\text{octanol}}/[\text{drug}]_{\text{water}}$). Thus, the ability to chemically modify a *synzyme* metal complex so as to optimize the partitioning and clearance characteristics of a metal-based drug could be a very key aspect to delivering a safe and effective agent. For example, perhaps highly lipophilic drugs would be desired for agents in treatment of Parkinson's disease so that the drugs may permeate the blood/brain barrier. In contrast, an agent to treat reperfusion injury following a myocardial infarct would probably be most available to the site of injury if it stayed in the circulation (water phase) longer and did not rapidly partition into the lipid phase. Obviously, the chemist must of necessity design ligands capable of providing high-activity catalysts, with excellent stability, and with the capability of varying functional substituents which can allow for structure/activity studies in real biological models of human disease.

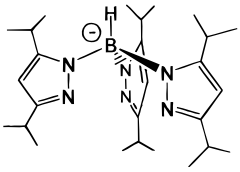
An example of such design considerations is found in the use of SOD mimics to treat reperfusion injury. This is a condition in which an area of tissue is deprived of oxygen for periods up to an hour before cell death occurs. During this hypoxic event, the cell is continuing to build reducing equivalents in the absence of the terminal oxidant, O_2.[52,53] As the ischemia develops, the pH in the region decreases ($\sim$5) apparently due to lactic acid buildup.[54] This is significant as this is the pH a metal complex must survive to be a drug for such an application, and it is significant in that this pH is near the pK_a of superoxide. This means that much of the superoxide generated upon reperfusion with fresh oxygenated blood to ischemic tissue (e.g., following dissolution of a clot with TPA in MI) will be present as $HO_2^{\bullet}$. This results in a strongly oxidizing media. If a putative catalyst was known to have the reduction of an oxidized complex, as a rate-determining (r-d) step in a catalytic cycle, then it could be expected that the pH dependence of the rate of the catalyst could play a role and, in fact, in this case the catalyst would be less effective at pH's lower than physiological (pH = 7.4).

3. Manganese Complexes as SOD Mimics

A. General Examples

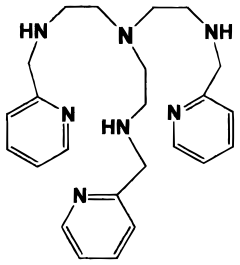
The use of manganese complexes as SOD mimics for use as human pharmaceuticals has been reviewed recently.[55,56] Many complexes of manganese have been reported to be capable of functioning as catalysts for the dismutation of superoxide including those with desferal,[57,58] 8-quinolol,[59] cyclam,[60] and aminopolycarboxylate complexes such as Mn^{III}(EDTA), Mn^{III}(CyDTA),[61] Mn^{II}(NTA), and Mn^{II}(EDDA).[62] All of these early reports based their claims of SOD catalytic activity utilizing indirect methodologies, such as those described above. Analysis of the SOD activity of these complexes in our laboratories using stopped-flow kinetic methodology[34,35] under the conditions defining true catalysis ($[O_2^{-\bullet}] \gg$ [Complex]) demonstrated that these complexes have no detectable catalytic SOD activity greater than catalytic rates (k_{cat}) of $10^{5.5}$ M^{-1} s^{-1} at pH = 7.4 or greater.[34] We concluded that it is most likely that such complexes react stoichiometrically with superoxide, resulting in their apparent activity in the indirect assays, although it cannot be ruled out that a lower level catalytic activity ($<10^{5.5}$) is operative with these complexes.

Another recent example of a Mn(II) complex reported to be an SOD mimic is the Mn complex with the tris-pyrazolylborate ligand, Mn(Obz)(3,5-*i*Pr_2-pzH)(HB3,5-*i*Pr_2pz)$_3$, which is reported to have an SOD acitivity of about 0.4% of Cu/Zn SOD based on the nitro blue tetrazolium indirect assay method.[63] Without additional data obtained under catalytic conditions, it is impossible to know whether this is a true catalyst. No information was reported regarding this complex's stability or reactivity with other biologically relevant small molecules other than that it is air sensitive. No biological data has been reported for the Mn(pyrazolylborate) complexes.



HB(3,5-*i*Pr_2pz)$_3$

Recently, a seven-coordinate Mn(II) complex with the tris[2-[*N*-(2-pyridylmethyl)amino]ethyl] ligand was synthesized and the complex showed to be a stoichiometric scavenger of superoxide.[64] The significance of this paper is that the authors thoroughly explored the reactivity of this complex with superoxide following their initial discovery using the ferricytochrome *c* indirect SOD activity assay that the complex was a very "active" SOD catalyst. The authors noted that the indirect assays can lead to false positives for catalytic SOD activity, and they further probed the catalytic activity of this Mn complex using pulse radiolysis under conditions of true catalysis, i.e., $[O_2^{-\bullet}] \gg$ [catalyst]. In fact, they found that the complex exhibits no catalytic activity under conditions of catalysis (i.e., the decay of superoxide is an unperturbed second-order process), and subsequent studies revealed that the Mn(II) complex actually forms a transient intermediate complex with superoxide ($k_{formation} \approx 10^{+7}$ M^{-1} s^{-1}) which disappears without formation of Mn(III). In any event, this study clearly emphasizes that the indirect assays cannot distinguish true catalytic SOD activity unambiguously.



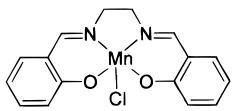
Other complexes of Mn(II) have been reported to possess modest catalytic SOD activity as monitored by the indirect assay methods. These include the $Mn^{II}(HL)_2$ complex [H_2L = 2,6-bis(benzamidazol-2-yl)pyridine],[65] metal saccharinates {$[M^{II}(Sac)_2(H_2O)_4$, where M = Mn, Fe, Co, Ni, Cu, and Zn},[66] and a Japanese patent which claims that the Mn^{III}bis(2,6-pydinedicarboxylate) complex is an SOD mimic.[67] For these reports the assay utilized to monitor SOD catalytic activity was the ferricytochrome *c* indirect assay, and without corroborative studies, it cannot be unequivocally stated that these are true SOD catalysts. In any event, complexes with such kinetically labile ligands as a mondentate saccharin ligand would not be expected to be useful as a human pharmaceutical due to stability concerns. Further, the catalytic activity of the Mn saccharinate complex is questionable as the same study reported that a complex with a nonredox-active metal, the Zn saccharinate complex, was an even more active SOD mimic. No biological data has been reported for any of these complexes.

B. Mn^{III}(salen) Complexes

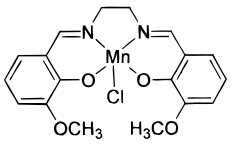
The Mn^{III}(salen) complexes, developed by Jacobson as catalysts for chiral epoxidations, have been reported to be superoxide scavengers/SOD mimics and possess catalase activity.[68,69] Eukarion, Inc., has patents covering the Mn^{III}(salen) structures as therapeutic agents,[70] and in February of 1997, Glaxo-Wellcome announced a collaboration with Eukarion for development of these synthetic superoxide scavengers (SCS's) for acute and chronic diseases excluding stroke or other neurological indications for which Eukarion has retained the rights.[71] Although mechanistic details have not been reported for these complexes, there has been some insight into the stability of the complexes in aqueous media. On the basis of indirect assays of SOD activity, added EDTA was observed to diminish activity in some cases, suggesting that the Mn is removed from the complex or otherwise tied up from coordination, although this last point is less likely as bovine serum albumin (BSA), which binds to Mn, does not inhibit the activity of these salen derivatives. Earlier work by Taylor et al. suggested these complexes undergo

quasireversible reduction in cyclic voltammetry experiments in DMSO solvent in which the complexes behave as 1:1 electrolytes dissociating the axial ligand, e.g., chloride,[72] thus establishing that reduction of Mn(III) to Mn(II) is thermodynamically a viable step in a catalytic SOD cycle with these complexes. No additional mechanistic studies regarding the putative SOD activity of these complexes has been published.

Biological studies have been published for two of these complexes, EUK-8 and EUK-134. EUK-8 has



EUK-8



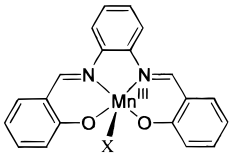
EUK-134

been tested in a range of disease models related to oxidative stress, and EUK-134, claimed to possess combined SOD and catalase mimic functions, has recently been tested in a model for stroke and earlier in a model of ischemia/reperfusion injury. Many neurological disorders have been linked to oxidative stress, and these Mn^{III}(salen) complexes have shown efficacy in models of neurological disorders. EUK-8 was shown to preserve synaptic function in hippocampal slices subjected to anoxia/reoxygenation,[73] and it protected organotypic hippocampal cultures from toxicity by amyloid peptide in a model of Alzheimer's disease[74] (see Table 1 for comparison of in vitro and ex vivo data). In two mouse models of Parkinson's disease, EUK-8 protected striatal dopaminergic neurons.[75] In the first model, a neurotoxin, 6-hydroxydopamine (6-OHDA) was given to mice intracerebrally. Protection was provided by intraperitoneal dosing of EUK-8 at 40 mg/kg. In the second model, mice were protected from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity by EUK-8 in the drinking water (5 mg/mL) starting 5 days prior to MPTP treatment (see Table 2 for comparison of in vivo data). EUK-8 prevented paralysis in mice subjected to experimental allergic encephalomyelitis (EAE), a T-cell-mediated autoimmune disease and animal model for multiple sclerosis in humans.[76] Induction of EAE was carried out in mice which were then treated with EUK-8 at 100 mg/kg at immunization and thereafter every 48 h. The treated mice began to show recovery at 30 days with full recovery at 40 days. EUK-134 was effective in a rat stroke model. In this model the middle cerebral artery was occluded, and after 3 h, EUK-134 was administered (0.25−2.5 mg/kg iv). The rats dosed at 2.5 mg/kg showed approximately 90% reduction in infarct volume compared to controls. EUK-8 provided some protection at 1.9 mg/kg and, at that dose, was comparable to EUK-134 at 0.25 mg/kg. The increased catalase activity of EUK-134 is proposed as the reason for improved efficacy over EUK-8.[77]

The Mn^{III}(salen) compounds have shown efficacy in several other disease models. EUK-8 protected pulmonary function in a porcine model of adult respiratory distress syndrome (ARDS).[78] This model used lipopolysaccharide (LPS) to induce ARDS. Treatment with EUK-8, along with a challenge dose of LPS, 18 h after introduction of LPS resulted in reduction of lung injury in a dose-dependent manner. Dosing for this experiment consisted of an iv 10 mg/kg bolus followed by 1 or 3 mg/kg/h.

A rat model of renal ischemia-reperfusion injury showed protection by EUK-134. Following 75 min of warm renal ischemia, 0.2 mg/kg of EUK-134 was injected into the rats just prior to reperfusion. Recovery of renal function was seen following this severe ischemic episode.[79] Several isolated heart studies have displayed beneficial effects for EUK-8. For example, EUK-8 modestly reduced the effects of ischemia-reperfusion on isolated rat hearts when added (50 mmol/L) 90 s prior to reperfusion. In this study the severity of arryhythmias was lowered in treated hearts compared to control hearts.[80] In another study, EUK-8 at 50 mmol/L was found to be protective against ultrastructural alterations induced by ischemia and reperfusion in isolated rat hearts which are overloaded with iron. The iron(II) exacerbates the oxidative damage by reacting with hydrogen peroxide to form hydroxyl radical in a Fenton reaction;[81] the proposed catalase activity of these complexes should indeed provide protective effects.

A related salen derivative, the Mn^{III}(salophen) complex, has also been reported by Liu et al.[82] to be active in the ferricytochrome *c* indirect assay for monitoring SOD activity. As with the Eukarion



Mn^{III}(Salophen)

complexes, considerable SOD activity is lost in the presence of EDTA but not BSA (bovine serum albumin). Interestingly, Mn^{III}(salophen) restores the ability of double mutant *E. coli sodA−sodB* to grow aerobically in a minimal glucose medium at concentrations of added complex up to 10 mM, but at higher concentrations the complex is toxic. Additionally, incubation of the complex with the cells prior to admission of oxygen reveals that the complex loses substantial activity in the cell culture. This suggests that the complex may not be stable in the biological milieu and the toxicity may be due to either free ligand or free aquo manganese, which is toxic in cultures at those concentrations.

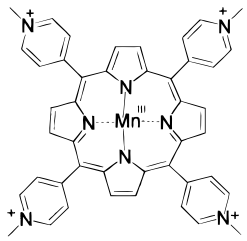
Clearly, a substantial body of biological data has been generated suggesting that the Mn^{III}(salen) complexes have protective effects in models of oxidative stress. It is important to determine the key chemical properties of these molecules which are the source of the biological effect. In our own laboratories we have investigated the catalytic properties of the Mn^{III}(salen) derivatives via stopped-flow analysis of superoxide decays.[83] Importantly, we observe that these complexes are of a very low true catalytic activity with the best rates observed being less than $8 \times 10^5\ M^{-1}\ s^{-1}$ at pH = 8.0 with the EUK-8 complex.

The matter of determining rates with these compounds and the ability to assess their biological activity is also complicated by very low water solubility of these types of complexes. This necessitates using organic cosolvents to aid in the dissolution of the complex. It is important to note that prior work by Taylor et al.[84] has shown that Mn^{III}(salen) complexes do not react with superoxide anion. In contrast, the pentadentate salen analogues do react with superoxide and they form long lifetime intermediates. Additionally, it has been reported that the Mn^{III}-(salen) complexes do not exhibit SOD-like activity based on electrochemical and spectrophotometric studies.[85] Thus, the conclusion is that the Mn^{II}(salen) complexes cannot be true catalytic SOD mimics but must owe their biological effects to another mechanism. Since the Eukarion studies indicate that the Mn^{III}(salen) derivatives are catalases, it is intriguing to speculate that the observed biological activity of these compounds may well arise from a combination of reactions, none of which are pure SOD catalysis. Thus, reactivity with hydrogen peroxide may give an intermediate which is capable of scavenging superoxide. Clearly, the chemistry in vivo could be very complicated, but it is also clear that the protective effects of these compounds are likely due to something other than true SOD catalytic activity, e.g., catalase plus stoichiometric superoxide scavenging.

C. Mn^{III}(porphyrinato) Complexes

Mn^{III}(porphyrinato(2−)) complexes have been identified as SOD mimics and studied by several groups. The earliest report of SOD activity by a manganese porphyrin complex was reported by Pasternack and co-workers nearly 20 years ago with the tetrakis(4-*N*-methylpyridyl)porphine complex of Mn^{III}.[86] In this work this complex was shown by both an indirect detection system using nitro blue tetrazolium and by a direct detection of oxygen production via an O_2 electrode to possess a catalytic SOD activity. Subsequent studies by Weinraub et al. document the ability of superoxide to react with and reduce the Mn(III) center to Mn(II) in this complex and other Mn(III)-(porphyrinato) complexes including tetrakis-*o*-(*N*-methylisonicotinamidophenyl)porphyrin (PFP) and [tetrakis-(4-*N*,*N*,*N*,-trimethylanilinium)porphyrin] (TMAP) ligands.[87] Additional studies on this highly cationic complex by the group at Duke University reveals that the complex is reversibly reduced by one electron in aqueous media and retains its activity in the presence of EDTA, suggesting that the complex is stable.[88] This complex has been reported to possess about 0.2% of the catalytic activity of the native Mn SOD enzymes based on data from the indirect assays.[88] In a recent report, a slightly lower k_{cat} for Mn^{III}(TMPyP) (3) is observed ($\sim 4 \times 10^6$ M^{-1} s^{-1}) when the rates were measured using the cytochrome *c* assay at concentrations of complex up to 6×10^{-6} M and utilizing a corrected rate contant for reaction of $O_2^{-\bullet}$ with cytochrome *c*.[88,89]

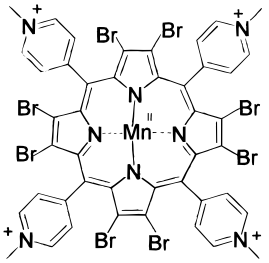
We have studied this complex via the stopped-flow catalytic assay in which a large excess of [superoxide]/[Mn(TMpyP)] was utilized. We find that this



complex is a catalyst for the dismutation of superoxide with an apparent rate constant of about 1×10^7 M^{-1} s^{-1} at pH = 7.4−7.8 and 21 °C—a value consistent with the number reported from indirect assays. This data was obtained at fairly low concentrations of catalyst ($\sim 0.5-2.0 \times 10^{-6}$ M). We observed that as the concentration of catalyst is increased up to 1×10^{-5} M, the catalyst loses activity—in other words the rate does not increase in a linear fashion as the catalyst concentration increases. This phenomenon could be due to saturation of the catalyst, but to ensure this was not so, the $[superoxide]_i$/[Mn-(TMpyP)] was maintained at a high consistent initial ratio. It is more likely that in the strong oxidizing media that the formation of oxo- or hydroxo-bridged dimers occurs. The dimers may not possess the SOD catalytic activity, so as the concentration of complex increases, the dimer formation reaction becomes favored, thereby lowering the apparent catalytic activity. Such dimer formation is, of course, well-known for the oxidized complexes of Mn and Fe with porphyrinato ligands and can, of course, be diminished by building steric constraints onto the porphyrin ligands blocking the approach of metal centers, i.e., "picket-fence" porphyrins.

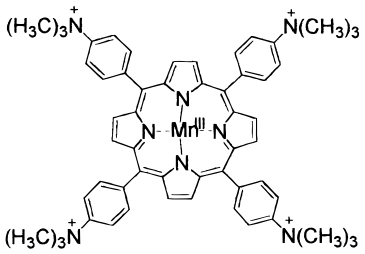
Duke University has patents covering the structure and therapeutic use of some of these Mn(III) porphyrins.[90] Recently, these manganic porphyrin complexes were reported to possess catalase activity[91] and a peroxynitrite reductase (or peroxidase) activity.[92] The catalase activity was studied in vitro in the protection of endothelial cells from injury due to hydrogen peroxide. The two complexes in this study, manganese tetrakis(1-methyl-4-pyridyl)porphyrin, Mn(TMPyP), and manganese tetrakis(4-benzoic acid)-porphyrin, Mn(TBAP), were compared with the respective inactive zinc complexes which have no protective effects.[91] Additionally, this same group has reported the octabromo porphyrinato derivative,[89] manganese(II)(octabromo-*meso*-tetrakis-(*N*-methylpyridinium-4-yl)porphyrin), which possesses increased activity (~60-fold or approximately 12% of the native Mn SOD enzyme). The stability of the Mn(II) state of the complex is low, although the Mn(III) state is quite stable to added EDTA. Studies of this octabromo derivative with *E. coli* showed protection for SOD-null strain at 0.05 mM, but toxicity was noted at 1.0 mM. In other experiments, Mn(TMPyP) was shown to inhibit the antibiotic resistance typically seen under oxidative stress and protected *E. coli* lacking Mn SOD and Fe SOD (termed sodA sodB) against aerobic heating and stationary-phase death.[93] Mn(TMPyP) has been shown to be taken up by *E. coli* until its intracellular concentration is 20-fold greater than the extracellular concentration. Most of

the intracellular complex was bound to protein and found to be inactive. The small percentage of complex that is free acts to protect the cells from $O_2^{-\bullet}$ damage. In this study, Mn(TMPyP) was also shown to inhibit the uptake of paraquat by *E. coli*.[94] It has also been reported that Mn(TMPyP) will decrease the growth inhibitory effect of superoxide produced in the cytoplasm or mitochondria of cultured mammalian cells and does so by an apparent oxidoreductase superoxide scavenging mechanism for its action in vitro.[95]

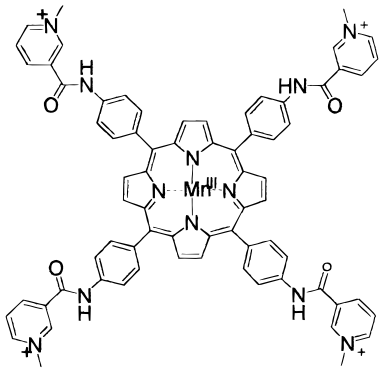


Mn(Br$_8$TMPyP)

Additionally, it was found that the manganic TMPyP complex itself exhibited cellular toxicity and appeared to promote autoxidation. In a separate study, a number of manganese porphyrin complexes, Mn(TpyP) (4-pyridyl), Mn(TMPyP) (1-Me-4-pyridyl), Mn(TMAP) [tetrakis-(4-*N*,*N*,*N*,-trimethylanilinium)-porphyrin], Mn(TBAP)(4-benzoic acid), Mn(TMINP), and tetrakis-*o*-(*N*-methylisonicotinamido-4-phenyl)-porphyrin were studied and the SOD activity determined using indirect the xanthine, ferricytochrome *c* assay, and then using SOD-null cell growth. Mn-



Mn(TMAP)



Mn(TMINP)

(TMPyP) appears to act intracellularly as a nicotinamide adenine dinucleotide phosphate (NADPH):$O_2^{-\bullet}$ oxidoreductase and as a glutathione (GSH):$O_2^{-\bullet}$ oxidoreductase.[88]

Very recently, a series of three isomeric manganic porphyrin derivatives, the ortho, meta, and para isomers of TMPyP, were reported and their activity assessed as SOD catalysts by the indirect cytochrome *c* method at pH = 7.8.[96] These complexes are very acid and base stable, as well as stable to large excesses of EDTA, but appear to suffer degradation of the porphyrin ring in the presence of hydrogen peroxide with half-lives measured in seconds, the order of stability being ortho:meta:para, 3:1:1. The catalytic activities were reported as 6×10^7 4.1×10^6 and 3.8×10^6 M^{-1} s^{-1} for ortho, meta, and para, respectively. It was found that the ortho and meta isomer complexes associate but weakly in vivo and in vitro with RNA and DNA and are protective in vivo in that they promote the growth of SOD-deficient *E. coli* in culture. But the para isomer interacts with DNA strongly and in fact inhibits growth of SOD-deficient *E. coli*.

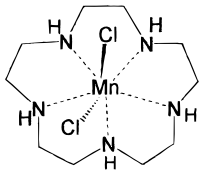
The Mn^{III}(tetrakis(4-benzoic acid)) complex, Mn-(TBAP), mentioned above has been the subject of a recent study in which both SOD activity and catalytic activity for the destruction of peroxynitrite are reported. Additionally, in vivo assays utilizing a carrageenan-induced pleurisy showed that the complex (10 mg/kg prior to carrageenan administration) prevented in a dose-dependent manner the degree of pleural exudation and neutrophil migration into lung tissue of the rat. Additionally, lung myeloperoxidase levels and histological studies show that organ injury was markedly less with drug treatment. The rats treated with Mn(TBAP) showed no nitrotyrosine formation, an indication that in vivo the complex destroys peroxynitrite and/or blocks peroxynitrite formation. Thus, the antinflammatory effects of the complex could be due to multiple scavenging roles, namely, superoxide dismutation and peroxynitrite destruction.[97]

Finally, an interesting derivative of the manganese porphyrins that has recently been described is a Mn complex of a spheroidal bis-porphyrin designed and proposed to be a SOD and catalase mimic. No data on activity is provided.[98] The Mn^{III}(porphyrinato) complexes have clearly been shown to possess true catalytic SOD mimic function and biologic function by many researchers. The issue confronting the use of porphyrin complexes as human pharmaceuticals appears to be one of potential side effects. The myriad redox pathways in which these manganic complexes can participate suggests the possibility of side effects, and the apparent toxicity of certain complexes due to DNA/RNA binding is of concern too. Indeed, it is known that metal porphyrin complexes suffer from a number of potential lethal side effects, including phototoxicity,[99,100] hepatotoxicity,[101,102] and those mentioned above. It is important to note that the porphyrin ligands are amenable to functionalization so that compounds showing toxicity can, at least potentially, be structurally altered so as to mitigate it. Although porphyrin ligand syntheses are notoriously low-yield syntheses, considerable progress in this regard has been made in the application of metalloporpyhrins to magnetic resonance imaging agents and to applications in photodynamic therapy. Fur-

ther, porphyrin ligands allow for a considerable span in redox potentials to be realized and allow for a broad range of complex lipophilicities to be achieved as well. Both factors could well be important in the development of any successful human pharmaceutical agent.

D. Mn^{II}(1,4,7,10,13-pentaazacyclopentadecane) Derivatives

The Mn^{II}(pentaazacyclopentadecane)([15]aneN_5) complexes have been demonstrated to possess catalytic SOD activity by stopped-flow kinetic analysis at [superoxide]/[Mn complex] > 100 and in vivo activity in a range of models involving oxidative stress (some preliminary reviews of this work have appeared).[103−105] The parent unsubstituted complex,[103] Mn([15]aneN_5)Cl_2, crystallizes as a seven-coordinate Mn(II) complex with *trans*-dichloro ligands and has been shown to be an excellent catalyst for the dismutation of superoxide with a rate at pH = 7.4 of 4×10^7 M^{-1} s^{-1}. The complex possesses

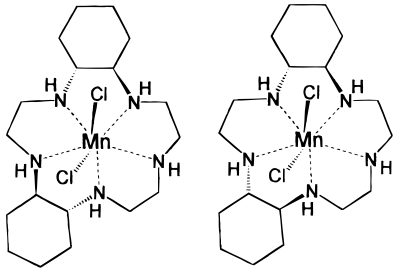


Mn([15]aneN_5)Cl_2

reasonable thermodynamic stability at pH = 7.4 (log K = 10.7) and excellent kinetic stability with the complex completely intact with no metal dissociation for up to days even in the presence of EDTA, which possesses a higher binding affinity for Mn(II) than does the macrocycle. The complex stability was thus adequate to assess this synthetic SOD mimic in a variety of in vitro and in vivo models of superoxide-mediated injury.

This complex is a potent antiinflammatory agent in vivo[106] and inhibits neutrophil-mediated aortic endothelial cell injury in vitro.[107] Subsequent studies with this agent shows that it is effective in potentiating the lifetime of nitric oxide in vitro,[47] presumably, by blocking the diffusion-controlled reaction of NO with superoxide, producing the toxic agent peroxynitrite. This provided the basis for testing the compound in an animal model of platelet-mediated (NO blocks platelet aggregation) thrombosis in injured and stenotic arteries. The complex was shown to be protective in that restenosis of the damaged vessel was inhibited by the agent when administered over a 2 week period.[108] Subsequent studies reveal that the complex is effective in blocking reperfusion injury in an isolated rabbit heart model[109] and in vivo in a canine model of cardiac reperfusion injury.[110]

Confirming that the concept of a *synzyme* for SOD activity had pharmacological relevance to human disease states provided the basis for pursuing this activity for the development of a pharmaceutical agent. The key factors necessary for improving the Mn([15]aneN_5)Cl_2 complex for use as a human pharmaceutical agent were to increase chemical stability and SOD activity, where increasing catalytic activity could conceivably translate into a lower dose of agent. Considerable progress has been made in understanding the mechanism of catalysis[111] and in demonstrating that the chemical stability can be increased by increasing the number of substituents on the carbons of the inner-ring of the macrocycle, while substituents on the nitrogens destroy activity.[112] The structure activity studies reveal that a single *trans*-cyclohexano substitution improves stability and activity, as evidenced by a k_{cat} of 9.09×10^7 at pH 7.4, and improved thermodynamic stability (log K = 11.6) and kinetic stability (2×). One remarkable aspect of the effect that substituents exert on the catalytic rate is revealed with the complex of the macrocycles containing two *trans*-fused cyclohexano groups differing in the stereochemistry of the substitution. Such a change can dramatically increase stability and activity. For example, k_{cat} for the all *R* complex, [Mn(2*R*,3*R*,8*R*,9*R*-biscyclohexano[15]aneN_5)Cl_2], at pH 7.4 is 1.2×10^8 M^{-1} s^{-1} and possesses an increased thermodynamic stability (log K = 13.3) and an enhanced kinetic stability (at any pH over a 500-fold slower rate of dissociation results when compared to the unsubstituted complex). The isomeric complex



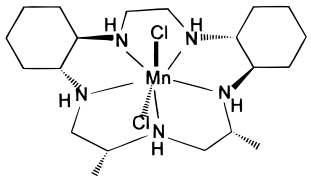
all-*R* $k_{cat} = 1.2 \times 10^{+8}$ M^{-1} s^{-1}

R,R,S,S- $k_{cat} < 5.5 \times 10^{+6}$ M^{-1} s^{-1}

containing the 2*R*,3*R*,8*S*,9*S*-biscyclohexano[15]aneN_5 ligand possesses a similar stability profile but has virtually no catalytic SOD activity.[111]

This contrast in rates, owing to a stereochemical change, made it imperative to better understand the mechanism of catalysis with these complexes so that a highly active and highly substituted (highly stable) complex could be designed and synthesized. The mechanism of the SOD dismutation reaction with these complexes involves two pathways, each of which possesses a rate-determing step which involves oxidation of Mn(II) to Mn(III).[111,113] The major pathway is proton-dependent: in which oxidation of an aquo Mn(II) complex to Mn(III)(hydroxo) occurs via an outer-sphere proton-assisted electron transfer, i.e., H-atom transfer. The less important path involves an inner-sphere path in which a vacant coordination site on Mn(II) must be generated so that superoxide can bind to Mn(II). This pathway is limited to the rate of axial ligand exchange on Mn(II), which for aquo ion exchange is on the order of 1×10^7 s^{-1}. From theoretical arguments, rate data, and labeling studies, we have shown that the faster outer-sphere path as well as the inner-sphere one involve a Mn(II) complex which is preorganized to be in a six-coordinate pseudo-octahedral geometry with a folded pentaaza ligand.[111] The substituents on the macro-

cycle dictate the folding of the ligand about the spherically symmetrical Mn(II) ion, thus making the system particularly well-suited for probing the degree/ease of folding utilizing computational techniques. Modeling studies employing molecular mechanics (MM) calculations reveal that excellent correlations exist for the catalytic rate with the ability of the macrocycle to accommodate such a folded geometry and adopt a six-coordinate nonplanar geometry about Mn(II), i.e., a pseudo-octahedral complex. These MM calculations show that the all *R* complex favors a folded geometry, while the *R,R,S,S* isomer is contrained to a planar seven-coordination mode.[113] Sub-



All-*R*

sequent modeling studies allowed us to design a highly substituted biscyclohexyl−bisdimethyl derivative which was predicted to be highly active as a catalyst and be much more stable owing to the large number of C-substituents (six) on the macrocyclic ring, affording a high degree of preorganizational rigidity. Indeed, the subsequent synthesis and characteriztion studies have shown that the Mn(II) complex with a highly substituted all *R*-bis-2,3,8,9-cyclohexyl-11,14-dimethyl[15]aneN$_5$ ligand affords a complex with an even greater catalytic activity than that of parent unsubstituted complex, $k_{cat} = 5 \times 10^7$ M^{-1} s^{-1} at pH = 7.4). Additionally, this complex possesses a greatly enhanced stability, log $K > 17$, with a kinetic stability of over 12 000 times that of the parent unsubstituted complex. The stability of this complex actually exceeds that of the Gd MRI imaging agents and was found to be completely stable *in vivo* (rodents) for 24 h.[113]

Inhibition of myocardial ischemic and reperfusion injury has been shown in anesthetized cats with the highly active all *R* complex, [Mn(2*R*,3*R*,8*R*,9*R*-biscyclohexano[15]aneN$_5$)Cl$_2$],[114] and both the [Mn-(2*R*,3*R*,8*R*,9*R*-biscyclohexano[15]aneN$_5$)Cl$_2$] complex and its dimethyl derivative, [Mn(all *R*-bis-2,3,8,9-cyclohexyl-11,14-dimethyl[15]aneN$_5$)Cl$_2$], are efficacious in an endotoxin-induced model of intestinal damage.[115]

4. Iron Complexes as SOD Mimics

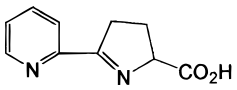
A. General Survey

In many respects the use of iron complexes as synzymes of SOD would seem highly attractive. Iron-based natural enzymes exist, namely, that all anaerobic prokaryotes, if they possess SOD activity, will contain FeSOD exclusively while facultative anaerobes contain both FeSOD and MnSOD. Iron(III) complexes would be attractive since they would exhibit greater kinetic and thermodynamic stability than Mn(II) or Cu(II) complexes. One complicating issue bearing on the iron complexes is that aquo iron-(II,III) and complexes of iron are prone to react with hydrogen peroxide (Fenton chemistry), generating hydroxyl radical.[116,117] This presents a dilemma in that hydrogen peroxide is one of the products of the dismutation of superoxide; thus, a drug candidate which reacts with hydrogen peroxide producing a potentially lethal agent, hydroxyl radical, will of course present serious toxicity concerns. It is for this reason that it is imperative to understand the reactivity of such synthetic iron complexes with H_2O_2 so as to be able to adequately assess their potential as a human pharmaceutical. Since free aquated iron is so reactive with H_2O_2 producing hydroxyl radical, it is also critical that the complexes be completely stable in vivo and excreted intact.

B. Iron Complexes with Non-Macrocyclic Polydentate Ligands

Aminopolycarboxylate complexes of iron(III) (such as EDTA, NTA, etc.) have been reported to possess SOD activity.[118,119] It is also known that such complexes are highly reactive with H_2O_2, generating hydroxyl radicals, and that they are toxic. This reactivity with hydrogen peroxide has been shown to be the source of damage that these complexes inflict on DNA, and this reactivity has been exploited for this use as DNA cleavage agents. These early reports of SOD activity for such Fe complexes point out the difficulties involved in determining unambiguously that a putative catalyst is truly an SOD catalyst. The pitfalls and controversy surrounding the claims of SOD catalytic activity with these complexes has been recounted elsewhere in considerable detail.[56] Nevertheless, a few points need to be made. Early assessments of activity failed to account for hydrogen peroxide reactivity which could readily provide an oxidative bypass to true SOD catalysis. Additionally, most studies relied upon indirect assays without careful corroboration of activity (i.e., utilization of pulse radiolysis or stopped flow direct assays) and many of the reports relied upon the measurement of rate constants for individual reaction steps believed to be the key steps in a catalytic cycle without measuring and observing the catalysis itself.

An iron(II)-containing pigment (neopurpuratin) isolated from bacterial culture was shown to contain iron(II) associated with the D-pyrimine ligand.[120]

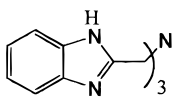


D-Pyrimine

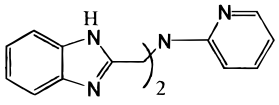
Solution mixtures of this potential ligand with iron-(II) (3:1 [L]/[Fe]) have been claimed to possess SOD activity based on the indirect ferricytochrome *c* assay, but no characterization of the solution species nor isolation of an pure iron complex were reported.[121]

A number of Fe(III) complexes with tripodal ligands favoring trigonal-bipyramidal coordination modes have been synthesized and been claimed to show SOD-like reactivity with superoxide anion, as shown by electrochemical studies.[122] The authors' studies

show that superoxide will not reduce Fe^{III}(salen) complexes at a rate fast enough for them to be competent SOD catalysts but do show that trigonal-bipyramidal-type structures with ligands such as ntb and pb2 can be reduced rapidly with superoxide, producing oxygen and the Fe^{II} complex.



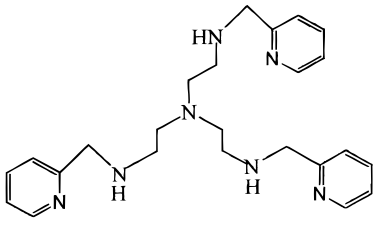
ntb



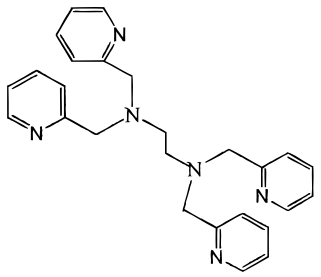
pb2

These studies do not reveal whether such complexes can be real catalysts as the nature and rate of a reoxidation step has not been reported, and no data regarding catalytic efficacy has been reported.

A related group of iron complexes based on the ligands TPEN and TPAA have been reported by Nagano et al. to be SOD mimics, and this area of iron mimics was the subject of a review.[123] On the basis



TPAA



TPEN

of the ferricytochrome *c* indirect assay, the Fe^{II}(TPEN) complex was claimed to possess about a 10-fold greater SOD activity than the Fe^{III}(TPAA). In contrast, the same authors reported that the Fe^{II}(TPEN) complex was about 100-fold less active in protecting cells in a superoxide-mediated cell-killing assay than the Fe^{III}(TPAA) complex.[124,125]

This anomaly between the in vitro cell killing and the results of the indirect activity assay can be readily rationalized based on the results of measuring the catalytic activities of these two complexes via stopped-flow analysis. We independently synthesized and tested these complexes for catalytic activity and found that the Fe(TPAA) complex is a competent catalyst for the dismutation of superoxide with a rate at pH = 7.8 of 2.15×10^6 M^{-1} s^{-1}, whereas the Fe(TPEN) complex displayed no measurable catalytic rate.[34] We further showed that the Fe(TPEN) complex rapidly oxidizes reduced cytochrome *c* in a H_2O_2-dependent reaction which explains the false positive result for Fe(TPEN) in the indirect assay.[34] Fe(TPAA) also oxidizes reduced cytochrome *c* under conditions of the assay but at a much slower rate than Fe(TPEN), which also explains why Fe(TPEN) is more active than Fe(TPAA) in the cytochrome *c* assay. Iuliano et al. carried out pulse radiolysis experiments probing the reaction of Fe(TPEN) with superoxide but utilized a high relative concentration of complex ($[Fe(TPEN)]/[O_2^{-\bullet}] \approx 10$). The ability to distinguish whether the Fe(TPEN) was a true catalyst for the dismutation of superoxide could not be determined from their study.[126] Clearly, the determination of true catalytic activity is necessary for the assessment of SOD activity of any putative SOD enzyme mimic and is critical for any evaluation of a *synzyme's* biological efficacy.

C. Fe^{III}(porphyrinato) Complexes

One of the earliest reported classes of "non-copper" based SOD mimics are based on Fe^{III}(porphyrinato) complexes described by Pasternack et al.[127] The porphyrin ligand which was utilized in these studies is the TMPyP (tetrakis-4-*N*-methylpyridyl)porphyrin)) ligand discussed above as its Mn(III) complex. On the basis of a combination of indirect and direct pulse radiolysis assays, it was shown that this iron(III) complex is indeed a catalyst for the dismutation of superoxide with a reported efficiency of about 3% that of the Cu/Zn enzyme. Subsequent studies have shown that the most active form of this complex is that which possesses an aquo ligand in an axial site. In these studies using pulse radiolysis detection methods in which [superoxide] > 10 [Fe(TMPyP)], a pseudo first-order superoxide dependence was observed indicating true catalysis and a rate higher than that observed in the Pasternack studies was measured.[128] Interestingly, the concentration of catalyst was also less in these studies. We also investigated this catalyst using the stopped-flow methodology previously discussed.[34] We observe that between pH = 7.4 and 8.1 that the Fe^{III}(TMPyP) complex is indeed a catalyst for the SOD reaction, but the observed rate constant was not linearly dependent on the concentration of the catalyst at constant initial superoxide concentration. The rate deviated negatively from linear behavior as catalyst concentration increased. This is consistent with the formation of an Fe(III)−μ-oxo (hydroxo) bridged dimer which possesses less SOD activity. We also observed that this complex oxidizes reduced cyctochrome *c* under the standard assay conditions, thus interfering with the indirect assay measurement.

More recently, two other Fe(III) porphyrin complexes have been reported, and one of these possesses a so-called picket fence like orientation of the 4-substituted porphyrin substituents: Fe^{III}(PFP), where PFP = tetra-*o*-(*N*-methylisonicotinamidophenyl)-porphyrin. The aquo iron(III) complex of this ligand was reported to possess a catalytic rate of 7.6×10^7 M^{-1} s^{-1} for the SOD reaction as monitored by pulse radiolysis with the reduction step being about an order of magnitude slower than reoxidation.[129] Pseudo first-order reaction kinetics were observed in these studies when catalyst concentration (5.7×10^{-7} M) was ~10-fold lower than [superoxide]. Further stud-

ies on the reoxidation of the reduced catalyst revealed that the rate was independent of [Fe(PFP)]. This last result is significant in that it suggests that this more sterically encumbered iron complex is less likely to dimerize, and hence, it does not lose activity at higher concentrations of catalyst. Nevertheless, these active iron catalysts have been shown to possess an inherent limitation in that they promote the Fenton reaction producing hydroxyl radicals from hydrogen peroxide.[130] Not surprisingly, Fe(TMPyP) was reported to be an efficient DNA nicking agent[131] and has been reported to be toxic to *E. coli*.[88] It appears that this class of mimic is limited in its therapeutic potential by its inherent toxicity.

D. Fe^{III}(1,4,7,10,13-pentaazacyclopentadecane) Derivatives

In section 3D of this review, the ability of the Mn^{II}-(1,4,7,10,13-pentaazacyclopentadecane) derivatives to function as SOD catalysts was discussed. These high-spin d^5 Mn(II) complexes function with a rate-determining step in the catalytic cycle being oxidation to Mn(III). The corresponding iron complexes with this same family of symmetrical 15-membered ring pentaaza ligands also crystallize as seven-coordinate *trans*-dichloro complexes and are also catalysts but function with catalytic activities about 10−100-fold less activity than their corresponding Mn(II) analogues.[132] As with the Mn(II) complexes, the stable oxidation state of the complex is that which favors the high-spin d^5 electronic configuration. Thus, the Fe(III) complexes with these ligands function as catalysts in which reduction to iron(II) is the rate-determining step of the catalytic cycle, not oxidation, as with Mn(II). Further mechanistic studies reveal that these complexes are present in aqueous solution at pH = 7.4 predominantly as their Fe^{III}(L)(aquo)-(hydroxo)$]^{2+}$ complex. This is the species whose concentration correlates with catalytic activity. The rate is actually dependent upon generation of a vacant coordination site on the Fe(III) so that an inner-sphere superoxide binding/reduction step can occur. The hydroxo ligand trans to the coordinated water labilizes the water to dissociation and gives rise to the active reducible precursor complex. The reduction occurs via an inner-sphere coordination of superoxide anion to the Fe(III) center. The rates observed correlate very well with the aquo ion exchange rates on the Fe(III) of these complexes, lending additional support to the mechanistic interpretation that the rate of catalysis is limited by the generation of a vacant coordination site on the iron(III) ion.

Subsequent studies in our laboratories reveals that these complexes suffer from a problem similar to that of the Fe^{III}(porphyrinato) complexes, namely, they react with hydrogen peroxide to produce hydroxyl radicals. Not surprisingly, these iron(III) complexes are quite toxic to human endothelial cells that are exposed to activated human neutrophils in culture.

These studies and those of other workers would suggest that the development of iron complexes as human therapeutics possessing the SOD function is constrained by the inherent tendency of iron complexes to react with hydrogen peroxide, generating hydroxyl radicals. This problem has yet to be surmounted and offers the difficult challenge to the chemist of how to design a high-activity iron catalyst for the SOD reaction, which is stable to dissociation but does not react with the product of the dismutation, hydrogen peroxide, producing hydroxyl radical.

5. Conclusion

Catalytic drugs/*synzymes* hold much promise for treating conditions in which the damaging molecule is continuously overproduced. In particular, the overproduction of superoxide following an insult such as occurs with ischemia followed by reperfusion, or following exposure to radiation,[133] or the onslaught of superoxide produced by activated neutrophils in an autoimmune condition such as arthritis can have serious, even lethal consequences to the organism. Functional SOD mimics offer a potential for treating such diseases produced under conditions of oxidative stress.[134] The advantages of a low molecular weight mimic over the enzyme have been recognized in terms of eliminating immunogenicity problems associated with protein administration, lower cost of goods, tissue permeability, potential for oral delivery, and potential for greater in vivo stability.

Of the SOD mimics currently under development, the advantage goes to those with the highest activity and biocompatibility, including biodistribution. Both the Mn^{III}(salen) complexes and the Mn^{II}([15]aneN_5) derivatives have excellent potential as treatments for a wide variety of disease states. Of all the complexes reported to date, these two classes of "mimics" have the most reported data in which the complexes were tested in animal models of human disease.

Research in the area of SOD mimics has been particularly fascinating because the discovery of these new catalysts has evolved in parallel with a greater understanding of the biological roles of superoxide, peroxynitrite, and nitric oxide. We can anticipate that a better understanding of the role of oxidative stress in disease will continue to develop. Such agents as those described here will play an important role in not only developing the fundamental mechanisms involved in the genesis of oxidative stress-induced disease, but ultimately in their treatment.

6. Abbreviations

BSA	bovine serum albumin
CYCLAM	1,4,8,11-tetraazacyclopentadecane
CyDTA	1,2-cyclohexanediaminetetraacetic acid
EAE	experimental allergic encephalomyelitis
EDDA	1,2-ethylendiaminediacetic acid
EDTA	ethylenediaminetetraacetic acid
MI	myocardial infarct
MM	molecular mechanics
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	magnetic resonance imaging
NBT	nitro blue tetrazolium
NTA	nitrilotriacetic acid
ROI	reactive oxygen intermediates
salen	salicyldialdehyde ethylenediimine
SOD	superoxide dismutase

SO	superoxide
TNF_a	tissue necrosis factor alpha
TPA	tissue plasminogen activator

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