

## Salen–Manganese Complexes as Catalytic Scavengers of Hydrogen Peroxide and Cytoprotective Agents: Structure–Activity Relationship Studies

Susan R. Doctrow,<sup>\*,†</sup> Karl Huffman,<sup>†</sup> Catherine Bucay Marcus,<sup>†</sup> Georges Tocco,<sup>†</sup> Evelyne Malfroy,<sup>†</sup> Christy A. Adinolfi,<sup>†</sup> Henry Kruk,<sup>‡</sup> Keith Baker,<sup>†</sup> Natalie Lazarowych,<sup>‡</sup> Julius Mascarenhas,<sup>‡</sup> and Bernard Malfroy<sup>†</sup>

*Eukarion, Inc., 6F Alfred Circle, Bedford, Massachusetts 01730, and Dalton Chemical Laboratories, Inc., Toronto, Ontario*

*Received May 14, 2002*

Synthetic catalytic scavengers of reactive oxygen species (ROS) may have broad clinical applicability. In previous papers, two salen–manganese complexes, EUK-8 and EUK-134, had superoxide dismutase (SOD) and catalase activities and prevented ROS-associated tissue injury. This study describes two series of salen–manganese complexes, comparing catalytic ROS scavenging properties and cytoprotective activities. The compounds vary widely in ability to scavenge hydrogen peroxide, with this activity most influenced by salen ring alkoxy substitution and aromatic bridge modifications. In contrast, all compounds show comparable SOD activities. The most active alkoxy-substituted catalase mimetics protected cultured cells from hydrogen peroxide, and a subset of these were also neuroprotective in a rodent stroke model. Thus, structural modification of the prototype EUK-8 yields compounds with enhanced catalase activity and, in turn, biological effectiveness. This supports the concept that salen–manganese complexes represent a class of SOD and, in particular, catalase mimetics potentially useful against ROS-associated diseases.

### Introduction

Oxygen, though essential for aerobic metabolism, can be converted to toxic metabolites, such as the superoxide anion and hydrogen peroxide, collectively known as reactive oxygen species (ROS). Increased ROS formation occurs under numerous pathological conditions including, broadly, ischemic and inflammatory processes.<sup>1</sup> When ROS generation exceeds the capacity of endogenous antioxidant defense systems to neutralize them, a condition known as oxidative stress,<sup>2</sup> tissues become vulnerable to damage. Antioxidant therapies have long been considered for a wide variety of disorders in which oxidative stress is believed to play a significant role.<sup>1,3</sup>

Endogenous antioxidant enzymes include the superoxide dismutases (SODs) (Cu-, Zn-, and Mn-containing forms) and catalase, which catalyze the neutralization of superoxide and hydrogen peroxide, respectively. Other enzymes acting against hydrogen peroxide include peroxidases and glutathione peroxidase. Exogenously administered SOD has been tested in various clinical indications with limited success. This has led to an interest in developing synthetic SOD mimetics with more favorable pharmaceutical properties than a protein such as SOD. The first low molecular weight SOD mimetics to be described were copper complexes that showed antitumor-promoting activity.<sup>4</sup> Subsequently, several classes of manganese complexes purported to mimic manganese SOD were described. These include complexes formed between the manganese and the chelator desferrioxamine,<sup>5</sup> a series of manganese–porphyrin complexes,<sup>6,7</sup> a class of manganese–macro-

cyclic ligand complexes,<sup>8,9</sup> and the salen–manganese complexes that are the focus of this research.<sup>10</sup> Certain of these manganese complexes have shown protective activity in various experimental disease models.<sup>9,11–15</sup> One class of nonmetal-containing compounds, carboxy-fullerenes, have also been reported to have catalytic ROS scavenging as well as neuroprotective activities.<sup>16</sup> A low molecular weight selenium complex known as Ebselen has been developed as a mimetic for glutathione peroxidase and has also shown protection in various models for oxidative stress.<sup>17</sup>

We have previously reported<sup>10</sup> that synthetic salen–manganese complexes are SOD mimetics. The series of compounds described in that study was either inactive or exhibited a range of SOD activities over 2 orders of magnitude. These compounds included EUK-8, one of the more active and the most water soluble of the series. EUK-8 was subsequently shown to exhibit catalase<sup>18,19</sup> and peroxidase<sup>19</sup> activities, showing that salen–manganese complexes can scavenge hydrogen peroxide in addition to superoxide. (Of the other classes of Mn-containing SOD mimetics listed above, the metalloporphyrins have also been found to exhibit hydrogen peroxide scavenging activities,<sup>7</sup> while the macrocyclic complexes reportedly show specificity for superoxide and are being designed to optimize SOD activity.<sup>9</sup>) Because hydrogen peroxide is produced *in vivo* during oxidative stress, is cytotoxic, and is stable in solution as compared to superoxide, the ability to consume hydrogen peroxide is regarded as an advantageous property of the salen–manganese complexes.<sup>19</sup> Despite this, relatively little effort has focused on the development and optimization of catalase mimetics for possible therapeutic use.

In support of the potential clinical value of these multifunctional catalytic ROS scavengers, EUK-8 is protective in numerous pharmacological models for

\* To whom correspondence should be addressed. Tel: 781-275-0424. Fax: 781-275-0752. E-mail: s.doctrow@eukarion.com.

<sup>†</sup> Eukarion, Inc.

<sup>‡</sup> Dalton Chemical Laboratories, Inc.

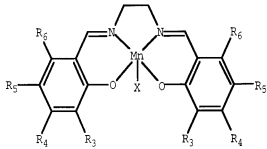
ROS-associated tissue damage. For example, EUK-8 protected organotypic hippocampal cultures from toxicity by the  $\beta$ -amyloid peptide.<sup>20</sup> Administered in vivo, it inhibited the degeneration of dopaminergic neurons in two murine models for Parkinson's Disease<sup>19</sup> and suppressed the development of paralysis in mouse experimental autoimmune encephalomyelitis, a model for multiple sclerosis.<sup>21</sup> Intravenously infused EUK-8 preserved pulmonary function in a highly stringent porcine endotoxin-induced model for the adult respiratory distress syndrome.<sup>18,22</sup> Subsequently, EUK-134, a 3,3'-methoxy salen ring-disubstituted EUK-8 analogue with equivalent SOD activity but enhanced catalase activity, was described and shown to be more neuroprotective than EUK-8 in a rodent stroke model.<sup>23</sup> EUK-134 protected hippocampal neurons and suppressed several indicators of stress and tissue damage in a kainic acid-induced seizure model in rats,<sup>24</sup> and both EUK-8 and EUK-134 extended the lifespan in the nematode worm *Caenorhabditis elegans*.<sup>25</sup> More recently, EUK-8 and EUK-134 were shown to prolong survival and attenuate indicators of spinal cord oxidative stress in a mouse model for familial amyotrophic lateral sclerosis,<sup>26</sup> and three salen-manganese complexes were found to prolong survival and rescue oxidative pathologies in mice lacking manganese SOD.<sup>27</sup>

Thus, a substantial body of evidence exists for the protective efficacy of salen-manganese complexes, as exemplified by EUK-8 and EUK-134, in models for ROS-associated pathologies. In this paper, we describe the synthesis and evaluation of two series of salen-manganese complexes, all structural analogues of EUK-8. The evaluation includes comparing their hydrogen peroxide and superoxide scavenging properties as well as their cytoprotective activities in a cell culture model. In addition, a subset of compounds are evaluated for neuroprotective efficacy in a rodent stroke model.

## Results and Discussion

**Salen Ring Substitutions Affect Hydrogen Peroxide Scavenging Activities of Salen-Manganese Complexes.** Figure 1 shows the structures of the ring-substituted series of salen-manganese complexes evaluated in this study. The Schiff base ligands used to complex manganese(III) are derivatives of the tetradentate ligand bis(salicylaldehyde)ethylenediamine (salen-H<sub>2</sub>).<sup>28</sup> Compounds were synthesized as described in the Experimental Section, with either chloride or acetate as axial ligand. In all cases, those compounds with an axial acetate ligand were found to be more water soluble than the corresponding chlorides. The prototype compound, EUK-8, contains a chloride axial ligand and unsubstituted salen ligand. It has previously been reported to exhibit SOD activity of about 700 units/mM.<sup>10</sup> The other complexes contain ligands with symmetrical substitutions on the salen rings as shown in Figure 1. This includes several chloride and acetate pairs, which differ only in the axial ligand. The two members of each pair showed similar, if not identical, activity in the various assay systems, as discussed further below. Two compounds, EUK-160 and EUK-189, are represented only as the more water soluble acetate complexes.

We have previously reported that salen-manganese compounds, including the unsubstituted prototype com-



Compound	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	R <sub>6</sub>	X
EUK-8	H	H	H	H	Cl
EUK-108	H	H	H	H	OAc
EUK-122	F	H	H	H	Cl
EUK-121	F	H	H	H	OAc
EUK-15	H	H	OMe	H	Cl
EUK-123	H	H	OMe	H	OAc
EUK-134	OMe	H	H	H	Cl
EUK-113	OMe	H	H	H	OAc
EUK-124	H	OMe	H	OMe	Cl
EUK-118	H	OMe	H	OMe	OAc
EUK-115	H	OMe	H	H	Cl
EUK-114	H	OMe	H	H	OAc
EUK-160	OMe	H	OMe	H	OAc
EUK-189	OEt	H	H	H	OAc

**Figure 1.** Structures of salen-manganese complexes. Substituents are as indicated in the table. OMe, methoxy (CH<sub>3</sub>O); OEt, ethoxy (CH<sub>3</sub>CH<sub>2</sub>O); OAc, acetoxy (CH<sub>3</sub>COO).

pound EUK-8, have SOD activity<sup>10</sup> but, subsequently, EUK-8 was also shown to have catalase activity, as detected by the generation of a stoichiometric excess of oxygen in the presence of hydrogen peroxide.<sup>18,19</sup> The catalase activity of EUK-8 is not saturable over a wide range of hydrogen peroxide concentrations (0.1–100 mM). Similarly, kinetic analyses of mammalian catalases indicate that the enzymes lack a *K<sub>m</sub>* for hydrogen peroxide and therefore exhibit increased activity as the intracellular hydrogen peroxide concentration increases.<sup>29</sup> (This is in contrast to dimanganese-containing bacterial catalases, which reportedly display saturation kinetics.<sup>30</sup>) EUK-8 also exhibits peroxidase activity,<sup>19</sup> which is consistent with its function as a catalase. Catalases are well-known to be capable of undergoing peroxidative as well as catalytic reactions.<sup>29,31</sup> In this manner, in the presence of a suitable electron donor, catalase driven decomposition of hydrogen peroxide proceeds via partitioning between catalytic and peroxidatic reactions.<sup>31</sup> EUK-8 and other salen-manganese complexes have a broader peroxidase substrate specificity than that reported for catalase proteins, for example, enabling the convenient assay of this activity with colorimetric substrates such as 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS). As with its catalase activity, the peroxidase activity of EUK-8 was not saturable with respect to hydrogen peroxide. Both catalase and peroxidase activities of EUK-8 were found to be pH sensitive, with activity increasing at increasing pH in the range of pH 6 to pH 9.

Catalase and peroxidase activities of the salen ring-substituted analogues are compared in Table 1. Catalase activity was assayed by monitoring the conversion of hydrogen peroxide to oxygen as described in the Experimental Section. The table presents initial rates as well as the maximal amount of oxygen produced,

**Table 1.** Catalytic Hydrogen Peroxide Scavenging Activities of Ring-Modified Salen–Manganese Complexes<sup>a</sup>

compd	catalase rate ( $\mu\text{M O}_2/\text{min}$ )	endpoint (maximal $\mu\text{M O}_2$ )	peroxidase rate ( $\mu\text{M ABTS}/\text{min}$ )
EUK-8	148 $\pm$ 27	27 $\pm$ 6	22.4 $\pm$ 4.1
EUK-108	149 $\pm$ 21	25 $\pm$ 4	23.6 $\pm$ 4.7
EUK-121	201 $\pm$ 39	28 $\pm$ 2	22.3 $\pm$ 0.7
EUK-122	197 $\pm$ 27	28 $\pm$ 1	23.6 $\pm$ 0.9
EUK-15	186 $\pm$ 2	84 $\pm$ 1	25.3 $\pm$ 5.5
EUK-123	112 $\pm$ 14	55 $\pm$ 4	19.9 $\pm$ 0.5
EUK-134	243 $\pm$ 18	81 $\pm$ 2	37.8 $\pm$ 9.6
EUK-113	260 $\pm$ 45	79 $\pm$ 9	36.0 $\pm$ 7.0
EUK-114	70 $\pm$ 8	11 $\pm$ 3	0.8 $\pm$ 0.2
EUK-115	81 $\pm$ 14	12 $\pm$ 0.2	0.8 $\pm$ 0.2
EUK-124	nd	nd	nd
EUK-118	35 $\pm$ 6	9 $\pm$ 4	−0.1 $\pm$ 0.1
EUK-160	235 $\pm$ 47	57 $\pm$ 9	44.8
EUK-189	180 $\pm$ 7	80 $\pm$ 3	30.0

<sup>a</sup> Catalase and peroxidase activities were conducted as described in the Experimental Section. Where indicated (nd), the assay was not performed because of poor solubility of the compound EUK-124.

calculated from time-dependent plots as described. The latter provides an indication of total substrate turnovers completed prior to inactivation of the compound.<sup>18</sup> As previously reported for EUK-8, the compounds are inactivated under the catalase assay conditions. The mechanism of inactivation may involve peroxidative decomposition of the compound and/or formation of a  $\mu$ -oxo or similar higher oxidation species,<sup>32</sup> but this has not been studied and is currently unknown. At present, we regard both parameters, initial rate and maximal oxygen production, as being useful to compare the hydrogen peroxide scavenging properties of various analogues, and consequently, both parameters are reported here. As shown in Table 1, salen ring substitution influences catalase activity in this series of compounds. For example, analogues with methoxy substitutions at the 3- (EUK-113, EUK-134) or 5-positions (EUK-15, EUK-123) completed 2–3 times as many reaction turnovers as EUK-8. The 3-substituted compounds had, in addition, an initial rate about twice as fast as EUK-8 and comparable analogues and were, therefore, the most active catalase mimetics of this series. The compound with the lowest catalase activity was EUK-118, the 4,6-methoxy-substituted compound. The relative peroxidase activities of the salen ring-substituted analogues showed a good correlation to their relative catalase activities (Table 1), as might be expected based upon the proposed relationship between catalytic and peroxidatic reactions. EUK-113 and EUK-134 were the fastest peroxidases while EUK-118 was the poorest, in this case having undetectable activity. For comparison, under these catalase assay conditions, bovine liver catalase (29 units/mL) produced oxygen at the rate of  $\sim 0.33$  mM/min with no apparent inactivation. Under the peroxidase assay conditions, the same concentration of bovine liver catalase showed no peroxidase activity toward ABTS.

Overall, it is apparent that catalase and peroxidase activities differ markedly among the various compounds in this series. Most notably, the presence of methoxy substituents at the 3-positions, as exemplified by EUK-113 and EUK-134, increases the catalase and peroxidase

activities as compared to the unsubstituted EUK-8 and EUK-108. The presence of methoxy groups at the 4-positions, in EUK-114 and EUK-115, markedly reduces the catalase and peroxidase activities relative to the unsubstituted analogues. The activity is even further weakened in EUK-118, with methoxy groups at the 4,6-positions. The 3,5-disubstituted analogue, EUK-160, showed catalytic activities approximately equivalent to those of EUK-113 and EUK-134, as did a 3-ethoxy compound, EUK-189.

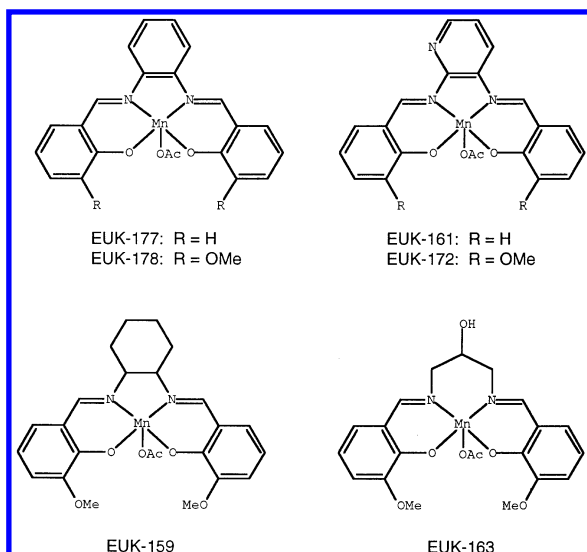
These studies yielded no evidence that the catalase and peroxidase activities are dissociable from one another since the rank order of the compounds was the same for both activities. This was not unexpected since the two reactions are hypothesized to arise, as alternative arms, from the same catalytic cycle. However, it is possible that some analogues will show differences in preference for one reaction pathway over the other. For example, EUK-118 showed measurable catalase activity but no detectable peroxidase activity, even though the latter assay is the more sensitive. Furthermore, we have found that analogues with longer chain 3-alkoxy substituents retain catalase activity but exhibit decreased peroxidase activity (data not shown). While the likelihood exists that certain structural alterations might produce a catalase without peroxidase activity, or vice-versa, in this ring-modified series of salen–manganese complexes, we conclude that the catalase and peroxidase activities are closely correlated with one another.

It has been reported that some peroxidases use organic peroxides as alternative substrates to hydrogen peroxide. In an analogous spectrophotometric peroxidase assay, EUK-8 exhibited weak peroxidase activity with 1 mM *tert*-butylhydroperoxide, oxidizing ABTS at a rate about 0.5% of that observed with the same concentration of hydrogen peroxide. (In comparison, horseradish peroxidase utilized *tert*-butylhydroperoxide with an ABTS oxidation rate that was about 5.4% of the rate with hydrogen peroxide.) EUK-113 was about a 3-fold faster peroxidase with *tert*-butylhydroperoxide than EUK-8. Interestingly, EUK-121 and EUK-122 were both even faster peroxidases with *tert*-butylhydroperoxide, about twice as fast as EUK-113. This observation implies that the various salen–manganese complexes may, as peroxidases, differ in their relative hydroperoxide specificities. EUK-118 had less than 2% of the peroxidase activity toward *tert*-butylhydroperoxide as did EUK-8. The ability to consume organic peroxides may have some relevance to an ability of compounds to scavenge lipid peroxides in tissues.

**Bridge Modifications Further Affect Catalase Activities of Salen–Manganese Complexes.** A second series of salen–manganese complexes was next synthesized, containing bridge structures different from the ethano bridge of the previous series. On the basis of the structure–activity data obtained with the first series, some of these bridge-modified analogues were also prepared with 3-methoxy symmetrical salen ring substitution. The structures of the compounds in the bridge-modified series are shown in Figure 2.

The catalase activities of these compounds are compared in Table 2. Because the axial ligand was found not to influence the catalytic activities, these compounds are analyzed only in the more water soluble form with





**Figure 2.** Structures of bridge-modified salen–manganese complexes. Abbreviations as for Figure 1.

**Table 2.** Catalase Activities of Bridge-Modified Salen–Manganese Complexes<sup>a</sup>

compd	catalase rate ( $\mu\text{M O}_2/\text{min}$ )	endpoint (maximal $\mu\text{M O}_2$ )
EUK-108	149 $\pm$ 21	25 $\pm$ 4
EUK-161	890 $\pm$ 144	206 $\pm$ 19
EUK-177	552 $\pm$ 131	119 $\pm$ 13
EUK-113	260 $\pm$ 45	79 $\pm$ 9
EUK-172	1073 $\pm$ 174	300 $\pm$ 14
EUK-178	814 $\pm$ 155	230 $\pm$ 7
EUK-159	251 $\pm$ 29	91 $\pm$ 5
EUK-163	36 $\pm$ 6	5 $\pm$ 1

<sup>a</sup> Compounds were assayed for catalase activity as described for Table 1. Values for the appropriate reference compounds (EUK-108 and EUK-113) without bridge modification are included again for comparison.

acetate as the axial ligand. Reference compounds included in Table 2 from the previous series for comparison are EUK-108 and EUK-113. This series of compounds showed an even wider range of catalase activities than the ring-modified series. For example, compounds containing six-membered aromatic bridge structures (EUK-161 and EUK-177) exhibited dramatically increased catalase activity as compared to the reference compound EUK-108 as well as to the best compounds identified in the salen ring-substituted series. Addition of 3-methoxy substituents (EUK-172 and EUK-178, respectively) resulted in even greater catalase activity. No enhancement of activity was obtained with a six-membered aliphatic bridge (EUK-159), which showed catalase activity equivalent to that of the reference compound EUK-113. An analogue containing a three carbon aliphatic bridge (EUK-163) exhibited very low catalase activity, comparable to that of the weakest catalase in the salen-substituted series. These findings suggest that a more planar structure, as might be afforded by the aromatic bridges and lost in EUK-163, might facilitate optimal catalase activity. In addition, the electronic effects of the aromatic bridge and extended aromatic conjugation are likely to play a role in enhancing catalase activity. (Peroxidase activity values are not presented for the bridge-modified series of compounds because the high catalase activity of analogues with aromatic bridge structures quenched any

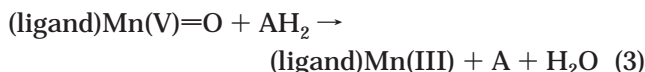
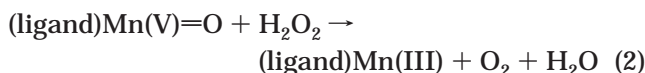
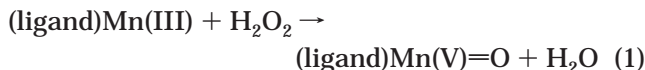
apparent peroxidase activity, as explained in the Experimental Section. This indicates that unlike the ethano-bridged compounds, these analogues show a preference of the catalytic vs the peroxidatic reaction pathway in the catabolism of hydrogen peroxide, at least under these experimental conditions.)

As in any research with metal complexes, the possibility exists that activities are attributable to the metal and/or the ligand liberated by dissociation of the complex. These possibilities were regarded as unlikely, primarily because the salen–manganese complexes are highly stable in aqueous solution, while the dissociated ligands are unstable. For example, EUK-8, EUK-134, and EUK-189 show no detectable decomposition, based on high-performance liquid chromatography (HPLC) analysis, over storage for many months at room temperature in aqueous solution. The aromatic bridge compounds have somewhat lower stability; for example, EUK-178 decomposes over several days in aqueous solution. In all cases, however, stability of the metal complexes is significantly greater than that of the metal free ligands. For example, the ligands for EUK-8 and EUK-134 have half-lives of less than 15 min in aqueous solution and the other ligands are similarly unstable. The hydrogen peroxide scavenging activities of these components were examined by assaying control compounds, comprising the metal, the ligands, or the breakdown products of the ligand, namely, the diamines and salicylaldehydes, for catalase and peroxidase activities as described in the Experimental Section. The following control compounds were tested for catalase activity: five different ligands (EUK-8/108, EUK-134/113, EUK-189, EUK-178, and EUK-172), 3-O-methoxysalicylaldehyde, 3-O-ethoxysalicylaldehyde, ethylenediamine, Mn(II) acetate, and Mn(III) acetate. None of these compounds showed oxygen generation in excess of background levels for the oxygen electrode assay with respect to the initial rates (calculated values for controls ranged from  $-7$  to  $8 \mu\text{M}/\text{min}$ ) or total oxygen generation (calculated values for controls did not exceed  $1 \mu\text{M}$ ). In addition, none of these compounds showed detectable peroxidase activity. Therefore, it is concluded that the catalytic hydrogen peroxide activities summarized in Tables 1 and 2 are attributable to the intact salen–manganese complexes.

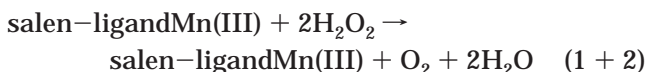
**Ring Substitutions and Bridge Modifications Do Not Affect SOD Activity of Salen–Manganese Complexes.** The SOD activities of both sets of compounds were determined as described in the Experimental Section. The  $\text{IC}_{50}$  values given for each compound in the SOD assay are equivalent to 1 international unit of SOD.<sup>6,10</sup> For all compounds except two, the SOD activities did not vary, with a mean  $\text{IC}_{50}$  value of  $0.7 \pm 0.2$  (SD) for the entire set. Two analogues, EUK-118 and EUK-124, showed slightly lower SOD activities, with  $\text{IC}_{50}$  values of 2 and  $5 \mu\text{M}$ , respectively (with EUK-124's poor solubility perhaps contributing to its lower apparent activity). Primarily, in comparison to other salen–manganese complexes tested previously, which had  $\text{IC}_{50}$  values ranging from about 0.3 to  $30 \mu\text{M}$ ,<sup>10</sup> all compounds in the present series might be regarded as having similar to identical SOD activity to one another and comparable SOD activities to the best molecules in the series reported by Baudry et al.<sup>10</sup> Their SOD activities

are also comparable to that of MnTMPyP, a metalloporphyrin SOD mimetic with a reported rate constant for reaction with superoxide of  $3.9 \times 10^7$ .<sup>6</sup> This compound, tested in parallel with the salen–manganese complexes in our assay system, had an IC<sub>50</sub> value of  $0.4 \pm 0.1 \mu\text{M}$ , as compared to the published value of  $0.7 \mu\text{M}$ .<sup>6</sup>

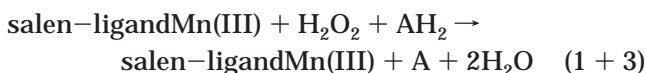
Thus, in contrast to the SOD activities of these and similar compounds, their catalytic hydrogen peroxide scavenging activities are profoundly influenced by the ligand structure. A potential mechanism for the catalase activity we've observed, taking into account the ability to catalyze a peroxidative pathway, can be described by the following scheme, where AH<sub>2</sub> is an oxidizable substrate such as ABTS:



Catalase reaction:

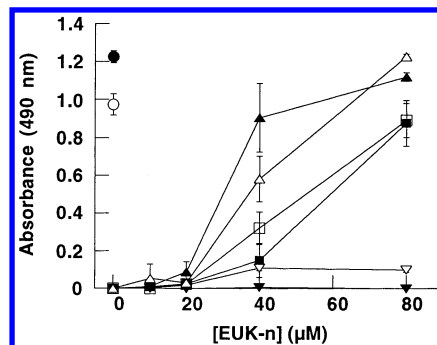


Peroxidase reaction:



In this proposed scheme, (ligand)Mn(V)=O is functionally analogous to “compound I”, the intermediate first described for the haem catalases.<sup>29</sup> While the exact structure of compound I is not known for this or for other metal complexes and metalloenzymes conducting such reactions, the intermediate is believed to result from a two electron oxidation of the catalytic complex by hydrogen peroxide.<sup>1</sup> When salen–manganese complexes are incubated with hydrogen peroxide, they undergo a spectral change consistent with formation of an oxidized intermediate (data not shown) and an analogous oxomanganese species has been proposed as the active intermediate in oxidative reactions by porphyrin–manganese<sup>33</sup> and salen–manganese<sup>34</sup> complexes. This scheme is also consistent with the observation that electron-donating alkoxy substituents enhance activity when located ortho or para to the metal complexation site of the salen ring (Table 1). Further enhancement of activity by aromatic bridge structures (Table 2) might also be predicted, through electronic contributions, most likely via resonance in the vicinity of the metal-binding nitrogen sites. The relative independence of these two effects is consistent with their apparent additivity (Table 2).

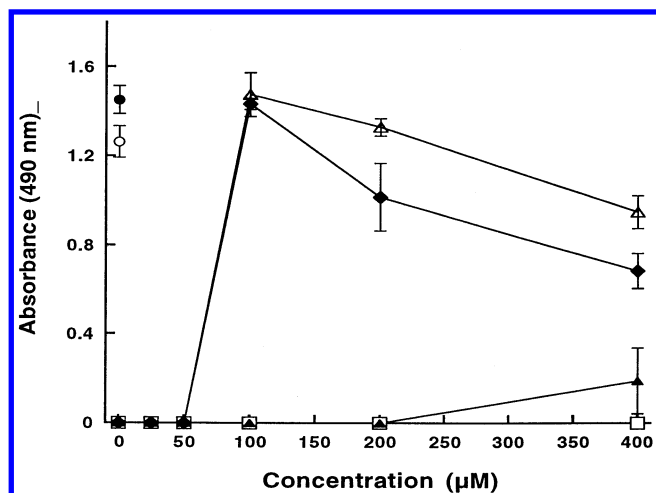
The relative involvement of different ROS in a given pathological setting is controversial. However, superoxide can dismutate spontaneously in solution to form hydrogen peroxide, which is long-lived, believed to freely diffuse across cell membranes, and is well-documented to be cytotoxic, either directly or by secondary produc-



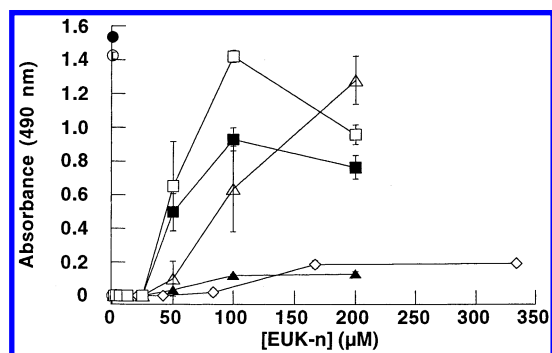
**Figure 3.** Cytoprotection by salen ring-substituted salen–manganese complexes. Cytoprotection studies were performed as described in the Experimental Section. Absorbance values given are the means  $\pm$  SD of triplicate samples. Control cells (open circle) received no glucose oxidase. Catalase-treated (solid circle) cells received glucose oxidase (0.02 units/mL) as well as bovine liver catalase (290 units/mL). Other samples received the same dose of glucose oxidase and the indicated concentrations of salen–manganese complex: EUK-134 (open triangle), EUK-113 (solid triangle), EUK-15 (open square), EUK-123 (solid square), EUK-8 (open inverted triangle), and EUK-118 (solid inverted triangle). EUK-108, EUK-121, EUK-122, EUK-114, and EUK-115 showed poor cytoprotective activity, comparable to that of EUK-8, and have been omitted from the figure for clarity. In separate experiments, EUK-160 (data not shown) and EUK-189 (Figure 5) showed equivalent cytoprotection activity to that of EUK-134. Inactivity in this assay was not found to be associated with cytotoxicity of the compounds for the cells, which was tested in parallel as described in the Experimental Section. For example, EUK-8 and EUK-15 showed no cytotoxicity at up to  $400 \mu\text{M}$ .

tion of hydroxyl radicals by the Fenton reaction.<sup>1</sup> While SOD and SOD mimetics are protective in some biological settings, they are also potentially deleterious due to their production of hydrogen peroxide. Overall, this implies that catalase activity would be a key attribute for synthetic ROS scavenging compounds. We next conducted studies to investigate the potential value of these catalase mimetics as cytoprotective agents. One goal was to assess the predictive value of catalase structure–activity relationships observed with these two series of salen–manganese complexes in such biological efficacy studies. We employed two biological models, an *in vitro* model for hydrogen peroxide-dependent cellular toxicity and an *in vivo* model for cerebral ischemia.

**Cytoprotective Activity.** Salen–manganese complexes were tested for protection of human dermal fibroblasts (HF cells) against glucose and glucose oxidase, a hydrogen peroxide-generating system. As shown in Figure 3, addition of glucose oxidase to the HF cell culture system resulted in complete lethality and bovine liver catalase afforded full protection. Most of the salen ring-modified salen–manganese complexes were essentially ineffective at protecting HF cells in this paradigm, with the exception of the 3- and 5-alkoxy-substituted compounds. Thus, cytotoxicity by glucose and glucose oxidase was highly discriminating between the various salen–manganese complexes. The most effective cytoprotective compounds, EUK-113 and EUK-134, were also the most active in catalase/peroxidase assays. EUK-160 (data not shown) and EUK-189 (Figure 4) showed equivalent cytoprotective activities. Only slightly less effective were EUK-15 and EUK-123. Altogether, these six compounds were highly efficacious,



**Figure 4.** Comparative cytoprotection activity of EUK-134 and control compounds. Cytoprotection studies were performed as described, with control and catalase-treated cells as indicated in the legend for Figure 3. The compounds tested were EUK-134 (open triangle), EUK-189 (solid diamond), Mn(II) acetate (open square), and 3-methoxysalicylaldehyde (solid triangle).



**Figure 5.** Cytoprotection by bridge-modified salen-manganese complexes. Cytoprotection studies were performed as described, with control and catalase-treated cells as indicated in the legend for Figure 3. The compounds tested were EUK-113 (open triangle), EUK-172 (solid square), EUK-178 (open square), EUK-161 (open diamond), and EUK-177 (solid triangle). EUK-163 was tested separately at up to 400  $\mu$ M and showed no cytoprotective activity (data not shown). Of the compounds shown, only EUK-178 showed some moderate cytotoxicity in the absence of glucose oxidase at 200  $\mu$ M while EUK-177 and EUK-172, as well as EUK-178, showed cytotoxicity only at 400  $\mu$ M.

displaying virtually complete protection. In contrast, the other complexes displayed little or no protection at any concentration tested. EUK-118 showed a complete lack of cytoprotection in the model, consistent with its virtual inactivity in the catalase/peroxidase assays. Control compounds lacking catalase activity were also inactive in this model. For example (Figure 4), 3-methoxysalicylaldehyde and Mn(II)acetate were inactive, as compared in parallel to the fully protective salen-manganese complexes EUK-134 and EUK-189. Ethylenediamine was cytotoxic for HF cells, as were various ligands, due most likely to their rapid dissociation to release the toxic diamine.

The bridge-substituted compounds were also evaluated for protective activity in the HF cell cytotoxicity model. The results (Figure 5) showed that compounds with aromatic bridges as well as 3-methoxy substituents (EUK-172 and EUK-178) were highly cytoprotective in

this model. Commensurate with their enhanced catalase activity, they were cytoprotective at lower doses than analogous compounds with the ethylenediamine bridge (e.g., EUK-113). However, interestingly, the aromatic bridged compounds lacking 3-methoxy substituents (EUK-161 and EUK-177) showed poor cytoprotective activity, despite their very high catalase activity. Taken together with data obtained with the ring-substituted series, this implies that both catalase activity and alkoxy substitution at the salen rings are required for cytoprotection in this HF model. Neither alone is sufficient, as evidenced, for example, by the poor cytoprotectiveness of methoxy-substituted compounds with poor catalase activity, such as EUK-163 and EUK-118. Lack of cytoprotective activity cannot be explained by toxicity of the compounds for HF cells. For example, of the bridge-modified compounds tested in Figure 5, EUK-161 was the least toxic, displaying no apparent cytotoxicity at 400  $\mu$ M. This does not, however, eliminate the potential role of a hydrogen peroxide-dependent toxicity caused by compounds lacking alkoxy substituents. In support of this possibility, in the presence of EUK-161 (200  $\mu$ M), HF cells were about 2–4-fold more sensitive to GO, as evidenced by cell death at lower GO concentrations. By comparison, EUK-161 at 100  $\mu$ M and EUK-8 at both concentrations did not significantly affect the dose-dependent sensitivity of HF cells to GO, while EUK-134 at both concentrations was protective (data not shown). While it is apparent that alkoxy substituents are advantageous for protective activity of salen-manganese complexes in this cellular model, the reasons for this element of the cytoprotection structure-activity relationship are not yet understood, but in some cases, a synergistic toxicity with hydrogen peroxide might play a role.

A relatively simple factor, such as lipophilicity, does not appear to play a role in this cytoprotection model, since EUK-134, the ethoxy analogue EUK-189 (Figure 4), and the cyclohexyl-bridged compound EUK-159 (data not shown) show identical cytoprotective activities, consistent with their equivalent catalase activities (Tables 1 and 2), and without regard to the greater lipophilicity of both EUK-189 and EUK-159 in octanol partitioning studies (data not shown). In a previously published study, EUK-134 and EUK-189 were highly protective and similarly effective at protecting rat primary neurons from hydrogen peroxide, in this case, administered directly as a bolus, rather than enzymatically.<sup>35</sup> Interestingly, in contrast to these observations with extracellular hydrogen peroxide, EUK-189 was recently shown to be approximately 2 orders of magnitude more potent than EUK-134 at inhibiting apoptosis in primary neurons<sup>36</sup> as well as being more effective in a mouse model for mitochondrial oxidative stress,<sup>27</sup> presumably via intracellular mechanisms more likely to be influenced by lipophilicity. It should also be noted that EUK-8, EUK-134, and EUK-189 have comparable activities and are all highly effective at protecting primary neurons and PC12 cells from toxicity by the peroxynitrite-generating agent sin-1.<sup>35</sup> Protection against sin-1 toxicity may be via a SOD activity and/or a direct interaction with the reactive nitrogen species nitric oxide or peroxynitrite. Manganese porphyrins, which, as noted above, have catalytic properties similar to those



of the salen–manganese complexes, are known to scavenge peroxynitrite,<sup>37</sup> and there has been indirect evidence that salen–manganese complexes can do so as well.<sup>38</sup> The details of this property, and structure–activity relationships of various salen–manganese complexes in potential interactions with reactive nitrogen species, are not known, but mechanisms analogous to the peroxidatic reactions described here are likely to contribute. The ability of mammalian catalase itself to scavenge nitric oxide in a hydrogen peroxide-dependent fashion has, in fact, been reported.<sup>39</sup> More directly, a very recent study now shows that EUK-8 and EUK-134 can react with peroxynitrite and nitric oxide via oxidant-dependent processes.<sup>40</sup> Altogether, the experience with these compounds or, indeed, with any multifunctional catalytic ROS scavenger, in cytoprotection models suggests that structure–activity relationships will be highly dependent on the nature of the model, including factors such as type(s) of ROS generated, intra- or extracellular site of ROS insult, and specific cell type, including available endogenous defense mechanisms. This is not at all surprising and, in fact, quite interesting. It demonstrates that the predictive value of any single *in vitro* model is limited and must be interpreted carefully in relationship to *in vivo* activities.

#### Neuroprotective Activity of Selected Salen–Manganese Complexes in a Rodent Stroke Model.

We previously showed that EUK-134 is highly neuroprotective in a rat stroke model involving permanent regional cerebral ischemia, with EUK-8 showing significantly less effectiveness.<sup>23</sup> We have now extended this study to test several other salen–manganese complexes. For comparison among analogues, all were administered at 5  $\mu\text{mol/kg}$ , with other dose groups also tested in some cases, as indicated. The results are summarized in Table 3. Three compounds, EUK-113, EUK-189, and EUK-172, exhibited neuroprotective activity comparable to that of EUK-134. Two compounds, EUK-161 and EUK-163, were not active. The lack of activity of EUK-161 is of interest, as it was with the HF cell model (Figure 4), considering its very high catalase activity. Again, this implicates the importance of alkoxy substituents for activity in this biological model. The lack of activity of EUK-163, which has 3-methoxy substituents but poor catalase activity, should also be noted. EUK-178 displayed a bell-shaped dose response, most likely due to its cytotoxicity, which is also evident in the dose–response behavior in the HF model (Figure 5) and in separate cytotoxicity studies in HF cells (Figure 5 legend). Overall, these findings demonstrate some association between cytoprotective activity in the HF cell model and neuroprotective activity in the rat stroke model. In particular, no compound lacking activity in the HF cell model showed significant neuroprotective activity *in vivo*. Not surprisingly, the structure–activity correlation between the two models was not absolute. For example, EUK-178 and EUK-172 were not more effective than EUK-134 in the stroke model, despite their much higher catalase activities and greater potencies in the HF cell model. Factors such as pharmacokinetics, *in vivo* stability, and blood–brain barrier permeability would influence the compounds' relative activities in the *in vivo* model while, of course, would play no role in the cell culture system.

**Table 3.** Neuroprotective Activity of Salen–Manganese Complexes in a Rodent Stroke Model<sup>a</sup>

treatment	dose	vehicle	n	% infarct (SD)	significant vs control
control		saline	43	7.4 (3.2)	
control (M)		5% mannitol	48	7.8 (3.0)	
control (M2)		5% mannitol, pH 7.6	4	7.7 (2.7)	
EUK-134	0.05	saline	10	6.3 (5.3)	ns
	0.5	saline	12	3.6 (3.2)	*
	5	saline	8	1.3 (1.8)	*
	5	5% mannitol	6	1.4 (2.5)	*
	53	5% mannitol	4	2.1 (1.5)	*
EUK-8	5	saline	8	3.7 (3.9)	ns
	39	5% mannitol	8	4.1 (3.2)	ns
EUK-113	5	saline	5	1.7 (1.3)	*
EUK-172	0.5	5% mannitol, pH 7.6	5	6.1 (3.4)	ns
	5	5% mannitol, pH 7.6	8	2.0 (1.6)	*
EUK-178	0.5	5% mannitol	7	2.5 (2.6)	*
	5	5% mannitol	4	5.7 (1.8)	ns
	17	5% mannitol	5	9.6 (3.8)	ns
EUK-163	5	saline	6	6.6 (5.7)	ns
EUK-189	5	5% mannitol	5	0.9 (1.0)	*
EUK-161	5	5% mannitol	7	5.2 (2.3)	ns

<sup>a</sup> The rat middle cerebral artery occlusion model was performed as described in the Experimental Section. Compounds were dissolved in the indicated vehicle for intravenous injection and compared to the appropriate vehicle control group as described (\* =  $p < 0.01$ ). Doses are in  $\mu\text{mol/kg}$ . In addition, two salicylaldehydes, each tested at 10  $\mu\text{mol/kg}$ , had no significant effect. Groups ( $n = 3$ ) treated with salicylaldehyde and 3-methoxysalicylaldehyde had mean percent infarcts of  $10.7 \pm 3.4$  and  $6.8 \pm 2.9$ , respectively.

Also, while the HF cell model was designed specifically to apply a hydrogen peroxide-dependent insult, it is extremely unlikely that hydrogen peroxide is the sole cause of neurological damage in the complex cascade that follows permanent ischemia. For example, as discussed earlier, reactive nitrogen species may contribute and the structure–activity relationships of reactive nitrogen scavenging properties of the salen–manganese complexes are not yet known. Nonetheless, it is well worth noting that the HF cell culture model does appear to have some predictive value in selecting compounds potentially active in the *in vivo* stroke model.

#### Conclusions

The salen–manganese complexes described in this study, all structural analogues of the prototype compound EUK-8, exhibit catalase and SOD activities *in vitro*. The hydrogen peroxide scavenging activities of these compounds can be structurally modulated. For example, catalase activity is increased by symmetrical 3- or 5-alkoxy substituents on the salen rings and by aromatic bridge structures. Such changes do not, however, affect SOD activity. Many of the more active catalases can effectively protect human fibroblasts in culture against exogenous hydrogen peroxide. However, at least within this series of compounds, this cytoprotection requires 3- and/or 5-alkoxy substituents, suggesting that these substituents confer some advantage, in addition to their enhancement of catalase activity, toward the biologically protective characteristics of salen–manganese complexes. Finally, four of the compounds that are protective in cell culture are also neuroprotective *in vivo* in a rat stroke model. Overall, these data show that salen–manganese complexes

represent a class of catalase mimetics whose hydrogen peroxide scavenging and cytoprotective activities can be substantially manipulated. These findings support the concept that compounds of this class, selected by biochemical and biological criteria such as those described here, have potential applications in the treatment of ROS-associated diseases, including ischemic tissue damage.

## Experimental Section

**Materials.** All chemicals, except for solvents, used in the synthesis of salen–manganese complexes were purchased from Aldrich Chemical Co. (Milwaukee, WI). All solvents used in synthesis of the compounds were reagent grade and were used without further purification and were obtained from either Caledon Laboratories (Georgetown, Ontario, Canada) or Commercial Alcohols (Toronto, Ontario, Canada). MnTMPyP was purchased from Alexis Corporation (San Diego, CA). The XTT reagent was obtained from Boehringer Mannheim, Inc. (Indianapolis, IN). All components of tissue culture media were purchased from BioWhittaker (Walkersville, MD), and tissue culture plasticware was from Corning (Corning, NY). Materials and chemicals used for the rodent stroke experiments were as described previously.<sup>23</sup> All other chemicals were obtained from Sigma Chemicals (St. Louis, MO).

**Synthesis and Characterization of Salen–Manganese Complexes.** EUK-8 and EUK-108 were prepared using a published procedure,<sup>28</sup> which was modified to produce the other complexes. The bis(salicylaldehyde)ethylenediamine (salen-H<sub>2</sub>)-substituted ligands were prepared by the addition of 1 equiv of ethylenediamine in absolute ethanol to a solution of 2 equiv of the substituted aldehyde in absolute ethanol (0.05–0.2 M solution). For the bridge-modified salen–manganese complexes, the corresponding diamine was substituted for ethylenediamine and reacted with either *o*-vanillin or salicylaldehyde in a similar manner. For EUK-159, the trans ( $\pm$ ) cyclohexyldiamine was used. For all ligands, the precipitate formed in the reaction was filtered, washed with ethanol, and air-dried to give the desired ligand in 79–96% yield. One equivalent of solid manganese(II) acetate tetrahydrate was added to a stirred suspension of 1 equiv of the ligand in 95% ethanol (0.025–0.03 M), either at ambient temperature or at reflux, and the reaction was then stirred for 1–2 h. The dark brown solutions were then evaporated to dryness under a stream of air. The crude product, generally a brown or dark green solid, was washed with acetone, filtered, and air-dried. The products were obtained as hydrates in 62–92% yield. The acetate complexes were converted to the corresponding chlorides by treating an aqueous solution (0.03–0.06 M) of the acetate, warmed to 50 °C, with 5 equiv of KCl dissolved in distilled water. A brown or dark green precipitate immediately formed. The suspension was cooled in an ice/water bath and then filtered, and the solid was washed with water and acetone. The products were obtained as hydrates in 66–78% yield.

<sup>1</sup>H NMR spectra of the ligands were obtained on a Bruker ARX 400 MHz instrument. The <sup>1</sup>H NMR data obtained for each ligand were as follows: EUK-113/134 ligand (CDCl<sub>3</sub>):  $\delta$  13.59 (bs, 2H), 8.35 (s, 2H), 6.8 to 6.92 (d's and t, 6H,  $J$  = 8.0 Hz), 3.98 (s, 4H), 3.90 (s, 6H); EUK-114/115 ligand (CDCl<sub>3</sub>):  $\delta$  13.70 (bs, 2H), 8.22 (s, 2H), 7.10 (d, 2H,  $J$  = 8.0 Hz), 6.44 (s, 2H), 6.39 (d, 2H,  $J$  = 8.0 Hz), 3.87 (s, 4H), 3.81 (s, 6H); EUK-15/123 ligand (CDCl<sub>3</sub>):  $\delta$  12.71 (bs, 2H), 8.32 (s, 2H), 6.91 (m, 4H), 6.75 (s, 2H), 3.96 (singlet, 4H), 3.77 (s, 6H); EUK-121/122 ligand (DMSO-*d*<sub>6</sub>):  $\delta$  13.94 (bs, 2H), 8.68 (s, 2H), 7.28 (m, 4H), 6.75 (m, 2H), 3.95 (s, 4H); EUK-118/124 ligand (CDCl<sub>3</sub>):  $\delta$  14.20 (bs, 2H), 8.46 (s, 2H), 5.97 (s, 2H), 5.71 (s, 2H), 3.79 (s, 10H), 3.75 (s, 6H); EUK-160 ligand (CDCl<sub>3</sub>):  $\delta$  8.25 (s, 2H), 6.54 (s, 2H), 6.28 (s, 2H), 3.95 (s, 4H), 3.86 (s, 6H), 3.74 (s, 6H); EUK-189 ligand (CDCl<sub>3</sub>):  $\delta$  13.61 (s, 2H), 8.34 (s, 2H), 6.76 to 6.94 (dd and t, 6H,  $J$  = 8.0 Hz), 4.13 (quartet, 4H,  $J$  = 7.0 Hz), 3.97 (s, 4H), 1.49 (t, 6H,  $J$  = 7.0 Hz); EUK-159 ligand (CDCl<sub>3</sub>):  $\delta$  13.8 (s, 2H), 8.26 (s, 2H), 6.73 to 6.89 (d's and t,

6H,  $J$  = 8.0 Hz), 3.89 (s, 6H), 3.34 (quartet, 2H,  $J$  = 4.0 Hz), 1.51 to 1.99 (m's, 8H); EUK-177 ligand (CDCl<sub>3</sub>):  $\delta$  13.05 (d, 2H,  $J$  = 6.6 Hz), 8.65 (d, 2H,  $J$  = 6.6 Hz), 6.81 to 7.58 (d's and t's, 12H,  $J$  = 7.5 Hz); EUK-178 ligand (DMSO-*d*<sub>6</sub>):  $\delta$  13.03 (s, 2H), 8.98 (s, 2H), 6.95 to 7.53 (d's and t's, 10H,  $J$  = 8 Hz), 3.87 (s, 6H); EUK-161 ligand (CDCl<sub>3</sub>):  $\delta$  13.38 (s, 1H), 12.85 (s, 1H), 9.55 (s, 1H), 8.68 (s, 1H), 8.45 (t, 1H,  $J$  = 2.0 Hz), 6.97 to 7.60 (d's and t's, 10H,  $J$  = 8.0 Hz); EUK-172 ligand (CDCl<sub>3</sub>):  $\delta$  13.46 (s, 1H), 12.91 (s, 1H), 9.50 (s, 1H), 8.67 (s, 1H), 8.43 (s, 1H), 6.90 to 7.53 (d's and t's, 9H,  $J$  = 7.0 Hz), 3.97 (s, 3H), 3.92 (s, 3H); EUK-163 ligand (CDCl<sub>3</sub>):  $\delta$  8.44 (s, 2H), 6.86 to 6.98 (d's and t's, 6H,  $J$  = 8.0 Hz), 3.93 (s, 6H), 3.75 to 3.89 (d and m, 5H,  $J$  = 4.7 Hz).

Elemental analyses of the final products were performed by Canadian Microanalytical Services (Delta, BC, Canada), and results were as follows: EUK-8, Anal. (C<sub>16</sub>H<sub>14</sub>ClMnN<sub>2</sub>O<sub>2</sub>, 0.25H<sub>2</sub>O) C, H, N; EUK-108, Anal. (C<sub>18</sub>H<sub>17</sub>MnN<sub>2</sub>O<sub>4</sub>, 0.5H<sub>2</sub>O) C, H, N; EUK-113, Anal. (C<sub>20</sub>H<sub>21</sub>MnN<sub>2</sub>O<sub>6</sub>, 3.0H<sub>2</sub>O) C, H, N; EUK-134, Anal. (C<sub>18</sub>H<sub>18</sub>ClMnN<sub>2</sub>O<sub>4</sub>, 3.2H<sub>2</sub>O) C, H, N; EUK-114, Anal. (C<sub>20</sub>H<sub>21</sub>MnN<sub>2</sub>O<sub>6</sub>, 1.0H<sub>2</sub>O) C, H, N; EUK-115, Anal. (C<sub>18</sub>H<sub>18</sub>ClMnN<sub>2</sub>O<sub>4</sub>, 2.5H<sub>2</sub>O) C, H, N; EUK-123, Anal. (C<sub>20</sub>H<sub>21</sub>N<sub>2</sub>MnO<sub>6</sub>, 2.5H<sub>2</sub>O) C, H, N; EUK-15, Anal. (C<sub>18</sub>H<sub>18</sub>ClMnN<sub>2</sub>O<sub>4</sub>, 1.2H<sub>2</sub>O) C, H, N; EUK-121, Anal. (C<sub>18</sub>H<sub>15</sub>F<sub>2</sub>MnN<sub>2</sub>O<sub>4</sub>, 2.5H<sub>2</sub>O) C, H, N; EUK-122, Anal. (C<sub>16</sub>H<sub>12</sub>ClF<sub>2</sub>MnN<sub>2</sub>O<sub>2</sub>, 1.25H<sub>2</sub>O) C, H, N; EUK-118, Anal. (C<sub>22</sub>H<sub>25</sub>MnN<sub>2</sub>O<sub>8</sub>, 0.75H<sub>2</sub>O) C, H, N; EUK-124, Anal. (C<sub>20</sub>H<sub>22</sub>ClMnN<sub>2</sub>O<sub>6</sub>, 0.4H<sub>2</sub>O) C, H, N; EUK-189, Anal. (C<sub>22</sub>H<sub>25</sub>MnN<sub>2</sub>O<sub>6</sub>, 1.8H<sub>2</sub>O) C, H, N; EUK-160, Anal. (C<sub>22</sub>H<sub>25</sub>MnN<sub>2</sub>O<sub>8</sub>, 2.8H<sub>2</sub>O) C, H, N; EUK-159, Anal. (C<sub>24</sub>H<sub>27</sub>MnN<sub>2</sub>O<sub>6</sub>, 2.4H<sub>2</sub>O) C, H, N; EUK-177, Anal. (C<sub>22</sub>H<sub>17</sub>MnN<sub>2</sub>O<sub>4</sub>, 0.5H<sub>2</sub>O) C, H, N; EUK-178, Anal. (C<sub>24</sub>H<sub>21</sub>MnN<sub>2</sub>O<sub>6</sub>, 1.6H<sub>2</sub>O) C, H, N; EUK-161, Anal. (C<sub>21</sub>H<sub>16</sub>MnN<sub>3</sub>O<sub>4</sub>) C, H, N; EUK-172, Anal. (C<sub>23</sub>H<sub>20</sub>MnN<sub>3</sub>O<sub>6</sub>, 1.9H<sub>2</sub>O) C, H, N; EUK-163, Anal. (C<sub>21</sub>H<sub>23</sub>MnN<sub>2</sub>O<sub>7</sub>, 3.33H<sub>2</sub>O) C, H, N.

**Catalase Activity.** Catalase activity was assayed by monitoring the conversion of hydrogen peroxide to oxygen using a Clark type polarographic oxygen electrode as described previously.<sup>23</sup> Salen–manganese complexes were tested at 10  $\mu$ M in reaction mixtures containing hydrogen peroxide at 10 mM, and initial rates and total amount of oxygen produced were determined as described.<sup>23</sup> Control compounds were tested at the appropriate equivalent concentrations, that is, 20  $\mu$ M for salicylaldehydes and 10  $\mu$ M for Mn salts, ligand, and ethylenediamine. Stock solutions of complexes and control compounds for this and other catalytic assays were prepared in water or methanol, depending on solubility. Where present, at a final concentration that did not exceed 5%, methanol did not affect assay activities. Blank reactions, containing all components except salen–manganese complex, yielded rate and end point values of  $1 \pm 34$  and  $3 \pm 2$ , respectively, and were not subtracted from the values obtained with compounds. For convenience and sensitivity, pH 8.1 was selected for catalase and peroxidase activity (below) assays. The use of millimolar hydrogen peroxide concentrations in the catalase assays was dictated by the sensitivity of the oxygen measurement system. However, under different assay conditions monitoring hydrogen peroxide disappearance, salen–manganese complexes also effectively neutralize lower concentrations of hydrogen peroxide at physiological pH, for example, under conditions of the cytoprotection assay described below.

**Peroxidase Activity.** Peroxidase activity was assayed by monitoring the hydrogen peroxide-dependent oxidation of ABTS spectrophotometrically. Assay mixtures consisted of 50 mM sodium phosphate, pH 8.1, 0.9% sodium chloride, 0.5 mM ABTS, 0.2 mM hydrogen peroxide, and 10  $\mu$ M salen–manganese complex. Assays were conducted at  $27 \pm 0.2$  °C. ABTS oxidation was monitored at 740 nm to eliminate interference by the salen–manganese complexes. The amount of oxidized ABTS was estimated using an  $\epsilon_{740}$  of  $20\,300\text{ M}^{-1}\text{ cm}^{-1}$  calculated based upon the published molar extinction coefficient at 414 nm.<sup>41</sup> There was no detectable ABTS oxidation in the absence of salen–manganese complex or hydrogen peroxide. The most active catalases, namely, EUK-161, EUK-172, EUK-177, and EUK-178, showed little or no activity in the peroxidase assay. This was attributed to rapid



destruction of hydrogen peroxide via their high catalase activity, as evidenced by the ability of these compounds to also quench apparent peroxidase activity of another analogue such as EUK-134, when added to the reaction mixture. Because of this artifact, peroxidase activities of the bridge-modified series of compounds were not included in Table 2.

**SOD Activity.** SOD activity was assayed by following the inhibition of the reduction of an electron acceptor molecule in the presence of the free radical generating system xanthine/xanthine oxidase as described by McCord and Fridovich,<sup>42</sup> using assay conditions essentially as described by Faulkner et al.,<sup>6</sup> except for the omission of ethylenediaminetetraacetic acid (EDTA), due to artifacts described previously.<sup>10</sup> Assays were conducted at  $27 \pm 0.2$  °C using a water-jacketed cell holder in a Beckman DU7400 spectrophotometer. Oxidized cytochrome c (0.13 mg/mL) was employed as acceptor, and its reduction was monitored spectrophotometrically at 550 nm.<sup>6</sup> To compare the SOD activities of salen–manganese complexes, their  $IC_{50}$  values were determined from concentration-dependent plots generated from 5 to 7 different concentrations. Control reactions to ensure that the compounds did not directly inhibit xanthine oxidase were performed by monitoring conversion of xanthine to urate at 292 nm<sup>43</sup> in reaction mixtures lacking cytochrome c. Selected salen–manganese complexes (EUK-8, EUK-134, EUK-15, EUK-115, EUK-122, EUK-172, EUK-159, EUK-177, EUK-178, EUK-163, EUK-161, and EUK-189) were tested for xanthine oxidase inhibition in this manner. While three compounds (EUK-8, EUK-15, and EUK-122) showed interference with the assay at 10  $\mu$ M, the others did not, showing no effect on the rate of urate production. It was concluded that the apparent SOD activity exhibited by these compounds was not attributable to an inhibition of xanthine oxidase. To eliminate the possibility of artifactual peroxide-dependent oxidation of cytochrome c interfering in this assay,<sup>44</sup> selected compounds (EUK-8, EUK-108, EUK-113, EUK-15, EUK-114, EUK-115, EUK-121, EUK-122, EUK-123, EUK-124, EUK-134, EUK-160, EUK-189, EUK-159, EUK-161, EUK-163, EUK-172, EUK-177, and EUK-178) were also assayed for SOD activity in the presence of bovine liver catalase (767 units/mL). In all cases, this was found not to affect the  $IC_{50}$  value or to significantly affect the rate of cytochrome c reduction in the xanthine/xanthine oxidase assay.

**Cytoprotection Assay.** This assay was conducted as described previously.<sup>23</sup> Essentially, human dermal fibroblasts (HF cells) were incubated in multiwell plates with the hydrogen peroxide-generating system glucose and glucose oxidase, in the presence of various doses of salen–manganese complexes or various control substances, as indicated in the figures. Stock solutions of salen–manganese complexes or control substances were prepared in water or nondenatured ethanol. Ethanol, where present, did not exceed 5% and was found to have no effect on cell viability. After 18 h, cells were washed and cell viability was assessed spectrophotometrically using the XTT reagent, a tetrazolium dye converted to a soluble colorimetrically detectable product by respiring cells, per manufacturer's instructions. Cells were examined under the microscope to confirm cytotoxicity readings as well as to eliminate any samples containing precipitated test agents. The amounts of glucose oxidase used in the cytoprotection assays corresponded to approximately 0.02 units/mL (0.8–1.0  $\mu$ g/mL). Because of normal variations associated with cell culture conditions, the concentration of salen–manganese complex required to overcome cytotoxicity also varied among individual experiments in the course of this study. Thus, to compare potencies of compounds tested on different days, an internal reference compound, EUK-134 or EUK-113, was included in each assay. The concentration of these reference compounds showing half-maximal cytoprotection ( $ED_{50}$ ) varied from 25 to 124  $\mu$ M. To assess cytotoxicity of the compounds alone, cells were incubated with salen–manganese complexes without glucose oxidase and cell viability was assessed as described above. The glucose and glucose oxidase system was utilized for these studies in order to expose cells for a period of hours to continuously generated hydrogen peroxide. We have found

this to be more reproducibly lethal in the HF cell system, in serum-containing medium, than bolus administrations of hydrogen peroxide. However, in other cellular systems, for example, rat primary neurons cultured in defined medium, salen–manganese complexes effectively protect against bolus-administered hydrogen peroxide.<sup>35</sup> To ensure that there were no direct effects of salen–manganese complexes on glucose oxidase, the enzyme activity was assayed in the presence or absence of EUK-134 or EUK-8. Glucose oxidase was assayed by monitoring hydrogen peroxide generation, using a modification of a horseradish peroxidase-coupled spectrophotometric assay described previously,<sup>45</sup> with horseradish peroxidase in excess to ensure that the peroxidase and/or catalase activities of the salen–manganese complexes did not interfere with the assay. Assay mixtures contained glucose oxidase (0.016 units/mL), glucose (1 g/L), ABTS (0.5 mM), horseradish peroxidase (77.5 units/mL), and sodium phosphate, pH 7.4, with and without added EUK-8 or EUK-134 (200  $\mu$ M). ABTS oxidation was monitored at 740 nm, as described above for the peroxidase assay. Reaction rates were readily detectable and did not differ significantly in the presence or absence of salen–manganese complexes.

**Rodent Stroke Model.** Middle cerebral artery occlusion was performed in Sprague–Dawley rats, and brain infarct volumes were quantitated 21 h after occlusion, as described previously.<sup>23</sup> Salen–manganese complexes were administered by intravenous bolus injection 3 h after occlusion. The choice of injection vehicle was determined by the solubility properties of the compound. For most of the compounds, either normal saline or 5% mannitol was used. For EUK-172, which has a pH-dependent solubility profile in the physiological range, the 5% mannitol vehicle was buffered with 20 mM sodium phosphate (pH 7.6) to prevent precipitation of the compound upon injection. Infarct volumes were not different for vehicle control animals receiving either of these vehicles (Table 3). The data are expressed as percent infarct volume, calculated by comparing the volume of infarcted brain tissue to the total brain volume of each animal. (As reported previously,<sup>23</sup> this corresponded to a mean infarct volume of about 100 mm<sup>3</sup> in the control groups.) Because of animal-to-animal variability in collateral circulation, success of the ligation, and other factors, data from brains showing no detectable infarct were omitted from analysis. Statistical significance was assessed by Dunnett's *t* test (*p* < 0.01), comparing to the appropriate vehicle control group.

**Acknowledgment.** This research was supported in part by a grant to Eukarion from the National Institutes of General Medical Sciences (R43 GM 57770).

## References

- (1) Halliwell, B. Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*, 2nd ed.; Clarendon Press: Oxford, 1989; p 543.
- (2) Sies, H. Biochemistry of oxidative stress. *Angew. Chem.* **1986**, *25*, 1058–1071.
- (3) Rice-Evans, C. A.; Diplock, A. T. Current status of antioxidant therapy. *Free Radical Biol. Med.* **1993**, *15*, 77–96.
- (4) Kensler, T. W.; Bush, D. M.; Kozumbo, W. J. Inhibition of tumor promotion by a biomimetic SOD. *Science* **1983**, *221*, 75–77.
- (5) Darr, D.; Zarilla, K. A.; Fridovich, I. A mimic of superoxide dismutase activity based upon desferrioxamine B and manganese (IV). *Arch. Biochem. Biophys.* **1987**, *258*, 351–355.
- (6) Faulkner, K. M.; Liochev, S. I.; Fridovich, I. Stable Mn(III) porphyrins mimic superoxide dismutase in vitro and substitute for it in vivo. *J. Biol. Chem.* **1994**, *269*, 23471–23476.
- (7) Patel, M.; Day, B. J. Metalloporphyrin class of therapeutic catalytic antioxidants. *Trends Pharmacol. Sci.* **1999**, *20*, 359–364.
- (8) Riley, D. P.; Weiss, R. H. Manganese macrocyclic ligand complexes as mimics of SOD. *J. Am. Chem. Soc.* **1994**, *116*, 387–388.
- (9) Salvemini, D.; Wang, Z. Q.; Zweier, J. L.; Samouilov, A.; Macarther, H.; Misko, T. P.; Currie, M. G.; Cuzzocrea, S.; Sikorski, J. A.; Riley, D. P. A nonpeptidyl mimic of superoxide dismutase with therapeutic activity in rats. *Science* **2000**, *286*, 304–306.

- (10) Baudry, M.; Etienne, S.; Bruce, A.; Palucki, M.; Jacobsen, E.; Malfroy, B. Salen-manganese complexes are superoxide dismutase-mimics. *Biochem. Biophys. Res. Commun.* **1993**, *192*, 964–968.
- (11) Bruce, A. J.; Najm, I.; Malfroy, B.; Baudry, M. Effects of desferrioxamine/manganese complex, a superoxide dismutase mimic, on kainate-induced pathology in rat brain. *Neurodegeneration* **1992**, *1*, 265–271.
- (12) Day, B. J.; Crapo, J. D. A metalloporphyrin superoxide dismutase mimetic protects against paraquat-induced lung injury in vivo. *Toxicol. Appl. Pharmacol.* **1996**, *140*, 94–100.
- (13) Melov, S.; Schneider, J. A.; Day, B. J.; Hinerfeld, D.; Coskun, P.; Mirra, S. S.; Crapo, J. D.; Wallace, D. C. A novel neurological phenotype in mice lacking mitochondrial manganese superoxide dismutase. *Nat. Genet.* **1998**, *18*, 159–163.
- (14) Macarthur, H.; Westfall, T. C.; Riley, D. P.; Misko, T. P.; Macvemin, D. Inactivation of catecholamines by superoxide gives new insights on the pathogenesis of septic shock. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 9753–9758.
- (15) Mackensen, G. B.; Patel, M.; Sheng, H.; Calvi, C. L.; Batinic-Haberle, I.; Day, B. J.; Liang, L. P.; Fridovich, I.; Crapo, J. D.; Pearlstein, R. D.; Warner, D. S. Neuroprotection from delayed postischemic administration of a metalloporphyrin catalytic antioxidant. *J. Neurosci.* **2001**, *21*, 4582–4592.
- (16) Dugan, L. L.; Turetsky, D. M.; Du, C.; Lobner, D.; Wheeler, M.; Alml, C. R.; Shen, C. K.; Luh, T. Y.; Choi, D. W.; Lin, T. S. Carboxyfullerenes as neuroprotective agents. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 9434–9439.
- (17) Sies, H.; Masumoto, H. Ebselen as a glutathione peroxidase mimic and as a scavenger of peroxynitrite. In *Antioxidants in Disease Mechanisms and Therapy*; Sies, H., Ed.; Advances in Pharmacology; Academic Press: San Diego, 1997; pp 229–246.
- (18) Gonzalez, P. K.; Zhuang, J.; Doctrow, S. R.; Malfroy, B.; Benson, P. F.; Menconi, M. J.; Fink, M. P. EUK-8, a synthetic superoxide dismutase and catalase mimetic, ameliorates acute lung injury in endotoxemic swine. *J. Pharmacol. Exp. Ther.* **1995**, *275*, 798–806.
- (19) Doctrow, S. R.; Huffman, K.; Marcus, C. B.; Musleh, W.; Bruce, A.; Baudry, M.; Malfroy, B. Salen-manganese complexes: combined superoxide dismutase/catalase mimics with broad pharmacological efficacy. In *Antioxidants in Disease Mechanisms and Therapeutic Strategies*; Sies, H., Ed.; Advances in Pharmacology; Academic Press: New York, 1997; pp 247–270.
- (20) Bruce, A. J.; Malfroy, B.; Baudry, M. Beta-amyloid toxicity in organotypic hippocampal cultures: protection by EUK-8, a synthetic catalytic free radical scavenger. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2312–2316.
- (21) Malfroy, B.; Doctrow, S. R.; Orr, P. L.; Tocco, G.; Fedoseyeva, E. V.; Benichou, G. Prevention and suppression of autoimmune encephalomyelitis by EUK-8, a synthetic catalytic scavenger of oxygen reactive metabolites. *Cell. Immunol.* **1997**, *177*, 62–68.
- (22) Gonzalez, P. K.; Zhuang, J.; Doctrow, S. R.; Malfroy, B.; Smith, M.; Menconi, M. J.; Fink, M. P. Delayed treatment with EUK-8, a novel synthetic superoxide dismutase (SOD) and catalase (CAT) mimetic, ameliorates acute lung injury in endotoxemic pigs. *Surg. Forum* **1995**, *46*, 72–73.
- (23) Baker, K.; Bucay-Marcus, C.; Huffman, C.; Kruk, H.; Malfroy, B.; Doctrow, S. R. Synthetic combined superoxide dismutase/catalase mimics are protective as a delayed treatment in a rat stroke model: a key role for reactive oxygen species in ischemic brain injury. *J. Pharmacol. Exp. Ther.* **1998**, *284*, 215–221.
- (24) Rong, Y.; Doctrow, S. R.; Tocco, G.; Baudry, M. EUK-134, a synthetic superoxide dismutase and catalase mimetic, prevents oxidative stress and attenuates kainate-induced neuropathology. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9897–9902.
- (25) Melov, S.; Ravenscroft, J.; Malik, S.; Gill, M. S.; Walker, D. S.; Clayton, P. E.; Wallace, D. C.; Malfroy, B.; Doctrow, S.; Lithgow, G. J. Extension of lifespan with superoxide dismutase/catalase mimetics. *Science* **2000**, *289*, 1567–1569.
- (26) Jung, C.; Rong, Y.; Doctrow, S.; Baudry, M.; Malfroy, B.; Xu, Z. Synthetic superoxide dismutase/catalase mimetics reduce oxidative stress and prolong survival in a mouse amyotrophic lateral sclerosis model. *Neurosci. Lett.* **2001**, *304*, 157–160.
- (27) Melov, S.; Doctrow, S. R.; Schneider, J. A.; Haberson, J.; Patel, M.; Coskun, P. E.; Huffman, K.; Wallace, D. C.; Malfroy, B. Lifespan extension and rescue of neurodegeneration in superoxide dismutase 2 nullizygous mice treated with superoxide dismutase/catalase mimetics. *J. Neurosci.* **2001**, *21*, 8348–8353.
- (28) Boucher, L. J. Manganese Schiff's base complexes II: synthesis and spectroscopy of chloro- complexes of some derivatives of (salicylaldehydeethylenediimato)manganese(III). *J. Inorg. Nucl. Chem.* **1974**, *36*, 531–536.
- (29) Chance, B.; Sies, H.; Boveris, A. Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* **1979**, *59*, 527–605.
- (30) Slebodnick, C.; Law, N. A.; Pecoraro, V. L. Non-heme peroxidases and catalases: mechanistic implications from the studies of manganese and vanadium model compounds. In *Biomimetic Oxidations Catalyzed by Transition Metal Complexes*; Meunier, B., Ed.; Imperial College Press: London, 2000; pp 215–268.
- (31) Oshino, N.; Oshino, R.; Chance, B. The characteristics of the "peroxidatic" reactions of catalase in ethanol oxidation. *Biochem. J.* **1973**, *131*, 555–567.
- (32) Dismukes, G. C. Polynuclear manganese enzymes. In *Bioinorganic Catalysis*; Reedijk, J., Ed.; Marcel Dekker: New York, NY, 1993; pp 317–346.
- (33) Robert, A.; Loock, B.; Momenteau, M.; Meunier, B. Catalase modeling with metalloporphyrin complexes having an oxygen ligand in a proximal position. Comparison with complexes containing a proximal nitrogen. *Inorg. Chem.* **1991**, *30*, 706–711.
- (34) Srinivasan, K.; Michaud, P.; Kochi, J. K. Epoxidation of olefins with cationic (salen)Mn(III) complexes. The modulation of catalytic activity by substituents. *J. Am. Chem. Soc.* **1986**, *108*, 2309–2320.
- (35) Anderson, I.; Adinolfi, C.; Doctrow, S.; Huffman, K.; Joy, K.; Malfroy, B.; Soden, P.; Rupniak, H. T.; Barnes, J. C. Oxidative signaling and inflammatory pathways in Alzheimer's disease. *Biochem. Soc. Symp.* **2002**, *67*, 141–149.
- (36) Pong, K.; Doctrow, S. R.; Huffman, K.; Adinolfi, C. A.; Baudry, M. Attenuation of staurosporine-induced apoptosis, oxidative stress, and mitochondrial dysfunction by EUK-134, a superoxide dismutase and catalase mimetic, in cultured cortical neurons. *Exp. Neurol.* **2001**, *171*, 84–97.
- (37) Crow, J. P. Peroxynitrite scavenging by metalloporphyrins and thiolates. *Free Radical Biol. Med.* **2000**, *28*, 1487–1494.
- (38) Doctrow, S. R.; Adinolfi, C.; Baudry, M.; Huffman, K.; Malfroy, B.; Marcus, C. B.; Melov, S.; Pong, K.; Rong, Y.; Smart, J.; Tocco, G. Salen manganese complexes, combined superoxide dismutase/catalase mimics demonstrate potential for treating neurodegenerative and other age-associated diseases. In *Critical Reviews in Oxidative Stress and Aging: Advances in Basic Science, Diagnostics, and Intervention*; Cutler, H. R. A. R., Ed.; World Publishing Company: Singapore, 2002; in press.
- (39) Poduslo, J. F.; Whelan, S. L.; Curran, G. L.; Wengenack, T. M. Therapeutic benefit of polyamine-modified catalase as a scavenger of hydrogen peroxide and nitric oxide in familial amyotrophic lateral sclerosis transgenics. *Ann. Neurol.* **2000**, *48*, 943–947.
- (40) Sharpe, M. A.; Olsson, R.; Stewart, V. C.; Clark, J. B. Oxidation of nitric oxide by oxomanganese salen complexes: a new mechanism for cellular protection by superoxide dismutase/catalase mimetics. *Biochem. J.* **2002**, *366*, 97–107.
- (41) Childs, R. E.; Bardsley, W. G. The steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) as chromagen. *Biochem. J.* **1975**, *145*, 93–103.
- (42) McCord, J. M.; Beauchamp, C. O.; Goscini, S.; Misra, H. P.; Fridovich, I. Superoxide and superoxide dismutase. In *Oxidases and Related Redox Systems*; King, H. S. M. T. E., Morrison, M., Ed.; University Press: Baltimore, 1973; pp 51–76.
- (43) Westerfield, W. W.; Richert, D. A.; Bloom, R. J. The inhibition of xanthine and succinic oxidases by carboxyl reagents. *J. Biol. Chem.* **1959**, *234*, 1889.
- (44) Weiss, R. H.; Flickinger, A. G.; Rivers, W. J.; Hardy, M. M.; Aston, K. W.; Ryan, U.; Riley, D. P. Evaluation of activity of putative superoxide mimics: direct analysis by stopped-flow kinetics. *J. Biol. Chem.* **1993**, *268*, 23049–23054.
- (45) Glucose Oxidase. In *Worthington Manual*; Worthington, C. C., Ed.; Worthington Biochemical Corporation: Freehold, NJ, 1988; pp 155–157.

JM020207Y