(54) Title: SYNTHETIC CATALYTIC FREE RADICAL SCAVENGERS USEFUL AS ANTIOXIDANTS FOR PREVENTION AND THERAPY OF DISEASE

(57) Abstract

     The invention provides antioxidant salen-metal complexes in a form suitable for pharmaceutical administration to treat or prevent a disease associated with cell or tissue damage produced by free radicals such as superoxide.

SYNTHETIC CATALYTIC FREE RADICAL SCAVENGERS USEFUL AS
ANTIOXIDANTS FOR PREVENTION AND THERAPY OF DISEASE

FIELD OF THE INVENTION

The invention provides pharmaceutical compositions
of synthetic catalytic small molecule antioxidants and free
radical scavengers for therapy and prophylaxis of disease,
methods for using the small molecule antioxidants in
prevention and treatment of pathological conditions, methods
for using the small molecule antioxidants for targeted
protection of tissues and/or cell types during cancer
chemotherapy, and methods for using the small molecule
antioxidants to prevent toxicologic damage to individuals
exposed to irritating oxidants or other sources of oxidative
damage, particularly oxygen-derived oxidative species such as
superoxide radical. The compositions and methods of the
invention are also used for preventing oxidative damage in
human transplant organs and for inhibiting reoxygenation
injury following reperfusion of ischemic tissues. The
compositions and methods of the invention are also useful for
chemoprevention of chemical carcinogenesis and alteration of
drug metabolism involving epoxide or free oxygen radical
intermediates.

BACKGROUND OF THE INVENTION

Molecular oxygen is an essential nutrient for
nonfacultative aerobic organisms, including, of course,
humans. Oxygen is used in many important ways, namely, as the
terminal electronic acceptor in oxidative phosphorylation, in
many dioxygenase reactions, including the synthesis of
prostaglandins and of vitamin A from carotenoids, in a host of
hydroxylase reactions, including the formation and
modification of steroid hormones, and in both the activation
and the inactivation of xenobiotics, including carcinogens.
The extensive P-450 system uses molecular oxygen in a host of
important cellular reactions. In a similar vein, nature
employs free radicals in a large variety of enzymic reactions.

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Excessive concentrations of various forms of oxygen
and of free radicals can have serious adverse effects on
living systems, including the peroxidation of membrane lipids,
the hydroxylation of nucleic acid bases, and the oxidation of
5 sulfhydryl groups and of other sensitive moieties in proteins.
If uncontrolled, mutations and cellular death result.

Biological antioxidants include well-defined
enzymes, such as superoxide dismutase, catalase, selenium
glutathione peroxidase, and phospholipid hydroperoxide
10 glutathione peroxidase. Nonenzymatic biological antioxidants
include tocopherols and tocotrienols, carotenoids, quinones,
bilirubin, ascorbic acid, uric acid, and metal-binding
proteins. Various antioxidants, being both lipid and water
soluble, are found in all parts of cells and tissues, although
15 each specific antioxidant often shows a characteristic
distribution pattern. The so-called ovothiols, which are
mercaptohistidine derivatives, also decompose peroxides
nonenzymatically.

Free radicals, particularly free radicals derived
20 from molecular oxygen, are believed to play a fundamental role
in a wide variety of biological phenomena. In fact, it has
been suggested that much of what is considered critical
illness may involve oxygen radical ("oxyradical")
pathophysiology (Zimmermen JJ (1991) Chest 100: 189S).
25 Oxyradical injury has been implicated in the pathogenesis of
pulmonary oxygen toxicity, adult respiratory distress syndrome
(ARDS), bronchopulmonary dysplasia, sepsis syndrome, and a
variety of ischemia-reperfusion syndromes, including
myocardial infarction, stroke, cardiopulmonary bypass, organ
30 transplantation, necrotizing enterocolitis, acute renal
tubular necrosis, and other disease. Oxyradicals can react
with proteins, nucleic acids, lipids, and other biological
macromolecules producing damage to cells and tissues,
particularly in the critically ill patient.
35 Free radicals are atoms, ions, or molecules that
contain an unpaired electron (Pryor, WA (1976) Free Radicals
in Biol. 1: 1). Free radicals are usually unstable and

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exhibit short half-lives. Elemental oxygen is highly
electronegative and readily accepts single electron transfers
from cytochromes and other reduced cellular components; a
portion of the O_2 consumed by cells engaged in aerobic

5 respiration is univalently reduced to superoxide radical ($\bullet O_2^-$)
(Cadenas E (1989) <u>Ann. Rev. Biochem. 58</u>: 79). Sequential
univalent reduction of $\bullet O_2^-$ produces hydrogen peroxide (H_2O_2),
hydroxyl radical ($\bullet OH$), and water.

Free radicals can originate from many sources,

10 including aerobic respiration, cytochrome P-450-catalyzed
monooxygenation reactions of drugs and xenobiotics (e.g.,
trichloromethyl radicals, $CCl_3\bullet$, formed from oxidation of
carbon tetrachloride), and ionizing radiation. For example,
when tissues are exposed to gamma radiation, most of the

15 energy deposited in the cells is absorbed by water and results
in scission of the oxygen-hydrogen covalent bonds in water,
leaving a single electron on hydrogen and one on oxygen
creating two radicals $H\bullet$ and $\bullet OH$. The hydroxyl radical, $\bullet OH$,
is the most reactive radical known in chemistry. It reacts

20 with biomolecules and sets off chain reactions and can
interact with the purine or pyrimidine bases of nucleic acids.
Indeed, radiation-induced carcinogenesis may be initiated by
free radical damage (Breimer LH (1988) <u>Brit. J. Cancer 57</u>: 6).
Also for example, the "oxidative burst" of activated

25 neutrophils produces abundant superoxide radical, which is
believed to be an essential factor in producing the cytotoxic
effect of activated neutrophils. Reperfusion of ischemic
tissues also produces large concentrations of oxyradicals,
typically superoxide (Gutteridge JMC and Halliwell B (1990)

30 <u>Arch. Biochem. Biophys. 283</u>: 223). Moreover, superoxide may
be produced physiologically by endothelial cells for reaction
with nitric oxide, a physiological regulator, forming
peroxynitrite, $ONOO^-$ which may decay and give rise to hydroxyl
radical, $\bullet OH$ (Marletta MA (1989) <u>Trends Biochem. Sci. 14</u>: 488;

35 Moncada et al. (1989) <u>Biochem. Pharmacol. 38</u>: 1709; Saran et
al. (1990) <u>Free Rad. Res. Commun. 10</u>: 221; Beckman et al.
(1990) <u>Proc. Natl. Acad. Sci. (U.S.A.) 87</u>: 1620). Additional

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sources of oxyradicals are "leakage" of electrons from
disrupted mitochondrial or endoplasmic reticular electron
transport chains, prostaglandin synthesis, oxidation of
catecholamines, and platelet activation.

5   Many free radical reactions are highly damaging to
cellular components; they crosslink proteins, mutagenize DNA,
and peroxidize lipids. Once formed, free radicals can
interact to produce other free radicals and non-radical
oxidants such as singlet oxygen ($^1O_2$) and peroxides.
10 Degradation of some of the products of free radical reactions
can also generate potentially damaging chemical species. For
example, malondialdehyde is a reaction product of peroxidized
lipids that reacts with virtually any amine-containing
molecule. Oxygen free radicals also cause oxidative
15 modification of proteins (Stadtman ER (1992) _Science_ 257:
1220).

          Aerobic cells generally contain a number of defenses
against the deleterious effects of oxyradicals and their
reaction products. Superoxide dismutases (SODs) catalyze the
20 reaction:

$$2 \cdot O_2^- + 2 H^+ \longrightarrow O_2 + H_2O_2$$
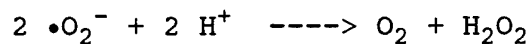
which removes superoxide and forms hydrogen peroxide. H_2O_2 is
not a radical, but it is toxic to cells; it is removed by the
enzymatic activities of catalase and glutathione peroxidase
25 (GSH-Px). Catalase catalyzes the reaction:

$$2 H_2O_2 \longrightarrow 2 H_2O + O_2$$
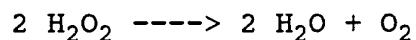
and GSH-Px removes hydrogen peroxide by using it to oxidize
reduced glutathione (GSH) into oxidized glutathione (GSSG)
according to the following reaction:

30          $$2 GSH + H_2O_2 \longrightarrow GSSG + 2 H_2O$$
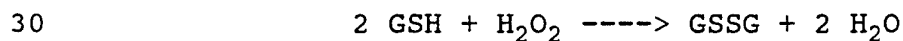
Other enzymes, such as phospholipid hydroperoxide glutathione
peroxidase (PLOOH-GSH-Px), converts reactive phospholipid
hydroperoxides, free fatty acid hydroperoxides, and
cholesterol hydroperoxides to corresponding harmless fatty
35 acid alcohols. Glutathione S-transferases also participate in
detoxifying organic peroxides. In the absence of these
enzymes and in presence of transition metals, such as iron or

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copper, superoxide and hydrogen peroxide can participate in
the following reactions which generate the extremely reactive
hydroxyl radical $\bullet OH^-$:

$$\bullet O_2^- + Fe^{3+} \; ----> \; O_2 + Fe^{2+}$$

5 $\qquad H_2O_2 + Fe^{2+} \; ----> \; \bullet OH + OH^- + Fe^{3+}$
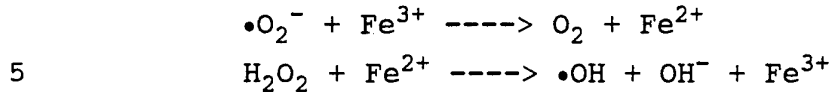
In addition to enzymatic detoxification of free
radicals and oxidant species, a variety of low molecular
weight antioxidants such as glutathione, ascorbate,
tocopherol, ubiquinone, bilirubin, and uric acid serve as

10 naturally-occurring physiological antioxidants (Krinsky NI
(1992) _Proc. Soc. Exp. Biol. Med._ 200:248-54). Carotenoids
are another class of small molecule antioxidants and have been
implicated as protective agents against oxidative stress and
chronic diseases. Canfield et al. (1992) _Proc. Soc. Exp._

15 _Biol. Med._ 200: 260 summarize reported relationships between
carotenoids and various chronic diseases, including coronary
heart disease, cataract, and cancer. Carotenoids dramatically
reduce the incidence of certain premalignant conditions, such
as leukoplakia, in some patients.

20 In an effort to prevent the damaging effects of
oxyradical formation during reoxygenation of ischemic tissues,
a variety of antioxidants have been used.

One strategy for preventing oxyradical-induced
damage is to inhibit the formation of oxyradicals such as

25 superoxide. Iron ion chelators, such as desferrioxamine (also
called deferoxamine or Desferol) and others, inhibit iron ion-
dependent $\bullet OH$ generation and thus act as inhibitors of free
radical formation (Gutteridge et al. (1979) _Biochem. J._ 184:
469; Halliwell B (1989) _Free Radical Biol. Med._ 7: 645; Van

30 der Kraaij et al. (1989) _Circulation_ 80: 158). Amino-steroid-
based antioxidants such as the 21-aminosteroids termed
"lazaroids" (e.g., U74006F) have also been proposed as
inhibitors of oxyradical formation. Desferrioxamine,
allopurinol, and other pyrazolopyrimidines such as oxypurinol,

35 have also been tested for preventing oxyradical formation in a
myocardial stunning model system (Bolli et al. (1989) _Circ._
Res. 65: 607) and following hemorrhagic and endotoxic shock

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(DeGaravilla et al. (1992) _Drug Devel. Res._ 25: 139).
However, each of these compounds has notable drawbacks for
therapeutic usage. For example, deferoxamine is not an ideal
iron chelator and its cellular penetration is quite limited.

5   Another strategy for preventing oxyradical-induced
damage is to catalytically remove oxyradicals such as
superoxide once they have been formed. Superoxide dismutase
and catalase have been extensively explored, with some
success, as protective agents when added to reperfusates in
10   many types of experiments or when added pre-ischemia (reviewed
in Gutteridge JMC and Halliwell B (1990) _op.cit._). The
availability of recombinant superoxide dismutase has allowed
more extensive evaluation of the effect of administering SOD
in the treatment or prevention of various medical conditions
15   including reperfusion injury of the brain and spinal cord
(Uyama et al. (1990) _Free Radic. Biol. Med._ 8: 265; Lim et al.
(1986) _Ann. Thorac. Surg._ 42: 282), endotoxemia (Schneider et
al. (1990) _Circ. Shock_ 30: 97; Schneider et al. (1989) _Prog._
Clin. Biol. Res. 308: 913, and myocardial infarction (Patel et
20   al. (1990) _Am. J. Physiol._ 258: H369; Mehta et al. (1989) _Am._
J. Physiol. 257: H1240; Nejima et al. (1989) _Circulation_ 79:
143; Fincke et al. (1988) _Arzneimittelforschung_ 38: 138;
Ambrosio et al. (1987) _Circulation_ 75: 282), and for
osteoarthritis and intestinal ischemia (Vohra et al. (1989) _J._
25   _Pediatr. Surg._ 24: 893; Flohe L (1988) _Mol. Cell. Biochem._ 84:
123). Superoxide dismutase also has been reported to have
positive effects in treating systemic lupus erythematosus,
Crohn's disease, gastric ulcers, oxygen toxicity, burned
patients, renal failure attendant to transplantation, and
30   herpes simplex infection.
          An alternative strategy for preventing oxyradical-
induced damage is to scavenge oxyradicals such as superoxide
once these have been formed, typically by employing small
molecule scavengers which act stoichiometrically rather than
35   catalytically. Congeners of glutathione have been used in
various animal models to attenuate oxyradical injury. For
example, N-2-mercaptopropionylglycine has been found to confer

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protective effects in a canine model of myocardial ischemia
and reperfusion (Mitsos et al. (1986) _Circulation_ 73: 1077)
and N-acetylcysteine ("Mucomyst") has been used to treat
endotoxin toxicity in sheep (Bernard et al. (1984) _J. Clin._
5 _Invest._ 73: 1772). Dimethyl thiourea (DMTU) and butyl-α-
phenylnitrone (BPN) are believed to scavenge the hydroxyl
radical, •OH, and has been shown to reduce ischemia-
reperfusion injury in rat myocardium and in rabbits (Vander
Heide et al. (1987) _J. Mol. Cell. Cardiol._ 19: 615; Kennedy et
10 al. (1987) _J. Appl. Physiol._ 63: 2426). Mannitol has also
been used as a free radical scavenger to reduce organ injury
during reoxygenation (Fox RB (1984) _J. Clin. Invest._ 74: 1456;
Ouriel et al. (1985) _Circulation_ 72: 254). In one report, a
small molecule chelate was reported to have activity as a
15 glutathione peroxidase mimic (Spector et al. (1993) _Proc._
Natl. Acad. Sci. (U.S.A.) 90: 7485).

Thus, application of inhibitors of oxyradical
formation and/or enzymes that remove superoxide and hydrogen
peroxide and/or small molecule oxyradical scavengers have all
20 shown promise for preventing reoxygenation damage present in a
variety of ischemic pathological states and for treating or
preventing various disease states associated with free
radicals. However, each of these categories contains several
drawbacks. For example, inhibitors of oxyradical formation
25 typically chelate transition metals which are used in
essential enzymatic processes in normal physiology and
respiration; moreover, even at very high doses, these
inhibitors do not completely prevent oxyradical formation.
Superoxide dismutases and catalase are large polypeptides
30 which are expensive to manufacture, do not penetrate cells or
the blood-brain barrier, and generally require parenteral
routes of administration. Free radical scavengers act
stoichiometrically and are thus easily depleted and must be
administered in high dosages to be effective.
35 Based on the foregoing, it is clear that a need
exists for antioxidant agents which are efficient at removing
dangerous oxyradicals, particularly superoxide and hydrogen

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peroxide, and which are inexpensive to manufacture, stable,
and possess advantageous pharmacokinetic properties, such as
the ability to cross the blood-brain barrier and penetrate
tissues. Such versatile antioxidants would find use as
5   pharmaceuticals, chemoprotectants, and possibly as dietary
supplements. It is one object of the invention to provide a
class of novel antioxidants which possess advantageous
pharmacologic properties and which catalytically and/or
stoichiometrically remove superoxide and/or hydrogen peroxide.
10   The references discussed herein are provided solely
for their disclosure prior to the filing date of the present
application. Nothing herein is to be construed as an
admission that the inventors are not entitled to antedate such
disclosure by virtue of prior invention. All publications
15   cited are incorporated herein by reference.

SUMMARY OF THE INVENTION
            In accordance with the foregoing objects, in one
aspect of the invention pharmaceutical compositions are
20   provided which have potent antioxidant and/or free radical
scavenging properties and function as in vivo antioxidants.
The pharmaceutical compositions of the invention comprise an
efficacious dosage of at least one species of salen-transition
metal complex, typically a salen-manganese complex such as a
25   salen-Mn(III) complex. In one embodiment, the pharmaceutical
composition comprises a salen-Mn complex which is a chelate of
Mn(III) with a diamine derivative, such as ethylenediamine
linked to two substituted salicylaldehydes. These
pharmaceutical compositions possess the activity of
30   dismutating superoxide (i.e., superoxide dismutase activity)
and, advantageously, also converting hydrogen peroxide to
water (i.e., catalase activity). The pharmaceutical
compositions are effective at reducing pathological damage
related to formation of oxyradicals such as superoxide and
35   peroxides and other free radical species.
            The invention also provides methods for treating and
preventing pathological conditions by applying or

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administering compositions of salen-transition metal complexes
in a therapeutic or prophylactic dosage. Salen-transition
metal complexes used in the methods of the invention are
typically salen-manganese complexes, such as Mn(III)-salen
5   complexes. The invention provides methods for preventing or
reducing ischemic/reperfusion damage to critical tissues such
as the myocardium and central nervous system. The invention
also provides methods for preventing or reducing cellular
damage resulting from exposure to various chemical compounds
10   which produce potentially damaging free radical species,
comprising administering a therapeutically or prophylactically
efficacious dosage of at least one species of salen-transition
metal complex, preferably a salen-manganese complex having
detectable SOD activity and preferably also having detectable
15   catalase activity. The antioxidant salen-transition metal
complexes of the invention are administered by a variety of
routes, including parenterally, topically, and orally.
        In one aspect of the invention, a therapeutic or
prophylactic dosage of a salen-transition metal complex of the
20   present invention is administered alone or combined with (1)
one or more antioxidant enzymes, such as a Mn-SOD, a Cu,Zn-
SOD, or catalase, and/or (2) one or more free radical
scavengers, such as tocopherol, ascorbate, glutathione, DMTU,
N-acetylcysteine, or N-2-mercaptopropionylglycine and/or (3)
25   one or more oxyradical inhibitors, such as desferrioxamine or
allopurinol, and/or one or more biological modifier agents,
such as calpain inhibitors. The formulations of these
compositions is dependent upon the specific pathological
condition sought to be treated or prevented, the route and
30   form of administration, and the age, sex, and condition of the
patient. These compositions are administered for various
indications, including: (1) for preventing
ischemic/reoxygenation injury in a patient, (2) for preserving
organs for transplant in an anoxic, hypoxic, or hyperoxic
35   state prior to transplant, (3) for protecting normal tissues
from free radical-induced damage consequent to exposure to
ionizing radiation and/or chemotherapy, as with bleomycin, (4)

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for protecting cells and tissues from free radical-induced
injury consequent to exposure to xenobiotic compounds which
form free radicals, either directly or as a consequence of
monooxygenation through the cytochrome P-450 system, (5) for
5   enhancing cryopreservation of cells, tissues, organs, and
organisms by increasing viability of recovered specimens, and
(6) for prophylactic administration to prevent:
carcinogenesis, cellular senescence, cataract formation,
formation of malondialdehyde adducts, HIV pathology and
10 macromolecular crosslinking, such as collagen crosslinking.
        In one aspect of the invention, salen-transition
metal complexes are formulated for administration by the oral
route by forming a pharmaceutical dosage form comprising an
excipient and not less than 1 μg nor more than about 10 grams
15 of at least one antioxidant salen-transition metal complex of
the invention. Dietary formulations are administered for
therapy of free radical-induced diseases and/or for the
chemoprevention of neoplasia and/or oxidative damage
associated with normal aerobic metabolism.
20   In another aspect of the invention, buffered aqueous
solutions comprising at least one antioxidant salen-transition
metal complex of the invention at a concentration of at least
1 nM but not more than about 100 mM is formulated for
administration, usually at a concentration of about 0.1 to 10
25 mM, typically by intravenous route, to a patient undergoing or
expected to undergo: (1) an ischemic episode, such as a
myocardial infarction, cerebral ischemic event,
transplantation operation, open heart surgery, elective
angioplasty, coronary artery bypass surgery, brain surgery,
30 renal infarction, traumatic hemorrhage, tourniquet
application, (2) antineoplastic or antihelminthic chemotherapy
employing a chemotherapeutic agent which generates free
radicals, (3) endotoxic shock or sepsis, (4) exposure to
ionizing radiation, (5) exposure to exogenous chemical
35 compounds which are free radicals or produce free radicals,
(6) thermal or chemical burns or ulcerations, (7) hyperbaric
oxygen, or (8) apoptosis of a predetermined cell population

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(e.g., lymphocyte apoptosis). The buffered aqueous solutions
of the invention may also be used, typically in conjunction
with other established methods, for organ culture, cell
culture, transplant organ maintenance, and myocardial

5   irrigation. Nonaqueous formulations, such as lipid-based
formulations are also provided, including stabilized
emulsions. The antioxidant salen-metal compositions are
administered by various routes, including intravenous
injection, intramuscular injection, subdermal injection,

10 intrapericardial injection, surgical irrigation, topical
application, ophthalmologic application, lavage, gavage,
enema, intraperitoneal infusion, mist inhalation, oral rinse,
and other routes, depending upon the specific medical or
veterinary use intended.

15   In another aspect of the invention, antioxidant
salen-transition metal complexes of the invention are employed
to modulate the expression of naturally-occurring genes or
other polynucleotide sequences under the transcriptional
control of an oxidative stress response element (e.g., an

20 antioxidant responsive element, ARE), such as an antioxidant
response element of a glutathione S-transferase gene or a
NAD(P)H:quinone reductase gene. The antioxidant salen-metal
complexes may be used to modulate the transcription of ARE-
regulated polynucleotide sequences in cell cultures (e.g., ES

25 cells) and in intact animals, particularly in transgenic
animals wherein a transgene comprises one or more AREs as
transcriptional regulatory sequences.
        The present invention also encompasses
pharmaceutical compositions of antioxidant salen-manganese

30 complexes, therapeutic uses of such antioxidant salen-
manganese complexes, methods and compositions for using
antioxidant salen-manganese complexes in diagnostic,
therapeutic, and research applications in human and veterinary
medicine.

35   The invention also provides methods for preventing
food spoilage and oxidation by applying to foodstuffs an
effective amount of at least one antioxidant salen-metal

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complex species. The invention also provides compositions for
preventing food spoilage comprising an effective amount of at
least one species of antioxidant salen-metal complex,
optionally in combination with at least one additional food
5   preservative agent (e.g., butylated hydroxytoluene,. butylated
hydroxyanisole, sulfates, sodium nitrite, sodium nitrate).
For example, an antioxidant salen-metal complex is
incorporated into a foodstuff subject to rancidification
(e.g., oxidation) to reduce the rate of oxidative
10 decomposition of the foodstuff when exposed to molecular
oxygen.

All publications and patent applications herein are
incorporated by reference to the same extent as if each
individual publication or patent application was specifically
15 and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the general structure of salen
deriviatives of the invention.
20   Fig. 2 shows a salen derivative according to the
structure shown in Figure 1, wherein n is 0.
Fig. 3 shows structures of preferred compounds of
the invention.
Fig. 4 shows schematically the effect of an
25 ischemic/reoxygenation episode on synaptic transmission in
isolated brain slices.
Fig. 5 shows the effect of a salen-Mn complex on
EPSP amplitude following an episode of ischemia/reoxygenation.
Fig. 6 shows the effect of a salen-Mn complex on
30 EPSP initial slope following an episode of
ischemia/reoxygenation.
Fig. 7 shows the effect of a salen-Mn complex on
brain slice viability following repeated episodes of
ischemia/reoxygenation.
35   Fig. 8 shows the protective effect of a salen-Mn
complex in an animals model of iatrogenic Parkinson's disease.
Fig. 9 shows that C7 protects hippocampal slices

from lactic acid-induced lipid peroxidation.

Fig 10 shows C7 protects dopaminergic neurons in
mouse striatum from 6-OHDA-induced degeneration.

Fig 11 shows C7 protects dopaminergic neurons in
5   mouse striatum from MPTP-induced degeneration.

Definitions

Unless defined otherwise, all technical and
scientific terms used herein have the same meaning as commonly
10   understood by one of ordinary skill in the art to which this
invention belongs. Although any methods and materials similar
or equivalent to those described herein can be used in the
practice or testing of the present invention, the preferred
methods and materials are described. For purposes of the
15   present invention, the following terms are defined below.

As used herein, an "antioxidant" is a substance
that, when present in a mixture or structure containing an
oxidizable substrate biological molecule, significantly delays
or prevents oxidation of the substrate biological molecule.
20   Antioxidants can act by scavenging biologically important
reactive free radicals or other reactive oxygen species ($\bullet O_2^-$,
H_2O_2, $\bullet OH$, HOCl, ferryl, peroxyl, peroxynitrite, and alkoxyl),
or by preventing their formation, or by catalytically
converting the free radical or other reactive oxygen species
25   to a less reactive species. An antioxidant salen-transition
metal complex of the invention generally has detectable SOD
activity. A salen-transition metal complex of the invention
has antioxidant activity if the complex, when added to a cell
culture or assay reaction, produces a detectable decrease in
30   the amount of a free radical, such as superoxide, or a
nonradical reactive oxygen species, such as hydrogen peroxide,
as compared to a parallel cell culture or assay reaction that
is not treated with the complex. Suitable concentrations
(i.e., efficacious dose) can be determined by various methods,
35   including generating an empirical dose-response curve,
predicting potency and efficacy of a congener by using QSAR
methods or molecular modeling, and other methods used in the

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pharmaceutical sciences. Since oxidative damage is generally
cumulative, there is no minimum threshold level (or dose) with
respect to efficacy, although minimum doses for producing a
detectable therapeutic or prophylactic effect for particular
5   disease states can be established. Antioxidant salen metal
complexes of the invention may have glutathione peroxidase
activity.

As used herein, a "salen-transition metal complex"
refers to a compound having a structure according to Structure
10 I, Structure II, Structure III, or Structure IV, Structure V,
Structure VI, Structure VII, Structure VIII, Structure IX
(see, infra) or any of the structures C1, C4, C6, C7, C9, C10,
C11, C12, C15, C17, C20, C22, C23, C25, C27, C28, C29, and C30
as shown in Fig. 3 and infra, preferably having a structure
15 corresponding to one of the structures shown in Fig. 3
selected from the group consisting of: C6, C7, and C12; more
preferably having a structure corresponding to the C7 or C12
structure for catalytic removal of superoxide. The transition
metal is typically selected from the group consisting of: Mn,
20 Mg, Co, Fe, V, Cr, and Ni; and is most conveniently Mn or Mg.

As used herein, "free radical-associated disease"
refers to a pathological condition of an individual that
results at least in part from the production of or exposure to
free radicals, particularly oxyradicals, and other reactive
25 oxygen species in vivo. It is evident to those of skill in
the art that most pathological conditions are multifactorial,
in that multiple factors contributing to the disease state are
present, and that assigning or identifying the predominant
causal factor(s) for any individual pathological condition is
30 frequently extremely difficult. For these reasons, the term
"free radical associated disease" encompasses pathological
states that are recognized in the art as being conditions
wherein damage from free radicals or reactive oxygen species
is believed to contribute to the pathology of the disease
35 state, or wherein administration of a free radical inhibitor
(e.g., desferrioxamine), scavenger (e.g., tocopherol,
glutathione), or catalyst (e.g., SOD, catalase) is shown to

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produce a detectable benefit by decreasing symptoms,
increasing survival, or providing other detectable clinical
benefits in treating or preventing the pathological state.
For example but not limitation, the disease states discussed
5   herein are considered free radical-associated diseases (e.g.,
ischemic reperfusion injury, inflammatory diseases, systemic
lupus erythematosis, myocardial infarction, stroke, traumatic
hemorrhage, spinal cord trauma, Crohn's disease, autoimmune
diseases (e.g., rheumatoid arthritis, diabetes), cataract
10 formation, uveitis, emphysema, gastric ulcers, oxygen
toxicity, neoplasia, undesired cell apoptosis, radiation
sickness, and other pathological states discussed in the
Background section and *infra*).

As used herein the terms "SOD mimetic", "SOD mimic",
15 "superoxide dismutase mimetic", and "superoxide catalyst"
refer to compounds which have detectable catalytic activity
for the dismutation of superoxide as determined by assay.
Generally, an SOD mimetic possesses at least about 0.001
percent of the SOD activity of human Mn-SOD or Zn,Cu-SOD, on a
20 molar basis, as determined by standard assay methods and/or
has at least 0.01 unit of SOD activity per mM according to the
SOD assay used hereinbelow, preferably at least 1 unit of SOD
activity per mM.

The term "alkyl" refers to a cyclic, branched, or
25 straight chain alkyl group containing only carbon and
hydrogen, and unless otherwise mentioned, contain one to
twelve carbon atoms. This term is further exemplified by
groups such as methyl, ethyl, n-propyl, isobutyl, t-butyl,
pentyl, pivalyl, heptyl, adamantyl, and cyclopentyl. Alkyl
30 groups can either be unsubstituted or substituted with one or
more substituents, e.g., halogen, alkyl, alkoxy, alkylthio,
trifluoromethyl, acyloxy, hydroxy, mercapto, carboxy, aryloxy,
aryl, arylalkyl, heteroaryl, amino, alkylamino, dialkylamino,
morpholino, piperidino, pyrrolidin-1-yl, piperazin-1-yl, or
35 other functionality.

The term "lower alkyl" refers to a cyclic, branched or
straight chain monovalent alkyl radical of one to six carbon

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atoms. This term is further exemplified by such radicals as
methyl, ethyl, n-propyl, i-propyl, n-butyl, t-butyl, i-butyl
(or 2-methylpropyl), cyclopropylmethyl, i-amyl, n-amyl, and
hexyl.

5   The term "aryl" or "Ar" refers to a monovalent
unsaturated aromatic carbocyclic group having a single ring
(e.g., phenyl) or multiple condensed rings (e.g., naphthyl or
anthryl), which can optionally be unsubstituted or substituted
with, e.g., halogen, alkyl, alkoxy, alkylthio,
10 trifluoromethyl, acyloxy, hydroxy, mercapto, carboxy, aryloxy,
aryl, arylalkyl, heteroaryl, amino, alkylamino, dialkylamino,
morpholino, piperidino, pyrrolidin-1-yl, piperazin-1-yl, or
other functionality.

           The term "substituted alkoxy" refers to a group
15 having the structure -O-R, where R is alkyl which is
substituted with a non-interfering substituent. The term
"arylalkoxy" refers to a group having the structure -O-R-Ar,
where R is alkyl and Ar is an aromatic substituent.
Arylalkoxys are a subset of substituted alkoxys. Examples of
20 preferred substituted alkoxy groups are: benzyloxy,
napthyloxy, and chlorobenzyloxy.

           The term "aryloxy" refers to a group having the
structure -O-Ar, where Ar is an aromatic group. A preferred
aryloxy group is phenoxy.

25   The term "heterocycle" refers to a monovalent
saturated, unsaturated, or aromatic carbocyclic group having a
single ring (e.g., morpholino, pyridyl or furyl) or multiple
condensed rings (e.g., indolizinyl or benzo[b]thienyl) and
having at least one heteroatom, defined as N, O, P, or S,
30 within the ring, which can optionally be unsubstituted or
substituted with, e.g., halogen, alkyl, alkoxy, alkylthio,
trifluoromethyl, acyloxy, hydroxy, mercapto, carboxy, aryloxy,
aryl, arylalkyl, heteroaryl, amino, alkylamino, dialkylamino,
morpholino, piperidino, pyrrolidin-1-yl, piperazin-1-yl, or
35 other functionality. The term "heteroaryl" or "HetAr" refers
to an aromatic heterocycle.

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"Arylalkyl" refers to the groups -R-Ar and
-R-HetAr, where Ar is an aryl group, HetAr is a heteroaryl
group, and R is straight-chain or branched-chain aliphatic
group. Examples of arylalkyl groups include benzyl and
5   furfuryl. Arylalkyl groups can optionally be unsubstituted or
substituted with, e.g., halogen, alkyl, alkoxy, alkylthio,
trifluoromethyl, acyloxy, hydroxy, mercapto, carboxy, aryloxy,
aryl, arylalkyl, heteroaryl, amino, alkylamino, dialkylamino,
morpholino, piperidino, pyrrolidin-1-yl, piperazin-1-yl, or
10   other functionality.

As used herein, the term "halo" or "halide" refers
to fluoro, bromo, chloro and iodo substituents.

As used in the structures that follow, the term
"OBn" means benzyloxy.

15   As used herein, the term "amino" refers to a
chemical functionality -NR'R", where R' and R" are
independently hydrogen, alkyl, or aryl. The term "quaternary
amine" refers to the positively charged group -N$^+$R'R"R"',
where R', R", and R'" are independently selected and are alkyl
20   or aryl. A preferred amino group is -NH$_2$.

The term "silyl" as used herein refers to
organometallic substituents, wherein at least one silicon atom
is linked to at least one carbon atom; an example of a silyl
substituent is the trimethylsilyl substituent, (CH$_3$)$_3$Si-.

25   The term "pharmaceutical agent or drug" as used
herein refers to a chemical compound or composition capable of
inducing a desired therapeutic effect when properly
administered to a patient.

Other chemistry terms herein are used according to
30   conventional usage in the art, as exemplified by The McGraw-
Hill Dictionary of Chemical Terms (ed. Parker, S., 1985),
McGraw-Hill, San Francisco, incorporated herein by reference).

DETAILED DESCRIPTION

35   Generally, the nomenclature used hereafter and the
laboratory procedures in cell culture, analytical chemistry,
organic synthetic chemistry, and pharmaceutical formulation

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described below are those well known and commonly employed in
the art. Standard techniques are used for chemical syntheses,
chemical analyses, pharmaceutical formulation and delivery,
and treatment of patients.

5   A basis of the present invention is the unexpected
finding that members of a class of compounds described
originally as epoxidation catalysts, the so-called salen-
transition metal complexes, also exhibit potent superoxide
dismutase activity and/or catalase activity and function as
10   catalysts for free radical removal both in vitro and in vivo.
The salen-transition metal complexes have been described as
chiral epoxidation catalysts for various synthetic chemistry
applications (Fu et al. (1991) J. Org. Chem. 56: 6497; Zhang W
and Jacobsen EN (1991) J. Org. Chem. 56: 2296; Jacobsen et
15   al. (1991) J. Am. Chem. Soc. 113: 6703; Zhang et al. (1990) J.
Am. Chem. Soc. 112: 2801; Lee NH and Jacobsen EN (1991)
Tetrahedron Lett. 32: 6533; Jacobsen et al. (1991) J. Am.
Chem. Soc. 113: 7063; Lee et al. (1991) Tetrahedron Lett. 32:
5055). However, salen-transition metal complexes are also
20   useful as potent antioxidants for various biological
applications, including their use as pharmaceuticals for
prevention or treatment of free radical-associated diseases.
Pharmaceutical formulations, dietary supplements, improved
cell and organ culture media, improved cryopreservation media,
25   topical ointments, and chemoprotective and radioprotective
compositions can be prepared with an effective amount or
concentration of at least one antioxidant salen-transition
metal complex species.
           The catalytic activity of salen-metal complexes to
30   interconvert epoxides may also be used to advantage to
scavenge or prevent formation in vivo of cytotoxic and/or
carcinogenic epoxide species, such as may be formed by the
cytochrome P-450 monooxygenation system (e.g., benzo-[a]-
pyrene diol epoxide). Catalytic salen-metal complexes may be
35   advantageously included into foodstuffs or dietary supplements
(or administered in other forms) to individuals who are at
risk of exposure to polycyclic hydrocarbon chemical

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carcinogens, such as workers in the petrochemical industry and
dyestuff manufacture. Moreover, catalytically active salen-
metal complexes may be formulated for administration to
smokers (including passive smokers) to enhance detoxification
5   of reactive epoxides formed from cigarette smoke.

The antioxidant salen metal complexes of the
invention can find use to partially or totally arrest the
progression of neurodegenerative diseases. For example,
mutations in Cu/Zn superoxide dismutase have been reported to
10   be strongly associated with amyotrophic lateral sclerosis
(ALS) (Rosen et al. (1993) Nature 362: 59; Deng et al. (1993)
Science 261: 1047). Similar defects in endogenous antioxudant
protection may be reponsible for multiple sclerosis,
peripheral neuropathies, and the like. Antioxidant salen
15   metal complexes of the present invention can be used for
treatment and prophylaxis of such neurodegenerative diseases
(e.g., ALS, MS).

Salen-Transition Metal Complexes

20   In accordance with a first aspect of the invention,
the salen-transition metal complex has the following
structure:

Structure I

30
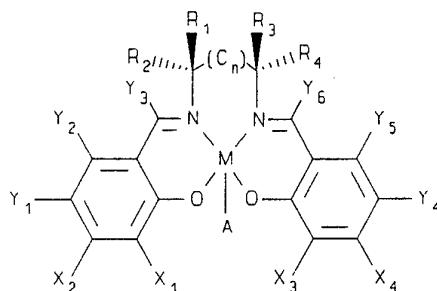
wherein M is a transition metal ion, preferably Mn; A is an
anion, typically Cl; and n is either 0, 1, or 2. X_1, X_2, X_3
and X_4 are independently selected from the group consisting of
hydrogen, silyls, aryls, arylalkyls, primary alkyls, secondary
35   alkyls, tertiary alkyls, alkoxys, aryloxys, aminos, quaternary
amines, heteroatoms, and hydrogen; typically X_1 and X_3 are from
the same functional group, usually hydrogen, quaternary amine,

20

or tertiary butyl, and X_2 and X_4 are typically hydrogen. Y_1, Y_2, Y_3, Y_4, Y_5, and Y_6 are independently selected from the group consisting of hydrogen, halides, alkyls, aryls, arylalkyls, silyl groups, aminos, alkyls or aryls bearing

5 heteroatoms; aryloxys, alkoxys, and halide; preferably, Y_1 and Y_4 are alkoxy, halide, or amino groups. Typically, Y_1 and Y_4 are the same. R_1, R_2, R_3 and R_4 are independently selected from the group consisting of H, CH_3, C_2H_5, C_6H_5, O-benzyl, primary alkyls, fatty acid esters, substituted alkoxyaryls,

10 heteroatom-bearing aromatic groups, arylalkyls, secondary alkyls, and tertiary alkyls.

According to one class of embodiments of the first aspect of the invention, at least one of the X_1 and X_3 sites, and preferably both X_1 and X_3 include a substituent selected

15 from the group of blocking substituents consisting of secondary or tertiary alkyl groups, aryl groups, silyl groups, heterocycles, and alkyl groups bearing heteroatom substituents such as alkoxy or halide. Preferably, the X_1 and X_3 sites bear the same substituent, which substituent is most preferably a

20 tertiary alkyl group, such as tertiary butyl. Preferably, when X_1 and X_3 bear a blocking substituent, then X_2 and X_4 are selected from a group of non-blocking substituents such as H, CH_3, C_2H_5, and primary alkyls, most preferably, H. Alternatively, either three or four of X_1, X_2, X_3, and X_4 can

25 be selected from the group of blocking substituents.

According to this first aspect of the invention, typically at least one and generally no more than two of R_1, R_2, R_3 and R_4 are selected from a group consisting of H, CH_3, C_2H_5, and primary alkyls. For convenience, this group will be

30 referred to as the non-blocking group. If R_1 is selected from the non-blocking group, then R_2 and R_3 are preferably selected from the blocking group, and typically R_2 and R_3 are identical and are phenyl or benzyloxy. If R_2 is selected from the non-blocking group, then R_1 and R_4 are preferably selected from the

35 blocking group. Likewise, if R_3 is selected from the non-blocking group, then R_1 and R_4 are preferably selected from the blocking group. Finally, if R_4 is selected from the non-

blocking group, then R_2 and R_3 are preferably selected from the blocking group. Phenyl and benzyloxy are particularly preferred blocking groups for substitution at any of R_1, R_2, R_3 and R_4. Typically, the blocking groups selected are
5 identical. A preferred class of embodiments have R_1 and R_4 as benzyloxy or phenyl and R_2 and R_3 as hydrogen.

Stated in other terms, one class of embodiments of the first aspect of the invention requires that, of the four sites available for substitution on the two carbon atoms
10 adjacent to nitrogen, at least one or two of these preferably will include a substituent from the non-blocking group.

Preferably, the non-blocking substituent is either hydrogen or methyl, but most preferably, hydrogen. Preferably, the blocking substituent is either a phenyl group,
15 a benzyloxy, or a tertiary butyl group, more preferably a phenyl group or a benzyloxy group, most usually a phenyl group.

Preferably, Y_3 and Y_6 are hydrogen, methyl, alkyl, or aryl. More preferably, they are hydrogen or methyl. Most
20 preferably, they are hydrogen.

The Y_1, Y_2, Y_4, and Y_5 sites are selected independently and are preferably occupied by hydrogen, although these sites may also be occupied by substituents independently selected from the group consisting of hydrogen,
25 halides, alkyls, aryls, alkoxy groups, substituted alkoxy groups, nitro groups, and amino groups. Y_1 and Y_4 are preferably occupied by methoxy, ethoxy, chloro, bromo, iodo, primary alkyl, tertiary butyl, primary amine, secondary amine, or tertiary amine substituents, most preferably methoxy,
30 chloro, tertiary butyl, or methyl.

In accordance with a second aspect of the invention, the salen-transition metal complex has the structure:

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Structure II
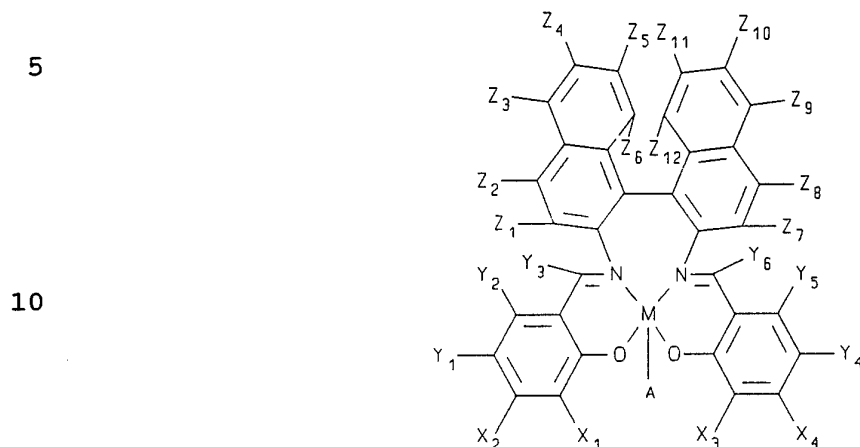
15 wherein M is a transition metal ion, preferably Mn, and A is
    an anion, typically Cl; where at least one of X_1 or X_2 is
    selected from the group consisting of aryls, primary alkyls,
    secondary alkyls, tertiary alkyls, and heteroatoms; where at
    least one of X_1 or X_3 is selected from the group consisting of
20 aryls, primary alkyls, secondary alkyls, tertiary alkyls,
    arylalkyls, heteroatoms, and hydrogen, preferably tertiary
    butyl or hydrogen; and where Y_1, Y_2, Y_3, Y_4, Y_5, Y_6, Z_1, Z_2, Z_3,
    Z_4, Z_5, Z_6, Z_7, Z_8, Z_9, Z_{10}, Z_{11}, and Z_{12} are independently
    selected from the group consisting of hydrogen, halides,
25 alkyls, aryls, amines, alkoxy, substituted alkoxy, arylalkyls,
    aryloxys, and alkyl groups bearing heteroatoms. Preferably Y_1
    and Y_4 are selected from the group consisting of lower alkyls,
    alkoxy, halide, and amino groups, more preferably from the
    group consisting of methoxy, chloro, and primary amine. One
30 preferred embodiment according to this second aspect is the
    species where: Y_1 and Y_4 are methoxy: X_1 and X_3 are
    independently selected and are hydrogen or tertiary butyl, and
    the remaining substituents are hydrogen.

35   In accordance with a third aspect of the invention,
    the salen-transition metal has the following structure:

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Structure III
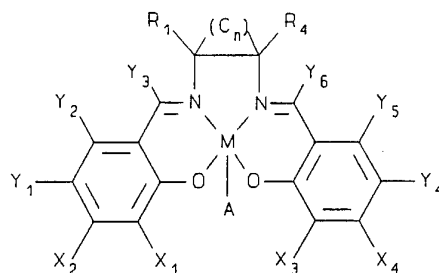
where M is a transition metal ion, typically Mn, and A is an
anion, typically Cl; where n is either 4, 5, or 6; where X_1,
X_2, X_3, and X_4 are independently selected from the group
consisting of aryls, arylalkyls, aryloxys, primary alkyls,
secondary alkyls, tertiary alkyls, alkoxy, substituted alkoxy,
heteroatoms, aminos, quaternary amines, and hydrogen;
preferably, at least one of X_1 or X_3 are selected from the
group consisting of aryls, primary alkyls, secondary alkyls,
tertiary alkyls, quaternary amines, arylalkyls, heteroatoms,
and hydrogen; preferably X_1 and X_3 are identical and are
hydrogen or tertiary butyl; where Y_1, Y_2, Y_3, Y_4, Y_5, and Y_6
are selected from the group consisting of aryls, arylalkyls,
primary alkyls, secondary alkyls, tertiary alkyls, alkoxys,
substituted alkoxys, aryloxys, halides, heteroatoms, aminos,
quaternary amines, and hydrogen; preferably at least one of Y_1
or Y_4 are selected from the group consisting of aryls, primary
alkyls, secondary alkyls, tertiary alkyls, substituted alkoxy,
heteroatoms, amines, and halides; more preferably Y_1 and Y_4 are
identical and are either methoxy, chloro, bromo, iodo,
tertiary butyl, or amine. R_1 and R_4 are independently selected
from the group consisting of hydrogen, halides, primary
alkyls, secondary alkyls, tertiary alkyls, fatty acid esters,
alkoxys, or aryls. Preferably R_1 and R_4 are identical; more
preferably R_1 and R_4 are hydrogen.
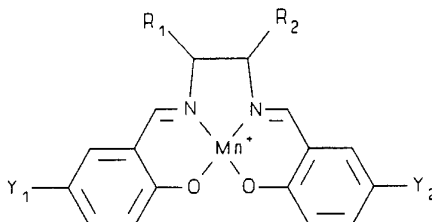
Preferred Antioxidant Salen-Metal Species

The following genera of antioxidant salen-metal
complexes are preferred for use in the compositions and
methods of the present invention, where substituents are not
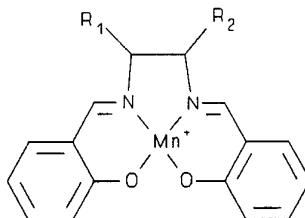
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shown they are hydrogen:

Structure IV

where Y_1 and Y_2 are independently selected from the group
consisting of methoxy, ethoxy, methyl, ethyl, t-butyl, chloro,
bromo, iodo, amino, quaternary amine, alkylamino,
dialkylamino, and hydrogen; R_1 and R_2 are independently
selected from the group consisting of: phenyl, benzyloxy,
chlorobenzyloxy, hydrogen, amino, quaternary amune, or fatty
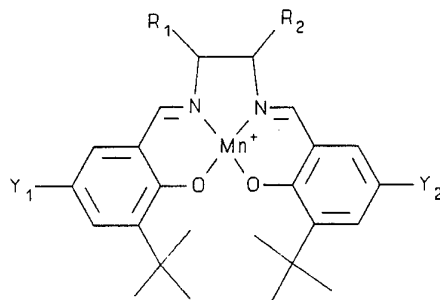acid ester. Preferably, Y_1 and Y_2 are identical.

Structure V

where R_1 and R_2 are selected independently from the group
consisting of: phenyl, benzyloxy, chlorobenzyloxy, hydrogen,
amino, quaternary amine, or fatty acid ester. Preferably, R_1
and R_2 are identical.

25

Structure VI

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where Y_1 and Y_2 are independently selected from the group
consisting of methoxy, ethoxy, methyl, ethyl, t-butyl, chloro,
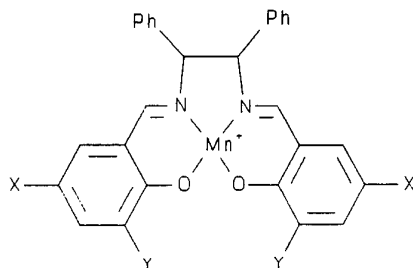bromo, iodo, amino, quaternary amine, alkylamino,
dialkylamino, and hydrogen; R_1 and R_2 are selected
independently from the group consisting of: phenyl, benzyloxy,
chlorobenzyloxy, hydrogen, amino, quaternary amine, or fatty
acid ester. Preferably, Y_1 and Y_2 are identical, and R_1 and R_2
are identical.

Structure VII

where X is selected from the group consisting of methoxy,
ethoxy, methyl, ethyl, t-butyl, chloro, bromo, iodo, amino,
quaternary amine, alkylamino, dialkylamino, and hydrogen; Y is
selected from the group consisting of t-butyl, quaternary
amine, amino, and hydrogen.

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Structure VIII
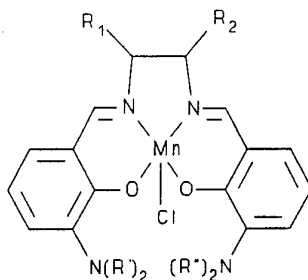
where R$_1$ and R$_2$ are independently selected from the group
consisting of aryloxys, alkoxys, aryls, and hydrogen; R' and
R" are independently selected from the group consisting of
alkyls, aryls, and hydrogen. Preferably, at least one of the
amino groups is protonated at physiological pH (i.e., pH 7.3-
7.8). Preferred R' or R'' alkyls include but are not limited
to: methyl, ethyl, and propyl. Preferred R$_1$ and R$_2$ aryloxys
include but are not limited to benzyloxy and chlorobenzyloxy.
Preferred R$_1$ and R$_2$ alkoxys include but are not limited to
ethoxy and methoxy.

        A preferred subgenus of Structure VIII includes, but
is not limited to:

Structure IX
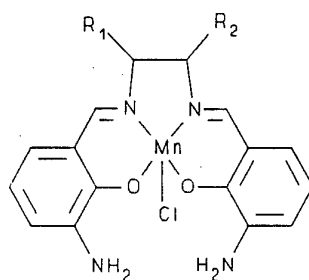
where R is selected from the group consisting of alkyls and
hydrogen. Preferably, at least one of the amino groups are
protonated at physiological pH (i.e., pH 7.3-7.8).

        The following species are preferred antioxidant
salen-transition metal complexes for formulation in
pharmaceutical compositions, dietary supplements, foodstuff

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preservatives, cosmetics, sunburn preventatives, and other
compositions of the invention, and are referenced by structure
number (e.g., C1 through C30) for clarity throughout.

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C1:

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C4:

20   C6:

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C7:

30   C9:
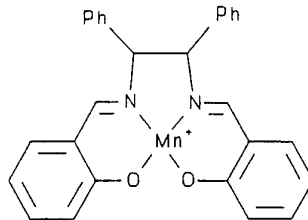
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C10:

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C11:

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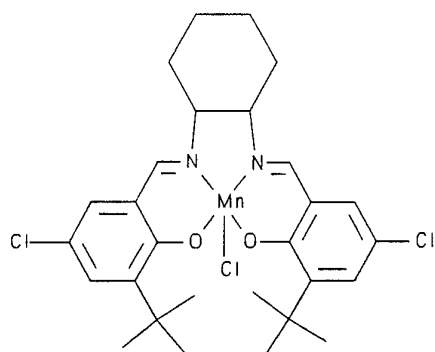
20   C12:

25

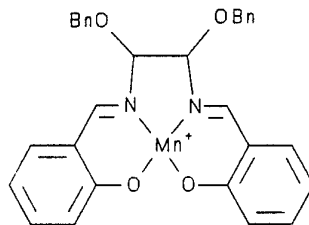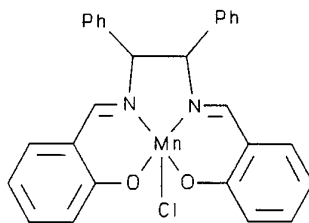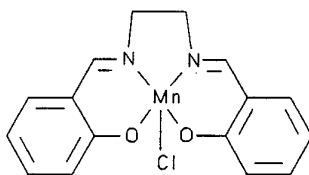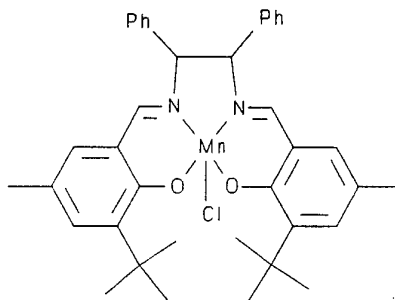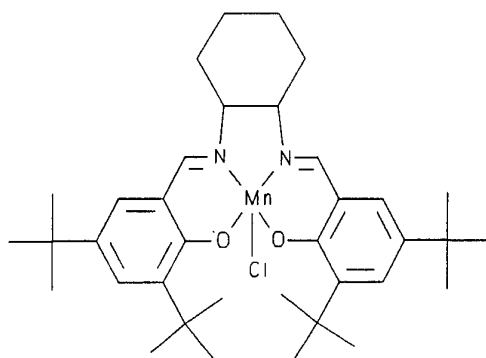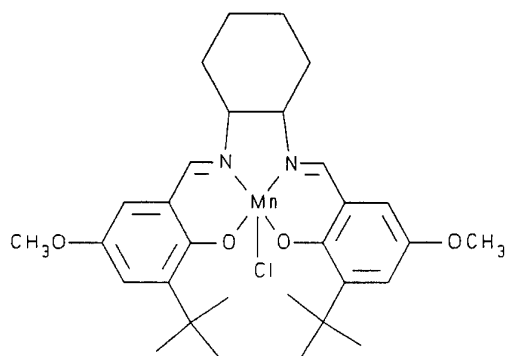30   C15:

35
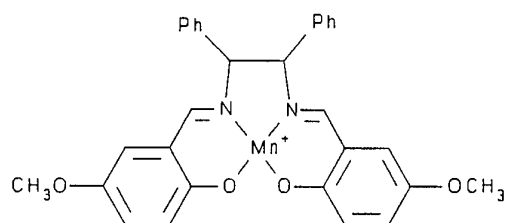
29

C17:

5
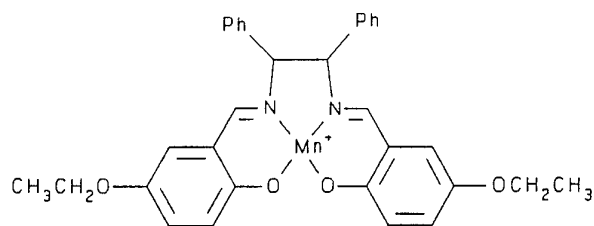
C20:

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C22:

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C23:

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C25:

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C27:

C28:
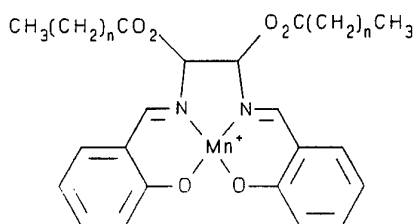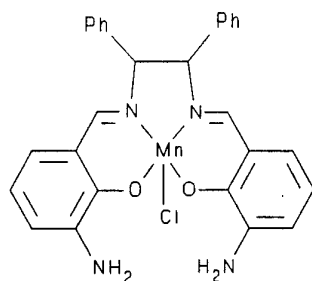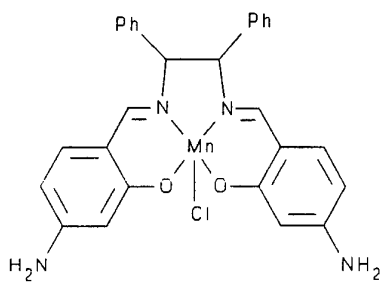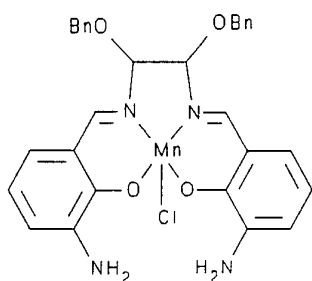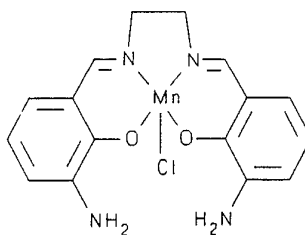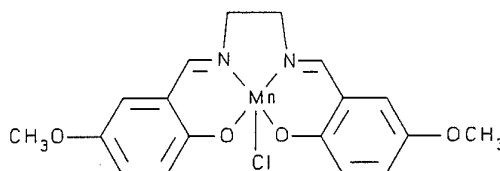
Pharmaceutical Compositions

The preferred pharmaceutical compositions of the present invention comprise a therapeutically or prophylactically effective dose of at least one salen derivative-based complex of a transition metal ion. The term "salen" is used herein to refer to those ligands typically formed through a condensation reaction of two molecules of a salicylaldehyde derivative with one molecule of a diamine derivative. While salen ligands are formed from ethylenediamine derivatives, propyl and butyl diamines may also be used to give analogous salen and salen derivatives. Salen derivatives are preferred and their general structure is shown in Fig. 1. A salen derivative where n is 0 is shown in Fig. 2.

As seen in Fig. 1, the two nitrogens and the two oxygens are oriented toward the center of the salen ligand and thus provide a complexing site for the transition metal ion M. Preferably, this metal ion is selected from the group consisting of Mn, Cr, Fe, Ni, Co, Ti, V, Ru, and Os. More preferably, the transition metal ion is selected from the group consisting of Mn, Mg, Cr, Fe, Ni, and Co. Most preferably, the metal ion is Mn.

Preferably, the anion is selected from the group consisting of PF_6, $(aryl)_4$, BF_4, $B(aryl)_4$, halide, acetate, triflate, tosylate, with halide or PF_6 being more preferred,

31

and chloride being most preferred.

Fig. 1 also shows the many sites available for substitution on the salen ligand. Of these sites, it is believed that R_1, R_2, R_3, R_4, and X_1, X_2, X_3, X_4, Y_3 and Y_6 are

5   the most important in this first salen-transition metal complex.

Structures I, III, IV, VI, VII, and VIII may have independently selected fatty acid ester substituents at the R_1, R_2, R_3, and R_4 positions. When present, the fatty acid

10   esters typically occupy no more than two substituent positions and are usually identical.

Examples of fatty acids suitable to produce the compounds of the instant invention are given in Tables I, II and III below:

15   Table I

$$CH_3-(CH_2)_f-(CH=CH)_g-(CH_2)_h-CO_2H$$
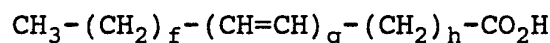
Carbons	f	g	h	Acid Name
16	5	1	7	Palmitoleic
18	7	1	7	Oleic
18	10	1	4	Petroselenic
18	5	1	9	Vaccenic
18	3	3	7	Punicic
18	1	4	7	Parinaric
20	9	1	7	Gadoleic
22	9	1	9	Cetoleic

35

                                     Table II

$$CH_3-(CH_2)_n-(CH=CH-CH_2)_m-(CH_2)_p-CO_2H$$
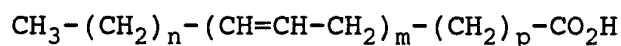
Carbons	f	g	h	Acid Name
18	4	2	6	Linoleic
18	1	3	6	Linolenic
20	4	4	2	Arachidonic

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Table III
$$CH_3-(CH_2)_w-CO_2H$$

Carbons	w	Acid Name
12	10	Lauric
14	12	Myristic
16	14	Palmitic
18	16	Stearic
20	18	Eicosanoic
22	20	Docosanoic

It will be appreciated that the unsaturated acids occur in isomeric forms due to the presence of the one or more unsaturated positions. The compounds of the present invention are intended to include the individual double bond isomers, as well as mixtures thereof. The fatty acid esters of the present invention can be obtained by known acylation techniques. *See, e.g.*, March, <u>Advanced Organic Chemistry</u>, 3rd Ed., John Wiley & Sons, New York (1985), pp. 299, 348-351, and 353-354, incorporated herein by reference.

<u>Preferred Antioxidant Salen-Transition Metal Complexes</u>

Figure 3 shows structures of preferred antioxidant salen-transition metal complexes of the invention. Example antioxidant salen-transition metal complexes are shown in Fig. 3. Compounds C1, C4, C6, C7, C9, C10, C11, and C12 are particularly preferred for formulation in pharmaceuticals and other antioxidant compositions of the invention. It is believed that C7 is particularly preferred because of its facile preparation and relatively hydrophilic nature which is well-suited to pharmaceutical usage.

A preferred salen-transition metal complex having high superoxide dismutase activity is the C12 compound having the structure:

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C12:
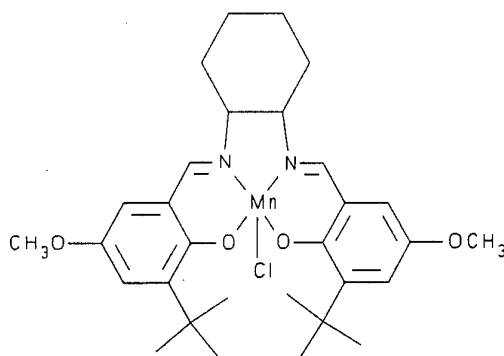
additional

preferred congeners of C12 are:

C29:

and
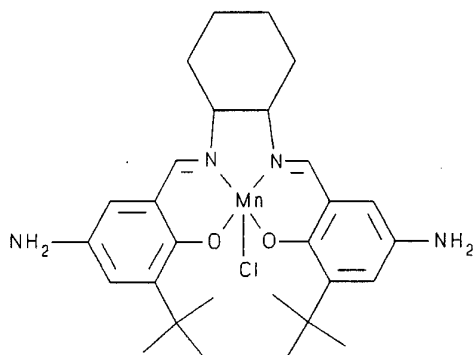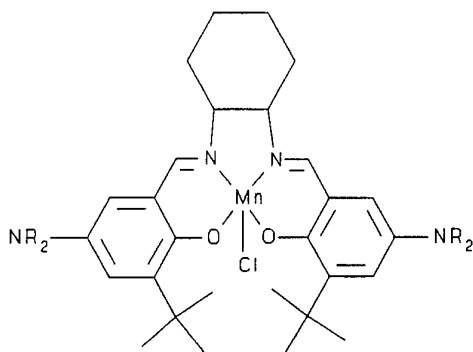
C30:

A particularly preferred antioxidant salen-metal
complex of the invention is C7:

C7:
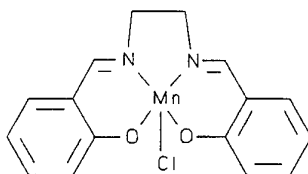
Antioxidant salen-transition metal complexes generally have
detectable superoxide dismutase activity and preferably also

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have catalase activity. Advantageously, C7 is both simple to
prepare and relatively hydrophilic, properties which make it
particularly well-suited for pharmaceutical use and
formulation in aqueous solution. The relatively hydrophilic
5 nature of C7 can be used to advantage in providing antioxidant
salen-metal complexes that are readily absorbed and
transported in the human body. One advantageous
pharmacokinetic property of C7 is believed to be the capacity
to cross the blood-brain barrier efficiently.
10

    Preparation of Antioxidant Salen-Transition Metal Complexes
         Preparation of salen-transition metal complexes are
performed essentially as described in US91/01915 filed 21
March 1991, Fu et al. (1991) J. Org. Chem. 56: 6497; Zhang W
15 and Jacobsen EN (1991) J. Org. Chem. 56: 2296; Jacobsen et
al. (1991) J. Am. Chem. Soc. 113: 6703; Zhang et al. (1990) J.
Am. Chem. Soc. 112: 2801; Lee NH and Jacobsen EN (1991)
Tetrahedron Lett. 32: 6533; Jacobsen et al. (1991) J. Am.
Chem. Soc. 113: 7063; Lee et al. (1991) Tetrahedron Lett. 32:
20 5055, each of which is incorporated herein by reference.
         Generally, the preferred route to prepare the
antioxidant salen-transition metal complexes of the present
invention is a condensation reaction with the substituted
salicylaldehyde and the substituted diamine. In general,
25 quantities of these compounds are reacted in a 2 to 1 molar
ration in absolute ethanol. The solutions are refluxed
typically for 1 hour, and the salen ligand is either
precipitated in analytically pure form by addition of water,
or the metal complex is generated directly by addition of the
30 metal as its acetate, halide, or triflate salt.

         The following procedure is general for the
preparation of antioxidant salen-Mn complexes of the formula:
35

35
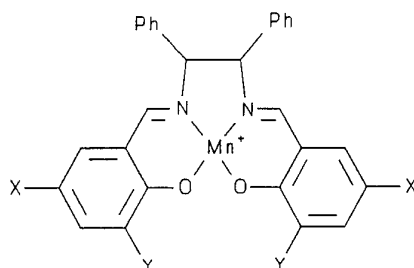
The salen ligand is redissolved in hot absolute
ethanol to give a 0.1 M solution.   Solid $Mn(OAC)_2 \cdot 4H_2O$ (2.0
equivalents) is added in one portion and the solution is
refluxed for 1 h.   Approximately 3 equivalents of solid LiCl
are then added and the mixture is heated to reflux for an
additional 0.5 h.   Cooling the mixture to 0°C affords the
Mn(III) complex as dark brown crystals which are washed
thoroughly with H_2O and isolated by filtration in
approximately 75% yield.   An additional crop of material can
be obtained by dropwise addition of H_2O to the mother liquor.
Combined yields of catalyst are typically about 80-95% for
this step, and about at least 80-90% overall from the
optically pure 1,2-diphenylethylene diamine.

Another example of the method of preparing the
antioxidant salen-Mn complexes are described as follows: Most
preferably, the starting diamine is R,R- or S,S-1,2-diamino-
1,2-diphenylethane and the starting salicylaldehyde is 3-tert-
butylsalicylaldehyde. A solution of 2.0 mmol of 3-tert-
butylsalicylaldehyde in 3 ml of absolute ethanol is added
dropwise to a solution of 1.0 mmol of (R,R)-1,2-diamino-1,2-
diphenylethane in 5 ml of ethanol. The reaction mixture is
heated to reflux for 1 h and then 1.0 mmol of $Mn(Oac)_2 \cdot 4H_2O$ is
added in one portion to the hot (60°C) solution. The color of
the solution immediately turns from yellow to brown upon
addition. It is refluxed for an additional 30 min and then
cooled to room temperature. A solution of 10% NaCl (5ml) is
then added dropwise and the mixture stirred for 0.5h. The
solvents are then removed in vacuo and the residue is
triturated with 50 ml of CH_2-Cl_2 and 50 ml of H_2O. The organic
layer is separated and the brown solution is washed with

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saturated NaCl. Separation of the organic phase and removal
of solvent resulted in a crude material which can be
recrystallized from C_6H_6/C_6H_{14} to give a (R,R)-salen-Mn
complex.

5   The synthesis of the antioxidant salen-transition
metal complexes of the invention may be routinely accomplished
by those of ordinary skill in the art according to the cited
publications.

          The SOD activity of the prepared salen-Mn complexes
10 is determined according to standard assay methods for SOD
activity known in the art and exemplified *infra*. Salen-metal
complexes having at least 0.01 unit of SOD activity per
millimole/liter in aqueous solution are antioxidant salen-
metal complexes; preferably antioxidant salen-metal complexes
15 have at least about 1 unit of SOD activity per
millimole/liter; and more preferably have at least about 100
units of SOD activity per millimole/liter; frequently having
more that 500 to 1000 units of SOD activity per mM or more.
For some medical uses where catalase activity is preferably
20 supplemented, it is advantageous that the SOD mimetic salen-
metal complex also possesses detectable catalase activity
(e.g., C4, C7, C9, C10, C11, C12); typically at least 10 units
of catalase activity per mM, and frequently at least 100 units
of catalase activity per mM.

25   Pharmaceutical Formulations
          Pharmaceutical compositions comprising an
antioxidant salen-transition metal complex of the present
invention are useful for topical and parenteral
administration, *i.e.*, subcutaneously, intramuscularly or
30 intravenously. The finding that salen-metal complexes possess
SOD activity *in vitro* as well as functioning *in vivo* indicates
that antioxidant salen-metal complexes are suitable SOD
mimetics for pharmaceutical use. The antioxidant salen-metal
complexes are suitable for administration to mammals,
35 including human patients and veterinary patients.
          The compositions for parenteral administration will
commonly comprise a solution of an antioxidant salen-

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transition metal complex or a cocktail thereof dissolved in an
acceptable carrier, preferably an aqueous carrier. Since many
of the salen-Mn complexes of the invention are lipophilic, it
is preferable to include in the carrier a hydrophobic base

5   (e.g., polyethylene glycol, Tween 20). A variety of aqueous
carriers can be used, _e.g._, water, buffered water, 0.4%
saline, 0.3% glycine and the like. These solutions are
sterile and generally free of particulate matter. These
compositions may be sterilized by conventional, well known

10   sterilization techniques. The compositions may contain
pharmaceutically acceptable auxiliary substances as required
to approximate physiological conditions such as pH adjusting
and buffering agents, toxicity adjusting agents and the like,
for example sodium acetate, sodium chloride, potassium

15   chloride, calcium chloride, sodium lactate, etc. The
concentration of the antioxidant salen-transition metal
complex(es) in these formulations can vary widely, _i.e._, from
less than about 1 nM, usually at least about 0.1mM to as much
as 100 mM and will be selected primarily based on fluid

20   volumes, viscosities, etc., in accordance with the particular
mode of administration selected. Most usually, the
antioxidant salen-metal complex is present at a concentration
of 0.1 mM to 10 mM. For example, a typical formulation for
intravenous injection comprises a sterile solution of an

25   antioxidant salen-metal complex (e.g., C7) at a concentration
of 5 mM in Ringer's solution. The generally hydrophobic
nature of some of the preferred antioxidant salen-metal
complexes indicates that a hydrophobic vehicle may be used, or
that an aqueous vehicle comprising a detergent or other

30   lipophilic agent (e.g., Tween, NP-40, PEG); alternatively, the
antioxidant salen complexes may be administered as a
suspension in an aqueous carrier, or as an emulsion.

Thus, a typical pharmaceutical composition for
intramuscular injection could be made up to contain 1 ml

35   sterile buffered water, and about 1-100 mg of antioxidant
salen-transition metal complex(es). A typical composition for
intravenous infusion can be made up to contain 250 ml of

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sterile Ringer's solution, and about 100-1000 mg of antioxidant salen-transition metal complex(es). Lipophilic agents may be included in formulations of lipophilic salen-metal complexes. Actual methods for preparing parenterally

5 administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th Ed., Mack Publishing Company, Easton, Pennsylvania (1980), which is incorporated herein by reference. A typical pharmaceutical

10 composition for topical application can be made with suitable dermal ointments, creams, lotions, ophthalmic ointments and solutions, respiratory aerosols, and other excipients. Excipients should be chemically compatible with the antioxidant salen-transition metal complex(es) that are the

15 active ingredient(s) of the preparation, and generally should not increase decomposition, denaturation, or aggregation of active ingredient(s). Frequently, excipients will have lipophilic components such as oils and lipid emulsions.

The antioxidant salen-transition metal complex(es)
20 of this invention can be lyophilized for storage and reconstituted in a suitable carrier prior to use. It will be appreciated by those skilled in the art that lyophilization and reconstitution can lead to varying degrees of antioxidant activity loss, and that use levels may have to be adjusted to

25 compensate.

The compositions containing the present antioxidant salen-transition metal complex(es) or cocktails thereof can be administered for prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a

30 patient already affected by the particular free radical-associated disease, in an amount sufficient to cure or at least partially arrest the condition and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose" or "efficacious dose."

35 Amounts effective for this use will depend upon the severity of the condition, the general state of the patient, and the route of administration, but generally range from about 1 mg

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to about 10g of antioxidant salen-transition metal complex(es)
per dose, with dosages of from 10 mg to 2000 mg per patient
being more commonly used. For example, for treating acute
myocardial ischemia/reoxygenation episodes, about 100 to 1000
5 mg of a antioxidant salen metal complex (e.g., C7) may be
administered systemically by intravenous infusion; at least
about 10mg to 500 mg of antioxidant salen-metal complex(es)
may be administered by intrapericardial injection to provide
elevated local concentrations of SOD activity in the
10 myocardium.

In prophylactic applications, compositions
containing the antioxidant salen-transition metal complex(es)
or cocktails thereof are administered to a patient not already
in a disease state to enhance the patient's resistance or to
15 retard the progression of disease. Such an amount is defined
to be a "prophylactically effective dose." In this use, the
precise amounts again depend upon the patient's state of
health and general level of immunity, but generally range from
1 mg to 10 g per dose, especially 10 to 1000 mg per patient.
20 A typical formulation of an antioxidant salen-metal complex
such as C7 will contain between about 25 and 250 mg of the
salen-metal complex in a unit dosage form.

Single or multiple administrations of the
compositions can be carried out with dose levels and dosing
25 pattern being selected by the treating physician. In any
event, the pharmaceutical formulations should provide a
quantity of the antioxidant salen-transition metal complex(es)
of this invention sufficient to effectively treat the patient.

Kits can also be supplied for use with the subject
30 antioxidant salen-transition metal complex(es) for use in the
protection against or therapy for a free radical-associated
disease. Thus, the subject composition of the present
invention may be provided, usually in a lyophilized form or
aqueous solution in a container, either alone or in
35 conjunction with additional antioxidant salen-transition metal
complex(es) of the desired type. The antioxidant salen-
transition metal complex(es) are included in the kits with

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buffers, such as Tris, phosphate, carbonate, etc.,
stabilizers, biocides, inert proteins, e.g., serum albumin, or
the like, and a set of instructions for use. Generally, these
materials will be present in less than about 5% wt. based on

5   the amount of antioxidant salen-transition metal complex(es),
and usually present in total amount of at least about 0.001%
based again on the concentration. Frequently, it will be
desirable to include an inert extender or excipient to dilute
the active ingredients, where the excipient may be present in

10   from about 1 to 99.999% wt. of the total composition.
        Salen-Mn complexes, preferably compound C12 or C7,
can be incorporated into a hypothermic cardioplegia solution
at a concentration of at least about 1 mM into a solution
formulation according to Amano et al. (1982) Jpn. J. Surg. 12:

15   87, incorporated herein by reference. Most preferably, C7 is
included in the cardioplegia solution.
        The dosage of SOD-mimetic salen-metal complex(es)
will vary with each particular application. Typically, the
composition is administered either systemically or topically.

20   Systemic administration includes per os and parenteral routes;
topical administration includes in situ applications. The in
situ means includes, for example, administering an SOD-mimetic
salen-metal complex by endoscopic bolus wash and/or paravenous
injection, or in the case of lower GI treatments, by enema.

25   Parenteral routes may include, for example, subcutaneous,
intradermal, intramuscular, and intravenous routes. The
amount of SOD-mimetic salen-metal complex(es) will range from
about 2 to 5,000 mg or more, typically 10 to 1000 mg,
depending on the administration interval and route, which can

30   range from a single oral dose, parenteral dose and/or topical
dose to multiple oral doses, parenteral doses, and/or topical
doses over a few days or greater than 5 weeks. The dosage may
also vary with the severity of the disease.

35   In Vitro and Research Administration
        In another aspect of the invention, antioxidant
salen-transition metal complexes of the invention are employed

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to modulate the expression of naturally-occurring genes or
other polynucleotide sequences under the transcriptional
control of an oxidative stress response element (e.g., an
antioxidant responsive element, ARE), such as an antioxidant
5   response element of a glutathione S-transferase gene or a
NAD(P)H:quinone reductase gene (Rozen et al. (1992) Arch.
Biochem. Biophys. 292: 589; Favreau and Pickett (1991) J.
Biol. Chem. 266: 4556; Rushmore and Pickett (1991) Methods
Enzymol. 206: 409; Rushmore and Pickett (1990) J. Biol. Chem.
10   265: 14648; Keyse et al. (1992) Nature 359: 644, incorporated
herein by reference). Transgenes, homologous recombination
constructs, and episomal expression systems (e.g., viral-based
expression vectors) comprising a polynucleotide sequence under
the transcriptional control of one or more ARE linked to a
15   promoter will be made by those of skill in the art according
to methods and guidance available in the art, as will
transformed cells and transgenic nonhuman animals harboring
such polynucleotide constructs. The antioxidant salen-metal
complexes may be used to modulate the transcription of ARE-
20   regulated polynucleotide sequences in cell cultures (e.g., ES
cells) and in intact animals, particularly in transgenic
animals wherein a transgene comprises one or more AREs as
transcriptional regulatory sequences. For transformed or
transgenic cell cultures, a dose-response curve is generated
25   by titrating transcription rate of the ARE-controlled
polynucleotide sequence against increasing concentrations of
antioxidant salen-metal complex(es), which will reduce the
transcription rate induced by oxidant agents (e.g., benzoyl
peroxide, glutathione-depleting agent) or oxidative stress.
30   Conversely, high levels of SOD-mimetic salen-metal complexes
may produce oxidative stress and free radical generation.
Similar dose-response titration can be performed in transgenic
animals, such as transgenic mice, harboring an ARE-controlled
transgene sequence.

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In Vivo Administration

According to this invention, a therapeutically or
pharmaceutically effective amount of an antioxidant salen-
transition metal complex is administered to a patient to treat
5   or prevent a free radical-associated disease. The required
dosage will depend upon the nature of the free radical-
associated disease, the severity and course of the disease,
previous therapy, the patient's health status and response to
the antioxidant salen-transition metal complex, and the
10 judgment of the treating physician. Typically, at least one
species of antioxidant salen-Mn complex is administered as the
sole active ingredient, or in combination with one or more
other active ingredients, typically selected from the group
consisting of: N-2-mercaptopropionylglycine, N-acetylcysteine,
15 glutathione, dimethyl thiourea, desferrioxamine, mannitol, α-
tocopherol, ascorbate, allopurinol, 21-aminosteroids, calpain
inhibitors, glutamate receptor antagonists, tissue plasminogen
activator, streptokinase, urokinase, nonsteroidal anti-
inflammatory agent, cortisone, and carotenoids. Antioxidant
20 salen-Mn complexes may also be administered in conjunction
with polypeptides having SOD and/or catalase activity,
particularly in view of the capacity of the salen-Mn
complexes, unlike SOD polypeptides, to cross the blood-brain
barrier and thereby complement systemic SOD administration.
25   The present invention includes a method of treating
patients, such as humans, who have a free radical-associated
disease with a prophylactically effective or therapeutically
effective amount of a antioxidant salen-transition metal
complex, typically a salen-Mn complex, preferably C7. This
30 method can be used to treat patients at various stages of
their diseases or to prevent development of free radical-
associated diseases in patients. In addition, the treatment
can be administered to prevent or reduce, as a prophylactic,
the age-adjusted probability of developing a neoplasm and/or
35 the age-adjusted mortality rate and/or the rate of senescence.
The antioxidant salen-metal complexes of the
invention can also be administered to patients who are

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infected with a human immunodeficiency virus (e.g., HIV-1) or
who are at risk of becoming infected with a human
immunodeficiency virus. The antioxidant salen-metal
complexes, typified by C7, can prevent or inhibit the

5   induction of HIV-1 replication in $CD4^+$ lymphocytes by tumor
necrosis factor (TNF) and/or prevent damage to or death of
$CD4^+$ cells as a consequence of HIV-1 infection. Without
wishing to be bound by any particular theory of HIV-1
replication or HIV-1 pathogenesis, it is believed that

10 administration of an antioxidant salen-metal complex, such as
C7, can inhibit and/or slow the development of HIV-1 related
pathology and/or can reduce the rate of decline of the $CD4^+$
lymphocyte population in HIV-infected individuals. The
antioxidant salen-metal complexes, such as C7, can also

15 inhibit pathology resulting from excessive or inappropriate
levels of TNF, both in AIDS and in other conditions (e.g.,
septic shock). Frequently, a dosage of about 50 to 5000 mg
will be administered to a patient with HIV and/or with
excessive or inappropriate levels of TNF, either in single or

20 multiple doses, to reduce or retard the development of
pathology and clinical symptoms. Antioxidant salen-metal
complexes may be administered therapeutically to treat viral
diseases other than HIV.

        Since oxidative damage occurs proportionately to the
25 abundance of free radicals and reactive oxygen species, it is
expected that administration of antioxidant salen-transition
metal complexes at even low levels will confer a protective
effect against oxidative damage; thus it is expected that
there is no threshold level below which antioxidant salen-Mn

30 complexes are ineffective.

        In general for treatment of free radical-associated
diseases, a suitable effective dose of the antioxidant salen-
Mn complex will be in the range of 0.01 to 1000 milligram (mg)
per kilogram (kg) of body weight of recipient per day,

35 preferably in the range of 1 to 100 mg per kg of body weight
per day. The desired dosage is preferably presented in one,
two, three, four or more subdoses administered at appropriate

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intervals throughout the day. These subdoses can be
administered as unit dosage forms, for example, containing 5
to 10,000 mg, preferably 10 to 1000 mg of active ingredient
per unit dosage form.

5   The composition used in these therapies can be in a
variety of forms. These include, for example, solid, semi-
solid and liquid dosage forms, such as tablets, pills,
powders, liquid solutions or suspensions, liposome
preparations, injectable and infusible solutions. The
10   preferred form depends on the intended mode of administration
and therapeutic application. Typically, a sterile solution of
a salen-metal complex in an aqueous solvent (e.g., saline)
will be administered intravenously. The compositions also
preferably include conventional pharmaceutically acceptable
15   carriers and adjuvants which are known to those of skill in
the art. See, e.g., Remington's Pharmaceutical Sciences, Mack
Publishing Co.: Easton, PA, 17th Ed. (1985). Generally,
administration will be by oral or parenteral (including
subcutaneous, intramuscular, intravenous, and intradermal)
20   routes, or by topical application or infusion into a body
cavity, or as a bathing solution for tissues during surgery.
          It should, of course, be understood that the methods
of this invention can be used in combination with other
antioxidant agents that have SOD activity, catalase activity,
25   GSH-Px activity, or are free radical scavengers or inhibitors
of free radical formation. While it is possible to administer
the active ingredient of this invention alone, it is believed
preferable to present it as part of a pharmaceutical
formulation. The formulations of the present invention
30   comprise at least one compound of this invention in a
therapeutically or pharmaceutically effective dose together
with one or more pharmaceutically or therapeutically
acceptable carriers and optionally other therapeutic
ingredients. Various considerations are described, e.g., in
35   Gilman et al. (eds) (1990) Goodman and Gilman's: The
Pharmacological Bases of Therapeutics, 8th Ed., Pergamon
Press; and Remington's supra, each of which is hereby

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incorporated herein by reference. Methods for administration
are discussed therein, e.g., for oral, intravenous,
intraperitoneal, or intramuscular administration, and others.
Pharmaceutically acceptable carriers will include water,
5   saline, buffers, and other compounds described, e.g., in the
Merck Index, Merck & Co., Rahway, NJ, incorporated herein by
reference.

The pharmaceutical compositions will be administered
by parenteral or oral administration for prophylactic and/or
10   therapeutic treatment. The pharmaceutical compositions can be
administered in a variety of unit dosage forms depending upon
the method of administration. For example, unit dosage forms
suitable for oral administration include powder, tablets,
pills, capsules, and dragees.

15   The pharmaceutical compositions will often be
administered intravenously. Thus, this invention provides
compositions for intravenous administration which comprise a
solution of the compound dissolved or suspended in an
acceptable carrier, preferably an aqueous carrier. A variety
20   of aqueous carriers can be used, e.g., water, buffered water,
0.4% saline, and the like. Often, the antioxidant salen-metal
complex(es), such as C7 or C12, may be dissolved in an organic
solvent (e.g., dimethylsulfoxide) and either applied directly
or diluted into an aqueous solvent. Typically, antioxidant
25   salen-metal complexes that are relatively lipophilic (e.g.,
C9, C12) are dissolved in an organic solvent such as DMSO and,
if desired, subsequently diluted into a more polar solvent,
such as water. These compositions will sometimes be sterilized
by conventional, well known sterilization techniques, or can
30   preferably be sterile filtered. The resulting aqueous
solutions can be packaged for use as is, or lyophilized, the
lyophilized preparation being combined with a sterile aqueous
solution prior to administration. The compositions can
contain pharmaceutically acceptable auxiliary substances as
35   required to approximate physiological conditions, such as pH
adjusting and buffering agents, tonicity adjusting agents,
wetting agents and the like, for example, sodium acetate,

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sodium lactate, sodium chloride, potassium chloride, calcium
chloride, sorbitan monolaurate, triethanolamine oleate, and
the like.

For solid compositions, conventional nontoxic solid
5   carriers can be used which include, for example,
pharmaceutical grades of mannitol, lactose, starch, magnesium
stearate, sodium saccharin, talcum, cellulose, glucose,
sucrose, magnesium carbonate, and the like. For oral
administration, a pharmaceutically acceptable nontoxic
10   composition is formed by incorporating any of the normally
employed excipients, such as those carriers previously listed,
and generally 0.001-95% of active ingredient, preferably about
20%.

The compositions containing the compounds can be
15   administered for prophylactic and/or therapeutic treatments.
In therapeutic applications, compositions are administered to
a patient already suffering from a disease, as described
above, in an amount sufficient to cure or at least partially
arrest the symptoms of the disease and its complications. An
20   amount adequate to accomplish this is defined as
"therapeutically effective amount or dose." Amounts effective
for this use will depend on the severity of the disease and
the weight and general state of the patient.

In prophylactic applications, compositions
25   containing the compounds of the invention are administered to
a patient susceptible to or otherwise at risk of a particular
disease. Such an amount is defined to be a "prophylactically
effective amount or dose." In this use, the precise amounts
again depend on the patient's state of health and weight.
30   For solid compositions, conventional non-toxic solid
excipients include, for example, pharmaceutical grades of
mannitol, lactose, starch, magnesium stearate, talcum,
celluloses, glucose, sucrose, magnesium carbonate, and the
like may be used. The active compound as defined above may be
35   formulated as suppositories using, for example, triglycerides,
for example, the Witepsols, as the carrier. Liquid
pharmaceutically administerable compositions can, for example,

be prepared by dissolving, dispersing, etc. an active compound
as defined above and optional pharmaceutical adjuvants in a
excipient, such as, for example, water, saline, aqueous
dextrose, glycerol, ethanol, and the like, to thereby form a

5   solution or suspension. If desired, the pharmaceutical
composition to be administered may also contain minor amounts
of nontoxic auxiliary substances such as wetting or
emulsifying agents, pH buffering agents and the like, for
example, sodium acetate, sorbitan monolaurate, triethanolamine

10   sodium acetate, triethanolamine oleate, etc. Actual methods
of preparing such dosage forms are known, or will be apparent,
to those skilled in this art; for example, see Remington's
Pharmaceutical Sciences, Mack Publishing Company, Easton,
Pennsylvania, 17th Edition, 1985. The composition or

15   formulation to be administered will, in any event, contain an
effective amount of the active compound(s).

     For oral administration, a pharmaceutically
acceptable non-toxic composition is formed by the
incorporation of any of the normally employed excipients, such

20   as, for example pharmaceutical grades of mannitol, lactose,
starch, magnesium stearate, talcum, celluloses, glucose,
sucrose, magnesium, carbonate, and the like. Such
compositions take the form of solutions, suspensions, tablets,
capsules, powders, sustained release formulations and the

25   like. Such compositions may contain 0.01-95% active
ingredient, preferably 1-70%.

     Parenteral administration is generally characterized
by injection, either subcutaneously, intramuscularly or
intravenously. Injectables can be prepared in conventional

30   forms, either as liquid solutions or suspensions, solid forms
suitable for solution or suspension in liquid prior to
injection, or as emulsions. Suitable excipients are, for
example, water, saline, dextrose, glycerol, ethanol or the
like. In addition, if desired, the pharmaceutical

35   compositions to be administered may also contain minor amounts
of non-toxic auxiliary substances such as wetting or
emulsifying agents, pH buffering agents and the like, such as

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for example, sodium acetate, sorbitan monolaurate, triethanolamine oleate, etc.

A more recently devised approach for parenteral administration employs the implantation of a slow-release or

5   sustained-release system, such that a constant level of dosage is maintained. See, e.g., U.S. Patent No. 3,710,795, which is incorporated herein by reference. Antioxidant salen-metal complexes may be administered by transdermal patch (e.g., iontophoretic transfer) for local or systemic application.

10   Once detectable improvement of the patient's conditions has occurred, a maintenance dose is administered if necessary. Subsequently, the dosage or the frequency of administration, or both, can be reduced, as a function of the symptoms, to a level at which the improved condition is

15   retained. When the symptoms have been alleviated to the desired level, treatment can cease. Patients can, however, require intermittent treatment on a long-term basis upon any recurrence of the disease symptoms or as a prophylactic measure to prevent disease symptom recurrence.

20   Antioxidant salen-metal complex(es) can also be added to extravasated blood for transfusion to inhibit oxyradical damage to the blood cells and components during storage; similarly, antioxidant salen-metal complexes can also reduce oxyradical damage to blood cells in vivo.

25   Antioxidant salen-metal complex(es) can also be added to rinse or storage solutions for organs and tissues, such as for organ transplantation or for surgical rinses. For example, excised organs are often placed in a preservation solution prior to transplant into a recipient. Inclusion of

30   at least one species of antioxidant salen-metal complex in a preservation solution, usually at a concentration of about 0.01 mM to 10 mM, is desirable for reducing damage due to ischemia during storage and reperfusion injury following reimplantation in the recipient. Various solutions described

35   in the art are suitable for the inclusion of a salen-metal complex, including but not limited to those described in U.S. Patent 5,145,771; Beyersdorf (1990) Chem Abst. 113: 84849w;

49

U.S. Patent 4,879,283; U.S. Patent 4,873,230; and U.S. Patent
4,798,824, incorporated herein by reference.

　　　Typically the antioxidant salen-metal complex is
present in the rinse or storage solution at a concentration of
5　about 10μM to about 10 mM, and most usually is present at 1
mM.　For example, but not to limit the invention, a suitable
rinse solution comprises Ringer's solution (102 mM NaCl, 4 mM
KCl, 3 mM CaCl$_2$, 28 mM sodium lactate, pH 7.0) or Ringer's
solution with 0.1 mM adenosine, and the antioxidant salen-Mn
10　complex C7 at a final concentration of 1 mM.　The rinse
solution can further comprise additional antioxidants (e.g.,
glutathione, allopurinol).　Preservation or rinse solutions
containing an antioxidant salen-metal complex can be used to
provide enhanced storage or irrigation of organs (e.g.,
15　kidney, liver, pancreas, lung, fetal neural tissue, heart,
vascular grafts, bone, ligament, tendon, skin) which is
believed to enhance the viability of the tissue and increase
resistance to oxidative damage (e.g., as a consequence of
ischemia/reperfusion).

20　　　　Without wishing to be bound by any particular theory
of antioxidant or catalytic oxyradical scavenger action, it is
believed that excessive dosages or concentrations of the
catalytic salen-metal complex(es) of the invention might
actually produce free radicals, such as superoxide, possibly
25　in a manner analogous to the presence of large amounts of
circulating free iron.　On this basis, it is believed that
prolonged administration of excessive doses of salen-metal
complexes are preferably avoided for antioxidant therapy.
However, it is also believed that administration of excessive
30　doses of a catalytically active salen-metal complex may be
used to advantage in generating free radicals, such as
superoxide, in local areas (e.g., for acne treatment, skin
cancer treatment, papillomas) or in cell cultures or
transgenic animals harboring a transgene under the
35　transcriptional control of a ARE.　For enhancing free radical
(e.g., superoxide) generation, it may be preferable to expose
the local site, cell culture, or transgenic animal to a

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hyberbaric environment and/or an oxygen-enriched atmosphere
(e.g., greater than about 21 percent molecular oxygen).

Alternatively, the capacity of the antioxidant
salen-metal complexes to catalyze the decomposition of
5   reactive oxygen species can be used to advantage to inhibit or
slow damage to biological tissues and cells. For example,
benzoyl peroxide is a widely used treatment for acne lesions;
excessive or inappropriate application of benzoyl peroxide
(e.g., accidental application to the eyes) may be treated by
10 local (or if desired, systemic) administration of an
antioxidant salen-metal complex (e.g., C7). Similarly,
oxyradical-induced damage to connective tissues (e.g.,
collagen) attendant to exposure to UV light, cigarette
smoking, and senescence may be reduced by administration of an
15 antioxidant salen-metal complex approximately concomitant with
the exposure to UV light, cigarette smoking, or other
oxyradical-generating process (e.g., cellular senescence).

Chemoprotection and Radioprotection
20   Antioxidant salen-transition metal complexes,
typically antioxidant salen-Mn complexes, such as compound C7,
are used to protect cells and tissues from free radical-
producing agents, such as ionizing radiation and
chemotherapeutic agents (e.g., bleomycin). Preferably, a
25 protective dosage comprising at least about 1μg of salen-Mn
complex/kg bodyweight is administered by one or more of
several routes (e.g., oral, intraveneous, intraperitoneal,
intragastric lavage, enema, portal vein infusion, topical, or
inhalation of mist), preferably by injection of liposomes or
30 immunoliposomes for targeted delivery of the antioxidant
salen-Mn complexes to protect normal cells, for example,
against free radical toxicity associated with chemotherapy or
radiotherapy of a neoplasm. The antioxidant salen-transition
metal complexes are preferably preadministered to the patient
35 prior to the commencement of the chemotherapy and/ or
radiotherapy, usually within about 24 hours of commencement,
and preferably within about 3-6 hours of commencement of the

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chemotherapy and/ or radiotherapy. Antioxidant salen-Mn may
be continually administered to the patient during the course
of therapy.

For example, a solution of an antioxidant salen-
5   metal complex can be encapsulated in micelles to form
immunoliposomes (U.S. Patent 5,043,164, U.S. Patent 4,957,735,
U.S. Patent 4,925,661; Connor and Huang (1985) J. Cell Biol.
101: 582; Lasic DD (1992) Nature 355: 279; Novel Drug Delivery
(eds. Prescott LF and Nimmo WS: Wiley, New York, 1989); Reddy
10   et al. (1992) J. Immunol. 148: 1585; incorporated herein by
reference). The immunoliposomes containing the antioxidant
salen-metal species will comprise a targeting moiety (e.g.,
monoclonal antibody) that targets the immunoliposomes to non-
neoplastic cells that are otherwise sensitive to radiotherapy
15   or chemotherapy. For example, immunoliposomes having a
monoclonal antibody that binds specifically to a hematopoietic
stem cell antigen not present on the cancer cells of the
individual may be used to target antioxidant salen-metal
complexes to hematopoietic stem cells and thereby protect said
20   stem cells against radiotherapy or chemotherapy used to treat
the cancer. Such a strategy is preferably employed when the
chemotherapeutic agent forms free radicals in vivo (e.g.,
bleomycin).

Antioxidant salen-Mn complexes are also administered
25   to individuals to prevent radiation injury or chemical injury
by free radical generating agents. Military personnel and
persons working in the nuclear, nuclear medicine, and/or
chemical industries may be administered salen-Mn complexes
prophylactically. Antioxidant salen-metal complexes may also
30   be used as chemoprotective agents to prevent chemical
carcinogenesis; particularly by carcinogens which form
reactive epoxide intermediates (e.g., benzo-[a]-pyrene,
benzanthracene) and by carcinogens or promoting agents which
form free radicals directly or indirectly (e.g.,
35   phenobarbital, TPA, benzoyl peroxide, peroxisome
proliferators: ciprofibrate, clofibrate). Persons exposed to
such chemical carcinogens are pretreated with an antioxidant

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salen-metal complex to reduce the incidence or risk of
developing neoplasia.

Antioxidant salen-metal complexes can also be
formulated into a lipophilic base (or, if desired, an aqueous
5   carrier) for topical application in cosmetics or sunburn-
prevention creams and lotions. A typical cosmetic or sunburn-
prevention cream or lotion will comprise about between 1 mg to
50 mg of antioxidant salen-metal complex per gram of cosmetic
or sunburn-prevention cream or lotion.
10   Antioxidant salen-metal complexes may also be
administered to deep-divers or individuals exposed to
hyberbaric environments were oxygen toxicity presents a health
risk. Administration of an efficacious dose of an antioxidant
salen-metal complex to an individual may permit the breathing
15 or hyberbaric and/or oxygen-enriched gases with a reduced risk
of oxygen toxicity. It is also believed that administration
of an efficacious dosage of an antioxidant salen-metal complex
can reduced toxicity and biological damage associated with
exposure to ozone. Prophylactic administration of an
20 antioxidant salen-metal complex to humans who are or will be
exposed to ozone is expected to confer an enhanced resistance
to ozone toxicity, such as the ozone-induced lung damage noted
in geographical areas with high ozone levels (e.g., Los
Angeles).
25

Utility, Testing and Administration

The compounds of the invention, antioxidant salen-
transition metal complexes, preferably salen-Mn complexes, are
useful treatments for protection against ischemic damage in
30 cardiac and non-cardiac states including myocardial
infarction, congestive heart failure, angina, arrhythmia,
circulatory disorders, and stroke. The compounds of the
invention inhibit the deleterious effects of ischaemia
(coronary infarction and reperfusion in the heart; transient
35 myocardial or CNS ischemia during surgery) without direct
depressant effects on myocardial contractility. Thus, the
compounds are effective in animal models for cardiovascular

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and CNS diseases, and will be useful for the treatment of
myocardial infarction, stroke, brain injury, and transplant
surgery, particularly with reperfusion of infarcted areas,
arrhythmias, variant and exercise-induced angina, congestive
5   heart failure, stroke and other circulatory disorders, in
mammals, particularly in human beings. The salen-Mn complexes
are also included in preservation solutions used to bathe
excised organs (e.g., heart, kidney, pancreas, liver, lung)
during transport and storage of the excised organ prior to
10   transplantion surgery, including skin grafting and corneal
grafting. The preservation solutions will typically comprise
at least about 1 μM of an antioxidant salen-metal complex,
preferably at least about 1 mM of an antioxidant salen-metal
complex.

15   Administration of the active compound and salts described
herein can be via any of the accepted modes of administration
for therapeutic agents. These methods include oral,
parenteral, transdermal, subcutaneous and other systemic
modes. The preferred method of administration is oral, except
20   in those cases where the subject is unable to ingest, by
himself, any medication. In those instances it may be
necessary to administer the composition parenterally. If the
composition comprises an antioxidant salen-metal species
having an amino substituent that can be protonated at
25   physiological pH, it is usually preferred that the antioxidant
salen-metal complex is dissolved or suspended in a solution
having a pH at which the amino substituent is protonated.

          The amount of active compound administered will, of
course, be dependent on the subject being treated, the
30   subject's weight, the severity of the affliction, the manner
of administration and the judgment of the prescribing
physician. However, an effective dosage is in the range of
0.01-50 mg/kg/day, preferably 0.5-25 mg/kg/day. For an
average 70 kg human, this would amount to 0.7-3500 mg per day,
35   or preferably about 35-1750 mg/day.

          Since all of the effects of the salen-Mn compounds
herein are achieved through a similar mechanism, dosages (and

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forms of administration) are within the same general and
preferred ranges for all these utilities.

The following examples are offered by way of
illustration, not by way of limitation.

EXPERIMENTAL EXAMPLES

In Vitro Catalytic Activities

The antioxidant catalytic activities of the C1, C4,
C6, C7, C9, C10, C11, and C12 salen-Mn complexes (see Fig. 3)
was determined; superoxide dismutase and catalase activities
were determined according to the following method.

Assay

The SOD activity of the compounds was determined by
evaluating the inhibition of the reduction of cytochrome C
produced by the oxygen free radical generating system,
xanthine plus xanthine oxidase. Cytochrome C reduction is
monitored spectrophotometrically at 550 nm according to the
method described in Darr et al. (1987) Arch. Biochem. Biophys.
258: 351, incorporated herein by reference. The concentration
of xanthine oxidase is adjusted such that it produces a rate
of reduction of cytochrome C at 550 nm of 0.025 absorbance
unit per minute. Under these conditions, the amount of SOD
activity required to inhibit the rate of cytochrome C
reduction by 50 percent (i.e., to a rate of 0.0125 absorbance
unit per minute) is defined as one unit of activity. Salen-
metal complexes are identified as antioxidants if they have at
least 0.1 unit of activity at a concentration of 1 mM under
these standard assay conditions.

Catalase activity was measured using a
spectrophotometric method in which the decomposition of
hydrogen peroxide is monitored at 240 nm according to the
method of Aebi et al. (1984) Methods Enzymol. 105: 121,
incorporated herein by reference. One unit of catalase
activity is defined as the amount of enzyme (or salen-metal
complex) required to decompose 1 μmole of hydrogen peroxide in
one minute.

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Each of the compounds was formulated in saline and
was stable with no loss of activity observed after several
weeks of storage at room temperature. Frequently, it is
desirable to first dissolve the salen-metal complex in an
5 organic solvent (e.g., DMSO) and then dilute the solution into
a more polar solvent such as water. This is particularly
preferred for salen-metal species that are relatively
hydrophobic (e.g., C12).

Table IV shows the in vitro SOD and catalase
10 activities of the various salen-Mn complexes tested. SOD and
catalase activities are expressed as units/mM.

Table IV

Salen-Mn Complex	SOD Activity	Catalase Activity
C1	308	262
C4	312	200
C6	812	0
C7	575	200
C9	111	20
C10	69	179
C11	101	46
C12	4397	144

In Vivo Biological Activities

A widely used assay to determine the therapeutic
25 potential of molecules in brain ischemia (stroke) consists of
evaluating their ability to prevent irreversible damage
induced by an anoxic episode in brain slices maintained under
physiological conditions. Rat brain slices were maintained at
35°C in an interface chamber in an artificial cerebrospinal
30 fluid containing: 124 mM NaCl, 3 mM KCl, 1.25 mM KH_2PO_4, 3 mM
CaCl, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 10 mM D-glucose, and 2 mM L-
ascorbate, continuously gassed with a mixture of $O_2:CO_2$ (95:5).
The atmosphere of the chamber was also continuously gased with
the mixture of $O_2:CO_2$ (95:5), except during the anoxic episode
35 when it was replaced by N_2. Axons were electrically
stimulated and the evoked excitatory post-synaptic potentials
(EPSPs) were recorded using microelectrodes.

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Fig. 4 shows the schematic of an EPSP recorded under normal conditions (A), five minutes following replacement of O_2 with N_2 (ischemic episode, B), and 30 to 40 minutes following reoxygenation (C). The extent of permanent damage

5 can be quantified by measuring both the amplitude (in mV) and the initial slope (in mV/msec) of the EPSP.

Figs. 5 and 6 show the protective effect of the antioxidant salen-Mn complex designated C7 in the rat brain slice ischemia EPSP system. Brain slices were incubated in

10 the absence or presence of 50 μM C7 and subjected to an episode of ischemia/reoxygenation. After 5 minutes of baseline recording, O_2 was replaced by N_2 for an average of 5 minutes. O_2 was then reintroduced and recording was continued for another 50 minutes. Samples with 50 μM C7 showed that both

15 the amplitude and slopes of the EPSPs recovered to pre-ischemia levels. In contrast, recovery in untreated brain slices was only about 40% of pre-ischemia levels.

As an additional assessment of efficacy, the percentage of viable slices following repeated ischemic

20 episodes was evaluated. Fig. 7 demonstrates that, while without any treatment this percentage is very low (6%), it was as high as 70% in slices treated with 50 μM C7. A slice was considered viable if an EPSP of 3 mV amplitude could be elicited by increasing stimulation intensity.

25

Animal Model Testing

An animal model of Parkinson's disease involving iatrogenic hydroxyl radical generation by MPTP (Chiueh et al. (1992) Synapse 11: 346, incorporated herein by reference) was

30 used to evaluate the protective effect of C7 on free radical-induced damage. The neurotoxin, MPTP, has been shown to lead to the degeneration of dopaminergic neurons in the brain, thus providing a good model of experimentally induced Parkinson's disease (e.g., iatrogenic toxicity). This model is now widely

35 accepted in the art and is used for evaluating potential therapeutic agents for this disease.

The number of dopaminergic neurons in brains of mice
treated with either: (1) MPTP alone, (2) the antioxidant
salen-metal complex C7 alone, (3) pretreatment with C7 and
then MPTP, or (4) untreated controls, were assayed by
5 measurement of the binding of the dopamine reuptake ligand,
mazindol. Tritiated mazindol was used for binding studies on
samples of the globus pallidus, caudate nucleus, and striatum
of mouse brain according to conventional methods; specific
binding of tritiated mazindol was determined
10 autoradiographically or by membrane binding (specific binding
to the membrane fraction). The experiment was performed over
a 7 day period. Mice in the MPTP group were treated
intraperitoneally with MPTP alone (40 mg/kg each day on days 1
and 2). Mice in the MPTP+C7 group were pretreated with C7 (33
15 mg/kg, i.p.) immediately prior to MPTP on days 1 and 2, and
were given C7 (33 mg/kg) alone on day 3. The animals were
sacrificed after 7 days. The results shown in Fig. 8 show a
significant protective effect conferred in vivo by the salen-
Mn complex, C7. Fig. 8 shows that the number of dopaminergic
20 neurons present in various regions of the mouse brain were not
adversely affected by the antioxidant salen-metal complex C7;
but dopaminergic neurons were reduced to about 15 percent of
control values in mice treated with MPTP alone; however
pretreatment with C7 approximately doubled the number of
25 surviving dopaminergic neurons present in mice subsequently
treated with MPTP. Lack of toxicity of C7 was shown by the
absence of adverse health effects in the C7-treated animals
over the 7 day test period.

These data demonstrate that the salen-Mn complexes
30 display therapeutic efficacy in vivo in rodent models of human
disease. and also indicate that the salen-Mn complexes cross
the blood-brain barrier efficiently. Taken together, these
data indicate a dramatic efficacy of salen-Mn complexes to
prevent free radical-induced damage and ischemia/reoxygenation
35 injury in the brain.

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Effect of C7 in isolated iron-overloaded rat hearts submitted
to ischemia and reperfusion

      Rats received an intramuscular injection of 0.25 ml
of an iron-dextran solution (100 g iron hydroxide, 99 g

5 dextran, water up to 1l) every third day during a 5-week
period to achieve a significant iron overload in cardiac
tissue. At the end of this treatment, rats were anesthetized
with sodium pentobarbital (40 mg/kg) and heparin (1,000 IU/kg)
was administered via a femoral vein. Hearts were then removed

10 and rapidly perfused through the aorta according to the
technique described by Langendorff [Langendorff, O., Pflügers
Arch. 61: 291, 1895] at a constant flow rate of 1l ml/minute.
The perfusion fluid was a modified Krebs-Henseleit buffer
containing (in mmol/l): NaCl 118, KCl 5.9, $NaHCO_3$ 25, $MgCl_2$

15 1.2, NaH_2PO_4 0.6, $CaCl_2$ 2.4, Glucose 1l. pH was maintained at
7.4 ± 0.05 when the perfusion medium was saturated with O_2-CO_2
(95%-5%) at 37°C. The perfusion apparatus was fully
thermostated such that the temperature of the perfusion medium
was 37.0 ± 0.5°C when it reached the aorta. An ultra-thin

20 balloon was inserted in the left ventricle immediately after
the initiation of aortic perfusion and was inflated so as to
obtain an end-diastolic pressure of 5 mm Hg. A 15 minute
stabilization period was initiated immediately following
balloon placement. At the end of this period, systolic and

25 diastolic ventricular pressures and heart beat rate (HR) were
recorded through a pressure transducer linked to the
ventricular balloon. Left Ventricular Developed Pressure
(LVDP) was calculated by the difference between systolic and
diastolic pressure and the product HR x LVDP was taken as an

30 index of oxygen consumption. Hearts were then subjected to a
15 minute total global normothermic ischemia, followed by 15
minutes of reperfusion with the perfusion medium used
initially. During this 15 minute reperfusion, heart rate, and
diastolic and systolic pressures were monitored. Early

35 ventricular fibrillations were analyzed 1 min. after the start
of the reperfusion.

Three experimental groups were studied. Group 1
(n=7) in which hearts were perfused with the standard
perfusion fluid (control group); group 2 (n=8) were perfused
in the presence of dimethylthiourea (DMTU, 10 mM; group 3
(n=8) were perfused in the presence of C7 (50 μM).

After the 15 minute reperfusion, 3 hearts in each
group were prepared for electron microscopy by perfusion with
2.5% glutaraldehyde. Ultra-thin slices (500-600Å thickness)
were examined.

Results

The following Table V shows heart rates (HR),
systolic pressures (SP), diastolic pressures (DP), and the
products HR x LVDP, in the three experimental groups, after 15
minutes of perfusion, before ischemia (Before), 1 minute after
reperfusion (1 After) and 15 minutes after reperfusion
(15 After). The table also shows the number of hearts
exhibiting episodes of ventricular fibrillation 1 minute after
reperfusion (VF).

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Table V

	HR (beats/min)	SP (mm Hg)	DP (mm Hg)	HR x LVDP (x 10.3)	VF
Controls:					
Before	276 ± 11	78 ± 7	6.3 ± 0.3	19.6 ± 1.6	-
1 After	96 ± 0	40 ± 6	23.3 ± 6.0	4.2 ± 1.7	5/7
15 After	232 ± 15	62 ± 10	13.6 ± 4.2	12.6 ± 2.3	-
+ DMTU					
Before	280 ± 10	97 ± 4	4.7 ± 0.3	24.1 ± 0.6	-
1 After	91 ± 10	62 ± 9*	37.2 ± 10.0	3.5 ± 1.2	3/8
15 After	226 ± 18	58 ± 6	27.8 ± 9.4	9.4 ± 2.0	-
+ C7					
Before	278 ± 7	90 ± 2	5.4 ± 0.3	23.5 ± 0.9	-
1 After	130 ± 13#	72 ± 8#	5.8 ± 0.5#±	9.9 ± 0.8#±	2/8
15 After	241 ± 15	92 ± 15	8.3 ± 0.6	21.7 ± 3.4¤±	-

*: $p < 0.01$, DMTU versus control at the same time.

#: $p < 0.01$, C7 versus control at the same time.

¤: $p < 0.05$, C7 versus control at the same time.

±: $p < 0.01$, C7 versus DMTU at the same time.

Table VI summarizes the results from the electron microscopy evaluation of the hearts. Mitochondria were classified into Type A (normal), Type B (swollen, unbroken), and Type C (ruptured membranes). Sarcomeres were classified into Type A (normal) and Type B (contacted and/or necrosis). The results are expressed as percentages. The numbers of mitochondria analyzed were 1293, 1632 and 1595 for controls, DMTU and C7 groups, respectively. The numbers of sarcomeres analyzed were 1046, 1173, and 1143 for controls, DMTU and C7 groups, respectively.

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Table VI

	Mitochondria			Sarcomeres	
	Type A	Type B	Type C	Type A	Type B
Controls	10.4	21.0	68.5	21.3	78.7
+DMTU	14.3*	19.5	66.2	13.7+	86.3+
+C7	31.0#±	15.2#¤	53.8#±	60.6#±	39.4#±

*: $p < 0.05$, DMTU versus control.
+: $p < 0.01$, DMTU versus control.
#: $p < 0.01$, C7 versus control.
¤: $p < 0.05$, C7 versus DMTU.
±: $p < 0.01$, C7 versus DMTU.

The data show that C7 effectively protected hearts
from ischemia/reoxygenation damage, both functionally and
structurally. In addition, C7 was significantly more
efficacious than DMTU, an antioxidant, even though it was used
at a concentration 200 times lower.

Experimental Autoimmune Encephalitis (EAE)
        EAE is an animal model of multiple sclerosis. 30
SJL female mice, aged 10 weeks, were divided into 2 groups of
20 mice (control) and 10 mice (C7 treated).
        Mice in both groups were immunized with an
encephalitogenic PLP peptide in complete Freund's adjuvant
subcutaneously, followed by Petrussis Toxin (IV). Petrussis
toxin was repeated on day 3 post immunization.
        Mice in the C7 group were treated daily (1 mg/mouse,
approximately 40 mg/kg) by IP injection, starting from 2 days
prior to immunization through day 14 after immunization.
        Animals were scored as follows:
        Stage I: Limp tail syndrome
        Stage II: Hind leg paralysis
        Stage III: Hind leg paralysis-Dragging movement
        Stage IV: Paralytic immobility, weight loss

Results

        During the third week following immunization, 8 of
20 mice in the control group developed symptomatic EAE: 2
Stage I, 4 Stage II/III, 2 Stage IV.

5   During that same period, only one of 10 mice in the
C7 treated group developed symptomatic EAE (Stage II).

        During the fifth week, i.e., three weeks after the
treatment with C7 was stopped, six mice in the C7 group
developed symptomatic EAE, 4 Stage II and 2 Stage IV.

10   These results indicate that C7 treatment prevented
the development of symptomatic EAE, and that the disease could
develop following interruption of the treatment.

Lipid peroxidation

15   Hippocampal slices (400 μm thick) were obtained from
Sprague-Dawley rats (150-200g) and collected in preoxygenated
(95% O_2 / 5% CO_2) Krebs-Ringer phosphate medium (pH 7.4)
containing NaCl 120 mM, KCl 5 mM, $CaCl_2$ 1.3 mM, $MgCl_2$ 1.2 mM,
NaPhosphate 16 mM (pH 7.4) and glucose 10 mM. After 15

20 minutes preincubation in a water bath at 35°C under agitation,
the buffer was replaced with the same buffer (control) or a
modified buffer (lactate buffer) containing NaCl 90 mM, KCl
5 mM, $CaCl_2$ 1.3 mM, $MgCl_2$ 1.2 mM, NaPhosphate 16 mM and lactic
acid 30 mM (pH 5.0). When present, C7 (50 μM) was added

25 during the preincubation and the incubation periods. After
100 minutes, slices were collected and homogenized in 0.9 ml
of TCA 5%, whereas 0.35 ml of TCA 5% was added to 0.5 ml of
the incubation medium. Lipid peroxidation was measured by
adding 0.25 ml of a thiobarbituric acid reagent (TBAR) to

30 0.85 ml of the TCA extracts and incubating the mixture for 60
minutes at 85-93°C. Lipids were then extracted with
2 x 0.5 ml 1-butanol by vortexing for 10 seconds, then
centrifuging at 2,000 rpm for 10 minutes. The absorbance of
peroxidized lipids in the alcohol phase was measured in a

35 spectrophotometer at 532 nm. Data were expressed as nmoles of
malondialdehyde (MDA) using authentic MDA to establish a
standard curve. Proteins were measured from an aliquot of the

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TCA extracts using the method of Bradford and the final
results were calculated as nmoles MDA formed/mg protein.

Results

5   The Fig. 9 shows lipid peroxidation at time 0
(immediately after sectioning), and after 100 minutes of
incubation at pH 7.4 (control), at pH 5.0 (lactate) in the
absence (LA) or presence (LA + C7) of 50 μM C7, in the slice
homogenates (hatched bars) and in the incubation medium dotted
10   bars). Data are means ± S.D. and the C-7 experimental group
were highly statistically significant as compared to control
(p < 0.01) while the small differences between LA and LA + C7
are not. Incubation of hippocampal slices with 30 mM lactate,
at a final pH of 5.0, resulted in a large increase in lipid
15   peroxidation, as measured by the thiobarbituric acid test.
Incubation of slices with C7 (50 μM) totally abolished the
increase in lipid peroxidation. Lactate-induced increases in
malondialdehyde concentration in both the incubation media
(dotted bars) and in the slice homogenates (hatched bars) were
20   blocked by C7. Incubation for 100 minutes without lactate,
either with or without C7, did not cause any appreciable
increase in lipid peroxidation.

           These data show that C7 prevents lipid peroxidation
induced by acidosis. Acidosis is known to induce extensive
25   oxidative damage. Lipid peroxidation is a consequence of such
oxidative damage, and has been found associated with a number
of human pathologies.

In vitro models of injury

30   Anoxia in hippocampal slices. Electrophysiological
experiments were performed on hippocampal slices (400 μm) from
adult Sprague-Dawley rats maintained at 35°C in 2 interface
chambers with or without 50 mM C7. A glass recording
micropipet was positioned in CA1 stratum radiatum to record
35   excitatory postsynaptic potential (EPSPs) generated by
electrical stimulation of the Schaffer-commissural pathway by
a bipolar stimulation electrode at a frequency of 0.033 Hz.

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During anoxic episodes, the oxygen supply was replaced with 100% N_2 gas. N_2 was supplied for 90 seconds following electrical silence after which O_2 was reintroduced. Recovery of EPSPs (both slope and amplitude) was recorded for 50

5 minutes, at which time the final viability of the slices was determined, with viability being defined as the ability of the slice to generate a 3 mV EPSP.

Acidosis in hippocampal slices. Hippocampal slices were collected in preoxygenated Krebs-Ringer phosphate buffer,

10 with or without 50 μM C7, at 35°C in a shaking water bath. After a 15 minute preincubation, slices were transferred into the same buffer or in a buffer containing 30 mM lactate, pH 5.0 (with or without C7). Slices from all groups were collected after a 100 minute incubation and tested for lipid

15 peroxidation, as indicated by malondialdehyde reaction with thiobarbituric acid.

In vivo model of neuronal injury

MPTP in mice. Adult male CFW mice (25-33 g) were

20 administered two injections of MPTP dissolved in normal saline (40 mg/kg, s.c.) 24 hours apart. A group of animals also received C7 in three injections (33 mg/kg, s.c.) administered 24 hours apart, starting 1 day before the onset of MPTP treatment. Animals were sacrificed 7 days after the first

25 MPTP injection, and neuronal pathology was assessed by the binding of ^{3}H-mazindol, a ligand for the dopamine transporter, to 10 mm frozen brain sections or to striatal homogenates.

6-OHDA in mice. Adult male CFW mice were anesthetized with ketamine and rumpun, and immobilized in a

30 stereotaxic device. 6-OHDA, as the hydrobromide salt, was dissolved in normal saline with 1% ascorbate, and 50 μg was administered in lateral ventricle by means of a 10 μl Hamilton syringe. C7 (66 mg/kg, i.p.) was administered daily for 4 days. Animals were sacrificed 7 days later, and neuronal

35 pathology was assessed by measuring ^{3}H-mazindol binding in striatal homogenates.

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RESULTS

C7 protects hippocampal slices from anoxia-induced damage

Hippocampal slices were subjected to anoxic
conditions with or without C7 (50 μM). C7 provided a
5   significant degree of protection against anoxia-induced
decrease in synaptic response in CA1. The decrease in both
EPSP slope (A) and amplitude (B) were prevented by C7.
Purified bovine SOD in the same assay provided no protection).

10   Fig. 1o shows I.c.v. injection of 6-OHDA (50 μg)
resulted in a 60-70% decrease in mazindol binding in
homogenates from the striatum ipsilateral from the injection
site and a 30% decrease from the contralateral striatum (Fig.
10). Treatment with C7 (4x66 mg/kg) produced a significant
15   reduction in the ipsilateral side and a complete protection in
the contralateral side.

MPTP administration (2x40 mg/kg, s.c.) resulted in a
75-80% decrease in mazindol binding. C7 treatment (3x33
mg/kg, i.p.) caused a significant ($p < 0.05$) degree of
20   protection against the reduction in ^{3}H-mazindol binding in
both the globus pallidus, and the caudate nucleus (panel A).
C7 treatment alone had no significant effect on ^{3}H-mazindol
binding. The same treatment also produced a significant
protection against the reduction in 3H-mazindol binding
25   measured in striatal homogenates (panel B).

CONCLUSIONS

These results illustrate the protective effects of a
Synthetic Catalytic Scavenger (SCS), C7, in various models of
30   neuronal damage. C7 was able to protect neurons from acute
early manifestations of neuronal damage, such as lipid
peroxidation and loss of synaptic viability, as well as long-
term manifestations of neuronal injury, such as neuronal loss
7 days after toxin injection.
35   In view of the positive effects obtained with
peripheral injections of C7 in the in vivo models of neuronal
injury, we conclude that the complex is stable in vivo and

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crosses the blood brain barrier as well as neuronal membranes.

The positive effects of C7 in various models of
neuronal injury indicate that reactive oxygen species,
especially the superoxide radical, play a significant role in
5   the pathology induced by ischemia and acidosis, and in MPTP-
and 6-OHDA-induced loss of nigrostriatal dopaminergic neurons.

Finally, in view of the wide range of pathological
conditions associated with overproduction of oxygen radicals,
these results support the idea that antioxidant salen-metal
10 complexess such as C7 might have a wide range of therapeutic
applications.

The foregoing description of the preferred
embodiments of the present invention has been presented for
purposes of illustration and description. They are not
15 intended to be exhaustive or to limit the invention to the
precise form disclosed, and many modifications and variations
are possible in light of the above teaching.

Such modifications and variations which may be
apparent to a person skilled in the art are intended to be
20 within the scope of this invention.

CLAIMS

        1.   A pharmaceutical composition comprising a
therapeutically effective dose of an antioxidant salen-metal
5   complex in a pharmaceutically acceptable form for prevention
or therapy of a free radical-associated disease.

        2.   A pharmaceutical composition according to claim
1, wherein the free radical-associated disease is selected
10 from the group consisting of: ischemic/reoxygenation episode
and iatrogenic free-radical toxicity.

        3.   A pharmaceutical composition according to claim
1, wherein the antioxidant salen-metal complex has the
15 structural formula:
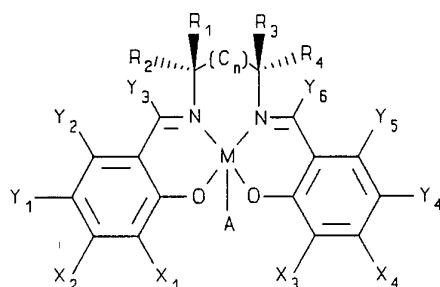
        wherein M is selected from the group consisting of Mn,
Co, Fe, V, Cr, and Ni; A is an anion;
25   n is either 0, 1, or 2;
        X_1, X_2, X_3 and X_4 are independently selected from the
group consisting of hydrogen, silyls, aryls, arylalkyls,
primary alkyls, secondary alkyls, tertiary alkyls, alkoxys,
aryloxys, aminos, quaternary amines, heteroatoms, and
30 hydrogen;
        Y_1, Y_2, Y_3, Y_4, Y_5, and Y_6 are independently selected from
the group consisting of hydrogen, halides, alkyls, aryls,
arylalkyls, silyl groups, aminos, alkyls or aryls bearing
heteroatoms, and halide; and
35   R_1, R_2, R_3 and R_4 are independently selected from the
group consisting of hydrogen, aryl, fatty acid esters,
substituted alkoxyaryls, heteroatom-bearing aromatic groups,

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arylalkyls, primary alkyls, secondary alkyls, and tertiary
alkyls.

4.   A pharmaceutical composition according to claim
3, wherein the antioxidant salen-metal complex is selected
from the group consisting of: Structure IV, Structure V,
Structure VI, Structure VII, Structure VIII, and Structure IX.

5.   A pharmaceutical composition according to claim
4, wherein the antioxidant salen-metal complex is selected
from the group consisting of: C1, C4, C6, C7, C9, C15, C17,
C20, C22, C23, C25, C27, and C28.

6.   A pharmaceutical composition according to claim
1, wherein the antioxidant salen-metal complex has the
structural formula:
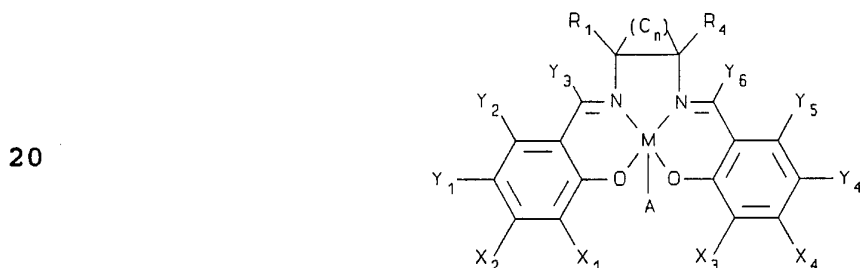
where M is a transition metal ion selected from the group
consisting of Mn, Co, Fe, V, Cr, and Ni; A is an anion;
     n is either 4, 5, or 6;
     X_1, X_2, X_3, and X_4 are independently selected from the
group consisting of aryls, arylalkyls, aryloxys, primary
alkyls, secondary alkyls, tertiary alkyls, alkoxy, substituted
alkoxy, heteroatoms, aminos, quaternary amines, and hydrogen;
     Y_1, Y_2, Y_3, Y_4, Y_5, and Y_6 are selected from the group
consisting of aryls, arylalkyls, primary alkyls, secondary
alkyls, tertiary alkyls, alkoxys, substituted alkoxys,
aryloxys, halides, heteroatoms, aminos, quaternary amines, and
hydrogen; and
     R_1 and R_4 are independently selected from the group
consisting of hydrogen, halides, primary alkyls, secondary

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alkyls, tertiary alkyls, fatty acid esters, alkoxys, or aryls.

7.   A pharmaceutical composition according to claim
4, wherein the antioxidant salen-metal complex is selected
from the group consisting of: C10, C11, C12, C29, and C30.

8.   A pharmaceutical composition according to claim
2, wherein the antioxidant salen-metal complex is the SOD-
mimetic C7.

9.   A pharmaceutical composition according to claim
8, wherein the pharmaceutical composition comprises at least
about 10 mg of C7 in a form suitable for parenteral
administration.

10.   A method for preventing, arresting, or treating
a free radical-associated disease state by administering to a
patient a therapeutically-effective dose of an antioxidant
salen-metal complex pharmaceutical composition according to
claim 1.

11.   A method according to claim 10, wherein the
free-radical associated disease is selected from the group
consisting of: ischemia/reoxygenation episode and iatrogenic
free-radical toxicity.

12.   A method according to claim 10, wherein the
antioxidant salen-metal complex pharmaceutical composition
comprises an antioxidant salen-metal complex selected from the
group consisting of: Structure I, Structure III, Structure IV,
Structure V, Structure VI, Structure VII, Structure VIII, and
Structure IX.

13.   A method according to claim 12, wherein the
antioxidant salen-metal complex is selected from the group
consisting of: C1, C4, C6, C7, C9, C10, C11, C12, C15, C17,
C20, C22, C23, C25, C27, C28, C29, and C30.

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14. A method according to claim 11, wherein the salen-metal complex is C7.

15. A composition for prevention of free radical-
associated radiation damage and chemical damage to a patient,
comprising a prophylactically-effective dose of at least one
species of an antioxidant salen-metal complex in a
pharmaceutically acceptable form.

16. A composition according to claim 15, wherein
the antioxidant salen-metal complex comprises the SOD mimetic
C7.

17. A composition according to claim 15, wherein
the antioxidant salen-metal complex is formulated for topical
administration to the skin of the patient.

18. A composition according to claim 17, wherein
the antioxidant salen-metal complex is formulated is a sun-
protective cream, a sun-protective lotion, or a cosmetic.

19. A method for preventing or reducing the
severity of free radical damage to a patient resulting from
treatment with ionizing radiation or administration of a free
radical-generating chemotherapeutic agent, comprising
administering to a patient a prophylactically-effective dose
of an antioxidant salen-metal complex.

20. A method according to claim 19, wherein the
antioxidant salen-metal complex is the SOD-mimetic C7.

21. A method according to claim 19, wherein the
free radical-generating chemotherapeutic agent is the
antineoplastic agent bleomycin.

22. A method according to claim 19, wherein the
antioxidant salen-metal complex is administered to said

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patient prior to administration of the ionizing radiation or
free radical-generating chemotherapeutic agent.

23.   A composition comprising a salen-metal complex
5   formulated for delivery to a human or veterinary patient.

24.   A composition according to claim 23, wherein
the salen-metal complex is formulated in immunoliposomes.

10   25.   A composition comprising a salen-metal complex
and mammalian blood.

26.   A non-human animal containing an antioxidant
salen-metal complex.
15

27.   A mammalian cell containing an antioxidant
salen-metal complex.

28.   A human organ or human blood comprising an
20   antioxidant salen-metal complex.

29.   A pharmaceutical composition comprising a
therapeutically effective dose of an antioxidant salen-metal
complex in a pharmaceutically acceptable form, wherein the
25   antioxidant salen-metal complex has the structural formula:
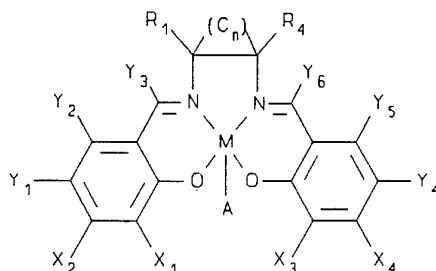
wherein M is manganese;
A is H or halogen;
35   n is 0, 4, 5, or 6, wherein Cn is absent if n = 0 and is
a saturated hydrocarbon chain if n = 4, 5, or 6;
R_1 and R_4 are independently selected from the group

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consisting of H, phenyl, lower alkoxy, and lower fatty acid
esters;

X_1 and X_3 are independently selected from the group
consisting of H, lower alkyl, amine, lower alkylamino, and
5   halogen;

X_2 and X_4 are H;

Y_1 and Y_4 are independently selected from the group
consisting of H, lower alkyl, halogen, and lower alkoxy;

Y_2, Y_3, Y_5 and Y_6 are H; and
10   all remaining substituent positions are H.

30.   A pharmaceutical composition according to claim
29, wherein the antioxidant salen-metal complex is selected
from the group consisting of: C1, C4, C6, C7, and C9.

15

31.   A method for preventing, arresting, or treating
neurological damage induced by MPTP or anoxia injury, said
method comprising the step of administering to a patient a
therapeutically-effective dose of an antioxidant salen-metal
20   complex pharmaceutical composition according to claim 29.

32.   A method according to claim 31, wherein said
antioxidant salen-metal complex pharmaceutical composition
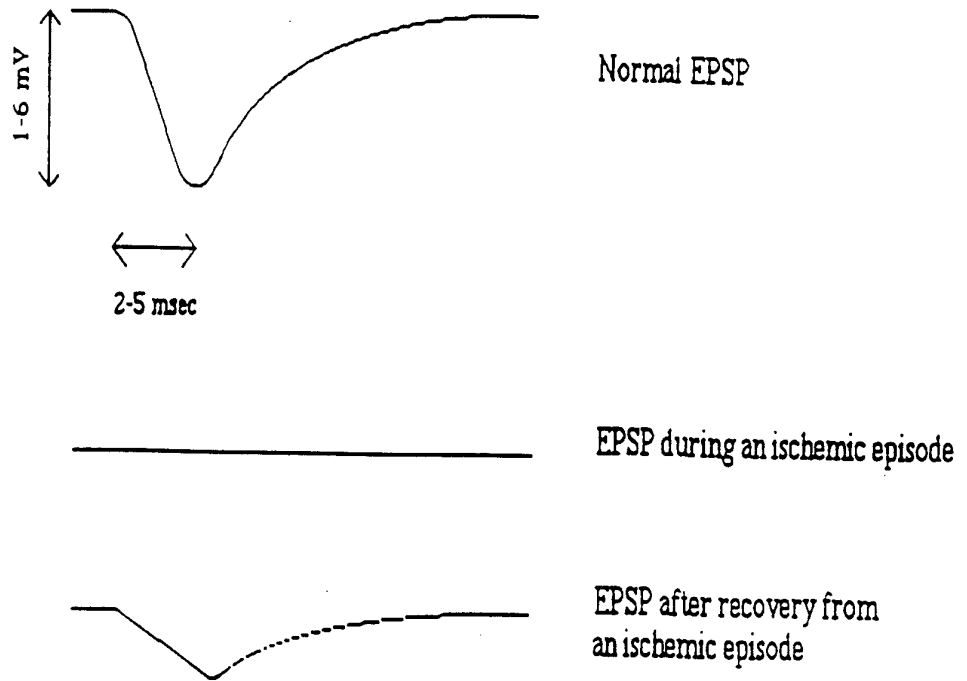comprises C7.

25
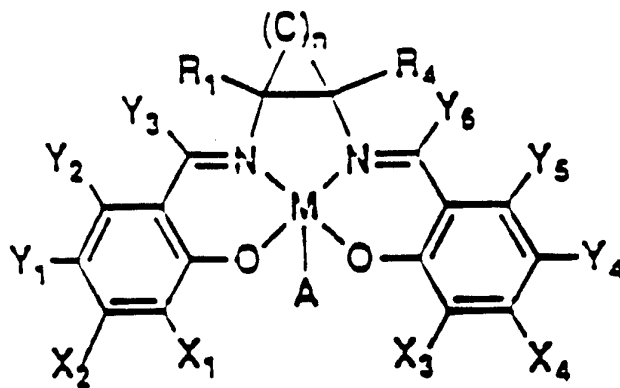
FIGURE 1

FIGURE 2

FIGURE 3

Normal EPSP

EPSP during an ischemic episode

EPSP after recovery from
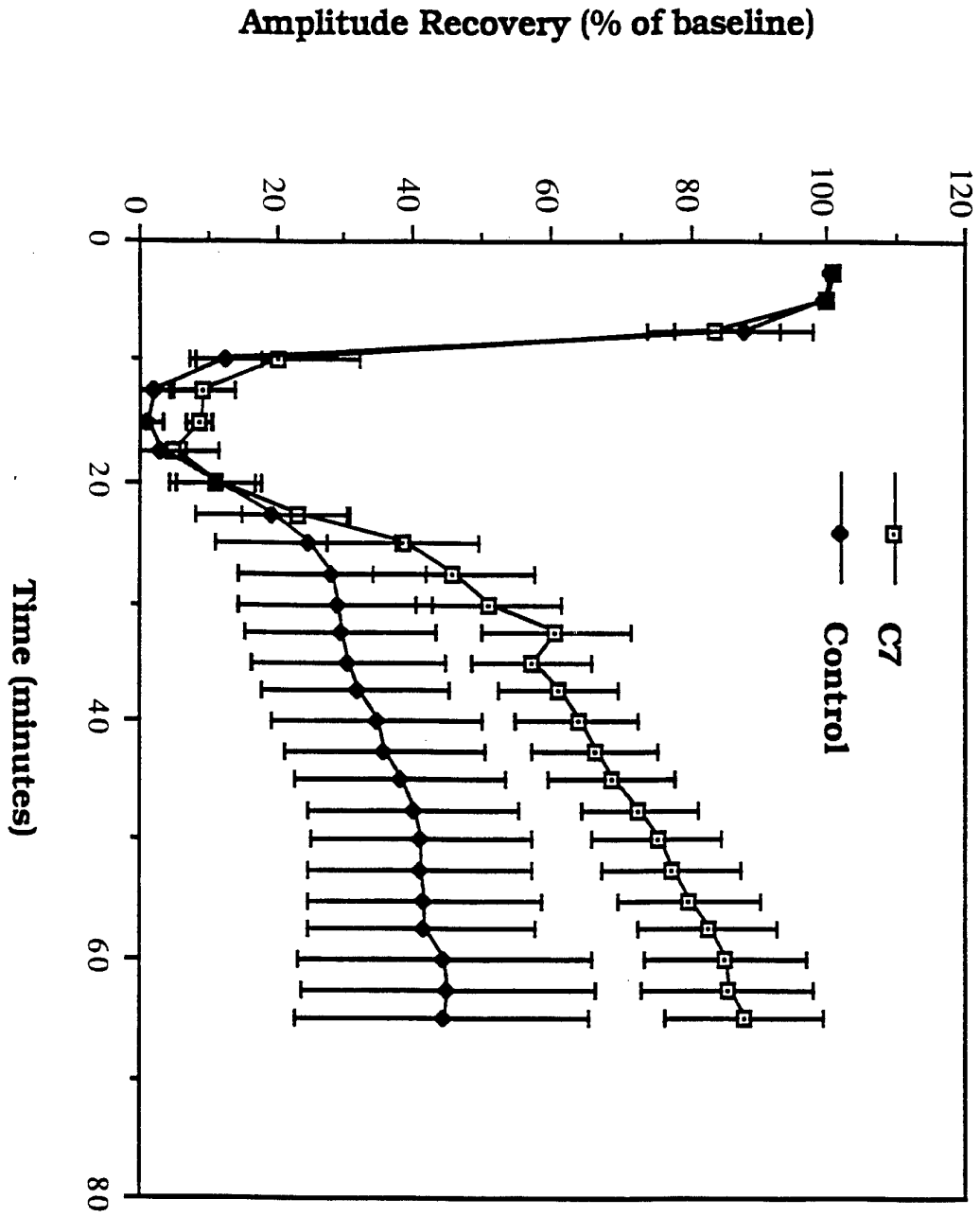an ischemic episode
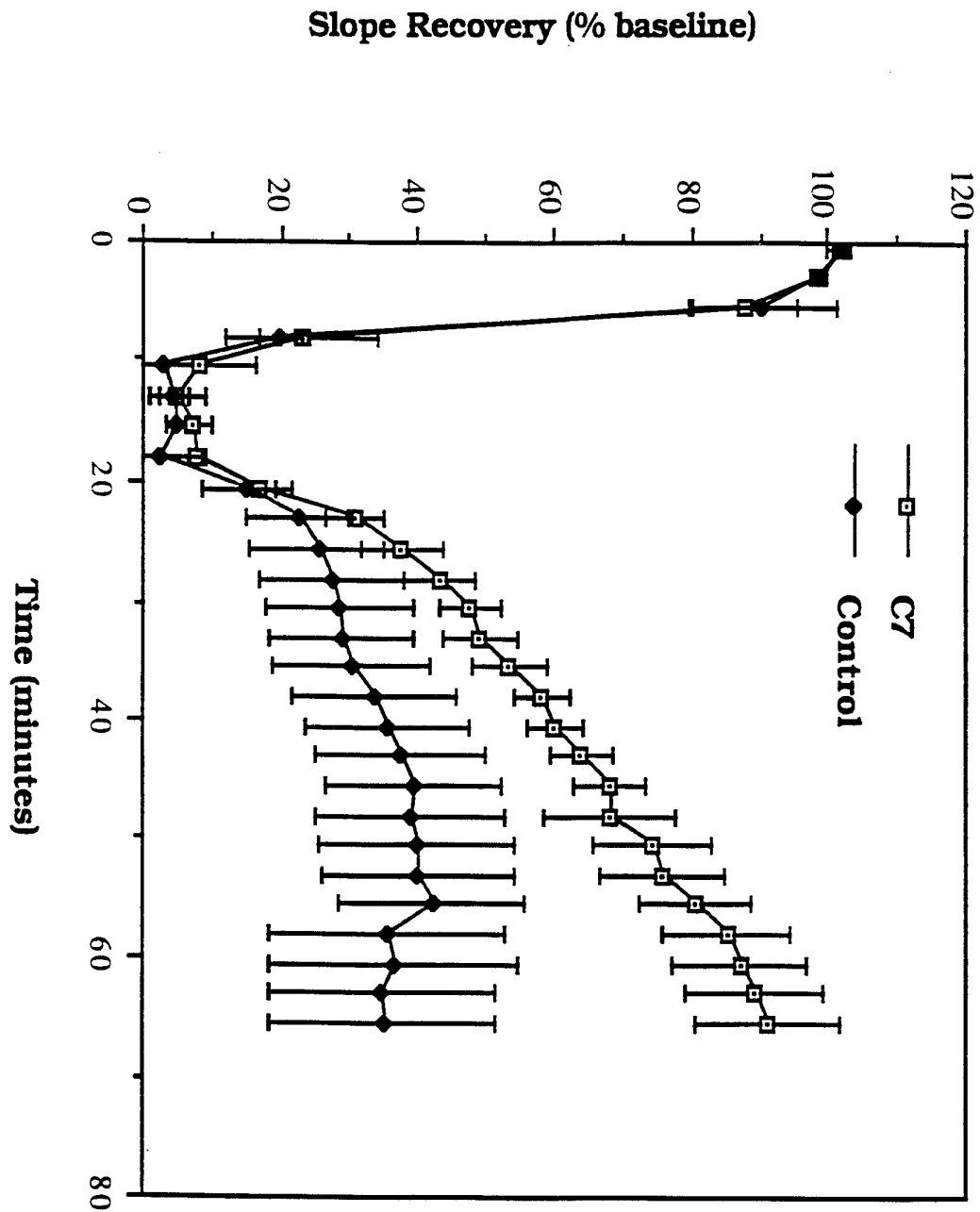
FIGURE 4

FIGURE 5

Slope Recovery (% baseline)
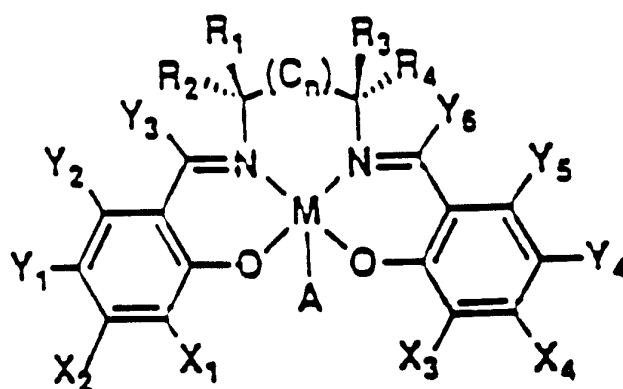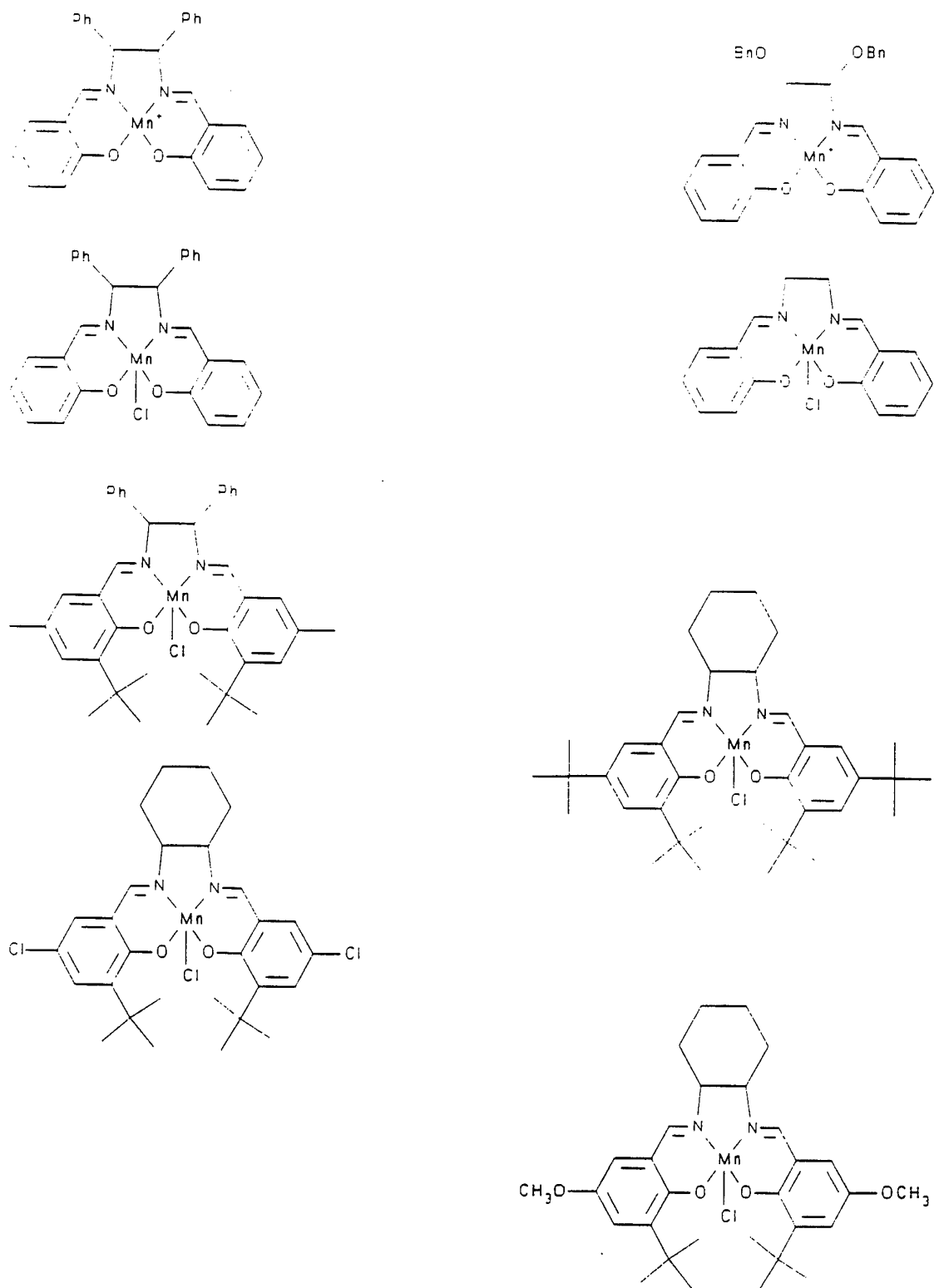
FIGURE 6

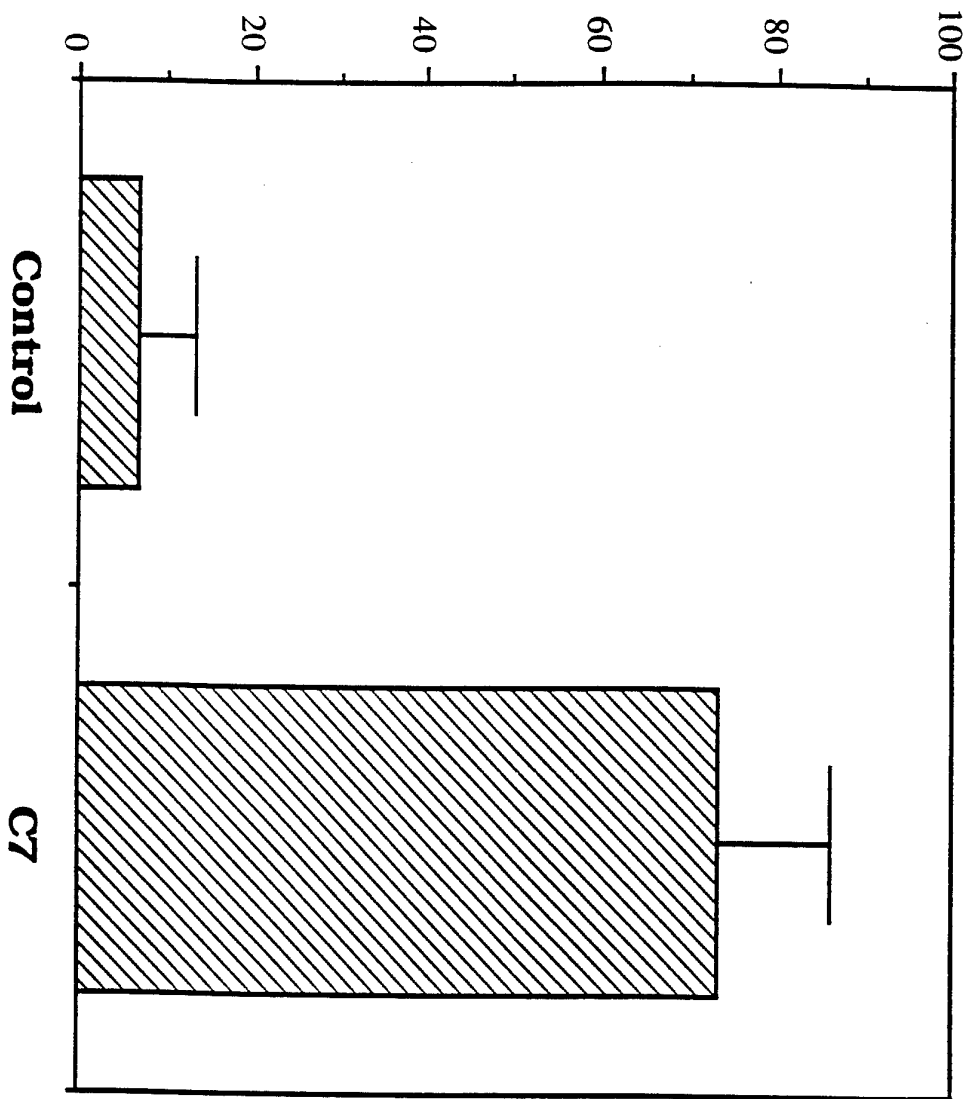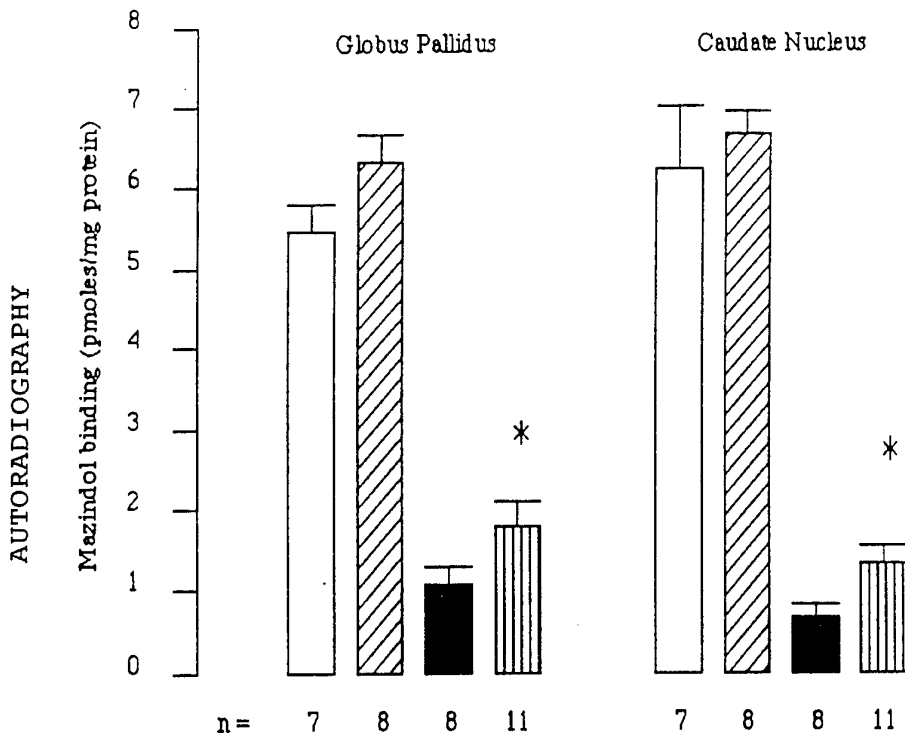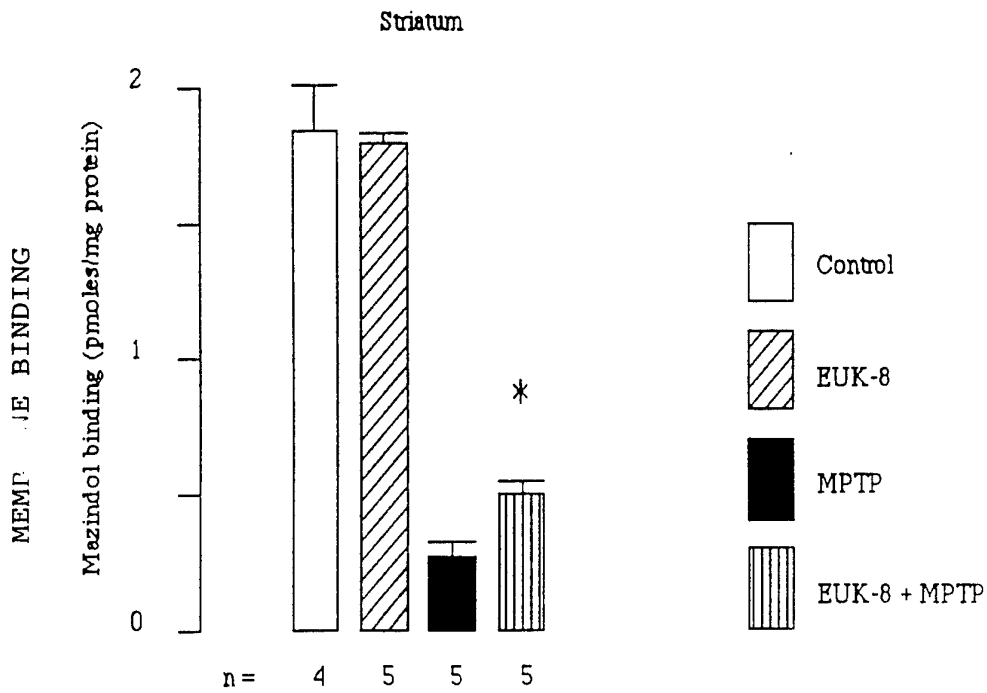## Percent of Viable Slices After One to Three Ischemic Episodes
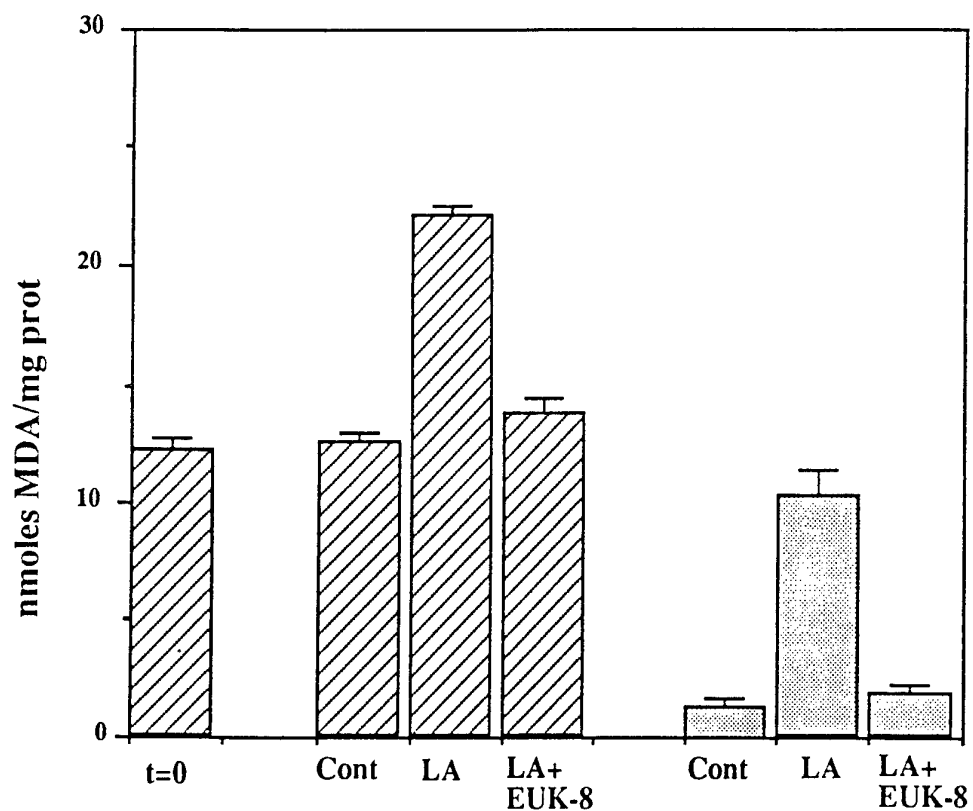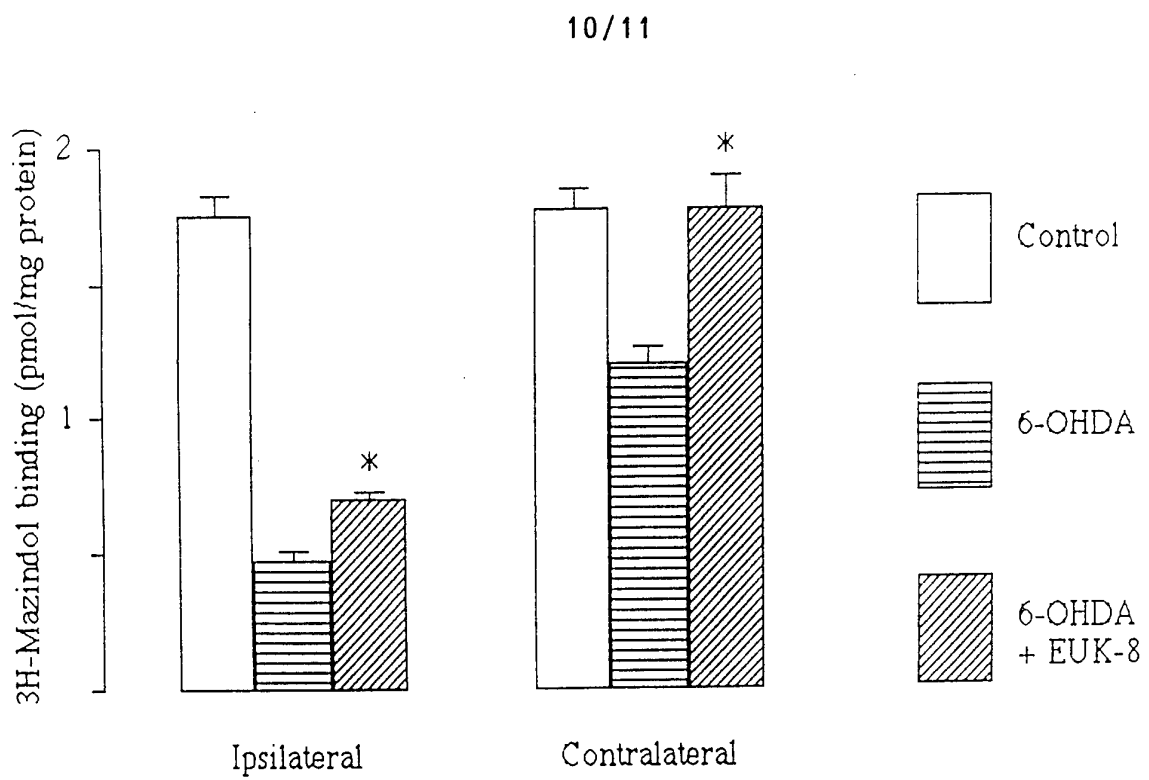

FIGURE 7

FIG. 8A

FIG. 8B

FIG. 8C

FIG. 9

FIG. 10
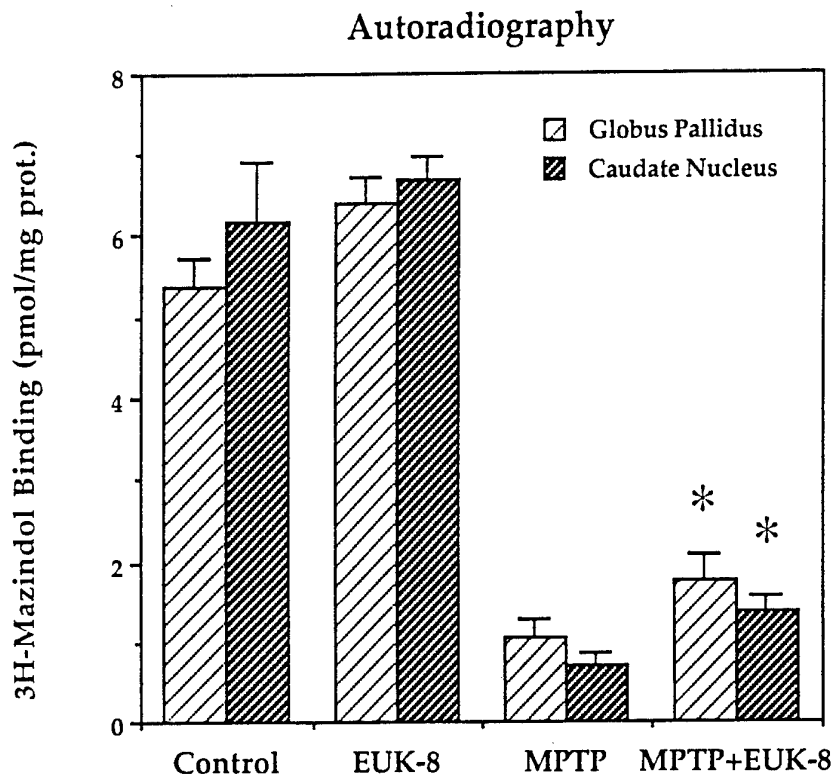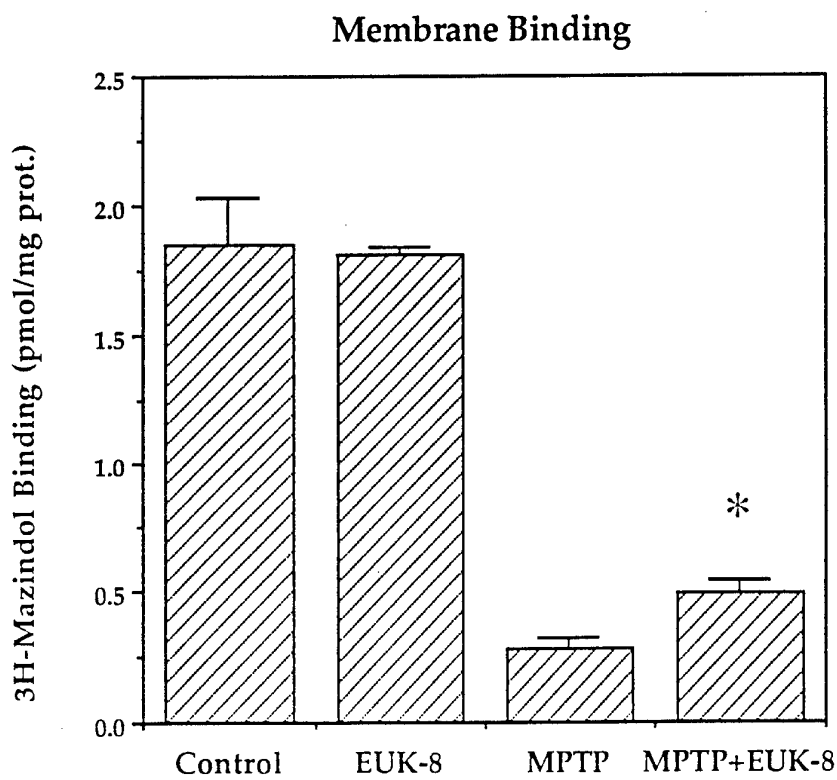
Autoradiography

FIG. 11A

Membrane Binding

FIG 11B

SUBSTITUTE SHEET

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :IPC(5): A61K 31/555

US CL :USCL: 514/185

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : USCL: 514/185

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS, Medline, search terms: structure compounds IV-IX and free radical or oxidize or radiation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	M. WOLFE, "BURGER'S MEDICINAL CHEMISTRY, FOURTH EDITION, PART III" published 1981 see pages 11, 22, 29-35, 39, 44	1-24, 29,30
Y	US, A, 4,540,573 (SCHEER) 21 APRIL 1992 see abstract and col 6, lines 44-46	1-24,29,30
Y	M. Sittig, "HANDBOOK OF TOXIC AND HAZARDOUS CHEMICAL AND CARCINOGENS" published 1985 by Noyes Publications, see pages 559-562,639-641,243-248.	1-24,29,30

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 MARCH 1994	MAR 28 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	GREG HOOK
Facsimile No. 703-305-3230	Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/11857

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
    (Form PCT/ISA/206 Previously Mailed.)
    Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-24,29,30

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)★

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-9,10-14,15-18,19-24,29-30, drawn to salen-metal pharmaceutical compositions.

II. Claims 25-28, drawn to salen-metal and cell compositions.

III. Claims 31-32, method of treating neurological disease induced by MPTP or anoxia.

Pharmaceutical compositions (Group I) and cell compositions (Group II) lack unity of invention because they are
different compositions related as combination and subcombination. The combination as claimed does not require the
particulars of the subcombination because the combination does not require a pharmaceutically acceptable form.

Methods of treating neurological damage (Group III) are to treating different medical conditions from that of Group I.
It is well known in the art that one method does not treat all medical conditions and that different medical condition
treatments are distinct and independent of each other.