

REVIEW

On the selectivity of superoxide dismutase mimetics and its importance in pharmacological studies

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The list of pathophysiological conditions associated with the overproduction of superoxide expands every day. Much of the knowledge compiled on the role of this radical in disease has been gathered using the native superoxide dismutase enzyme and, more recently, by the use of superoxide dismutase knockout models or transgenic models that overexpress the various isoforms of the enzyme. Although the native enzyme has shown promising anti-inflammatory properties in both preclinical and clinical studies, there were drawbacks and issues associated with its use as a therapeutic agent and pharmacological tool. Based on the concept that removal of superoxide modulates the course of inflammation, synthetic, low-molecular-weight mimetics of the superoxide dismutase enzymes that could overcome some of the limitations associated with the use of the native enzyme have been designed. In this review, we will discuss the advances made using various superoxide dismutase mimetics that led to the proposal that superoxide (and/or the product of its interaction with nitric oxide, peroxynitrite) is an important mediator of inflammation, and to the conclusion that superoxide dismutase mimetics can be utilized as therapeutic agents in diseases of various etiologies. The importance of the selectivity of such compounds in pharmacological studies will be discussed.

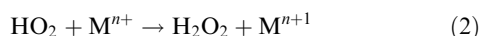
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Abbreviations: NFκB, nuclear factor κB; OONO⁻, peroxynitrite; PARP, poly-ADP-ribose polymerase; ROS, reactive oxygen species; SOD, superoxide dismutase; SODm, superoxide dismutase mimetics; TNFα, tumour necrosis factor α

Superoxide dismutase

The recognition that Orgotein, a CuZn metalloprotein prepared from animal tissues, has potent anti-inflammatory activity (Huber *et al.*, 1980) predates the recognition that the corresponding protein from bovine erythrocytes possesses an enzyme activity catalysing the dismutation of superoxide into hydrogen peroxide and molecular oxygen (Mc Cord & Fridovich, 1969). It is now well appreciated that, under normal circumstances, in healthy individuals, this radical burden is contained by superoxide dismutase (SOD) enzymes (Figure 1). SOD enzymes are a class of oxidoreductase enzymes, which, in mammals, contain either Cu or Mn at the active site and catalyse the dismutation of superoxide, the one-electron reduction product of molecular oxygen,



where Mⁿ is the metalloenzyme in the reduced state and Mⁿ⁺¹ is the enzyme in the oxidized state to oxygen and hydrogen peroxide. Three different isoforms of SOD have been characterized in mammals. The SOD enzymes have a distinct genomic structure and are well compartmentalized (Figure 2).

SOD1 or Cu/Zn SOD and SOD3 or EC-SOD have Cu and Zn in their catalytic centre, while SOD2 or Mn SOD has Mn in the catalytic centre. SOD1 has been found in the cytoplasm, nuclear compartments and in the inter membrane space of the mitochondria (Chang *et al.*, 1988; Keller *et al.*, 1991; Crapo *et al.*, 1992; Liou *et al.*, 1993) (Figure 2). SOD2 is localized in the mitochondria matrix (Weisiger & Fridovich, 1973) of the cells and SOD3 has been found in the extracellular compartments (Marklund, 1984), where it binds the extracellular matrix through its high-affinity carboxyterminus (Oury *et al.*, 1996; Bowler *et al.*, 2001) (Figure 2). The mammalian ECSOD is a tetrameric glycosylated CuZnSOD (Marklund, 1982). The importance of SOD2 is highlighted by the findings that, in contrast to SOD1 (Reaume *et al.*, 1996) and SOD3 (Carlsson *et al.*, 1995), the SOD2 knockout is lethal to mice (Li *et al.*, 1995; Lebovitz *et al.*, 1996; Melov *et al.*, 1999).

The use of the knockout models and systems where the SOD enzymes have been overexpressed by genetic manipulation have been crucial in understanding the importance of the SOD antioxidant system under physiological and pathological conditions (Figure 2). SOD1 maps to chromosome 21 (Tan *et al.*, 1973), a finding that was crucial in understanding the contribution of this enzyme, for example, in Down's syndrome (Avraham *et al.*, 1988; Groner *et al.*, 1994). Using mutant

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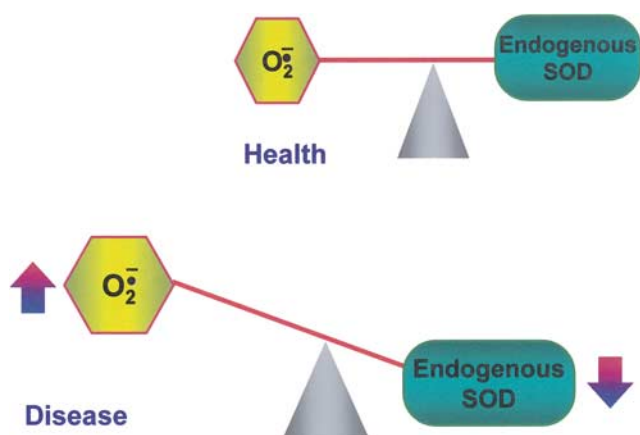


Figure 1 Superoxide and SOD in health and disease. Endogenous SODs are critical in keeping superoxide under tight control. In diseases, there is an imbalance between the amount of superoxide formed and in the ability of the enzymes to remove it (activity of the enzyme is reduced).

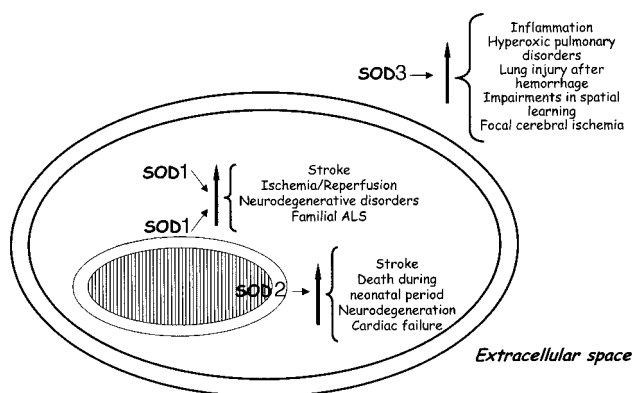


Figure 2 Relative roles of SOD isoforms in disease. SOD isoforms are well compartmentalized inside the cell (SOD1, cytoplasm; SOD2 in the mitochondria) and in the extracellular space (SOD3). The involvement of the three isoforms in several pathological conditions has been unravelled by modulating the expression of the three enzymes using knockout and transgenic models.

SOD1-expressing transgenic mice, several studies have pointed to a variety of functions of mutant SOD1, which enhances the catalytic activity of the peroxynitrite-mediated tyrosine nitration, readily releases the reactive Cu ions, induces apoptotic cell death, enhances peroxidase activity, damages the mitochondria to release Ca^{2+} , and forms SOD1-containing aggregates in the cytoplasm (Shibata *et al.*, 2000). Many of these studies have obtained evidence for increased oxidative damage in neurodegenerative disorders such as amyotrophic lateral sclerosis (Gurney, 1994; Brown, 1995; Wong *et al.*, 1995). In considering the findings of increased oxidative damage in mutant SOD1-expressing transgenic mice, it should be remembered that overexpression of mutant SOD1 may enhance oxidative stress generation from this enzyme (Shibata *et al.*, 2000). Downregulation of SOD1 *in vitro* and *in vivo* models has been associated with neuronal death (Troy *et al.*, 1996), while overexpression of SOD1 in transgenic mice has been associated with protection of the cerebral tissue in several pathological conditions such as ischaemia or Parkinson's

disease (Chan *et al.*, 1991; Przedborski *et al.*, 1992; Nakao, 1995).

SOD2 maps to chromosome 6 and the knockout of this gene is lethal (Lebovitz *et al.*, 1996). The deficiency of SOD2 resulted in an increased production of superoxide, which in turn inhibits the respiratory chain by inactivating complex I and complex II (Li *et al.*, 1995; Lebovitz *et al.*, 1996). Loss or reduction of the SOD2 activity has been associated with mitochondrial vacuolization and lipid peroxidation, which in turn leads to neurodegeneration and heart failure (Figure 2). SOD2 gene has several polymorphisms which have been associated with a reduction of the enzyme activity and in turn to increased risk of sporadic motor neuron disease, nonfamilial idiopathic cardiomyopathy, breast cancer and reduction of the tumour-suppressive effect of SOD2 (Zelko *et al.*, 2002). Several efforts have been made in order to determine whether an upregulation of SOD2 would be critical for the improvement of pathological conditions where the loss of SOD activity is known to contribute to the underlying pathology (for example, ischaemia and reperfusion injury). It has been shown that transgenic animals that overexpress SOD2 had a 35% reduction of infarct size compared to wild-type animals (Chen *et al.*, 1998). The SOD2 overexpression, or induction of the enzyme activity, lead to a reduction of lipid peroxidation and tyrosine nitration, protecting tissues from cellular death (Keller *et al.*, 1998). In addition, overexpression of SOD2 is critical for tissue protection against radiation-induced cytotoxicity, while HIV regulatory proteins induce a reduction of the expression and activity of SOD2 (Flores *et al.*, 1993; Westendorp *et al.*, 1995; Yan *et al.*, 1999).

SOD3 is the least characterized among the SOD family enzymes. It maps to chromosome 4 and part of chromosome 5, and has been shown to have high affinity for heparin. To date, the only known mutation has been demonstrated to be localized to the heparin-binding site (Folz *et al.*, 1994). Several polymorphisms have been described, but their roles need to be further clarified (Zelko *et al.*, 2002). SOD3 has been detected in several compartments such as the plasma, lymph, ascites, cerebrospinal fluid and lung (this organ has the highest concentration of this enzyme) (Marklund, 1982). The studies performed using knockout animals (Figure 2) highlighted the role of SOD3 in focal cerebral ischaemia, where the implications of the loss of this enzyme activity are in impaired spatial learning and increased sensitivity of the null mice to hyperoxia exposure (Carlsson *et al.*, 1995; Levin *et al.*, 1998). Transgenic animals with increased expression of SOD3, in particular, in compartments such as alveolar type II and nonciliated bronchial epithelial cells demonstrated the critical role of this enzyme during hyperoxic pulmonary disease. Thus, enhancement of the SOD3 expression and activity in the lung attenuates the hyperoxic lung injury response by attenuating neutrophil infiltration (Folz *et al.*, 1999). The overexpression of SOD3 seems to be critical during lung injury after haemorrhage (Bowler *et al.*, 2001). Here, overexpression improves lung injury, as shown by the overall inhibition of neutrophil infiltration, lipid peroxidation and pulmonary oedema. ECSOD has a number of other important properties, which are not discussed here but can be found in recent reviews by Crapo and co-workers (Bowler & Crapo, 2002a, b).

In summary, a number of animal models of disease have shown that genetically engineered mice that lack SODs are more sensitive and those that overexpress SODs are resistant.

Superoxide

Superoxide is formed from various sources, including normal cellular respiration, activated polymorphonuclear leucocytes, endothelial cells and mitochondrial electron flux (Mc Cord & Omar, 1993; Lenaz, 2001). Superoxide generation by phagocytic NADPH oxidase is known to be important in bacterial killing. In host defence, free radicals can serve, in some instance, as microbicidal and parasiticidal agents (Peterhans, 1997; Rojkind *et al.*, 2002), or they may facilitate/promote replication of parasites, depending on the cell and virus involved (Albrecht *et al.*, 1992; Pace & Leaf, 1995). What is clear and what has been reported since the discovery of the native superoxide enzyme (Mc Cord & Fridovich, 1969) is that superoxide contributes to the pathogenesis of a wide array of diseases (Salvemini & Cuzzocrea, 2002a, b; 2003). In acute and chronic inflammation, the production of superoxide is increased at a rate that overwhelms the capacity of the endogenous SOD enzyme defence system to remove it (Figure 1). The consequence of this imbalance results in superoxide-mediated damage (Figure 3).

Some important proinflammatory roles for superoxide include endothelial cell damage and increased microvascular permeability (Droy-Lefaix *et al.*, 1991; Haglind *et al.*, 1994), recruitment of neutrophils at sites of inflammation (Salvemini *et al.*, 1999a), autocatalytic destruction of neurotransmitters and hormones such as noradrenaline and adrenaline, respectively (Macarthur *et al.*, 2000), lipid peroxidation and oxidation, DNA damage (Dix *et al.*, 1996), activation of poly-ADP-ribose polymerase (PARP; Szabó *et al.*, 1996; Szabó & Dawson, 1999a), inactivation of nitric oxide (Gryglewski *et al.*, 1986) and formation of peroxynitrite (the reaction product of nitric oxide and superoxide, Beckman *et al.*, 1990), a potent cytotoxic and proinflammatory molecule (Ischiropoulos *et al.*, 1992; Beckman & Crow, 1993; Salvemini *et al.*, 1998; 1999a) (Figure 3). Peroxynitrite nitrates endogenous SOD, the enzyme that keeps superoxide under tight control. Once nitrated, MnSOD and/or CuZn SOD lose their enzymatic activity, an event favouring the accumulation of superoxide and superoxide-driven damage (Yamakura *et al.*, 1998; 2001; MacMillan-Crow & Thompson, 1999; Souza *et al.*, 2000; Matsunaga *et al.*, 2001). This is supported by the overwhelming body of evidence which shows that nitration of this enzyme is closely linked to those disease states driven by

overt production of superoxide, for example, ischaemia and reperfusion, organ transplantation, shock and inflammation, neurodegeneration such as Alzheimer's disease, amyotrophic lateral sclerosis or AIDS dementia complex (MacMillan-Crow & Cruthirds, 2001; Mollace *et al.*, 2001; Ischiropoulos & Beckman, 2003 for review).

SOD: preclinical experience

The list of pathophysiological conditions associated with the production of superoxide continues to expand. The most exciting realization is that there appears to be a commonality to the tissue injury observed in various disease states, namely, superoxide produces tissue injury (and associated inflammation) in most tissues in similar ways. Tissue injury and inflammation form the basis of many disease pathologies: ischaemia and reperfusion injuries, radiation injury, hyperoxic lung damage, asthma, atherosclerosis and so forth. This provides a unique opportunity to manipulate numerous disease states with an agent that *selectively* removes superoxide (*vide infra*). Protective and beneficial roles of SOD have been demonstrated in a broad range of diseases, both preclinically and clinically (Mc Cord, 1974; Halliwell & Gutteridge, 1985; Maxwell, 1995). For example, preclinical studies have revealed that SOD enzymes have a protective effect in animal models of ischaemia–reperfusion injury (including the heart, liver, kidneys and brain) (Werns *et al.*, 1988; Ando *et al.*, 1989; Chan *et al.*, 1991; Omar & McCord, 1991; Yang *et al.*, 1994), transplant-induced reperfusion injury (Zweier, 1997), inflammation (Oyanagui, 1976; Shingu *et al.*, 1994), Parkinson's disease (Przedborski *et al.*, 1992; Andreassen *et al.*, 2001), cancer (Church *et al.*, 1993; Safford *et al.*, 1994; Yoshizaki *et al.*, 1994), AIDS (Flores *et al.*, 1993; Edeas *et al.*, 1997; Mollace *et al.*, 2001; 2002), asthma (Bowler & Crapo, 2002a, b), chronic obstructive pulmonary diseases (Barnes, 2000; Salminen *et al.*, 2001) and respiratory syncytial virus infections (Wyde *et al.*, 1996). Furthermore, the native enzyme attenuates the intestinal injury induced by alcohol (Terano *et al.*, 1989), *Helicobacter Pylori* (Lamarque *et al.*, 2000) and nonsteroidal anti-inflammatory drugs including indomethacin, diclofenac and flurbiprofen (Evans & Whittle, 2001).

SOD: clinical experience

When tested in humans in various clinical trials, Orgotein® (bovine CuZnSOD) showed promising results in acute and chronic conditions associated with inflammation, including rheumatoid arthritis and osteoarthritis (Goebel *et al.*, 1981; Goebel & Storck, 1983; Lund-Olesen & Menander-Huber, 1983; Gammer & Broback, 1984; Mc Ilwain *et al.*, 1989; Mazieres *et al.*, 1991; Borigini & Paulus, 1995) as well as side effects (acute and chronic) associated with chemotherapy and radiation therapy (Marberger *et al.*, 1974; 1975; 1981; Edsmyr *et al.*, 1976; Menander-Huber *et al.*, 1978; Housset *et al.*, 1989; Delanian *et al.*, 1994; Sanchiz *et al.*, 1996). Thus, in clinical trials, the use of the native enzyme supported the concept that removal of superoxide had a beneficial outcome. There were drawbacks associated with its use. The main problem was the nonhuman origin of the enzyme bovine. This inevitably gave rise to a variety of immunological problems, which eventually

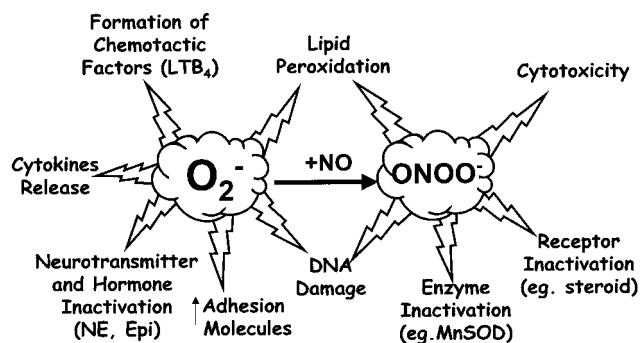


Figure 3 Impact of superoxide generation in inflammation. Excessive production of superoxide can lead to inflammation through various pathways, including the generation of destruction of beneficial nitric oxide (NO) and simultaneous generation of cytotoxic and proinflammatory peroxynitrite ($ONOO^-$).

led to its removal from the market, except in Spain where it is still clinically used to prevent radiation-induced side effects.

Based on the concept that removal of superoxide modulates the course of inflammation, others and we have pursued the concept of designing synthetic, low-molecular-weight mimetics of the SOD enzymes, which could overcome some of the limitations associated with Orgotein. This allows the synthetic SOD mimetics (SODm) to serve as pharmaceutical candidates in a variety of diseases in which the native SOD enzyme was found to be effective. This concept has proven to be one which a number of researchers and companies have been pursuing in recent years. A review of the patent literature in this arena was published (Henke, 1999).

Pharmacological use of SODm

Manganese(III) Metalloporphyrins

Manganese-based metalloporphyrin complexes scavenge of superoxide, hydrogen peroxide, peroxynitrite and lipid peroxyl radicals (Faulkner *et al.*, 1994; Day *et al.*, 1995; 1997; 1999; Szabó *et al.*, 1996a). The manganese moiety of the porphyrin-based SODm (Figure 4) functions in the dismutation reaction with superoxide by successive reduction followed by oxidation changes in its valence between Mn(III) and Mn(II), much like native SODs, whereas the catalase activity of metalloporphyrins could be attributed to their ability to also undergo oxidation to higher oxidation states as Mn(IV) or Mn(V) or Mn(IV) (porphyrin radical cation) oxidation levels. In general, metalloporphyrins with higher SOD activity possessed greater catalase activity. Metalloporphyrins have been shown to be protective in a wide variety of *in vitro* oxidative stress models involving the generation of superoxide, hydrogen peroxide and peroxynitrite alone or in concert. At micromolar levels, they protect cultured cells against the toxicity of superoxide generators paraquat (Day *et al.*, 1995) and pyocyanine (Gardner *et al.*, 1996), hydrogen peroxide generator, glucose oxidase (Day *et al.*, 1997) and peroxynitrite injury produced by endotoxin (Szabó *et al.*, 1996a) or peroxynitrite itself (Misko *et al.*, 1998). Metalloporphyrins such as Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) (Figure 4) are also potent inhibitors of lipid peroxidation (Day *et al.*, 1999), exerting a

protective effect against some of the detrimental effects associated with endotoxic and haemorrhagic shock (Zingarelli *et al.*, 1997; Szabó, 1998), as well as in acute liver failure (Ferret *et al.*, 2001; Malassagne *et al.*, 2001). Since these SODm scavenge other reactive oxygen species including peroxynitrite, the efficacy of MnTBAP in these models probably relates to its peroxynitrite-scavenging activity in addition to its superoxide-scavenging activity (Zingarelli *et al.*, 1997; Szabó, 1998).

Manganese(III)salen complexes

The Mn(III)salen complexes (Figure 4) have been reported to be superoxide scavengers/SODm, and possess catalase activity (Doctrow *et al.*, 1997). Biological studies have been published for two of these complexes, manganese *N,N'*-bis(salicylidene)ethylenediamine chloride (EUK-8) and manganese 3 methoxy *N,N'*-bis(salicylidene)ethylenediamine chloride (EUK-134), in several disease models related to oxidative stress including stroke, ischaemia–reperfusion injury, Parkinson's disease and experimental allergic encephalomyelitis (EAE) (Doctrow *et al.*, 1997; Malfroy *et al.*, 1997; Baker *et al.*, 1998; Pong *et al.*, 2000; Bianca *et al.*, 2002). However, Mn(III)salen complexes, in the presence of peroxynitrite and/or hypochlorite, become oxidized to oxoMn-salen. OxoMn-salens are potent oxidants that rapidly oxidize NO to NO₂ and also oxidize nitrite to nitrate (Sharpe *et al.*, 2002). These data support the evidence that the Mn(III)salen complex is a nonselective free radical scavenger that protects cells from oxidative stress, but are not useful pharmacological tools to dissect the relative importance of superoxide in disease. Evidence is available to support that EUK-8 significantly attenuated many of the features of a porcine model of LPS-induced adult respiratory distress syndrome (ARDS), where the role of superoxide has been well described by Kinnula and co-workers (Gonzalez *et al.*, 1995; Kinnula *et al.*, 1995). EUK-134 attenuates the multiple organ injury and dysfunction caused by endotoxin in the rat (Bianca *et al.*, 2002).

Furthermore, it has been shown that a small increase in myocyte oxidative stress due to partial inhibition of SOD1 leads to myocyte hypertrophy, whereas a higher level of oxidative stress caused by total inhibition of SOD1 results in apoptosis (Siwik *et al.*, 1999). The response of cardiac myocytes to low and high levels of mechanical stretch is similar to the effect of directly increasing myocyte oxidative stress, and the phenotype shift at each level of stretch is inhibited by EUK-8 (Pimentel *et al.*, 2001). EUK-8 and EUK-134 have been used to investigate the role of oxidative stress in several neurodegenerative diseases, and it has been shown, for example, that these compounds are able to protect microglial cells from beta-amyloid peptide (1–42 fragment) injury and to limit the output of prostaglandin E₂ produced from activated microglial cells (Anderson *et al.*, 2001). Furthermore, the compounds reduce the levels of oxidative stress and prolong the survival of mice expressing human mutant SOD1 G93A, which is a polymorphism of familiar amyotrophic lateral sclerosis (FALS; Jung *et al.*, 2001). Recently, it has been demonstrated that manganese (salen) complex compounds, when administered to MnSOD knockout mice, double the lifespan of these animals and inhibit the cardiomyopathies associated with the knockout phenotype (Melov *et al.*, 2001). These results suggest that these compounds enter the

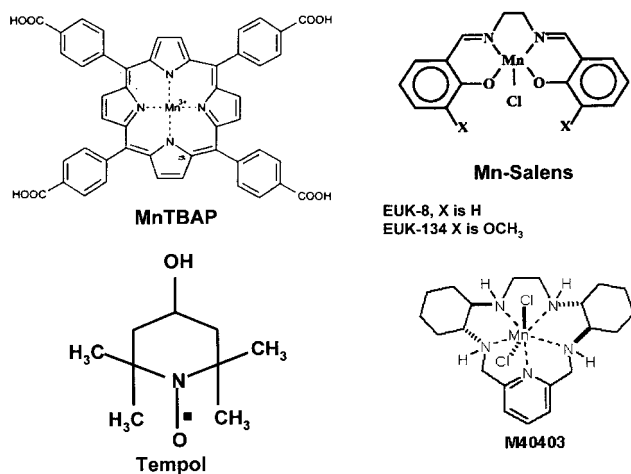


Figure 4 Structure of different classes of superoxide scavengers.

mitochondria, the intracellular location where the majority of superoxide is generated and where endogenous MnSOD enzyme is localized, to protect against the damage elicited by the deregulation of superoxide radical. Transforming growth factor β (TGF β) is directly involved in the development of tubulointerstitial fibrosis in chronic renal disease, and oxygen radicals can modulate the expression of TGF β . EUK-8 stimulates the transcription of TGF β gene as well as protein expression in treated mouse proximal tubular cells, whereas exogenous hydrogen peroxide suppressed the transcription of TGF β (Wolf *et al.*, 2001). EUK-8 has been shown to reduce arrhythmias induced by a 10 min regional ischaemia/reperfusion injury induced by the left coronary artery ligation and release (Tanguy *et al.*, 1996). Therefore, although these agents are protective in some models of diseases, the results obtained from the studies cannot be used to support the nature of the radical involved.

Nitroxides

Tempol(4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) (Figure 4) is a water-soluble analogue of the spin label TEMPO, which is widely employed in electron spin resonance spectroscopy. Tempol is a stable piperidine nitroxide of low molecular weight (MW: 172), which permeates biological membranes. There is now good evidence that tempol exerts beneficial effects in animal models of shock, ischaemia–reperfusion injury, inflammation, hypertension, diabetes and endothelial cell dysfunction (see Thiernemann (2003) for review). There is a controversy as to whether tempol and other stable nitroxides are ‘SOD-mimetics’ or act as stoichiometric scavengers of superoxide anions (Weiss *et al.*, 1993; Krishna *et al.*, 1996). Nevertheless, there is evidence that tempol does attenuate the effects of superoxide *in vitro* (Samuni *et al.*, 1991; Reddan *et al.*, 1992; Laight *et al.*, 1997; 1998). However, tempol also reduces the formation of hydroxyl radicals (Mitchell *et al.*, 1990; Monti *et al.*, 1996) and attenuates the cytotoxic effects of hydrogen peroxide, which is mediated by hydroxyl radicals (Bowes *et al.*, 1998; 1999), supporting its nonselectivity. Tempol also inhibits the peroxynitrite-mediated nitration of phenolic compounds in the presence of a large molar excess of peroxynitrite, suggesting a catalytic-like mechanism (Carroll *et al.*, 2000). Reactive oxygen species and peroxynitrite cause strand breaks in DNA, which in turn results in activating PARP. Activation of PARP catalyses the transfer of ADP-ribose moieties from NAD⁺ to various nuclear proteins including histones and PARP (automodification domain) itself. Continuous or excessive activation of PARP produces extended chains of ADP-ribose on nuclear proteins (poly-ADP-ribose chains or PAR chains), and results in a substantial depletion of intracellular NAD⁺ and subsequently ATP, which may ultimately cause cell death (Szabó, 1998; Szabó *et al.*, 1996b; Szabó & Dawson, 1999a). Tempol prevents the increase in PARP activity caused by hydrogen peroxide in cultured human cardiomyoblasts (Thiernemann (2003) for review).

There is now good evidence that tempol exerts beneficial effects in conditions associated with ischaemia–reperfusion of many organs including the heart, kidney and brain. For instance, administration of tempol prior to the onset of reperfusion reduces the infarct size caused by regional myocardial ischaemia and reperfusion in the rat and rabbit

in vivo. In addition, reperfusion of hearts with buffer containing tempol causes a 37% reduction in infarct size. These data provide convincing evidence that the administration of tempol prior to the onset of reperfusion reduces myocardial infarct size in rodents.

Renal ischaemia/reperfusion injury is one of the most common causes of acute renal failure. In a rat model of renal ischaemia and reperfusion (bilateral clamping of both renal arteries), tempol significantly reduced the glomerular dysfunction and the tubular injury and dysfunction. Tempol also reduced the histological evidence of renal damage associated with ischaemia/reperfusion, and caused a substantial reduction in the staining for nitrotyrosine and PARP, suggesting reduced nitrosative and oxidative stress. In primary proximal tubule cells of the rat, tempol significantly attenuates the H₂O₂-mediated decrease in mitochondrial respiration and increase in LDH release, indicating a reduction in cell injury and death.

Tempol also reduces the tissue injury associated with ischaemia and reperfusion of the brain. In the anaesthetized rat, tempol reduces the infarct volume caused by middle cerebral artery occlusion and reperfusion without affecting the regional cerebral blood flow or blood pressure, indicating that the observed protective effects are independent of any local or systemic haemodynamic effects (Cuzzocrea *et al.*, 2000a). In a gerbil model of bilateral occlusion and reperfusion of both common carotid arteries, tempol (30 mg kg⁻¹ i.p. at 30 min prior to reperfusion, and at 1 and 6 h after the onset of reperfusion) reduced the formation of postischaemic brain oedema, attenuated the increase in the cerebral levels of malondialdehyde and the hippocampal levels of myeloperoxidase. The immunohistochemical analysis of the hippocampal region of brains subjected to ischaemia-reperfusion exhibited a positive staining for nitrotyrosine (an indicator of the generation of the potent oxidant peroxynitrite) and for PARP. In gerbils subjected to both common carotid arteries, which were treated with tempol, the degree of staining for nitrotyrosine and PARP was markedly reduced. Tempol increased survival and reduced the hyperactivity (secondary to the ischaemia-induced neurodegeneration) caused by cerebral ischaemia and reperfusion. The loss of neurons from the pyramidal layer of the CA1 region caused by ischaemia and reperfusion was also attenuated by treatment of gerbils with tempol. This study, hence, provides evidence that the membrane-permeable, radical scavenger tempol reduces the cerebral injury caused by transient, cerebral ischaemia *in vivo* (Cuzzocrea *et al.*, 2000b).

Rats subjected to severe splanchnic ischaemia develop a significant decrease in the mean arterial blood pressure, a significant increase in tissue myeloperoxidase activity and a marked injury to the distal ileum. There is now evidence that tempol (30 mg kg⁻¹ bolus injection 5 min prior to reperfusion, followed by an infusion of 30 mg kg⁻¹ h⁻¹ intravenously) reduces (i) the infiltration of the reperfused intestine with neutrophils, (ii) the lipid peroxidation, (iii) the production of peroxynitrite, (iv) the degree of P-selectin and ICAM-1 staining in tissue sections from SAO-shocked rats, (v) histological signs of bowel injury and (vi) mortality at 2 h after reperfusion (Cuzzocrea *et al.*, 2000a).

There is good evidence that (i) both septic and haemorrhagic shock are associated with the generation of superoxide anions, hydrogen peroxide and peroxynitrite, and (ii) that tempol reduces the multiple organ injury in animal models of shock.

For instance, endotoxemia in the rat causes within 6 h hypotension, acute renal dysfunction, hepatocellular injury, pancreatic injury and an increase in the plasma levels of nitrite/nitrate. Pretreatment of rats with tempol (100 mg kg⁻¹ i.v. bolus injection, 15 min prior to LPS, followed by an infusion of 30 mg kg⁻¹) did not affect the circulatory failure, but attenuated the renal dysfunction and the hepatocellular injury/dysfunction caused by LPS. Tempol did not affect the rise in nitrite/nitrate caused by endotoxin (Leach *et al.*, 1998). Similarly, tempol also reduces the renal and liver injury and dysfunction caused by wall fragments of Gram-positive bacteria in the anaesthetized rat. Haemorrhage (sufficient to lower the mean arterial blood pressure to 50 mmHg for 90 min) and subsequent resuscitation with shed blood (in the rat) result within 4 h after resuscitation in a delayed fall in blood pressure, renal and liver injury, and dysfunction as well as lung and gut injury. In all organs, haemorrhage and resuscitation resulted in the nitrosylation of proteins (determined by immunohistochemistry for nitrotyrosine), suggesting the formation of peroxynitrite and/or reactive oxygen species. Treatment of rats upon resuscitation with tempol (30 mg kg⁻¹ bolus injection followed by an infusion of 30 mg kg⁻¹ h⁻¹ i.v.) attenuated the delayed circulatory failure as well as the multiple organ injury and dysfunction associated with haemorrhagic shock (Mota-Filipe *et al.*, 1999).

Following the observation by Sledzinski *et al.* (1997) that tempol exerts beneficial effects in a rodent model of pancreatitis, there is now good evidence that tempol reduces the degree of inflammation and the associated tissue injury in animal models of diseases associated with local or systemic inflammation. These include rodent models of carrageenan-induced pleurisy, colitis, zymosan-induced multiple organ injury and uveoretinitis (Thiemermann (2003) for review). The doses of tempol necessary to protect tissues against the injury associated with the above disorders are relatively high (ranging from 10 to 100 mg kg⁻¹ for i.v. bolus administration).

Thus, tempol is a membrane-permeable, radical scavenger, which interferes with the formation and/or the effects of many radicals including superoxide anions, hydroxyl radicals and peroxynitrite. There is now a substantial body of evidence from preclinical studies showing that tempol may be useful in the therapy of ischaemia-reperfusion injury, shock and inflammation.

Manganese(II) (pentaazamacrocyclic ligand)-based complexes

Our research group set out many years ago to design highly active and stable mimetics of the mammalian SOD enzymes for use as possible therapeutic agents in human disease. Initially, we sought to mimic the function of the enzymes with little emphasis on selectivity, but soon we found that many of the metal complexes capable of a fast dismutation of superoxide anion can also react with the hydrogen peroxide product of the dismutation in a Fenton-like reaction producing hydroxyl radical, an even more reactive and potentially destructive radical than superoxide anion. Thus, our earliest focus was to design functional SODm that would not react with oxidants like hydrogen peroxide or hypochlorite. An additional paradigm that we were working under was the constraint that we wanted to develop agents that could be used as human pharmaceutical agents, if they were indeed

efficacious in animal models of disease. To this end, we focused on metal complexes that would be stable under physiological conditions. From our research efforts, we indeed discovered and developed a class of agents that met the four major criteria that we felt were critical: high SOD activity, high stability, selectivity only for superoxide and *in vivo* efficacy. We focused on the design and synthesis of Mn(II) and Fe(III) complexes which possess high inherent chemical and thermodynamic stability, and at the same time are highly effective catalysts for the dismutation of superoxide anion (Figure 4). As can be seen by EPR studies, M40403 is very effective in removing superoxide generated by, for example, phorbol 12-myristate 13-acetate-stimulated human neutrophils (Figure 5). This dual-design goal of *high stability* and *high SOD activity* was achieved by utilizing a combination of computer-aided modelling studies (Henke, 1999; Riley *et al.*, 1999) and synthesis activities, and has led to the development of a novel class of highly active SOD catalysts which are also very stable complexes. These synthetic SODm are exemplified by the prototypical complex M40403 (Figure 4; Table 1), derived from the 15-membered macrocyclic ligand 1,4,7,10,13-pentaazacyclopentadecane, containing the added bis(cyclohexylpyridine) functionalities (Riley *et al.*, 1999). M40403 is a stable, low molecular weight, manganese-containing, nonpeptidic molecule, possessing the function and catalytic rate of native SOD enzymes, but with the advantage of being a much smaller molecule (MW 483 vs 30,000 for the mimetic and native enzymes, respectively) (Salvemini *et al.*, 1999b). Another important advantage of these synthetic enzymes is that they do not possess the bell-shaped curve that is a common characteristic to the native SOD enzyme. At low doses, the SOD enzymes are anti-inflammatory, whereas at high doses they exhibit proinflammatory effects (Dowling *et al.*, 1993). The proinflammatory effects of the SOD enzyme are not well understood, but it is speculated to be due to its reaction with the dismutation product hydrogen peroxide, to generate hydroxyl radicals *via* Fenton chemistry (Mao *et al.*, 1993). The lack of a bell-shaped dose-response curve with the SODm

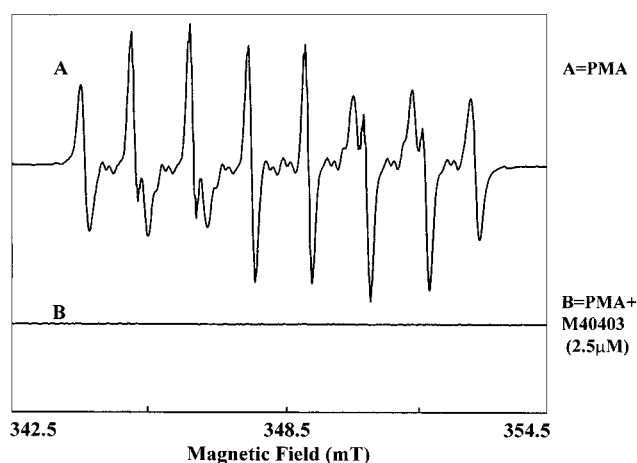
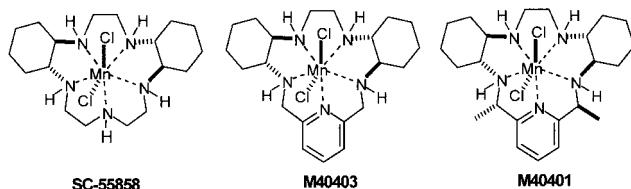


Figure 5 Effect of SOD mimic M40403 on superoxide production from human neutrophils. Spin-trapping measurements were performed using the spin trap DEPMPO, 10 mM, with 0.4 M PMN stimulated with PMA, 200 ng/ml. The spectra shown were recorded 30 min post activation. (a) Control-activated PMNs; (b) in the presence of 2.5 μ M M40403. EPR spectra were recorded in a flat cell on a Bruker ESP300 spectrometer, using a TM110 cavity with 100 kHz modulation frequency.

Table 1 Properties of Mn(II)-based selective superoxide dismutase mimetics (e.g. M40403)

Manganese-containing bicyclohexylpyridine
 Catalytic activity equivalent to that of the native enzyme
 Penetrates cells and wide organ distribution
 Selective for superoxide (no interaction with other biologically relevant molecules, for example, nitric oxide, hydrogen peroxide, peroxynitrite)
 Stable *in vivo*: no loss of manganese and excreted intact
 Not deactivated by peroxynitrite
 Suitable pharmacological tool to dissect the role of superoxide in physiopathological conditions
 Pharmacologically efficacious in numerous animal models of disease

**Figure 6** Structures of Mn(II)-based selective SODm (for example, M40403).

may be related to the selective reactivity of the SOD mimics with superoxide and the complexes' inability to react with hydrogen peroxide.

The Mn(II)-based SODm such as M40403 and M40401 (Figure 6) described herein are *catalytic agents* (drugs); that is, the compounds do not involve a stoichiometric interaction with a biological target, such as a receptor, but instead enhance the rate of conversion of superoxide to molecular oxygen and hydrogen peroxide, without the complex itself being consumed. The SODm have been rigorously characterized for SOD activity by stopped-flow kinetic analysis, for *in vitro* stability by kinetic and thermodynamic assays, and for *in vivo* stability by electron spin resonance and radiolabel studies using Mn-, H- and C-labelled mimics. The ability of the SODm to scavenge superoxide *in vivo* has also been demonstrated by electron spin resonance studies (Table 1). Their inability to react with hydrogen peroxide or peroxynitrite has been screened and demonstrated with a variety of techniques, including spectrophotometric diode array detection reactivity studies (for Mn(III) production from OCl^- , OONO^- and H_2O_2), *via* oxygen electrodes (for catalase activity), and in stopped flow experiments designed to follow the loss of peroxynitrite (Table 1).

We have published extensively in chemical literature on the mechanism and subsequent computer-aided design (CAD) (Riley *et al.*, 1999; Aston *et al.*, 2001) of these highly active and stable mimetics (Riley *et al.*, 1996). Three of the mimetics that we have used extensively in pharmacology studies are shown in Figure 6, and span a range of catalytic rate constants at $\text{pH} = 7.4$ ($\text{M}^{-1} \text{s}^{-1}$) (SC-55858 = 1.2×10^8 , M40403 = 1.6×10^7 and M40401 = 1.6×10^9), with similar hydrophobicities. It should be noted that each of these catalysts has a proton first-order dependence on the rate; that is, the rate increases as the pH drops. All the three agents have a catalytic rate greater than the native MnSOD enzyme at $\text{pH} = 6.5$, for example. Thus, by utilizing the CAD principles that we have developed, we have been able to synthesize not only highly active catalysts but also agents such as M40403 and M40401 that are stable *in*

Table 2 Summary of the anti-inflammatory properties of Mn(II)-based selective superoxide mimetics (e.g. M40403)

Inhibition of the upregulation of adhesion molecules (ICAM-1, P-selectin) and PMNs infiltration at the inflamed site
 Attenuation of proinflammatory cytokine release (TNF α , IL-1 β , IL-6)
 Protection of the inactivation of nitric oxide and preservation of its beneficial effects
 Inhibition of lipid peroxidation and cellular protection
 Attenuation of DNA damage and subsequent activation of poly-ADP-ribose polymerase
 Attenuation of peroxynitrite formation and subsequent peroxynitrite-mediated damage
 Protects against superoxide anion-driven deactivation of catecholamines – important immunoregulators
 Inhibition of the activation of transcription factors (NF κ B)

in vivo for extended periods, and are amphoteric with log *P*-values of ~ 0.3 – 0.4 (slightly hydrophilic). The recently developed M40401 (the *S,S*-dimethyl-substituted derivative of the M40403 bicyclohexylpyridyl class of mimetic) actually possesses a higher catalytic activity at $\text{pH} = 7.4$ than the native Mn SOD enzyme (Aston *et al.*, 2001). In fact, its catalytic rate exceeds $1 \times 10^{+9} \text{M}^{-1} \text{s}^{-1}$, comparable to the native Cu/Zn SOD enzymes.

In the light of the critical roles of superoxide in disease, these new *selective*, potent and stable synthetic enzymes of SOD, as represented by M40403, have broad potential as pharmacological tools to dissect the role of superoxide in disease models where other such relevant biological oxidants may be present and are expected to play a role.

Others and we have shown over the last several years that SODm are anti-inflammatory (Salvemini *et al.*, 1999b; 2001a, b; Cuzzocrea *et al.*, 2001a), and protective in models of septic shock (Cuzzocrea *et al.*, 2001a; Salvemini & Cuzzocrea, 2002a) and ischaemia-reperfusion injury (Cuzzocrea *et al.*, 2001a, b; Salvemini & Cuzzocrea, 2002b; 2003). The anti-inflammatory profile of these agents is shown in Table 2.

An important mechanism by which SODm attenuate inflammation is by reducing peroxynitrite formation by simply removing the superoxide before it can react with nitric oxide. This is important since the proinflammatory and cytotoxic effects of peroxynitrite are numerous (Squadrito & Pryor, 1995). For instance, removal of peroxynitrite by agents such as FeTMPS, a porphyrin-containing molecule, which increases the rate of isomerization of peroxynitrite to nitrate (Stern *et al.*, 1996) is cytoprotective (Misko *et al.*, 1998) and anti-inflammatory (Salvemini *et al.*, 1998). Peroxynitrite nitrates tyrosine residues in proteins, and nitrotyrosine formation, as monitored by immunofluorescence, has been used as a marker for the detection of the endogenous formation of peroxynitrite (Beckman, 1996). Using nitrotyrosine as a marker for the presence of ONOO $^-$ has been challenged by the demonstration that other reactions can also induce tyrosine nitration; for example, the reaction of nitrite with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide can lead to the formation of nitrotyrosine (Eiserich *et al.*, 1998). Thus, increased nitrotyrosine staining is considered as an indicator of 'increased nitrosative stress' rather than as a specific marker of the generation of peroxynitrite (Eiserich *et al.*, 1998). Therefore, our recent study (Fries *et al.*, 2003) and the studies with the peroxidase-deficient mice (Brennan *et al.*, 2002; Gaut *et al.*,

2002) reinforce previous discussions that a number of pathways apparently contribute to the nitration of tyrosine residues in proteins *in vivo* independently, or even simultaneously (Ischiropoulos, 1998; Hurst, 2002). With the combined judicious use of both pharmacological agents and molecular approaches, and depending upon the inflammatory stimuli and cell type, the sources and nature of nitrating agents *in vivo* are now being elucidated. Therefore, the conclusion of our recent publication is that superoxide contributes in a precise pattern of intracellular protein tyrosine nitration (Fries *et al.*, 2003).

In this regard, we have also found that SODm of the M40403 class block nitrotyrosine staining in models of inflammation, suggesting that superoxide anion-driven peroxynitrite formation is in fact responsible for the formation of nitrotyrosine, and that its inhibition could account for the anti-inflammatory effects of SODm (Salvemini *et al.*, 1999b; 2001a,b; Cuzzocrea *et al.*, 2001c). This in fact was the first evidence to show *in vivo* a superoxide-dependent nitration, since the M40403 class of SODm does not react with nitric oxide or peroxynitrite. A similar pattern of immunoreactivity for nitrotyrosine is observed in a lung model of pleurisy (Ischiropoulos *et al.*, 1995; Salvemini *et al.*, 2001a; Bianca *et al.*, 2002).

A substantial amount of data have been generated to support the concept that peroxynitrite generation plays an important part in the proinflammatory roles that have been ascribed to nitric oxide (Salvemini *et al.*, 1998). *Based on this premise, we propose that selective SODm should be considered as a therapeutic means to attenuate inflammatory responses.* In addition, superoxide, by interacting with nitric oxide, destroys the biological activity of this mediator (Gryglewski *et al.*, 1986), attenuating important antiinflammatory and tissue-protective properties of nitric oxide; namely, its maintenance of blood vessel tone, platelet reactivity, cytoprotective effects in numerous organs (including heart, intestine and kidney) (Moncada *et al.*, 1991; Szabo & Billiar, 1999b; Liaudet *et al.*, 2000) and release of anti-inflammatory and cytoprotective prostacyclin (*via* activation of the constitutive cyclooxygenase enzyme) (Salvemini *et al.*, 1993; 1996).

In addition, it has been demonstrated that NO derived from iNOS is necessary for a substantial antiviral effect of interferon- γ and for the ability of the body to fight viruses. Thus, inhibition of iNOS enhances viral replication and leads to an increase in lethality in systemic viral infection models (Karupiah *et al.*, 1993). In other words, the potential beneficial anti-inflammatory effects of iNOS inhibition are cancelled out by independent, deleterious actions of iNOS inhibition. However, in other types of viral infection, where clearance of the virus is independent of interferon- γ , the anti-inflammatory effect of iNOS inhibition becomes prominent. For example, viral pneumonitis induced by influenza A virus is abolished in iNOS-deficient mice (Karupiah *et al.*, 1998). Therefore, we have clearly demonstrated that M40403 or M40401 did not interfere with the activation of iNOS during acute inflammation, and did not reduce the NO production after ischaemia and reperfusion. Thus, removal of superoxide reduces the formation of the cytotoxic peroxynitrite and does not interfere with the beneficial effects of NO.

As indicated earlier, superoxide anion and peroxynitrite induce DNA single-strand damage that is the obligatory trigger for PARP activation (Inoue & Kawanishi, 1995; Salgo *et al.*, 1995), eventually leading to cell death (Szabó *et al.*, 1996b). Furthermore, substantial evidence exists to support the

fact that PARP activation is important in inflammation (Szabó & Dawson, 1999a). PARP is the target for therapeutic intervention, and its inhibitors such as nicotinamide and 3-aminobenzamide attenuate both acute and chronic inflammatory processes (Cuzzocrea *et al.*, 1998a,b; Virag & Szabo, 2002). SODm reduce PARP immunofluorescence and attenuate the reduction of NAD⁺ in models of acute and chronic inflammation (Salvemini *et al.*, 2001a,b; Cuzzocrea *et al.*, 2001c). In light of the role of PARP in inflammation, it is possible that PARP inhibition by these SODm accounts for their anti-inflammatory response.

Superoxide increases neutrophil adhesion and infiltration (Warren *et al.*, 1990; Salvemini *et al.*, 1999a; 2001a; Cuzzocrea *et al.*, 2001c), and generates potent chemotactic mediators such as leukotriene B₄ (Al-Shabanah *et al.*, 1999). Removal of superoxide inhibits the infiltration of neutrophils at sites of inflammation, as shown by the use of the native SOD enzyme (Niwa *et al.*, 1985), experiments performed in transgenic mice that overexpress the human CuZnSOD enzyme (Chan *et al.*, 1991) and by the use of SODm such as SC-55858 and M40403 (Lowe *et al.*, 1996; Salvemini *et al.*, 1999b; 2001a). This correlates well with an attenuation of lipid peroxidation and overall attenuation of acute and chronic inflammation. A possible mechanism by which SODm attenuate neutrophil infiltration is by downregulating adhesion molecules such as ICAM-1 and P-selectin. Thus, inhibition of neutrophil infiltration at sites of inflammation and reperfusion injury correlated well with the inhibition of both ICAM-1 and P-selectin (Hardy *et al.*, 1994; Cuzzocrea *et al.*, 2001c), supporting the involvement of superoxide in the regulation of adhesion molecules (through mechanisms yet to be defined). In addition to ICAM-1 and P-selectin, other adhesion molecules may be affected by superoxide. The release of a variety of proinflammatory cytokines is also regulated by superoxide. Thus, SODm inhibit a number of inflammatory cytokines, including tumour necrosis factor- α , interleukin-1 β and interleukin-6 (TNF α , IL-1 β and IL-6, respectively), as shown in models of acute and chronic inflammation (Cuzzocrea *et al.*, 2001c; Salvemini *et al.*, 2001a; Salvemini & Cuzzocrea, 2002b). The mechanism(s) through which superoxide regulates cytokines is under evaluation in various research groups. Recent data demonstrate that superoxide anions (generated from xanthine-xanthine oxidase) can directly release TNF α from macrophages (Volk *et al.*, 1999). Interestingly, the anti-inflammatory cytokine IL-10 is not affected (Salvemini *et al.*, 2001a). A role for reactive oxygen species in signal transduction during inflammatory stimuli is becoming appreciated. For example, the MAPK pathway and MAPK-mediated regulation of TNF α production (post LPS-stimulated alveolar epithelial cells) is redox-dependent, requiring at least in part a NF κ B/ROS-sensitive mechanism (Haddad & Land, 2002). McInnis and co-workers reported that *N*-methyl-D-aspartate-mediated neurotoxicity is associated with activation of the NF κ B system, an effect that is blocked by M40403, suggestive of a role for superoxide in the activation of this important transcription factor (Mc Innis *et al.*, 2002).

Thus, removal of superoxide impacts the inflammatory cascade through at least three major pathways: (1) inhibition of peroxynitrite formation and sparing of nitric oxide, (2) inhibition of neutrophil infiltration at the site of inflammation, and (3) inhibition of proinflammatory cytokine release.

Although limited information is currently available on this topic, it is possible that another mechanism that may reveal itself as being important through which superoxide might modulate the inflammatory response is through the deactivation of catecholamines (noradrenaline and adrenaline). Catecholamines are released as part of the body's 'survival' response to numerous exogenous and endogenous stimuli including, of course, inflammation. Besides their well-known vasoconstrictor (for noradrenaline) and bronchodilator (for adrenaline) properties, catecholamines are also well known in regulating the immune system (Van der Poll *et al.*, 1994; 1996; Van der Poll & Lowry, 1997). Once deactivated, catecholamines are no longer able to exert their effect. Deactivation of catecholamines accounts for the hyporeactivity and hypotension associated with shock (Macarthur *et al.*, 2000; 2003), contributing therefore to the development of both the vascular abnormalities with this disease. In addition to their pivotal role in transducing the sequential cardiovascular and haemodynamic crisis following severe Gram-negative bacteremic sepsis, endogenous catecholamines are instrumental in controlling the expression of cytokines. Nerve-derived noradrenaline and circulating adrenaline act *via* β -adrenergic receptors on macrophages and neutrophils downregulating inflammatory responses mediated specifically by TNF α and IL-1 β (Severn *et al.*, 1992; Monastra & Secchi, 1993; Van der Poll *et al.*, 1996; Guirao *et al.*, 1997). Deactivated catecholamines are no longer able to exert this effect, contributing to the increase in cytokine release during shock. M40403 or M404041 inhibits cytokine production by removing superoxide, at least in part through protection and preservation of endogenous catecholamines (Macarthur *et al.*, 2000; 2003). Furthermore, the production of superoxide by activated neutrophils and macrophages is also negatively modulated by catecholamines through β -adrenergic receptor activation (Baciu, 1988; Weiss *et al.*, 1996), underlining the potential immunoregulatory effects of catecholamines. This mechanism is potentially relevant to sepsis and multiorgan failure (Salvemini & Cuzzocrea, 2002a; 2003).

SODm- and hydrogen peroxide-driven toxicity

It is often asked whether SODm, which lack catalase activity, increase cellular damage by enhancing the local concentration of the so-called 'toxic' hydrogen peroxide. We will take this opportunity to discuss that the contention that H₂O₂ is more toxic than superoxide is not necessarily correct. This is based on the following. Hydrogen peroxide is itself not a radical but actually quite an inert oxidant, whose cellular toxicity is probably in the 100 μ M–mM range. The hydrogen peroxide's toxicity is likely due to the generation of reduced iron (Fe(III) is the oxidation state of iron in iron-storage sites) which, as Fe(II) reacts with hydrogen peroxide (Fenton reaction), undergoing homolytic cleavage to generate Fe(III)(OH) and hydroxyl radical. Iron(III) must first be reduced to 'free' soluble Fe(II) for this reaction to occur; one of the best reductants available in inflammatory or reperfusion disease states is superoxide, which has been shown to be an excellent kinetically competent reductant of Fe(III) in iron storage sites liberating Fe(II) (Keyer & Imlay, 1996). Thus, superoxide becomes (not hydrogen peroxide) the culprit leading to generation of conditions favourable for Fenton chemistry to be initiated. The statement that we would be creating a more

toxic condition by generating more hydrogen peroxide is also not correct when one inspects the stoichiometry of the reactions involved. Nearly all of the oxidizing reactions which superoxide enters into involve hydrogen atom abstraction from a biological target molecule such as a catecholamine, DNA, RNA, and an allylic CH of a fatty acid, steroids, and so forth. These oxidation reactions are free radical chain reactions and produce at least one hydrogen peroxide per oxidation; in fact, these are all free radical chain reactions that in the presence of oxygen will yield many molecules of hydrogen peroxide with one initiation from superoxide. When superoxide is dismuted, the stoichiometry is such that two superoxides and two protons generate one oxygen molecule and one hydrogen peroxide as the net reaction; thus, in effect, each mole of superoxide now leads to $\frac{1}{2}$ a mole of hydrogen peroxide. So, in effect, by dismuting superoxide one actually decreases the potential H₂O₂ burden – not increasing it. Furthermore, to date there is no evidence that *in vivo* Fenton reaction occurs (Koppenol, 1998; Toyokuni, 2002; Blokhina *et al.*, 2003). In fact, Koppenol and colleagues have indicated that no iron complex has been identified *in vivo* that participates in the Fenton reaction (Koppenol, 1998). Thus, the use of SODm does not lead to a toxic condition by generating more hydrogen peroxide. Furthermore, when these molecules have been tested in the *in vitro* study of neutrophil-mediated injury of human aortic endothelial cells, no toxic effect has been observed (Hardy *et al.*, 1994). This study clearly demonstrates not only that the compounds protect against the activated neutrophil-mediated killing of the human aortic cells, but also that the coadministration with catalase or glutathione peroxidase did not have any additional benefit either in the presence of the mimetics or in their absence (Hardy *et al.*, 1994). Thus, our data support that hydrogen peroxide toxicity is not an issue when efficient and selective superoxide dismutation is achieved. Furthermore, the chronic treatment *in vivo* with M40403 (10 days in a model of arthritis) did not exert any toxic effect (Salvemini *et al.*, 2001b).

Conclusions

There is no doubt that superoxide plays a role in various disorders. That superoxide plays a role in human disease has also been substantiated by encouraging results obtained in various clinical trials performed with Orgotein[®] (Niwa *et al.*, 1985; Flohe, 1988). Interestingly, the first clinical pilot studies with the native enzyme were carried out as early as the 1970s in rheumatoid arthritis (RA) and osteoarthritis (OA), with preliminary results demonstrating efficacy. Further studies then showed that Orgotein[®], when given by intra-articular injection, attenuates signs and symptoms (inflammation and pain) of RA and OA (Goebel *et al.*, 1981; Goebel & Storck, 1983; Lund-Olesen & Menander-Huber, 1983; Gammer & Broback, 1984; Mc Ilwain *et al.*, 1989; Mazieres *et al.*, 1991). Interestingly, in these studies, Orgotein[®] led to some 60% decrease in the consumption of analgaesics. Furthermore, Orgotein[®] was found to be effective when given by intra-articular injection in patients with temporomandibular joint (TMJ) dysfunction, who failed to respond to standard therapy (Lin *et al.*, 1994), and was found to reduce pain in patients with duodenal ulcer pain (Pascu & Dejica, 1987). Possibly, the most compelling data for the efficacy of SOD in human disease

come from a large body of data gathered since the early 1980s, showing the protective effects of Orgotein® (given by intramuscular injection) against acute and chronic side effects associated with chemotherapy and radiation therapy (Marberger *et al.*, 1974; 1975; 1981; Edsmyr *et al.*, 1976; Menander-Huber *et al.*, 1978; Housset *et al.*, 1989; Delanian *et al.*, 1994; Sanchiz *et al.*, 1996). It is of major importance that Orgotein® is able to reverse fibrosis once it is established (Niwa *et al.*, 1985; Flohe, 1988). Other clinical settings in which SOD (whether of recombinant or bovine origin) was used include patients with Crohn's disease, various forms of periarticular inflammation and Peyronie's disease (Babior, 1997). Despite the apparent clinical benefit, SOD enzymes suffer as drug candidates due to a number of reasons described in the previous sections, and were removed from the markets primarily because of immunogenic response. In certain cases where an enzyme of potential therapeutic benefit does not have the appropriate properties for a drug, a synthetic, small-molecule enzyme mimetic can conceivably be designed with chemical and physical characteristics suitable for a therapeutic.

In this review, we have discussed a number of agents that may fulfill this purpose: these provide an example of a unique approach for the development of artificial enzymes as future drugs. The Mn(II)-based SODm are true mimetics of the native enzyme – they are catalysts and are selective for superoxide. These, like the native SOD enzymes, are catalysts, and remove superoxide at a high rate, selectively, without reacting with other biologically relevant oxidizing species, such as nitric oxide, peroxynitrite, hydrogen peroxide, hypochlorite or oxygen (Riley *et al.*, 1996; 1997; Salvemini *et al.*, 1999b). The unique *selectivity* of mimetics such as M40403 resides in the nature of the manganese(II) centre in the complex. The resting oxidation state of the complex is the reduced Mn(II) ion; as a consequence, the complex has no reactivity with reducing agents until it is oxidized to Mn(III) by protonated superoxide, whereupon, the complex is rapidly reduced back to

the Mn(II) state by the superoxide anion at diffusion-controlled rates. Since the complex is so difficult to oxidize ($> +0.74$ V (vs SHE)), many one-electron oxidants cannot oxidize this and its related complexes (including nitric oxide and oxygen). Further, since the SODm operate *via* a facile one-electron oxidation pathway, other two-electron nonradical, but nevertheless, potent oxidants are not kinetically competent to oxidize the Mn(II) complex; for example, peroxynitrite, hydrogen peroxide or hypochlorite. It is important to realize that this property is not shared by other 'so-called and claimed classes of SODm' discussed in sections A through C. Although these agents are clearly protective (as discussed in this review) in animal models, one general limitation of these agents (if one is interested in dissecting the contribution of superoxide in physiopathological situations *versus* other reactive species) is that they react not only with superoxide but also with a wide variety of reactive oxygen species. From a pharmacological standpoint, such nonselectivity makes the interpretation of the result difficult to make, and caution needs to be taken before making general assumptions and claims on the type of the reactive oxygen species in the disease state under investigation. In general, such agents should not be accepted as pharmacological tools with which to investigate or validate the role(s) of the superoxide in *in vivo* model systems.

We propose that compounds that have been developed, and that will be developed in the future with SOD activity and selectivity for superoxide, will find clinical utility in diseases mediated, in part, by superoxide (Figure 7). To this end, M40403 has been developed with the intent to utilize it as a human pharmaceutical agent for the treatment of various diseases. M40403 has successfully completed a Phase I safety clinical trial in healthy human subjects.

We would like to thank Dr Alexandre Samouilov for his help in generating EPR data to support Figure 5.

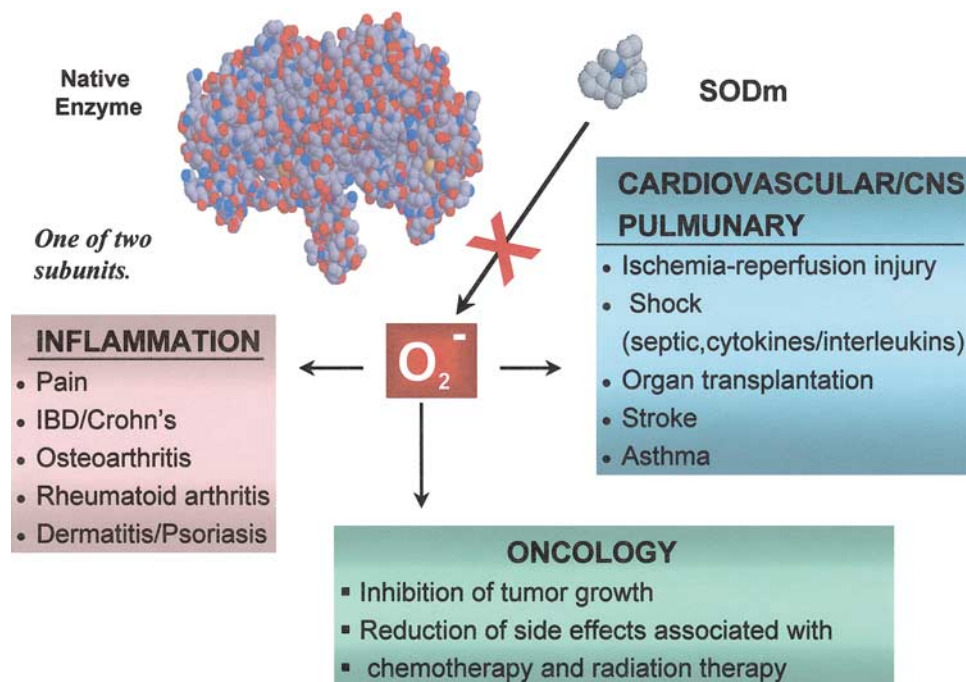


Figure 7 Therapeutic opportunities for SODm.

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