A Nonpeptidyl Mimic of Superoxide Dismutase with Therapeutic Activity in Rats

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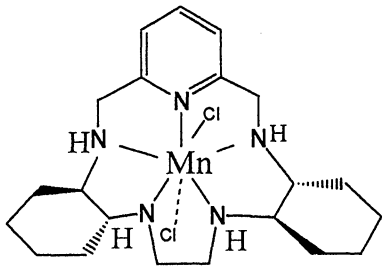
Many human diseases are associated with the overproduction of oxygen free radicals that inflict cell damage. A manganese(II) complex with a bis(cyclohexylpyridine)-substituted macrocyclic ligand (M40403) was designed to be a functional mimic of the superoxide dismutase (SOD) enzymes that normally remove these radicals. M40403 had high catalytic SOD activity and was chemically and biologically stable in vivo. Injection of M40403 into rat models of inflammation and ischemia-reperfusion injury protected the animals against tissue damage. Such mimics may result in better clinical therapies for diseases mediated by superoxide radicals.

Many diseases can be characterized as conditions in which the body fails to contain the overproduction of an undesired metabolic byproduct. Although all mammalian life consumes O_2 as the ultimate oxidant supporting cellular respiration, a considerable portion of this O_2 is reduced, through one-electron paths, to the superoxide anion ($O_2^{\bullet -}$). Under normal circumstances, this radical burden is controlled by SOD enzymes in the mitochondria (Mn based), in the cytosol (Cu and Zn), or on extracellular surfaces (Cu and Zn). The SODs (*1, 2*) are oxidoreductases that contain Cu, Fe, or Mn at the active site and catalyze the dismutation of $O_2^{\bullet -}$ to O_2 and hydrogen peroxide (H_2O_2). In certain diseases, the production of $O_2^{\bullet -}$ is enhanced, resulting in $O_2^{\bullet -}$-mediated cell injury. Examples of such oxidative stress-related diseases include reperfusion injury, such as that which occurs after acute myocardial infarction or stroke, and inflammatory processes, such as arthritis. Although administration of recombinant SOD has been beneficial in animal models of disease mediated, in part, by superoxide (for example, myocardial ischemia-reperfusion injury, inflammation, and cerebral ischemia-reperfusion injury), clinical trials of this enzyme had to be curtailed because of immunogenic responses (*3*). Further evidence implicating $O_2^{\bullet -}$ as a mediator of diseases such as neuronal apoptosis, cancer, and acquired immunodeficiency syndrome (*3*) continues to accrue.

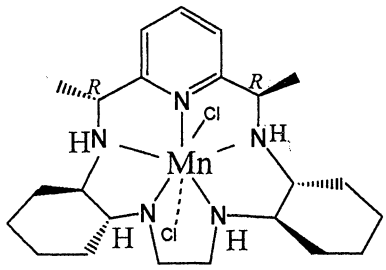
Because of the limitations associated with enzyme therapies (solution instability, limited cellular accessibility, immunogenicity, bell-shaped dose response curves, short half-lives, costs of production, and proteolytic digestion), we have synthesized SOD mimics with a low molecular weight (*4*). Through our previous work (*5–7*), we have discovered, using molecular modeling studies, a stable and active class of SOD mimic, exemplified by the prototypical complex, M40403. This mimic is derived from the macrocyclic ligand, 1,4,7,10,13-pentaazacyclopentadecane, containing the added bis(cyclohexylpyridine) functionalities. This complex catalyzes the dismutation of $O_2^{\bullet -}$ with rates approaching that of the native Mn SOD enzyme (*8*). M40403 (Fig. 1) has a molecular weight of 484.4 and a catalytic SOD rate $>2 \times 10^7$ M^{-1} s^{-1}, comparable to that of the Mn SODs at a pH of ~6. It is thermodynamically stable ($\log K > 17$; K, stability constant) and stable for up to 10 hours in whole rat blood at 37°C, although it was observed to partition into red blood cells. After intravenous (iv) injection into rats, M40403 distributes widely into the heart, lungs, brain, liver, and kidneys, while retaining its intact chemical identity. Moreover, the complex is excreted intact with no detectable dissociation and is recovered in urine and feces (*8*). M40403 does not react with nitric oxide (NO), H_2O_2, or peroxynitrite ($OONO^-$) (PN) (*5, 7*).

We evaluated the activity of M40403 in a rat model of inflammation. Intraplantar injection of carrageenan in rats (*9*) results in a time-dependent increase in paw volume that is maximal after 3 to 6 hours (*10*). Administration of M40403 (1 to 10 mg/kg, as iv bolus) 30 min before injection of carrageenan ($n = 6$) inhibited edema at subsequent time points (Fig. 2A), suggesting that $O_2^{\bullet -}$ is a critical mediator in the development of the inflammatory response.

Intraplantar injection of carrageenan also provokes a time-dependent infiltration of neutrophils at the inflamed site (*10*); a profound release of proinflammatory mediators, such as prostaglandin E_2 (PGE_2), tumor necrosis factor–α (TNF-α), and interleukin-1β (IL-1β) (*10–12*); and tissue damage, as evidenced by the release of lactate dehydrogenase (LDH) (*13*). Because these events are maximal at 6 hours after carrageenan injection (*8*), we evaluated the effects of M40403 at this time point. M40403 (1 to 10 mg/kg; $n = 6$), given 30 min before carrageenan injection, inhibited neutrophil infiltration and the release of TNF-α, IL-1β, and LDH in a dose-dependent manner (Fig. 2B). Thus, the biological properties of M40403 mimic those of the native enzymes that have also been shown to attenuate edema (*10, 14*) and neutrophil infiltration. This is consistent with a role for $O_2^{\bullet -}$ in eliciting neutrophil adhesion and infiltration (*10, 15*). Similar to results obtained with polyethylene glycol (PEG) SOD (*10*), M40403 had no effect on the release of PGE_2 (Fig. 2B), indicating that



M40403



M40404

Fig. 1. Structures of M40403 and M40404.

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inhibition of proinflammatory prostaglandins (PGs) (*11*) does not account for its beneficial effects. This contrasts with our observations of inhibitors of NO synthase, in which inhibition of inflammation was associated with a reduction in NO and PGs (*10, 16*). In addition, a monoclonal antibody to PGE_2 inhibits edema after intraplantar injection of carrageenan (*17*), indicating that PGE_2 plays a role in edema. We cannot explain at this stage why M40403 is anti-inflammatory, despite high levels of PGE_2. Nevertheless, these findings highlight the complex interactions between various mediators in inflammation. The SOD-inactive M40404, a structural analog of M40403 (Fig. 1) (*8*), at 10 mg/kg (n = 6) had no effect on edema (Fig. 2A), nor did M40404 have any effect on neutrophil recruitment or TNF-α, IL-1β, and LDH release (n = 6) (*8*).

M40403 was also evaluated in a rat model of ischemia-reperfusion injury and shock, namely the splanchnic artery occlusion (SAO) model. In this model, circulatory shock occurs when reperfusion follows prolonged ischemia of the splanchnic circulation (*18*). The end result is a high mortality, with most animals dying within the first 2 hours after reperfusion (*18*). Occlusion of the splanchnic arteries produced an increase in mean arterial pressure (from 115 ± 4 to 127 ± 4 mm of Hg), which, upon reperfusion, decreased until death (mean survival time was 90 ± 5 min; n = 27) (*8*).

To evaluate the effects of M40403 on local and systemic changes associated with reperfusion injury, we collected blood and tissue samples after the period of ischemia or 70 min after reperfusion. The local alterations in this model include neutrophil infiltration into the intestine (Fig. 3B) and a profound peroxidation of membranes resulting in high plasma levels of lipid peroxidation products, such as malondialdehyde (MDA) (Fig. 4A). The systemic alterations include an increase in plasma levels of TNF-α and IL-1β (Fig. 4, B and C), infiltration of neutrophils into the lung and intestine (Fig. 3), and severe hypotension. These events are most likely triggered by $O_2^{\bullet -}$ generated during the reperfusion phase, because no changes were observed when blood or tissues were removed after the period of ischemia before reperfusion (n = 6) (*8*). When infused for 15 min before reperfusion, M40403 (0.1 to 1 mg/kg; n = 6), but not M40404 (1 mg/kg; n = 6), inhibited, in a dose-dependent manner, the increase in plasma levels of MDA (45, 61, and 67% at 0.1, 0.3, and 1 mg/kg, respectively), TNF-α (88, 95, and 100% at 0.1, 0.3, and 1 mg/kg, respectively), and IL-1β (68, 96, and 98% at 0.1, 0.3, and 1 mg/kg, respectively) (Fig. 4, A through C), as well as the infiltration of neutrophils, as measured by

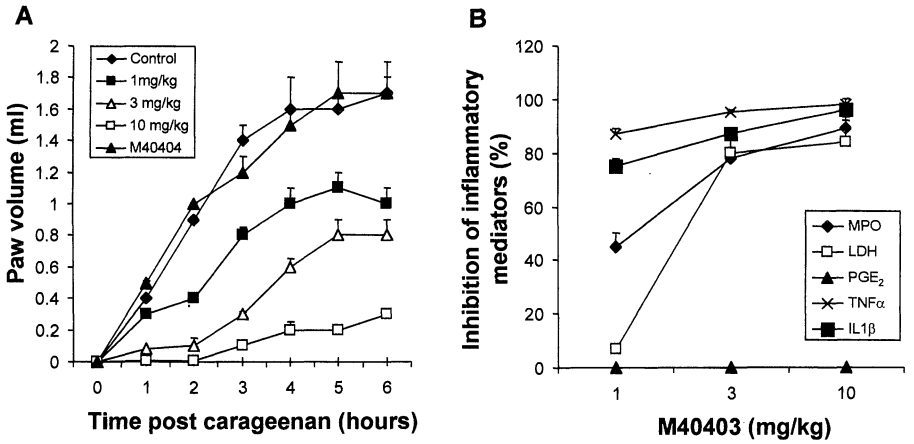

Fig. 2. (**A**) Intraplantar injection of carrageenan causes a time-dependent increase in paw edema, and this is blocked in a dose-dependent manner by M40403 (1 to 10 mg/kg, given as an iv bolus). The catalytically inactive SOD mimic, M40404 (10 mg/kg, given as an iv bolus), had no effect. (**B**) Increases in neutrophil infiltration (indexed by MPO amounts in paw tissue), TNF-α, IL-1β, and LDH, but not in PGE_2, at 6 hours after carrageenan injection are also inhibited by M40403 (1 to 10 mg/kg) (B). TNF-α, IL-1β, LDH, and PGE_2 were measured in paw exudates. The error bars represent the mean ± SEM for six experiments.

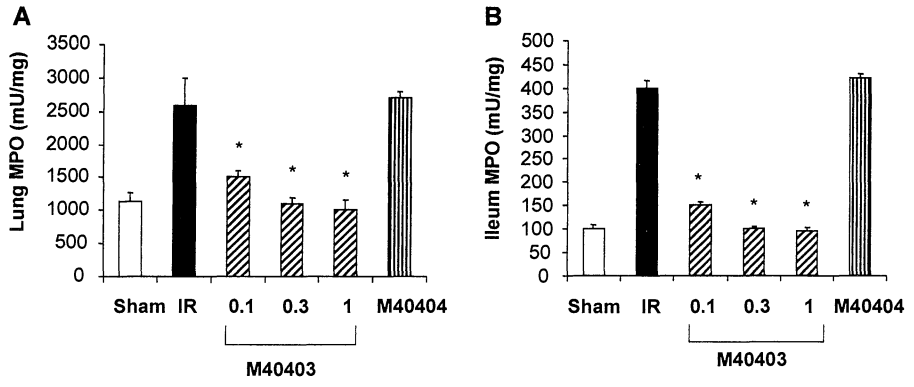

Fig. 3. Reperfusion of the ischemic splanchnic circulation (IR) results in the infiltration of neutrophils in the (**A**) lung and (**B**) ileum, as evaluated by MPO levels, and this is inhibited in a dose-dependent manner by M40403 (0.1 to 1 mg/kg), but not by M40404 (1 mg/kg). The error bars represent the mean ± SEM for six experiments. *P < 0.05 when compared to basal values. Sham, animals that underwent the same surgical procedure but did not undergo ischemia and reperfusion (controls).

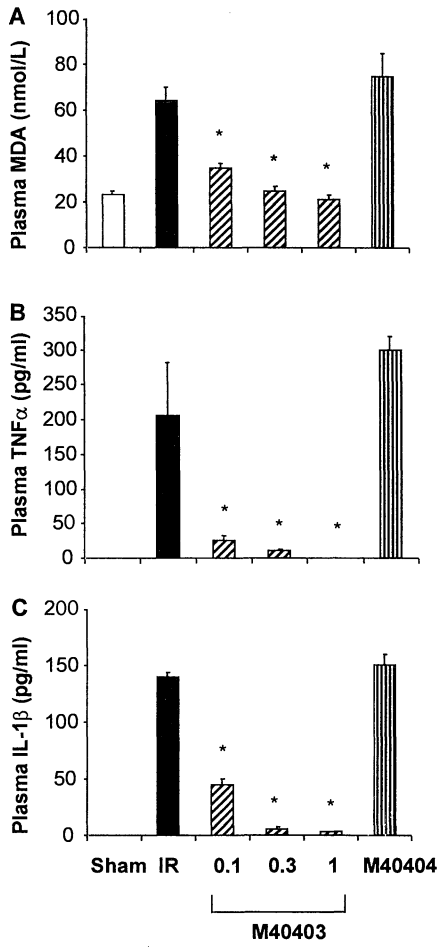

Fig. 4. Reperfusion of the ischemic splanchnic circulation results in increases in (**A**) plasma MDA, (**B**) TNF-α, and (**C**) IL-1β, and this is inhibited in a dose-dependent manner by M40403 (0.1 to 1 mg/kg), but not by M40404 (1 mg/kg). The error bars represent the mean ± SEM for six experiments. *P < 0.05 when compared to basal values.

myeloperoxidase (MPO) levels, into the ileum (62, 75, and 76% at 0.1, 0.3, and 1 mg/kg, respectively) and lung (42, 56, and 61% at 0.1, 0.3, and 1 mg/kg, respectively) (Fig. 3, A and B).

These results with M40403 are consistent with observations from transgenic mice overexpressing human SOD (Cu and Zn) (*19*), indicating that neutrophil infiltration in the lung and intestine in the SAO model is prevented. Likewise, administration of PEG SOD also exhibited a protective effect in this model (*20*). Furthermore, M40403 ($n = 8$), but not M40404 ($n = 4$), both given at 1 mg/kg, prevented the fall in blood pressure seen after reperfusion (*8*) and increased the survival time (90 ± 5% survival at 4 hours for rats treated with M40403 versus 0% survival at 4 hours in untreated rats and those treated with M40404).

In summary, our results demonstrate that M40403 is a stable SOD mimic with therapeutic activity in models of inflammation and ischemia. In addition to the direct effects of $O_2^{\bullet -}$ in these models, there are likely to be indirect effects mediated by the formation of PN. It is possible that some of the beneficial anti-inflammatory and cytoprotective effects of M40403 are due to the prevention of PN formation by the removal of $O_2^{\bullet -}$ before it reacts with NO (*21*). The mechanism or mechanisms by which $O_2^{\bullet -}$ modulates events such as neutrophil influx at inflamed sites or cytokine production and release have yet to be defined.

Understanding the signal transduction mechanisms used by free radicals to modify the course of disease will undoubtedly elucidate important molecular targets for future pharmacological intervention. SOD mimics such as M40403 can serve as tools to dissect these mechanisms. In addition, these molecules may have potential for the treatment of diseases ranging from acute and chronic inflammation to cardiovascular disease and cancer.

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Anaerobic Microbes: Oxygen Detoxification Without Superoxide Dismutase

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Superoxide reductase from the hyperthermophilic anaerobe *Pyrococcus furiosus* uses electrons from reduced nicotinamide adenine dinucleotide phosphate, by way of rubredoxin and an oxidoreductase, to reduce superoxide to hydrogen peroxide, which is then reduced to water by peroxidases. Unlike superoxide dismutase, the enzyme that protects aerobes from the toxic effects of oxygen, SOR does not catalyze the production of oxygen from superoxide and therefore confers a selective advantage on anaerobes. Superoxide reductase and associated proteins are catalytically active 80°C below the optimum growth temperature (100°C) of *P. furiosus*, conditions under which the organism is likely to be exposed to oxygen.

Aerobic organisms have an efficient metabolism as compared to that of most anaerobes because of the high reduction potential of molecular oxygen, which serves as the terminal electron acceptor for respiration. This advantage comes with a price, because both the chemical and the metabolic reduction of oxygen result in the production of highly toxic and reactive oxygen species (*1*, *2*). The univalent reduction product of oxygen, superoxide (O_2^-), reacts with hydrogen peroxide in the presence of transition metals to produce the reactive hydroxyl radical $OH^{\cdot}$, which is most likely responsible for the toxic effects of molecular oxygen (*3*). Aerobic organisms have developed mechanisms to protect themselves from oxygen toxicity. These involve the enzymes superoxide dismutase (SOD) (Eq. 1) (*4*), catalase (Eq. 2) (*5*), and nonspecific peroxidases (Eq. 3) (*5*, *6*).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (2)$$

$$H_2O_2 + RH_2 \rightarrow 2H_2O + R \quad (3)$$

For normal growth, aerobic organisms require molecular oxygen at near-atmospheric concentrations (21% v/v), whereas anaerobic organisms vary in their responses to oxygen, ranging from the extremely sensitive methanogens (*7*) to the more aerotolerant, sulfate-reducing *Desulfovibrio* (*8*), some species of which may be microaerophilic (*9*, *10*). Although most anaerobes inhabit ecosystems that are periodically exposed to air, they are unlikely to contain SOD or catalase, because both enzymes generate molecular oxygen (Eqs. 1 and 2) and thereby potentially prop-

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[11] **Pharmacological and Biochemical Demonstration of the Role of Cyclooxygenase 2 in Inflammation and Pain**

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[13] **Peroxynitrite Decomposition Catalysts: Therapeutics for Peroxynitrite-Mediated Pathology**

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[21] **Apparent Hydroxyl Radical Production by Peroxynitrite: Implications for Endothelial Injury from Nitric Oxide and Superoxide**

Joseph S. Beckman; Tanya W. Beckman; Jun Chen; Patricia A. Marshall; Bruce A. Freeman

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