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Research article

The protective effect of M40401, a superoxide dismutase mimetic, on post-ischemic brain damage in Mongolian gerbils

Vincenzo Mollace*[1], Michelangelo Iannone[2], Carolina Muscoli[1,3], Ernesto Palma[1], Teresa Granato[2], Andrea Modesti[4], Robert Nisticò[5], Domenicantonio Rotiroti[1] and Daniela Salvemini[3]

Address: [1]Faculty of Pharmacy, University of Catanzaro "Magna Graecia", Roccelletta di Borgia, Catanzaro Italy, [2]Institute of Neurological Science ISN – Section of Pharmacology, CNR, Roccelletta di Borgia, Catanzaro, Italy, [3]Metaphore Pharmaceuticals, 1910 Innerbelt Business Center Dr, St Louis MO 63114, USA, [4]Department of Experimental Medicine and Biochemical Science, University of Rome "Tor Vergata", Rome 00161, Italy and [5]Faculty of Pharmacy, University of Calabria – Arcavacata di Rende (CS), Italy

Email: Vincenzo Mollace* - mollace@libero.it; Michelangelo Iannone - iannone@unicz.it; Carolina Muscoli - cmuscoli@metaphore.com; Ernesto Palma - palma@unicz.it; Teresa Granato - granato@cs.cnr.it; Andrea Modesti - modesti@uniroma2.it; Robert Nisticò - gnistico@europarl.eu.int; Domenicantonio Rotiroti - rotiroti@unicz.it; Daniela Salvemini - cmuscoli@metaphore.comt

* Corresponding author

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Abstract

Background: Overproduction of free radical species has been shown to occur in brain tissues after ischemia-reperfusion injury. However, most of free radical scavengers known to antagonize oxidative damage (e.g. superoxide dismutase, catalase), are unable to protect against ischemia-reperfusion brain injury when given *in vivo*, an effect mainly due to their difficulty to gain access to brain tissues. Here we studied the effect of a low molecular weight superoxide dismutase mimetic (M40401) in brain damage subsequent to ischemia-reperfusion injury in Mongolian gerbils.

Results: In animals undergoing ischemia-reperfusion injury, neuropathological and ultrastructural changes were monitored for 1–7 days either in the presence or in the absence of M40401 after bilateral common carotid artery occlusion (BCCO). Administration of M40401 (1–40 mg/kg, given i.p. 1 h after BCCO) protected against post-ischemic, ultrastructural and neuropathological changes occurring within the hippocampal CA1 area. The protective effect of M40401 was associated with a significant reduction of the levels of malondialdehyde (MDA; a marker of lipid peroxidation) in ischemic brain tissues after ischemia-reperfusion.

Conclusion: Taken together, these results demonstrate that M40401 provides protective effects when given early after the induction of ischemia-reperfusion of brain tissues and suggest the possible use of such compounds in the treatment of neurological dysfunction subsequent to cerebral flow disturbances.

Background

Transient or permanent restriction of cerebral blood flow results in ischemic stroke, the third leading cause of death in industrialized countries. Many pathophysiological

mechanisms involved in post-ischemic damage of nervous tissues have been widely explored over the last few years in order to develop selective and more suitable strategies for the treatment of ischemia/reperfusion-related neurological disorders [1–6]. In particular, mounting evidence suggests that a crucial role in triggering and maintaining the post-ischemic insult to brain tissues is represented by the oxidative stress which follows the reperfusion phase of stroke. Reperfusion of the ischemic brain, excessive release of excitatory amino acids, such as glutamate, and infiltration by neutrophils are major sources of reactive oxygen species (ROS) generation. These, in turn, amplify the profound imbalance found in the neurons and astroglial cells of the ischemic core and penumbra [7]. In particular, the reaction of superoxide anions with nitric oxide (NO), leads to the formation of peroxynitrite ($ONOO^-$) [8], a powerful damaging oxidant and nitrating agent, which may induce many of the permanent ultra structural changes of ischemic brain tissue [9,10].

Despite the large amount of evidence showing the clear relationship between oxidative stress and post-ischemic brain damage, neuroprotection by free radical-scavenging molecules *in vivo* is not as straightforward as that observed when using *in vitro* models. In particular, superoxide dismutase (SOD) as well catalase, the catalytic scavengers for superoxide anions or hydrogen peroxide respectively, while producing a significant neuroprotective effect *in vitro*, were found unsatisfactory when used in experimental stroke models *in vivo* [11–14]. The reasons for such failures are not clear, but could be related to various factors including rapid clearance, lack of cellular penetration, short half-life and lack of blood brain barrier penetration [15–17]. On the other hand, these enzymes show modest protective effects when treatment is administered before ischemia, but little to no protection in a delayed-treatment protocol [14].

To overcome some of the limitations associated with the native SOD enzymes, low molecular weight synthetic enzymes possessing improved cellular and tissue penetration, with wide organ bio-distribution have been identified. One of these, EUK 134, a low molecular weight salen manganese complex was found to be protective when given in a rat model of focal cerebral ischemic without reperfusion [18]. Since EUK 134 possesses both SOD and catalase activity, these results suggest a role for both superoxide and hydrogen peroxide in brain damage evoked by ischemia without reperfusion.

Recently, a class of non peptidic low-molecular weight compounds proven to possess comparable catalytic activity to that of the native superoxide dismutase (SOD) enzymes has been reported, and the use of these compounds has been suggested for assessing an improved therapeutic approach in diseases mediated by superoxide overproduction [19,20]. In particular, evidence exists suggesting that M40401 [21], an SOD mimetic compound able to gain access to brain tissues, produces protective effect against oxidative damage generated in the brain of paraquat-treated rats, when given peripherally [22]. These new SOD mimetics represent a breakthrough in chemical design in that they are stable *in vivo*, possess high activity, and are selective for superoxide with no activity toward H_2O_2, peroxynitrite, nitric oxide, or hypochlorite [23–25].

The present experiments have been carried out in order to study 1) the possible role of superoxide in electrocortical and ultra structural changes subsequent to brain damage associated with ischemia and reperfusion induced by unilateral occlusion of carotid artery (BCCO) in Mongolian gerbils, and 2) the protective effect of a SOD mimetic (M40401) which lacks catalase activity against ischemia-reperfusion brain damage.

Results

Effects of ischemia-reperfusion in the hippocampus of Mongolian gerbils

Induction of BCCO in Mongolian gerbils produced significant early neuropathological and ultrastructural changes, which were followed by significant neuronal loss within the CA1 area of gerbil's hippocampus. In particular, histological and ultrastructural evaluation carried out at day 1 after induction of brain ischemia showed significant incidence, compared to sham operated animals (n = 10; Figure 1A), of eccentric pycnotic nuclei, intracellular oedema and vacuolar degeneration (n = 10; Figure 1B) found in CA1 and partially in CA2 areas of gerbil's hippocampus. CA3, CA4 and Dentate gyrus (DG) showed a normal cytoarchitecture (n = 10; not shown). Furthermore, the examination of brain tissues by electron microscopy carried out 1 day after the ischemic insult showed, within CA1 area of the hippocampus of gerbils, the presence of vacuolated neurons with irregular shaped nuclei composed of euchromatin with margination of heterochromatin; in a few cells, large electron-light vacuoles occupied the entire cytoplasm and the cell shape was not maintained. Moreover, the mitochondria were dense and disorganized. In the same area, a few glial cells showed a suffering phenotype characterized by dispersion of the chromatin into the cytoplasm due to the absence of the nuclear membrane, nucleoli still prominent, dense cytoplasm with small vacuolization and endoplasmic reticulum preserved; the plasma membrane was not always visible and often interrupted and in the extracellular space myelinic fibers with alteration of the myelinic structure are visible (n = 10; Figure 2A,2B,2C). These features were not visible in sham animals where neurons and glial cells showed a normal cytoarchitecture.

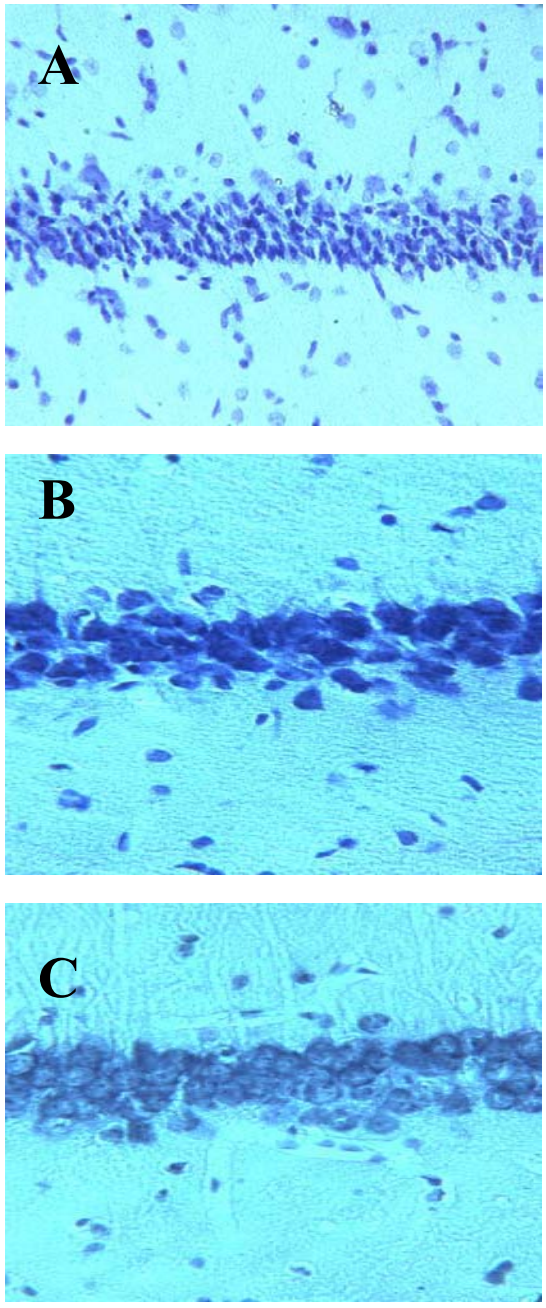

Figure 1
Temporary (5 min) BCCO in Mongolian gerbils leads to damage in the hippocampal CA1 area, compared to sham-operated animals (A), characterized by neurons showing eccentric pycnotic nuclei, intracellular edema and vacuolar degeneration (B). M40401 (40 mg/Kg) given i.p. 1 h after BCCO protected against ischemia-reperfusion hippocampal early lesion (C)

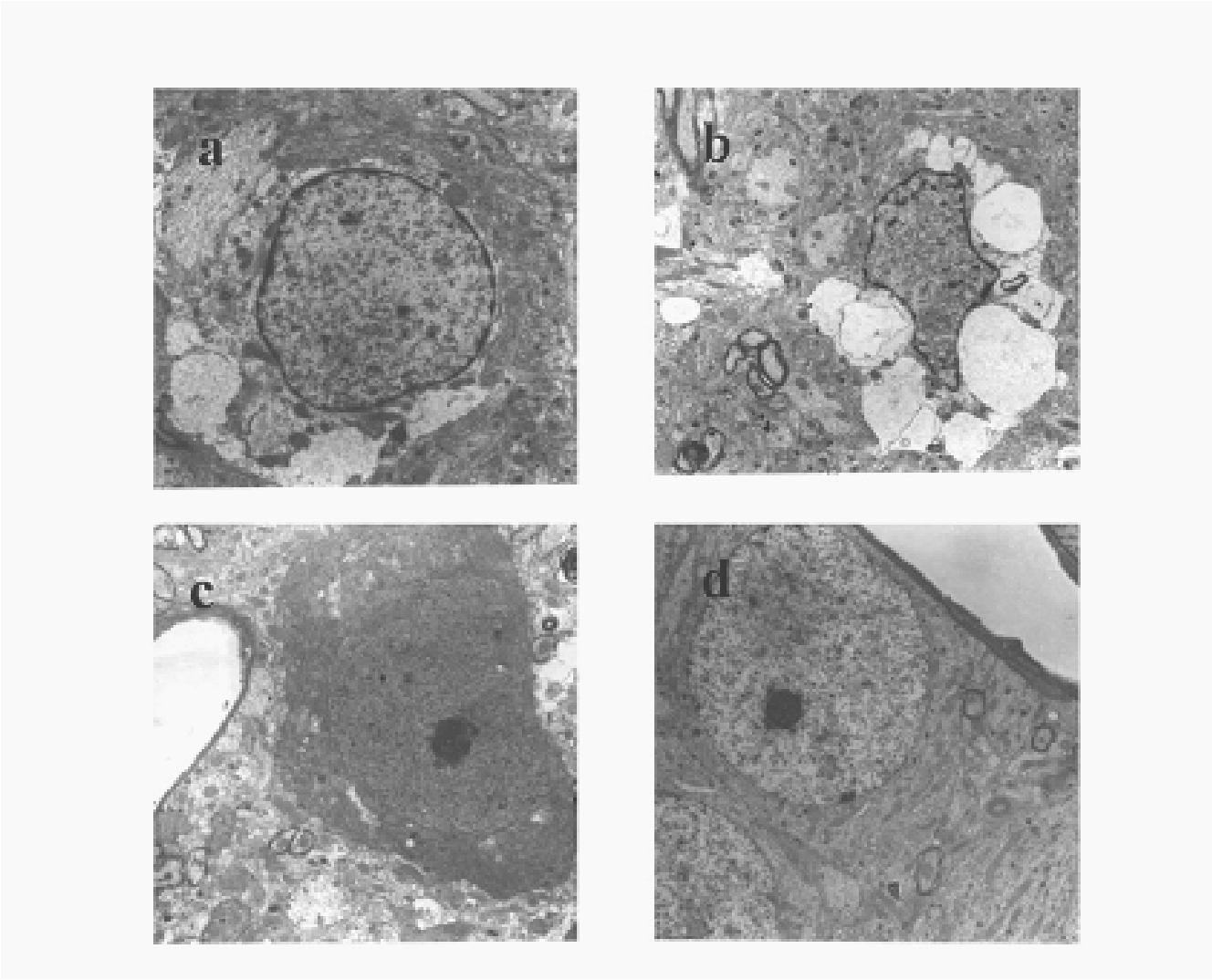

Figure 2
(A-C): ischemia + 1 day recovery: prominent ultrastructural damages of CA1 area of the hippocampus in the site of carotid artery occlusion. In particular, are displayed vacuolated neurons with irregular nuclei and margination of the heterochromatin (pre-apoptotic neurons). Large electron light vacuoles occupy the cytoplasm and the mitochondria are dense and disorganized. In many cells, vacuoles occupy the entire cytoplasm, cell shape is not maintained and the chromatin is dispersed into the cytoplasm (see A). (magnification × 3800). (D) M40401 (40 mg/kg) protected against damage. Indeed, the micrographs show the presence, in the hypothalamic CA1 area of normal neurons with large nuclei with euchromatin and prominent nucleoli. In addition, developed mitochondria, rough endoplasmic reticulum and normal cytoskeleton components are distinguished. In the extracellular space are found preserved myelinic fibers and blood vessels. (magnification × 3800)

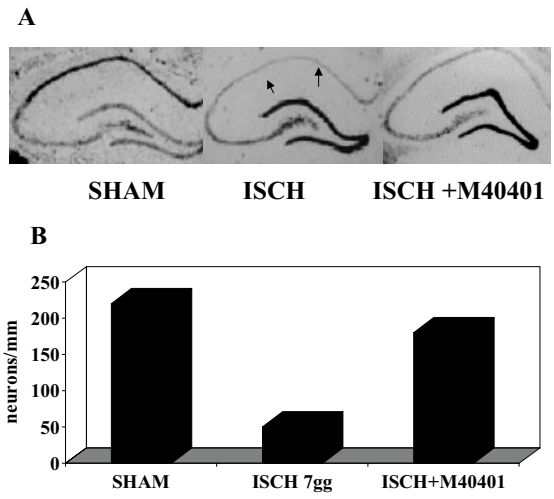

Figure 3
Panel A. Representative histological examination of brain hippocampus of Mongolian gerbil's showing significant neuronal loss within CA1 area, compared to sham operated animals, when evaluated 7 days after induction of BCCO. Panel B shows quantitative analysis for CA1 hippocampal neurons detected in sham-operated and BCCO-operated animals either untreated or treated with M40401. In particular, M40401 (40 mg/Kg given i.p. 1 h after BCCO; n = 10) showed protective effect against ischemia-reperfusion-induced reduction of neuronal cell number detected within CA1 hippocampal area.

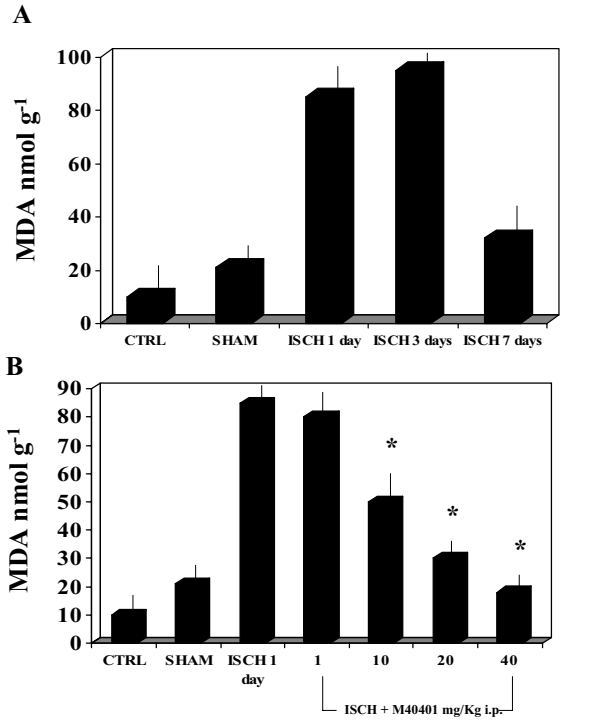

Figure 4
Time-dependent elevation of malondialdehyde levels (MDA, nmol/g tissue) within the hippocampus of Mongolian gerbils (A). The increase of MDA found 1 day after BCCO was attenuated dose-dependentently by M40401 (1–40 mg/kg; n = 10 for each dose; B). * $P < 0.05$ ischemic *vs* M40401-treated gerbils.

The early post-ischemic lesion seen 1 day after BCCO was accompanied by loss of neurons within CA1 area when evaluated 7 days after induction of global ischemia. Indeed, in a group of 10 animals undergoing BCCO, histological examination of brain slices carried out at the day 7 after brain ischemia showed significant reduction in the number of neurons detected within CA1 area of gerbil's hippocampus compared to sham operated animals (n = 10; Figure 3A and 3B).

Effects of M40401

Administration of M40401 (1–40 mg/Kg i.p. 1 h after BCCO; n = 10 for each dose), produced a significant and dose-dependent reduction of neuropathological and ultrastructural early changes within the hippocampus of gerbils undergoing ischemia-reperfusion (Figure 3B and 4D). Thus, the administration of M40401 (1–40 mg/Kg given i.p, after BCCO, n = 10 for each dose) in Mongolian gerbils reduced by 18 ± 4, 36 ± 2, 72 ± 4 and 100%, respectively, the number of cells showing ultrastructural changes in the hippocampus found 1 days after induction of BCCO. Sham operated Mongolian gerbils treated with 1–40 mg/kg i.p. of M40401 (n = 10 for each dose) did not show any significant histopathological and ultrastructural modification when compared to sham non-treated Mongolian gerbils (data not shown). Results showing lack of BCCO-related early damage in the hippocampal area after the highest dose of M40401 (40 mg/kg, i.p, n = 10) are shown in Figure 1C and 2D. In addition, M40401 (40 mg/Kg given i.p. 1 h after BCCO; n = 10), lead to protective effect against neuronal loss seen 7 days after BCCO within the CA1 area of the gerbil's hippocampus (Figure 3A and 3B).

Effect of M40401 in the post-ischemic elevation of MDA in brain tissues

Ischemia-reperfusion was followed by significant elevation of the concentration of MDA in ischemic brain, mainly within the hippocampus. This effect was observed

3–6 h after brain ischemia, peaked 1–3 days after occlusion then decreased toward the baseline value (Figure 4A). M40401 (1–40 mg/kg; Figure 4; n = 10 for each dose), dose-dependently attenuated the increase of MDA levels detected in the hippocampus 1 day after BCCO suggesting that, in this model, the SOD mimetic was able to reduce lipid peroxidation in injured brain (Figure 4B).

Discussion

It is known that ischemia-reperfusion of brain tissue is followed by an increased production of reactive oxygen species (ROS) (mainly superoxide anion, hydroxyl radical and hydrogen peroxide) which, in turn, participate in the mechanisms leading to post-ischemic neuronal cell death and apoptosis of neuronal and non neuronal cells [26,27]. Importantly, accumulation of free fatty acids and adenine nucleotides has been described over the ischemic period after both global and focal ischemia in the brain. Thus, during reperfusion, metabolism of free fatty acids (via cycloxygenase and lipoxygenase activation) and of adenine nucleotides (via xanthine oxidase activity) leads to ROS overproduction [8,10,28–31]. This is further accompanied by leukocyte infiltration, which generates both ROS and nitric oxide which, in turn, contribute to the generation of highly reactive and neurotoxic nitrogen free radical species, such as peroxynitrite [8,10].

The antioxidant status of the tissue affected by ischemia-reperfusion is of great importance for the primary endogenous defense against the free radical-induced injury. In particular, evidence exists that the SOD activity in serum is reduced in stroke patients, and replacement of antioxidant activity could be beneficial in the acute treatment of cerebral ischemia [32]. In addition, it has been suggested that O_2^- overproduced in a mitochondrial compartment, when uncoupled from antioxidant defenses, induces impairment of mitochondrial function and causes exacerbation of cerebral infarction after ischemia [33]. Moreover, it has also been shown that the endogenous antioxidant activity is differentially affected by the intensity of ischemic challenge and this suggests that the regional effects of ROS vary substantially following ischemia-reperfusion [34]. Thus, changes in the antioxidant status of brain tissues before, during and after ischemia-reperfusion greatly affects post-ischemic brain damage and has been widely studied in the last few years. In fact evidence has been acquired showing that extracellular superoxide dismutase deficiency worsens outcomes from focal cerebral ischemia in the mouse [35]. In particular, it has been shown that delayed cell injury after transient global ischemia is exacerbated in mutant mice deficient in Cu/Zn SOD [36]. Also, evidence exists showing that reduction of Cu/Zn-superoxide dismutase activity exacerbates neuronal cell injury, apoptotic cell death and edema formation after transient focal cerebral ischemia [37]. An enhanced expression of endogenous antioxidants significantly attenuates ischemia-reperfusion injury of brain tissues, however, the use of SOD enzyme is limited by its pharmacological profile [33,38–43].

On the basis of this evidence, it is likely that an additional supply of antioxidant moieties over and above the natural SOD enzyme levels is crucial for a selective protection of brain tissue against ischemic injury [43,44]. In particular, the discovery that metalloporphyrin complexes of Mn and Fe that possess the capacity to destroy superoxide, peroxynitrite, and hydrogen peroxide show efficacy in models of disease states involving free radical overproduction has heightened interest in the development of novel antioxidants which have been found to possess selective catalytic superoxide dismutase mimetic activity [19,20,22,23,45]. In the present experiments we demonstrate that M40401, one of the most active non-peptidic SOD mimetic reported, produces a relevant and significant protective effect against neuropathological and ultrastructural changes elicited by temporary BCCO in Mongolian gerbils. This confirms the involvement of superoxide-overproduction in ischemia-reperfusion injury and suggests a potential therapeutic role of such free radical scavengers in early stages of the retrograde and anterograde spread of neurotoxicity. The evidence that M40401 produces a significant protection of brain tissues against post-ischemic brain injury is relevant since this indicates that this compound reaches concentrations in the brain able to develop pharmacological SOD mimetic activity when given peripherally. Antioxidant effect at the level of the brain was in fact confirmed by the reduction of lipid peroxidation as shown by the reduction of MDA formation in brain tissues of ischemic Mongolian gerbils treated with M40401.

In conclusion, our results indicate that low molecular weight synthetic SOD mimetic which lack catalase activity may represent a novel and potentially useful approach in the treatment of many neurodegenerative disorders, such as ischemia/reperfusion brain injury, in which an abnormal release of free radicals has been shown to occur.

Methods

Bilateral Common Carotid Artery Occlusion and electrocortical recordings

Adult male Mongolian gerbils (*Meriones unguiculatus*, 50–70 g; Charles River, Milan, Italy), housed in a temperature (20°C) and humidity (60%) – controlled colony room, were used for this study. The colony was maintained in a 12 h light/dark cycle with light on at 7:00 a.m. and both laboratory food and tap water were available *ad libitum*. Under chloral hydrate (400 mg/Kg i.p.) anesthesia, 4 stainless steel wire electrodes were chronically implanted onto each fronto-parietal cortex under stereotaxic

guidance; the ground electrode was anchored to the nasal bone. Animals were allowed one week recovery before testing. Before experiments, the animals were placed individually in a perspex cage and allowed 30 min acclimatization to the new environment. Electrocortical (ECoG) recordings were made via an EEG machine (Mod. ERA-9; OTE Biomedica, Florence, Italy). A 30 min period of EcoG recording under basal conditions, as well as before induction of brain ischemia, was taken as basal value. Brain ischemia was induced by temporary bilateral occlusion of the common carotid artery (BCCO). Under anesthesia with ether, the common carotid arteries were dissected via a ventral neck incision and occluded with ligatures for 5 min (day 0). Then, the ligatures were removed, the skin incision sutured and animals were allowed to recover. A complete flattening of ECoG activity, was found for about 10 minutes after BCCO indicating the occurrence of brain ischemia. Animals that displayed no post-ischemic ECoG flattening were discarded from the study. Rectal temperature was monitored and kept between 36°C and 37°C during surgery by means of a heating pad. In addition, mean arterial blood pressure (MABP) and blood gases were evaluated immediately before, during, 5 minutes and 1, 2, 4 and 6 hours after BCCO either in untreated or M40401-treated gerbils.

Neuropathological studies

1, 3 or 7 days after BCCO, the gerbils were re-anesthetized with pentobarbital and transcardially perfusion-fixed with 4% buffered formaldehyde (pH 7.4) after a brief rinse with saline and heparin (0.1%) at room temperature. The brains were removed, kept in cold fixative for 2 hours, and stored in phosphate-buffered saline (PBS) overnight.

Histological stainings for nerve cells were performed to evaluate general brain morphology and tissue damage. Briefly, coronal slices of the dorsal hippocampus 400 μm thick were cut with a vibratome, dehydrated in graded alcohols and embedded in paraffin. Coronal sections 6 to 7 μm were stained with cresyl violet. The sections were coverslipped with permount and examined using a light microscope (Leica). Sham operated animals were used as a control group: these underwent similar handling but no BCCO was induced.

For Electron Microscopy studies, coronal sections (10 μm thick) collected every 50 microns were stained using Nissl method (cresol fast violet) and examined in a Leitz-orthoplan photomicroscope. In particular, animals were perfusion-fixed with 0.01 M PBS (pH 7.4) followed by 3% paraformaldehyde and 0.1% glutaraldehyde in 0.15 M PB (pH 7.4) at room temperature. The brain was removed, blocks of the areas of interest were made and these were postfixed in osmium tetroxide 1.33% for 2 h at 4°C. After several washes in PBS, the tissue fragments were dehydrated in graded alcohol, transferred into toluene, and embedded in Epon 812 resin. The resin was allowed to polymerize in a dry oven at 60°C for 24 h. Thin sections were cut with a glass knife Reichert microtome, stained with toluidine blue and examined on Axioscope microscope (Zeiss). Ultra-thin sections were cut on a Reichert microtome using a diamond knife, stained with uranyl-acetate-lead-hydroxide and evaluated and photographed on a Philips electron microscope CM10 (Philips, Endhoven, The Netherlands).

Malondialdehyde Determinations

Malondialdehyde (MDA; used as a biochemical marker for lipid peroxidation) was measured by a method previously described [46]. Levels of MDA were measured 1, 3 and 7 days after induction of brain ischemia in untreated or M40401-treated Mongolian gerbils. Briefly, hippocampal regions of perfused Mongolian gerbils were surgically identified, removed and then frozen in liquid nitrogen, and homogenized in potassium chloride (1.15%). Chloroform (2 ml) was then added to each homogenate and then spun for 30 min.

The organic layer of the sample was removed and dried under nitrogen gas and re-constituted with 100 μl of saline. MDA generation was evaluated by the assay of thiobarbituric acid (TBA)-reacting compounds. The addition of a solution of 20 μl of sodium dodecyl sulphate (SDS; 8.1%), 150 μl of 20% acetic acid solution (pH3.5), 150 μl of 0.8% TBA and 400 μl of distilled water, produced a chromogenic product which was extracted in n-butanol and pyridine. Then, the organic layer was removed and MDA levels read at 532 nm and expressed as nmol MDA/g wet tissue.

Drug administration and experimental groups

M40401 (Metaphore Pharm. Inc., St. Louis) was dissolved in 26 mM sodium bicarbonate buffer (pH = 8.1–8.3). M40401 (1–40 mg/kg) was given intraperitoneally (i.p.; 0.3 ml) 1 h after BCCO (day 0). ECoG changes and histopathological studies were carried out 1, 3 and 7 days after M40401 administration. The same drug administration protocol was performed in sham animals.

Selectivity of M40401

It has been shown that the pentaaza macrocyclic ligand complexes of Mn(II) can not only be highly active catalysts for the dismutation of O_2^-, but that they are also highly selective (25). In particular, evidence exists that this complex and others of this pentaaza macrocyclic ligand class, such as M40403 or M40401 do not react with hydrogen peroxide under the same conditions [25], nor do they react with other biologically relevant oxidants such as $ONOO^-$ or nitric oxide [25].

Materials

All the reagents used for this study were purchased from Sigma (Milan). M40401 was kindly provided by Dr. D.P. Riley (Metaphore Pharm. Inc, St. Louis, Mo, USA).

Statistics

Results are expressed as means ± s.e.m. for n experiments. The results were analysed independently by at least three observers and Student's unpaired t test was used to determine the significance between means. A P value of < 0.05 was taken as significant.

Author's contributions

VM conceived the study and participated in the sequence alignment and drafted the manuscript. MI carried out the surgery and the immunohistochemistry and participated in the drafted of the manuscript. CM carried out the surgery and biochemical analysis and conceived the study. EP participated in the sequence alignment and performed the statistical analysis. TG and AM carried out the electron microscopy studies. RN participated in the design of the study. DR and DS participated din the design of the study and its coordination.

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