Salen Mn Complexes are Superoxide Dismutase/Catalase Mimetics that Protect the Mitochondria

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Abstract: Salen Mn complexes, including EUK-134, EUK-189 and a cyclized analog EUK-207, are synthetic superoxide dismutase (SOD) and catalase mimetics that are beneficial in many models of oxidative stress. Though not designed to target the mitochondria, salen Mn complexes show "mito-protective" activity, that is, an ability to attenuate mitochondrial injury, in various experimental systems. Treatment with EUK-134 prevents respiratory chain abnormalities induced by ionizing radiation in rat astrocyte cultures. Treatment with salen Mn complexes also prolongs survival, protects mitochondrial enzymes and prevents oxidative pathologies in Sod2-/- mice, which lack the mitochondrial form of superoxide dismutase. Recently, EUK-207 was shown to attenuate ischemia reperfusion injury, including mitochondrial dysfunction, in hearts from *ABC-me*-/+ mice, which are deficient in a mitochondrial transporter and more vulnerable to oxidative stress. Since mitochondrial dysfunction has been implicated in many forms of injury and degeneration, this "mito-protective" property may explain some of the cytoprotective effects of salen Mn complexes *in vivo*, and may also enhance their potential therapeutic value.

Keywords: Antioxidant, mitochondria, mito-protection, superoxide dismutase mimetic, catalase mimetic, Mn ligand complex.

INTRODUCTION: OVERVIEW OF THE BIOLOGICAL EFFECTS OF SALEN MN COMPLEXES *IN VITRO* AND *IN VIVO*

Salen Mn complexes are a class of synthetic low molecular weight agents that mimic the antioxidant enzymes superoxide dismutase (SOD) and catalase, scavenging superoxide and hydrogen peroxide, respectively [1, 2]. Data, including structure-activity relationship findings *in vitro* and *in vivo*, indicate that the hydrogen peroxide scavenging property, namely catalase activity, is a more important parameter than SOD activity in determining certain cytoprotective effects of salen Mn complexes, though other factors such as pharmacokinetics and cytotoxicity also play a significant role [1]. In addition, it has been shown that salen Mn complexes can scavenge reactive nitrogen species (RNS) through mechanisms analogous to their catalase activity [3], a property also of potential relevance to their cytoprotection, including their ability to attenuate protein nitration in oxidative injury models [4, 5]. Overall, this combination of properties (i.e. low molecular weight, catalytic scavenging mechanism, and activity against multiple damaging species) provides advantages for salen Mn complexes over other antioxidants, such as noncatalytic ROS scavengers or proteinacious antioxidant enzymes [6, 7]. Prototype salen Mn complexes EUK-8 and the more active catalase mimetics EUK-134 and EUK-189 (Fig. **1**) were originally described and shown to be cytoprotective in various systems [1, 2, 8]. EUK-207 (Fig. **1**) is a "second generation" cyclized salen Mn complex that has catalytic properties equivalent to EUK-134 and EUK-189, but was designed for greater stability [2, 9, 10] and shows a longer plasma half-life in rats [11]. Salen Mn complexes have shown beneficial effects in many *in vivo* models for several neurodegenerative disorders [12-17], stroke and other forms of excitotoxic and ischemic injury [4, 18, 19], radiation injury [11, 20, 21], endotoxemia [22], and age-related impairments [9, 23-25]. Most recently, EUK-207 was found to mitigate delayed radiation injury to the lung [11, 26], kidney and skin [11] in rats and to protect from cardiac ischemia-reperfusion in mice [27]. In many of these efficacy models, salen Mn complexes were not only functionally protective, but also suppressed biochemical indicators of oxidative stress, such as oxidative modifications of protein, lipids and nucleic acids [4, 9, 12, 22, 23, 26, 27]. A summary of the antioxidant effects of salen Mn complexes in various experimental models is given in Table **1**. While most of these previous studies did not focus on the mitochondria, the Table does include one example of a lipid peroxidation measurement in isolated cardiac mitochondria, which was decreased in hearts treated with EUK-207.

Many disorders involving oxidative stress also involve inflammation, and inflammatory cells are a well-established source of damaging reactive oxygen species (ROS) [8]. Increasingly, however, the mitochondria, and particularly when dysfunctional, are also regarded as being major sources of ROS. A proportion of the oxygen consumed during normal respiration is converted to superoxide within the mitochondria, with various studies yielding estimates of 0.15 to 4% [28-32]. Though it has been suggested that some of these values over-estimate the *in vivo* situation [33], it is nonetheless clear that substantial levels of ROS can be produced by

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mitochondria, especially in highly metabolic tissues such as the brain, heart and muscle. Mitochondrial dysfunction, resulting from genetic modification or damage, can lead to more ROS production or can otherwise expose the vulnerability of cells to endogenous oxidative stress. Key mitochondrial enzymes, including respiratory chain components and certain tricarboxylic acid cycle enzymes are themselves vulnerable to oxidative injury [34]. Thus, the mitochondrion is not only a source, but also a likely target of oxidative injury. It is a reasonable hypothesis, therefore, that a cycle of mitochondrial injury/oxidative stress can contribute to chronic injury and degeneration. There is, indeed, much evidence implicating oxidative stress and mitochondrial dysfunction in many neurodegenerative diseases and in aging and age-associated decline [35-44]. There is also some evidence suggesting that mitochondrial damage is a significant contributor to some forms of radiation-induced injury [45-47].

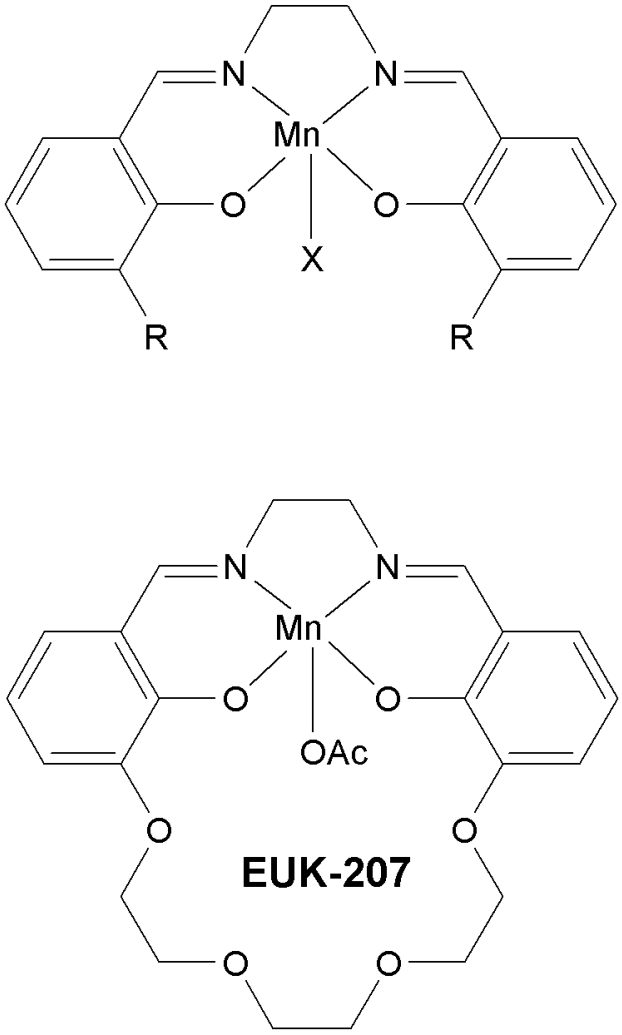

Fig. (1). Structures of salen Mn complexes: EUK-8, EUK-134, EUK-189 and EUK-207. SOD and catalase activities, cytoprotective, and other properties of EUK-207 [2, 10] and the non-cyclized salen Mn complexes [1] have been described. EUK-8, R=H, X=Cl; EUK-134, R = methoxy, X = acetoxy; EUK-189, R = ethoxy; X = acetoxy; EUK-207: OAc = acetoxy

Table 1. Summary of Antioxidant Effects of Salen Mn Complexes in Experimental Models

Catalytic ROS scavenging activities *in vitro*:
• Superoxide and hydrogen peroxide [1,18,22].
• Nitric oxide and peroxynitrite [3].
Decreases in biological samples from animal models:
• DCFH-DA fluorescence [24].
• Lipid peroxidation (malonyldialdehyde) [9. 12, 22-24, 27].
• Lipid peroxidation (F_4-neuroprostanes and F_4-neurofurans) [13].
• Protein nitration (3-NT) [4,12].
• Activation of ROS-associated transcription factors [4,16,23].
• GSSG/GSH [23].
• Nucleic acid oxidation (8-OHdG) [9,24,26].
• Protein oxidation (protein carbonyls) [9,12].
• SERCA oxidation (Cys^{674} sulfonylation) [27].

Treatment with salen Mn complexes, in addition to causing functional improvements in a variety of experimental models, also decreases biochemical indicators of oxidative or nitrosative stress. This Table summarizes examples such antioxidant effects. Unless otherwise indicated, the measurements were made on tissue homogenates or sections from the animal injury models. In one case (malonyldialdehyde measurement in reference [27]) oxidative injury in isolated cardiac mitochondria was the endpoint.

To address disorders in which the mitochondria are implicated, a number of agents, particularly antioxidant compounds, have been designed to specifically target the mitochondria [46, 48-50]. Generally, the approach has involved linking an antioxidant moiety to a lipophilic cation, with the membrane potential of the mitochondria enabling sequestration of the antioxidant. Unlike such agents, the salen Mn complexes were not designed to be targeted to the mitochondria. Many of the ligands of lead salen Mn complexes, including EUK-189 and EUK-207, are neutrally charged and, in addition, the compounds are only slightly lipophilic [51]. Interestingly, Keaney *et al.* [52] found that a mitochondrial preparation from C elegans that had been administered the salen Mn complexes EUK-8 or EUK-134 showed elevated SOD activity, whereas the cytosolic fraction did not. While promising, these data must be interpreted carefully because many Mn-containing compounds, including possible breakdown products of the salen Mn complexes, have SOD activity. Catalase activity, indicative of intact salen Mn complexes of selected structures [1], was found not to be elevated in the treated nematodes. Thus, while the Keaney *et al.* report is intriguing, no study has, to date, directly addressed accumulation of intact salen Mn complexes, versus, for example, breakdown products, in the mitochondria. Regardless, in addition to their broader antioxidant effects, salen Mn complexes have shown substantial protective efficacy in various models for mitochondrial injury/dysfunction, indicating that they have sufficient subcellular accessibility to afford significant "mito-protection". In the absence of evidence for specific localization of salen Mn complexes to the mitochondria, it is possible that their ability to protect mitochondria is indirect. For example, they might neutralize total cellular ROS during injury scenarios, thereby protecting the mitochondria as well as other organelles from oxidative in-

jury. The ability of salen Mn complexes to suppress a breadth of oxidative effects *in vivo* (Table **1**) is consistent with this hypothesis, though it does not eliminate the possibility that the compounds, at least to some extent, also directly access the mitochondria.

SALEN MN COMPLEXES ATTENUATE RADIATION-INDUCED MITOCHONDRIAL INJURY IN RAT ASTROCYTES AND MITIGATE DELAYED RADIATION INJURY *IN VIVO*

Radiation-induced injury to normal CNS tissue is of significant concern and is the major dose-limiting factor in the treatment of gliomas and other head and neck tumors. The CNS damage resulting from irradiation is most often characterized by vascular abnormalities, demyelination and, ultimately, necrosis, which can be expressed years after radiotherapy [53]. Although this form of radiation-induced normal tissue injury has been well described in terms of histological and functional criteria [54, 55], its pathogenesis remains poorly understood. Delayed cell death and tissue injury can result from a number of reactive processes, but accumulating data suggest that an initiating source of these delayed effects can be mitochondrial dysfunction. CNS lesions following radiation share some similarity to other CNS injuries, such as trauma and ischemia, that have been associated with mitochondrial damage [56]. The ultimate consequences of mitochondrial injury include insufficient ATP production and increased generation of ROS and the CNS, with its high energy demands and rate of oxidative metabolism, is particularly susceptible. Astrocytes are the most prevalent cell type in the CNS and play a critical role in the protection of endothelial cells, oligodendrocytes and neurons from oxidative or excitotoxic injury [57-59]. Given their key functions, it would be expected that the response of astrocytes to radiation would have a major impact on the radiation response of the CNS as a whole. Therefore, rat astrocyte cultures were used to investigate the role of mitochondrial dysfunction in CNS radiation injury. As described previously [11], these cells, irradiated at 5, 10, or 30 Gy, showed no significant loss of viability, consistent with their non-proliferative state, but developed mitochondrial abnormalities, particularly at the higher radiation doses. These abnormalities included decreased ATP levels and increased mitochondrial mass per cell both known consequences of mitochondrial impairment (Fig. **2**). The irradiated astrocytes also developed changes in respiratory chain activities, namely decreases in complex II and III and, atypical of other forms of mitochondrial toxicity, an increase in complex I. When given to the cells immediately after 30 Gy irradiation, the salen Mn complex EUK-134 attenuated, in a dose-dependent manner, radiation-induced effects on respiratory complex activities (Fig. **3**). This effect was significant even when EUK-134 (50 μM) was added to cultures up to 12 hr after irradiation (30 Gy) (Fig. **4**). These data provide direct evidence for radiation-induced non-lethal mitochondrial abnormalities in astrocytes, potentially sufficient to cause sustained ROS production and other injurious effects. The ability of EUK-134 to normalize respiratory chain activities in irradiated astrocytes strongly suggests that salen Mn complexes are "mito-protective" in this context, and may have value in mitigating radiation injury to the mitochondria. More research is needed to determine whether salen Mn complexes protect the mitochondria directly from oxidative damage or indirectly via suppression of total cellular oxidative stress, or act via a combination of both. Prior data showing that expression of antioxidant enzymes in the mitochondria decreases cellular radiation sensitivity [47, 63] do, however, support the likely importance of mitochondrially located antioxidants in mitigating radiation injury. Whether the findings with salen Mn complexes in astrocyte cultures extend to key target cells for delayed radiation injury in other tissues remains to be investigated. However, Vorotnikova *et al*. [60] showed that several compounds with SOD and catalase activities, including the salen Mn complexes EUK-189 and EUK-207, inhibited radiation-induced apoptosis in bovine adrenal capillary endothelial cell cultures. More recently, EUK-207 was found to prevent several radiation-induced injuries in human microvascular endothelial cell cultures [61]. These findings may be relevant to the potentially broad applicability of these agents as radiation mitigators, since the vascular endothelial cell is regarded as being an important target for radiation injury in several normal tissues [62]. Overall, the ability of salen Mn complexes such as EUK-189 or EUK-207 to mitigate radiation injury in a number of *in vivo* systems [11, 20, 26] may relate to their "mito-protective" properties, but this hypothesis remains to be tested. Whether the delayed mitochondrial injury occurring after radiation in astrocytes is a consequence of oxidative stress is not yet proven, though its mitigation by EUK-134 does support this possibility. Also consistent with a role for oxidative stress in this mitochondrial injury are findings, noted above, that various strategies to increase expression of antioxidant enzymes (SODs or catalase) in the mitochondria decreases cellular sensitivity to radiation [47, 63]. In addition, a mitochondrial-targeted nitroxide antioxidant decreased radiation sensitivity and mitochondrial lipid oxidation in irradiated mouse embryonic and human epithelial cells [46]. While mechanisms whereby intracellular oxidative stress would be increased after irradiation are not fully understood, these findings have, overall led to the speculation that irradiation of cells causes a delayed oxidative injury to mitochondria that, in turn, generate increased ROS, mediating a cycle of chronic oxidative injury.

SALEN MN COMPLEXES PROLONG SURVIVAL AND ATTENUATE CNS AND OTHER MITOCHONDRIAL INJURIES IN *SOD2-/-* MICE

As discussed above, ionizing radiation causes mitochondrial injury that is mitigated by salen Mn complexes, but the mechanism of such injury, including the role of oxidative stress, is not yet clear. However, salen Mn complexes have also been found "mito-protective" in mouse models involving deliberate induction of mitochondrial impairment. One such approach relies upon the deletion of a key mitochondrial antioxidant enzyme. Endogenous antioxidant defenses [8] include three types of SOD enzymes. The cytosolic and extracellular forms (SOD1 and SOD3, respectively) are Cu and Zn containing enzymes. The mitochondrial form (SOD2, or MnSOD) instead has Mn in its active site. Mice lacking SOD2 (*Sod*2-/- mice) develop severe, lethal pathologies attributable to severe mitochondrial oxidative injury [64-66]. Treatment of one such strain (on a CD1 background, $Sod^{2tm1Cje}$ - *Sod*2 nullizygous mice) [65] with daily injections

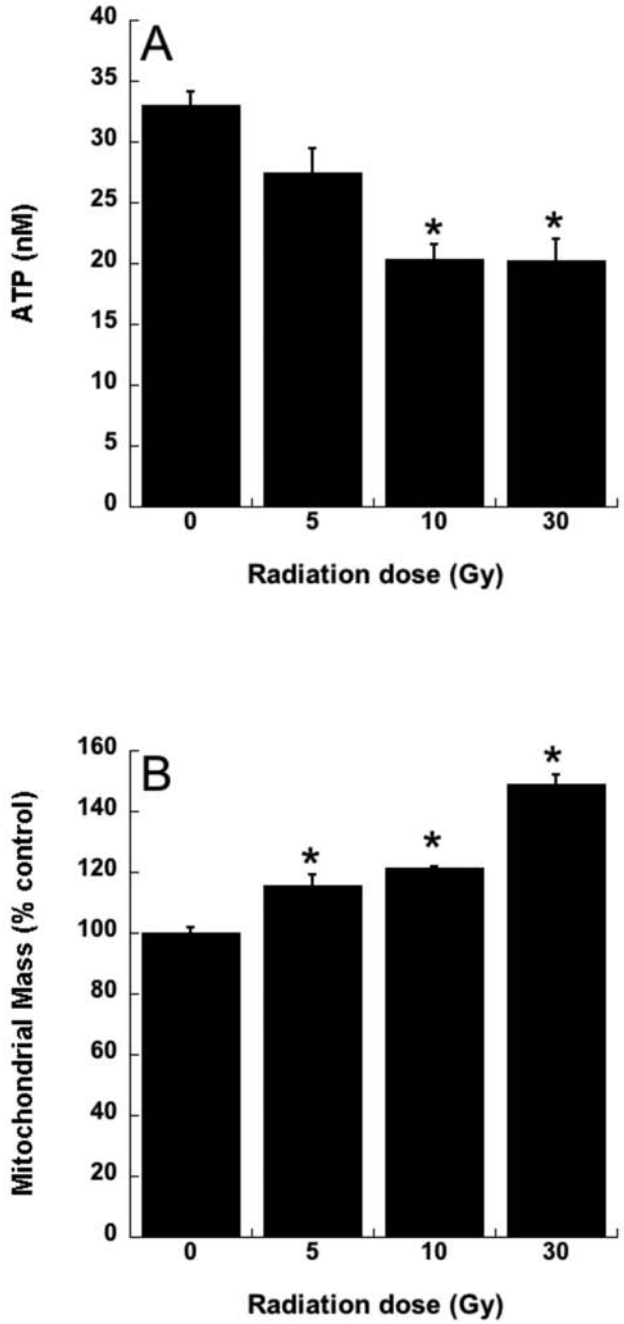

Fig. (2). Mitochondrial impairments caused by ionizing radiation in astrocytes. Rat astrocytes were cultured and exposed to ionizing radiation and mitochondrial impairments monitored as described [11]. A. ATP levels, assayed 4 days after irradiation using the luciferin/luciferase assay. B. Mitochondrial mass, assayed 4 days after irradiation, using MitoTracker Green fluorescence measured with flow cytometry. Data are means ± SEM for n = 3; * indicates significantly different from unirradiated group ($p<0.05$). Reprinted from [11].

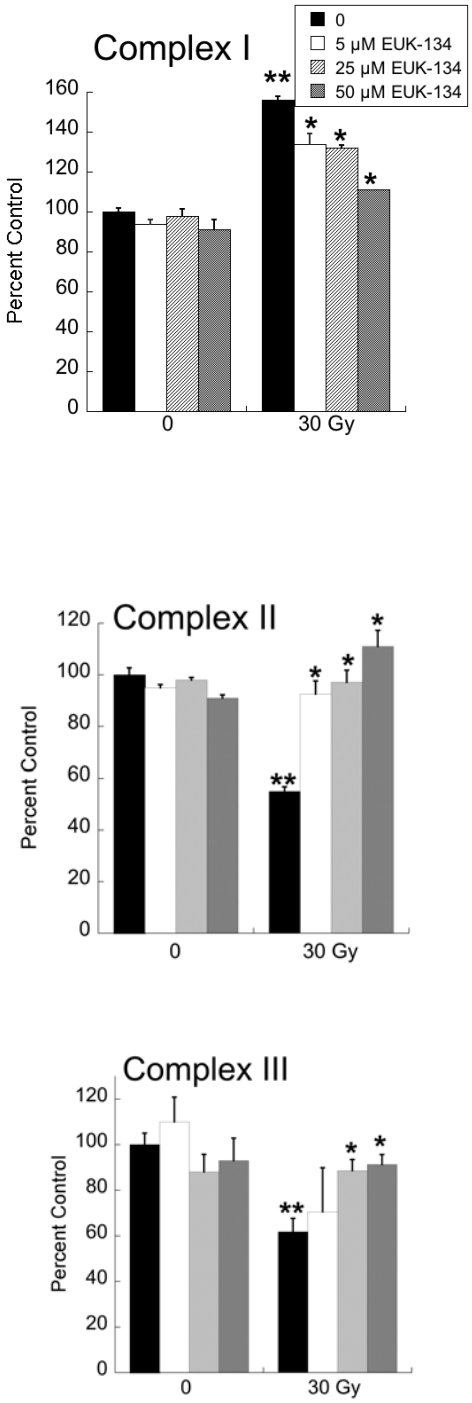

Fig. (3). Dose-dependent mitigation of respiratory chain effects in irradiated astrocytes by EUK-134. Confluent, non-proliferating astrocytes were cultured and exposed to ionizing radiation as described for Fig. **2**. EUK-134 was added immediately after irradiation, where indicated. On day 4 after irradiation, cells were harvested and respiratory chain complex activities measured as previously described for Complex I [80], Complex II [81], and Complex III [82]. Y axis indicates percent of the activity of the unirradiated control cells. Data are means ± SEM for n=3. ** indicates significantly different from unirradiated (no EUK-134); * indicates significantly different from 30 Gy (no EUK-134) ($p<0.05$). Reprinted from [11].

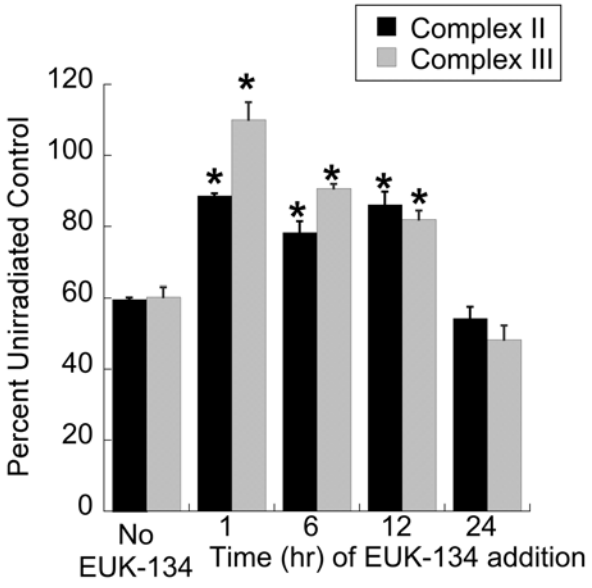

Fig. (4). Time course of EUK-134 mitigation of mitochondrial effects in irradiated astrocytes. Experiment was conducted as described for Fig. **3**, except that EUK-134 (50 uM) was added at the indicated time, in hours, after irradiation. * indicates significantly different from 30 Gy (no EUK-134). Reprinted from [11].

of salen Mn complexes prolonged survival up to 3-fold, protected mitochondrial enzymes, and prevented oxidative pathologies to brain, heart and liver [34, 67]. Salen Mn complexes were more effective than any other agent tested in *Sod*2-/- mice, namely, the Mn porphryin SOD/catalase mimetic MnTBAP, the Mn macrocyclic SOD mimetic M40403, and the mitochondrial metabolites alpha lipoic acid and L-acetyl-carnitine [2]. Subsequent studies testing a number of additional antioxidant agents (Table **2**), including two antioxidants designed to target the mitochondria [68] continue to demonstrate that salen Mn complexes are most effective at prolonging survival of *Sod*2-/- mice. This is despite the fact that many of the ineffective agents have been reported as having beneficial effects in a variety of other disease models. Of all agents tested, only salen Mn complexes and MnTBAP showed any prolongation of survival in the *Sod*2-/- mice. This strain of mice, untreated, had a median lifespan of about 8 days, exhibiting a variety of oxidative pathologies including hepatic lipid accumulation and a lethal cardiomyopathy [69]. MnTBAP treatment approximately doubled their lifespan, ameliorating dilated cardiomyopathy and hepatic lipid accumulation. In extending the life of the *Sod*2-/- mice beyond 2 weeks of age, through protection of peripheral tissues, MnTBAP uncovered a severe neurological disorder, attributable to the inability of the agent to cross the intact blood-brain barrier and protect against ROS-induced mitochondrial injury within the brain [69]. The neurological phenotype seen in the MnTBAP-treated *Sod*2-/- mice is characterized by a severe motor disturbance, with an underlying pathology of a spongiform change predominantly within the frontal cortex and focally in brainstem nuclei [69]. An analogous neurological phenotype occurs in the small percent of longer-lived "outlier" untreated *Sod*2-/- mice (Melov, unpublished) as well as in *Sod*2-/- mice on certain other genetic backgrounds [64]. In a subsequent study [67], the salen Mn compounds EUK-8, EUK-134 and EUK-189 were found to increase the lifespan of *Sod*2-/- mice even further than MnTBAP, eliminating the peripheral (not shown) and CNS spongiform (Fig. **5**) pathologies. The cause of the delayed death in the salen Mn complex treated *Sod*2-/- mice appeared to be yet another unmasked neurological syndrome, but one not involving spongiform histopathology. The cardioprotective effects of EUK-8 were subsequently confirmed by another laboratory, using a strain of mice with *SOD*2 deleted in the heart and skeletal muscle [70].

Table 2. Survival Data for *Sod*2-/- Mice Treated with Synthetic Antioxidants

Treatment (mg/kg)	N	Median Survival (Days)
Untreated	244	8
MnTBAP (5)	167	17*
EUK-8 (1)	121	19*
EUK-8 (30)	167	25*
EUK-134 (1)	24	15*
EUK-134 (30)	31	25*
EUK-189 (1)	231	19*
EUK-189 (30)	98	30*
EUK-189 (100)	5	27*
EUK-207 (30)	9	23*
M40403 (10)	17	8 - NS
ALCAR (400) and LA (150)	7	6 - NS
5% Mannitol	4	9 - NS
5% Glucose	2	6 - NS
Methylene Blue (0.1)	16	6 - NS
Methylene Blue (0.3)	12	6 - NS
Methylene Blue (3)	6	4*
Methylene Blue (30)	6	4*
MitoQ	2	11 - NS
MitoE	4	10 - NS
Idebenone (5)	4	9 - NS

These data summarize the results of Kaplan-Meier survival analysis as previously reported [67]. Statistical difference from untreated group: (* $p<0.0001$), NS - not significant. All mice were from a reduced litter size [67, 69]. All received ip injections at the indicated dose, except for LA which was given sc, and MitoQ, and MitoE, where the mother was orally gavaged with compound to administer the lipophilic compounds via the milk to the nursing pups. This table represents a cumulative update that includes previously published data [2, 67, 69], as well as unpublished data from the Melov laboratory up to June 2011.

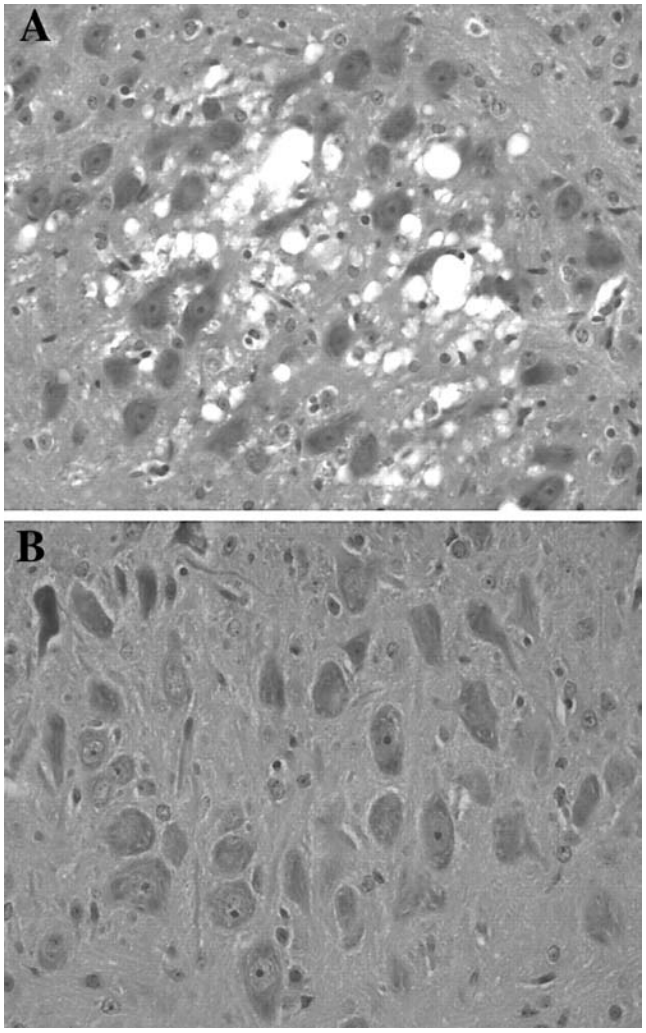

Fig. (5). Dose-dependent rescue of spongiform encephalopathy in *Sod*2-/- mice. **5A** shows the trigeminal motor nuclei (hematoxylin and eosin) (400× magnification) of a 3-week-old *Sod*2-/- mouse treated with EUK-8 at 1 mg/kg; prominent spongiform changes are seen that are consistent with those reported previously in MnTBAP-treated *Sod*2-/- mice [69]. **5B** shows the equivalent regions from a 3-week-old *Sod*2 -/- mouse treated with EUK-8 at 30 mg/kg, demonstrating the rescue of spongiform changes. While salen Mn treated mice eventually developed a severe movement disorder, even the longest lived mice receiving 30 mg/kg salen Mn complex did not show spongiform pathology (not shown). Reprinted from [67], which reports the magnification scale (400X for a single column (1/2 journal page) width illustration.

It was further noted by Melov *et al.* [67] that EUK-189 was significantly more effective than EUK-134 though both compounds have equivalent ROS scavenging activities. It is hypothesized that the greater lipophilicity of EUK-189 increased its intracellular and mitochondrial access, which could explain its greater effectiveness [67]. Consistent with this interpretation, EUK-189 is more potent than EUK-134 against an intracellular insult, staurosporine-induced cell death [71] but has equivalent cytoprotective activity against extracellular hydrogen peroxide [1]. Of further interest, EUK-8, with a lipophilicity similar to that of EUK-134, was found to be at least as effective in *Sod*2-/- mice. EUK-8 and EUK-134 have equivalent *SOD* activities, but EUK-134 is more active as a catalase, and was far more effective than EUK-8 in hydrogen peroxide cytotoxicity and rodent stroke models [1]. Such data indicate that salen Mn complexes show different structure-activity relationships in different experimental systems. It is tempting to speculate that factors such as the relative importance of superoxide and hydrogen peroxide in each experimental injury will help to determine which analogs are most effective. Subsequent testing of the more stable cyclized analog EUK-207 (Table **2**) indicated that, at the doses tested, it had an effectiveness comparable to, no greater than, the other salen Mn complexes. EUK-189 and EUK-207 show similar potency and efficaciousness in preventing age-related cognitive impairment in wild type mice [9, 24]. While EUK-207 is less lipophilic than EUK-189, it has greater stability and is more potent against staurosporine induced cell death [51]. Such observations suggest that the increased stability of EUK-207 and greater lipophilicity of EUK-189 may balance one another out, leading to comparable activities in some *in vivo* models. In an interesting contrast, EUK-207 significantly mitigates renal radiation injury in rats, while EUK-189 does not [11]. A potential reason suggested was that the mitigation of renal radiation injury may occur at the level of the vasculature, and EUK-207 has a longer plasma half-life than EUK-189. While their mechanism(s) and sites of action in the various *in vivo* injury models require further study, it is, nonetheless, clear that several salen Mn complexes show substantial protective properties against the severe oxidative mitochondrial injury occurring in *Sod*2-/- mice. Besides enhancing survival, there is biochemical evidence that salen Mn complexes act to directly protect the mitochondria in *Sod*2-/- mice. As compared to wild type mice, *Sod*2-/- mice show reduced activities of certain mitochondrial enzymes known to be sensitive to oxidative damage, including cis-aconitase [67] and respiratory chain components [34, 66]. Mitochondria isolated from EUK-8 treated, as compared to untreated, *Sod*2-/- mice showed significantly higher activities of these enzymes. These data indicate at least partial protection of these mitochondrial proteins by salen Mn complexes. With any biologically active metal-ligand complex, including salen Mn complexes, it is possible that its function *in vivo* involves delivery of metal to some crucial site of action [11], for example, supplying it to metalloenzymes. It is, therefore, interesting to note that this is an unlikely explanation for the protective effects of salen Mn complexes in *Sod*2-/- mice, which lack the *Sod*2 apoenzyme that would, presumably, be a key protective site for Mn delivery. However, the possibility that salen Mn complexes act, at least in part, through altering the homeostasis of Mn or another metal (for example, a metal displaced by Mn) does exist in this or in any other experimental system, particularly in any highly complex *in vivo* model.

THE SALEN MN COMPLEX EUK-207 FACILITATES RECOVERY FROM ISCHEMIA-REPERFUSION AND PREVENTS CARDIAC MITOCHONDRIAL INJURY IN *ABCME* +/- MICE

Oxidative stress and mitochondrial dysfunction are central mediators of cardiac dysfunction after ischemia/reperfusion [72-75]. ATP binding cassette mitochondrial erythroid (ABC-me) is a mitochondrial transporter highly induced during erythroid differentiation and predominantly expressed

in bone marrow, liver and heart [76]. Its role in the heart has been unknown until recently, but previous studies demonstrated that *ABC-me* yeast orthologue (MDL1) protected cells from increased oxidative stress [77]. Liesa *et al.* [27] recently showed that ABC-me also plays a protective role against cardiac injury, including mitochondrial damage, during ischemia/reperfusion in mice. This report also demonstrated a cardioprotective, including "mito-protective" effect of the cyclized salen Mn complex EUK-207 in ABC-me deficient (*ABC-me*+/-) mice. This study used isolated perfused hearts from *ABC-me*+/- and wild-type mice. The ABC-me heterozygotes showed a normal cardiac phenotype under basal conditions. However, when their hearts were subjected to ischemia/reperfusion, they exhibited a more severe cardiac dysfunction than those from wildtypes, as indicated by a marked decrease in recovery, during reperfusion, of systolic and diastolic left ventricular function. Furthermore, hearts from *ABC-me* +/- mice showed mitochondrial injury after ischemia reperfusion, as indicated by respiratory dysfunction and increased lipid oxidation of isolated cardiac mitochondria. Mitochondria from wild type hearts showed better respirometry after ischemia reperfusion than those from *ABC-me* +/- mice, indicating that the normal levels of ABC-me protect the mitochondria during this insult. Hearts from both strains of mice showed immunohistochemical evidence for oxidative injury to the sarcoplasmic reticulum Ca AT-Pase (SERCA), though this oxidation-dependent staining was more intense in the ABCme+/- than in the wild type hearts [27]. As summarized in (Fig. **6A**), EUK-207 pretreatment facilitated recovery of cardiac function after ischemia/reperfusion in both strains of mouse. (Fig. **6B**) shows the mitochondrial dysfunction observed in the *ABC-me*+/- hearts following ischemia reperfusion, and prevention of this dysfunction by EUK-207. As shown in (Fig. **6C**), after ischemia/reperfusion, the hearts from the EUK-207 treated ABCme-/+ mice showed greater total ATP levels than those from untreated heterozygotes, further indicative of improved mitochondrial function with EUK-207 treatment. The rate of ATP production was also greater in isolated cardiac mitochondria from the EUK-207 treated, versus untreated, *ABC-me*+/- mice after ischemia/reperfusion [27]. Taken together, the data show not only that ABC-me deficiency leaves the heart more vulnerable to oxidative mitochondrial injury caused by ischemia/reperfusion, but also that the salen Mn complex EUK-207 protects the cardiac mitochondria under this circumstance, as it did in the *Sod*2-/- mice. It is possible that some of the beneficial effects of EUK-207 in this model were not mitochondria-specific, for example: EUK-207 treatment improved recovery of cardiac function after ischemia/reperfusion in wild type mice (Fig. **6A**), while not appearing to change their mitochondrial function based on respirometry; and EUK-207 treatment inhibited the oxidation of SERCA, a sarcoplasmic reticulum protein [27]. Such findings indicate that EUK-207 could have a broader antioxidant effect though, of course, it remains possible that some of these injuries are secondary to excess ROS produced by damaged, even subtly altered, mitochondria. Nonetheless, a broader array of potential antioxidant actions by EUK-207 would be consistent with the fact that salen Mn complexes were not designed to specifically target the mitochondria but, instead, have the potential to protect cells more globally. Interestingly, the study in *ABC-me*+/- mice is consistent with an earlier study showing that the prototype salen Mn complex EUK-8 preserved cardiac function after ischemia-reperfusion in iron-overloaded rat hearts [78]. In that model, EUK-8 also prevented much of the tissue damage, including severe mitochondrial swelling and disruption that had been observed morphologically in the control iron-overloaded hearts subjected to ischemia-reperfusion (Fig. **7**).

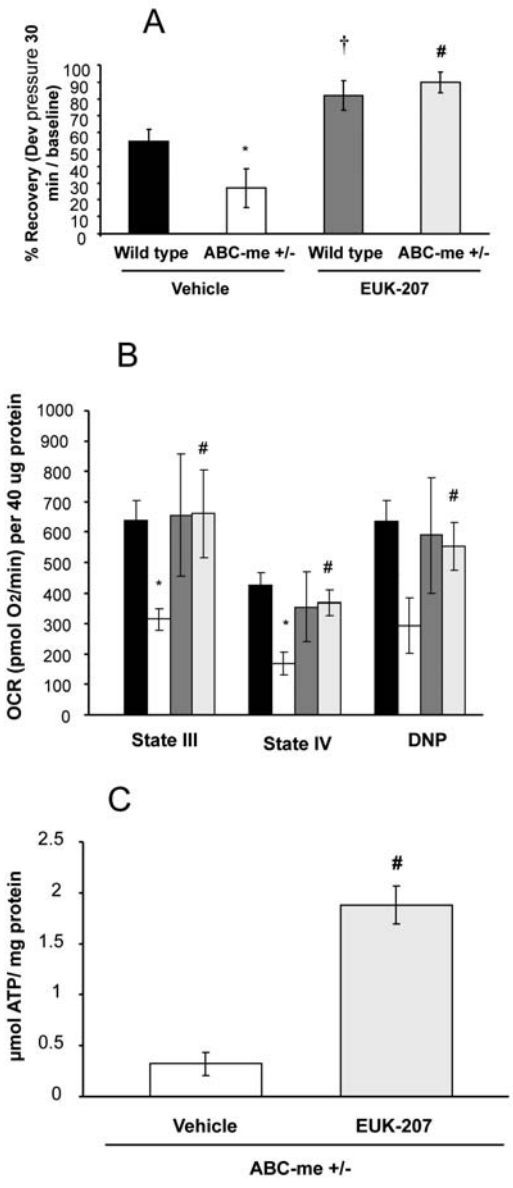

Fig. (6). EUK-207 improves cardiac and mitochondrial function following ischemia-reperfusion. Isolated perfused hearts from wild type and *ABC-me* +/- mice (with inactivation of one allele of the mitochondria-specific transporter, ABC-me [76]) were subjected to 10 min ischemia, after 20 min pretreatment with or without EUK-207 (50 uM) in the perfusate, and allowed to recover with 20 min of reperfusion. They were then analyzed for: **6A**. Cardiac function, measured by left ventricular developed pressure; **6B**. Mitochondrial function, measured as oxygen consumption (driven by complex II substrates) in isolated heart mitochondria; and **6C**. Total ATP levels in the heart tissue. State III respiration induced with ADP, State IV with oligomycin (inhibiting ATP synthase) and uncoupled respiration with the proton uncoupler 2,4-dinitrophenol (DNP). Reprinted from [27].

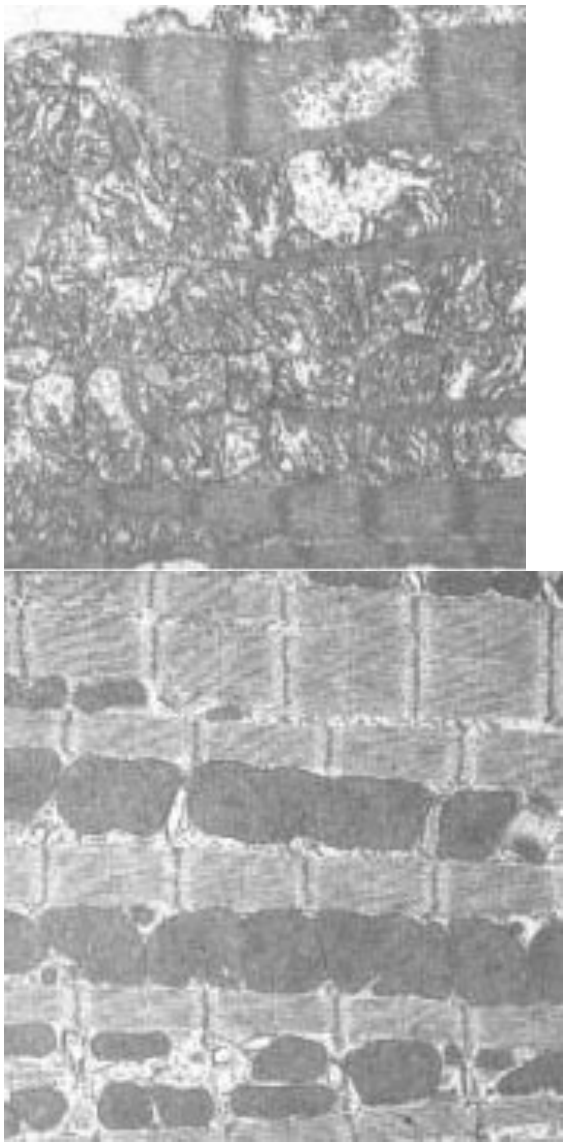

Fig. (7). EUK-8 prevents morphological damage induced by ischemia-reperfusion in the iron-overloaded rat heart. Hearts from iron-overloaded rats were perfused by the Langendorff method and subjected to 15 min ischemia followed by 15 min reperfusion, then processed for electron microscopy as described [78]. The figure shows representative micrographs from hearts perfused with no further additions (top) or 50 μM EUK-8 (bottom) in the perfusion medium. Reprinted from [78], which reports the magnification scale (11,300X for a single column (1/2 journal page) width illustration).

CONCLUSIONS

Salen Mn complexes have long been known to have catalytic ROS and RNS scavenging activities and protective efficacy in numerous models for oxidative stress [2, 8]. However, the unexpected "mito-protective" actions of salen Mn complexes might further enhance their potential therapeutic value, since mitochondrial dysfunction is implicated in multiple diseases. Furthermore, an increased understanding of the mechanisms whereby salen Mn complexes protect cells and organisms could shed some light on whether mitochondrially-targeted therapeutic approaches are necessary to combat such diseases, or whether a broader antioxidant strategy accessing the mitochondria along with other cellular sites is sufficient, or even more desirable. The data reviewed here suggest that there may be no need to improve mitochondrial selectivity of salen Mn complexes, as they already show strong protective activity in different models of increased mitochondrial oxidative damage, two induced by genetic changes selective for mitochondria. In fact, as discussed earlier, it is still possible that the "mito-protective" effects of salen Mn complexes are an indirect consequence of their suppressing total cellular oxidative stress, rather than requiring their accumulation in the mitochondria. Consistent with this, Dessolin *et al.* [79] synthesized mitochondrially-targeted versions of various antioxidants, including EUK-134, and concluded that they were no more anti-apoptotic than the parent antioxidant compounds. Furthermore, as with any metal-ligand complex, these compounds might act *in vivo*, at least in part, by altering metal homeostasis. Future work aimed at understanding the precise molecular mechanisms by which salen Mn complexes protect mitochondria is required, and might potentially unravel novel strategies to produce new therapeutic agents that mimic their "mito-protective" mode of action. For example, the ability of salen Mn complexes to protect the mitochondria without accumulating therein could support the existence of a novel mitochondrial antioxidant pathway whose activation could take place in the cytosol. Therefore, apart from any potential therapeutic use, salen Mn compounds and similarly protective antioxidant agents can be used as "probes" to better understand basic and novel cellular processes regulating mitochondrial protection from oxidative stress.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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