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Catalytic antioxidants to treat amyotropic lateral sclerosis

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Abstract

Catalytic antioxidants are comprised of specialised classes of organometallic complexes that can catalyse the decomposition of injurious biological oxidants. These complexes have been shown to prevent the formation of several oxidative markers in spinal cord of G93A amyotropic lateral sclerosis mice and markedly extend survival, even when administered at symptom onset; however, it is now clear that some complexes lacking in antioxidant activity are also protective. New proteomics data suggest that these complexes also induce a broad spectrum of endogenous cellular defense mechanisms. The combination of antioxidant and adaptive resistance effects may explain the remarkable potency of these compounds and may also suggest wide applicability for them in a number of neurodegenerative diseases.

Keywords

adaptive resistance; AEOL10150; G9; G93A mice; gadolinium texaphyrin; malondialdehyde; manganese porphyrin; metalloporphyrin; motexafin gadolinium; nitrotyrosine; onset administration paradigm; peroxynitrite; preconditioning; redox cycling

1. Introduction

Organometallic complexes that possess a stably bound redox active metal (e.g., iron, manganese and copper) have the potential to be catalytic antioxidants; however, many factors contribute to the ability to catalytically decompose and quench biological oxidants [1,2] and few classes of organometallics have the requisite collective properties to function as effective catalytic antioxidants *in vivo*. Nature has opted to make catalytic antioxidants via the use of proteins that harbour active site metals (e.g., Cu–Zn superoxide dismutase 1 (SOD1) and manganese superoxide dismutase) and/or generic metallic prosthetic groups that are finetuned by the surrounding protein (e.g., haem-containing catalase and glutathione peroxidase). Indeed, haem (iron protoporphyrin IX) is the perfect example of a redox catalyst that can be utilised to reduce an oxidant (e.g., hydrogen peroxide by catalase) or to produce one (e.g., hypochlorous acid by myeloperoxidase) depending on the microenvironment created by proximal amino acid residues. Thus it is no wonder that the most effective catalytic antioxidants to date are basically analogues of haem.

Amyotropic lateral sclerosis (ALS) is a fatal neurodegenerative disease that typically begins with weakness in a limb or difficulty in swallowing and slurred speech (bulbar-onset ALS). The disease rapidly progresses to total body paralysis but leaves cognition largely intact; death due to respiratory complications usually occurs within 1 – 5 years of diagnosis [3,4]. There are no effective treatments for ALS and, with the exception of the 2% of cases related to mutations to Cu–Zn SOD1, there are no reliable predictors of disease that would facilitate preventative

treatments. Thus treatments must currently be aimed at slowing disease progression once symptoms appear and differential diagnosis is made.

The seminal 1993 discovery that mutated SOD1 caused one form of familial ALS was quickly followed with transgenic mouse models of ALS based on ubiquitous expression of a human SOD1 mutant [5,6]. The first successful model was based on the G93A (glycine to alanine substitution at position 93 of the SOD1 protein) mutation and (as such) it has become the standard model for testing new therapeutic agents [7]. Scores of human clinical trials have been initiated based on results in this model but, thus far, the results in humans have been disappointing [101]. However, it must be emphasised that most G93A mouse studies to date have used presymptomatic administration of test agents, something that cannot be accomplished in human patients. Thus the lack of predictive power of the mouse model to date may be related – at least in part – to the fundamental difference in study design [8].

Several lines of evidence unequivocally demonstrated that SOD1 mutant-mediated ALS is not related to a loss of SOD1 activity but rather to a gained toxic function of the mutant enzymes [3]. Although the precise mechanism of toxicity of the SOD1 mutants has remained elusive, it is clear that in the absence of bound zinc human SOD1 – whether mutant or wild type – is both pro-oxidative and pro-aggregatory [9-13]. Mutations appear to adversely affect zinc-binding affinity and thereby make mutants more likely to exist in a zinc-deficient form [14,15]. However, conditions that limit zinc availability to wild-type SOD1 could, theoretically, result in toxicity (and ALS) via the same mechanism. The zinc-deficiency hypothesis provides a way to explain how so many different mutations could all result in a common toxic phenotype and leaves open the intriguing possibility that all forms of ALS could be related to SOD1 [16,17]. In any case, it is clear that neuronal death converges at some point into common signalling pathways [18,19]. Thus it seems reasonable that agents that act on these common pathways should be effective for treating ALS, regardless of the initiating stimulus.

Evidence for pathologically relevant oxidative injury in both human ALS and in mouse models of ALS is undeniable; however, as with so many other human disease conditions where oxidative injury is occurring, it is difficult to determine whether the oxidation of proteins, DNA and lipids is a primary cause of motor neuron death in ALS or secondary to some other more fundamental pathological process. In any case, given the magnitude of the oxidative changes and the fact that they are primarily localised to the affected tissue (spinal cord), it seems reasonable that prevention of the concomitant oxidative injury would be beneficial in limiting local neuronal injury/death and/or propagation. Many of the oxidative markers seen in ALS and mouse models – particularly nitrated proteins [8,20-23] and nitrated tocopherol [24,25] – are indicative of peroxynitrite. A number of antioxidants that scavenge peroxynitrite reasonably well *in vitro* (e.g., tocopherol, glutathione ester, *N*-acetylcysteine and ebselen) have been tested in ALS mice but only recently have more efficient catalytic antioxidants (such as iron and manganese porphyrins) been examined [8,26-28]. The significance of this lies in relative reaction rates and broad specificity; many of the metalloporphyrins react with peroxynitrite and other biologically relevant oxidants at rates > 10,000-fold faster than antioxidants tested previously [2,26,28-32,34].

It is important to emphasise the critical differences between these newer organometallic antioxidants and classical antioxidants (such as ascorbate, *N*-acetylcysteine and tocopherol) as the latter group has proven to have little or no benefit in human clinical trials. Faster reaction rates, reactivity with a broader range of oxidants, and catalytic (rather than sacrificial) activity make these compounds fundamentally different from endogenous/dietary antioxidants. In addition, as discussed in Sections 7 and 8, the catalytic antioxidants may possess other properties that classical antioxidants lack, such as the ability to upregulate endogenous defence mechanisms.

As mentioned in Section 1, few classes of organometallic compounds have the properties needed to be good pharmacological agents. When considered from the standpoint of rational drug design concepts, thorough biochemical and physical characterisation, a broad range of reactivity with biologically relevant oxidants and demonstration of beneficial effects in a number of biological model systems, the series of novel manganese porphyrins that originated in the laboratory of Fridovich I (Duke University) [29] represent the state of the art in terms of pharmacologically viable catalytic antioxidants. Many of these manganese porphyrins have been shown to react with superoxide, peroxynitrite, carbonate radical and nitric oxide (NO) at rates fast enough to out-compete other biomolecular targets [30]. In general, the manganese porphyrins require endogenous reductants – such as ascorbate, glutathione, NADPH and so on – to regenerate the metal-reduced (Mn^{3+} or Mn^{2+}) forms [31,32] and to quench secondary reactive species produced [2,33-35]. However, by reacting with oxidants such as peroxynitrite (and then being re-reduced by endogenous reductants such as ascorbate and glutathione) faster than the direct reaction of peroxynitrite with these reductants, the metalloporphyrins greatly amplify the effectiveness of endogenous reductants at detoxifying reactive species.

MnTCPP (Mn^{3+} meso-tetrakis[4-carboxyphenyl]porphyrin; often referred to in the literature with the trivial name of MnTBAP (Mn^{3+} tetrabenzoic acid porphyrin)) was among the first catalytic antioxidants to show protection from oxidative stress in biological systems [1,2]; however, relatively high concentrations were needed and some preparations were more effective (on a milligram per milligram basis) than others (Fridovich I, Day B and Crapo J, personal communications; and Crow JP and Estevez A, unpublished observations). The second order rate constant for the reaction of FeTCPP (Fe^{3+} meso-tetrakis[4-carboxyphenyl] porphyrin, the iron analogue of TBAP) with peroxynitrite is ~ 100-fold greater than for MnTCPP [34]. Thus contamination of MnTCPP preparations with differing amounts of FeTCPP has been considered as a possible explanation for the variability in biological activity observed with MnTCPP from different sources. Unlike MnTCPP, FeTCPP (and other iron porphyrins) can catalytically decompose peroxynitrite even in the absence of added reductants [33,36]. For all of these reasons, FeTCPP was chosen as the first metalloporphyrin to be tested in the G93A ALS mouse model. FeTCPP was among the first compounds to show a significant survival benefit when given at symptom onset [26]. FeTCPP was the first metalloporphyrin reported to extend survival of G93A ALS mice; since that time, other metalloporphyrins including Mn^{3+} meso-tetrakis(*N*,*N*′-diethylimidazolium-2-yl)porphyrin (abbreviated as Mn^{3+} TDE-2-ImP^{5+} and designated AEOL 10150) [8,27,28], Fe^{3+} meso-tetrakis(4-acetoxyphenyl)porphyrin (abbreviated as FeTAPP) and Mn^{3+} meso-tetrakis(*N*-hexylpyridinium-2-yl)porphyrin (abbreviated as Mn^{3+} TH-2-PyP^{5+}) have shown even more pronounced survival effects in these mice (Crow Laboratory, University of Arkansas College of Medicine, 2005 – 2006, unpublished results). Mn^{3+} TDE-2-ImP^{5+} is now in multidose Phase I trials in ALS having successfully completed single dose Phase I trials.

2. Superoxide dismutase mimetics and catalytic antioxidants

Many of the manganese porphryins, which are now being examined as therapeutics in ALS (and other model systems), originated as part of an effort to rationally design low molecular weight ‘mimics’ of SOD (i.e., compounds with catalytic activity similar to SOD but which lacked the disadvantages of large proteins). After many years of work, newer-generation manganese porphyrin analogues possess SOD activities approaching that of the human enzyme (on a milligram per milligram basis) [30]. Due to this history and the designation of these manganese porphyrins as SOD mimetics, there has been a strong tendency to ascribe any and all protective properties of these compounds to their SOD-like activity. In that regard, the G93A ALS mouse model is an ideal test. These mice contain many copies of the G93A SOD1 mutant gene and the total SOD1 activity in virtually all tissues is ~ 8-fold (800%) greater than normal. Considering the overwhelming SOD1 activity already present in these mice, it is highly

unlikely that the small increase in SOD1 activity afforded by pharmacological concentrations of metalloporphyrins contributes in any way to their therapeutic effect. Thus the answer must lie elsewhere.

In the mid 1990s, the ability of this class of compounds to react very rapidly with peroxynitrite (second-order rate constants of ~ $10^6 – 10^8$ M^{-1} s^{-1}) and to catalytically decompose it became evident [31,33,36,37]. By this time, peroxynitrite was being recognised as a potent endogenous oxidant and cytotoxin, which could account for much of the oxidative injury previously ascribed to superoxide, NO, hydrogen peroxide and even the hydroxyl radical [38-40]. The demonstration that NO reacted with superoxide at a near-diffusion-limited rate to form peroxynitrite [41], and that peroxynitrite could modify biomolecules in virtually all of the ways previously ascribed to hydroxyl radical [42,43], led to a true paradigm shift in the oxidative stress community, with the advent of phrases such as ‘reactive nitrogen species’, ‘nitrosative stress’ and ‘nitro-oxidative stress’, as well as a blurring of the lines between classical free radical biology and NO physiology.

Many of the manganese and iron porphyrins possess the ability to decompose hydrogen peroxide, but the catalytic turnover rate (K_{cat}) values are many orders of magnitude slower than catalase itself [44]. In addition, hydrogen peroxide can diffuse across membranes into compartments where catalase and/or glutathione peroxidase exist, making it unlikely to accumulate in specific subcellular compartments that could somehow only be accessed by synthetic porphyrins. Thus there is considerable debate as to whether the catalase-like activity of the manganese and iron porphyrins contributes to their overall biological properties in any significant way. Nevertheless, recognition of the broad spectrum of oxidant-scavenging activities of these complexes led to the evolution away from their characterisation solely as ‘SOD mimics’ and in favour of ‘catalytic antioxidants’. Because biochemical mechanisms exist to recycle endogenous antioxidants (such as ascorbate, tocopherol and glutathione), they could also be considered – in the strictest sense – to be catalytic antioxidants; however, reduction of these endogenous compounds is typically slower or otherwise less efficient than the initial oxidation and, therefore, rate limiting. By contrast, reduction of the oxidised (Fe^{4+} or Mn^{4+}) porphyrins is at least as fast as the initial oxidation by peroxynitrite [32,36] such that the effective concentration of the active reduced forms is maintained during redox cycling. This is one reason why these compounds can function as effective antioxidants at nanomolar to low micromolar concentrations [2,36].

3. Possible mechanism of protection: acute prevention of oxidative modifications of biomolecules

Catalytic antioxidant porphyrins have been shown to prevent oxidative modifications to proteins, particularly protein tyrosyl nitration, in a number of model systems [45-49] when given shortly before the injurious stimulus. Nitration of aromatic residues such as tyrosines in proteins (and, to a lessor extent, nitration of tocopherol and guanine) is the most distinctive modification associated with peroxynitrite production but by no means the only one; oxidation of critical cysteine, methionine and tryptophan residues by peroxynitrite may ultimately be more pathologically relevant. Depending on which biomolecular targets are modified and the effect these modifications have on their function, such oxidative modifications in and of themselves could be the basis for oxidative injury and motor neuron death. Compounds that catalytically decompose peroxynitrite would protect against all types of modifications and nitrotyrosine is a useful marker to assess the extent to which all peroxynitrite-mediated oxidative modifications have been prevented.

4. Possible mechanism of protection: chronic inhibition of oxidative signalling

In addition to direct oxidative inactivation of key proteins and enzymes, production of oxidants could result in neuronal injury and death via activation and/or suppression of signalling cascades; for example, peroxynitrite potently (nanomolar concentrations) inactivates thiol-dependent tyrosine phosphatases *in vitro* [50] and has been shown to produce marked broad-spectrum increases in tyrosine phosphorylation in cell models [51-54]. Peroxynitrite is also known to acutely activate apoptotic signalling in neurons [55-57]. In both of these situations, scavenging of peroxynitrite by fast-reacting catalytic antioxidants would prevent such signalling cascades. Chronic, low-level oxidative stress is known to stimulate production and release of pro-inflammatory cytokines as well as turning on various oxidant-sensitive genes [58-61]. This may be related to the oxidation of zinc thiolate moieties in a number of zinc-finger transcription factors [62]. In any case, chronic treatment with catalytic antioxidants could decrease the overall level of inflammatory amplification via this process.

As discussed in Sections 7 and 8, the therapeutic effect of the metalloporphyrins, particularly in ALS mice, may involve more than catalytic antioxidant activity. First, it is important to provide some characterisation of the ALS mouse model and the way in which this model has been used to test the metalloporphyrins (ways which differ somewhat from testing of many other compounds to date).

5. G93a ALS mouse model: comparison of littermates and normalisation of survival results

Evaluation of the potential therapeutic value of the catalytic antioxidant compounds in ALS, particularly in relation to other compounds, is dependent on the manner in which the ALS mouse results are examined and normalised. As discussed in Section 1, many positive results involving presymptomatic treatment in the mice have failed to yield comparable results when human patients are treated at symptom onset [8,28]. Due to the need for a definitive diagnosis of ALS (and even more stringent enrolment criteria), human trials frequently involve treatment of individuals at relatively advanced stages of disease. In an effort to better mimic the human clinical situation, researchers at the Crow Laboratories have carried out all mouse studies using the onset administration paradigm; that is, each mouse is assessed for changes in gait (due to hind limb weakness) and administration of test compounds is begun at the symptom onset in each mouse. With this study design, the first day of symptom onset and the first day of drug treatment are the same. For this reason, the more relevant measure of survival is the time from onset to death, termed the survival interval (SI) [8,17,28].

A careful inspection of survival data in the G93A mouse model reveals that symptom onset is strikingly similar from laboratory to laboratory and from generation to generation within the same laboratory [8] [102]. However, there is considerable variation in survival after onset (SI); the SI varies from as little as 10 days [63,64] to as much as 45 days [103] for vehicle-treated control mice. The Amyotrophic Lateral Sclerosis Therapy Development Foundation lists age of onset and ultimate survival for hundreds of groups of G93A mice and the variability in SI is quite large even in the same laboratory. Given that the age of symptom onset is relatively constant (and variability exists primarily in ultimate survival), the more scientifically sound approach is to normalise survival data to either SI or total lifespan [8,65]; that is, one can ratio the mean SI of the treated group to the mean SI of the control group or do the same with mean lifespan. With presymptomatic treatment, one must distinguish between a delay in disease onset, extension of survival after onset or both. Even when such distinctions are made, the interpretation of presymptomatic studies is more complicated and cannot be directly

extrapolated to onset studies in humans. Normalisation to total lifespan neglects this critical distinction, whereas SI automatically measures only the extension of survival after onset (the measure that is meaningful to humans) [8]. It must be emphasised that the ratio of drug-treated SI:control SI will always yield a larger number due to the demoninator being smaller. What matters is relevance to human ALS, and the SI ratio is equivalent to asking 'how much longer will I live with drug treatment than without it?'.

Because variable survival is so critical to interpreting results with test agents (and making decisions as to which agents warrant study in humans) in this mouse model, efforts have been made to understand what factors might be involved. The level of SOD1 mutant transgene expression has been examined at both the level of mRNA and protein (immunoblot), as well as by SOD1 activity measurements: researchers in the Crow laboratories have seen no evidence of changes. Environmental variables (e.g. food, water, bedding, cages, vivarium temperature, light and dark cycles and so on) have all been examined, but no variable has been found that correlates with ultimate survival of these mice [83]. Despite the lack of explanation for differences in SI, it is clear that the differences are scientifically sound and not simply a matter of different criteria for sacrifice; for example, in mice that had a mean SI of 12.5 or 17.8 days (2 different control groups), vehicle-treated cohort mice from the same litters – sacrificed 10 days after onset – showed marked paralysis and had only 38% of the spinal neurons of healthy non-transgenic mice [8,28]. By contrast, in control mice with a mean SI of ~ 42 days, cohort mice retained 65% of spinal neurons at a time point 25 days after symptom onset [27]. Thus the difference in survival after onset was objectively reflected by the numbers of remaining spinal neurons, which (in turn) correlate well with overall motor function.

In the absence of a rigorous scientific explanation for variability in survival, the only recourse is to compare control and drug-treated mice derived from the same pooled littermates, run concurrently side by side. When pooled littermates and a randomisation protocol based on symptom onset are used, the test compound becomes the only variable in determining ultimate survival. Adequate statistical power can be achieved with smaller group sizes relative to studies involving groups of mice from obtained from commercial sources, where precise ages and parentage for each mouse are not tracked.

6. G93a mouse model: survival and histopathology with different metalloporphyrins

By comparing the mean SI of drug-treated mice with vehicle-treated littermate controls, one obtains a SI ratio. In the case of FeTCPP, the authors found that treatment with 1 mg/kg/day via intraperitoneal injection extended the SI by ~ 1.55-fold [26]. In this study, three oxidative markers were examined via immunohistochemistry: nitrotyrosine (protein nitration), malondialdehyde-adducted protein and protein carbonyls. As malondialdehyde is derived from oxidised lipid, it is an indirect marker of lipid oxidation. In all three cases, pronounced increases in oxidative markers were observed in vehicle-treated mice (increases that were largely prevented by FeTCPP treatment) [26].

Using the newer-generation manganese porphyrin Mn^{3+} TDE-2-ImP^{5+}, developed by Batinic-Haberle I and colleagues (Duke University) [66] as part of the SOD mimetic series, researchers at the Crow Laboratories have obtained some of the best survival effects so far using the onset administration paradigm in the ALS mice (for a comprehensive review of these studies, see [8,28]). In 3 separate studies in the mice, a reproducible survival effect equivalent to as much as a 3-fold increase in SI was seen [8,28]. In the published study [8], histopathological markers were examined in cohort mice 10 days after symptom onset; both vehicle-treated and Mn^{3+} TDE-2-ImP^{5+}-treated mice were sacrificed 10 days after onset (and after initiation of drug administration) and compared with age-matched, non-transgenic SJLb6 mice. This time point

was chosen based on the dramatic difference between treated and control groups in terms of motor function; vehicle-treated mice showed moderate to severe hind-limb paralysis, whereas drug-treated mice retained motor function similar to the first day of symptom onset. As with FeTCPP, nitrotyrosine- and malondialdehyde-adducted protein were almost totally prevented by Mn^{3+} TDE-2-ImP^{5+} treatment; the morphology of motor neurons and the extent of reactive gliosis in drug-treated mice were similar to healthy non-transgenic mice [8].

Both Mn^{3+} TDE-2-ImP^{5+} and Mn^{3+} TE-2-PyP^{5+} have a formal charge of +5; Mn^{3+} TDE-2-ImP^{5+} has 4 symmetric *N*-*N*′-diethylimidazolium-2-yl substituents and Mn^{3+} TE-2-PyP^{5+} has 4 *N*-ethylpyridium groups. These alkyl groups may help shield the positive charges and may aid in membrane penetration. However, a new analogue of Mn^{3+} TDE-2-ImP^{5+} that has 4 *N*-hexyl groups instead of the *N*-ethyl groups (Mn^{3+} TH-2-PyP^{5+}) has been found to produce similar survival effects in the ALS mice at 5 – 10 times lower doses than Mn^{3+} TDE-2-ImP^{5+} (Crow Laboratory, University of Arkansas College of Medicine, 2005 – 2006, unpublished results). In addition, the tetramethylester form of the iron porphyrin FeTCPP (FeTAPP) enhanced survival (SI) by ~ 2-fold, relative to 1.55-fold for the free acid form [26]. At present, the reasons for these enhanced effects with either the newer manganese or newer iron porphyrin analogue are not known but better CNS penetration is reasonable in both cases due to the greater lipophilicity of these analogueues.

7. Possible mechanism of protection: preconditioning (adaptive resistance) effects

As discussed in Sections 3 and 4, the beneficial effect of the iron and manganese porphyrins in ALS mice could be related to their novel antioxidant properties. This assumption has led many investigators to use these complexes only in very short-term pretreatment designs (i.e., addition of metalloporphyrin just minutes before challenge with the injurious stimulus). Other studies in cell models have hinted of differences in the extent of protection depending on the length of the metalloporphyrin pretreatment interval (i.e., pretreating for several hours afforded greater protection than pretreating immediately prior to challenge) [67,68]. In the ALS mouse model, immunoblot analysis suggests that haem oxygenase-1 (HO-1, also known as heat-shock protein 32 [HSP-32]) and HSP-70 are upregulated by metalloporphyrins (Crow JP, University of Arkansas College of Medicine and Calingasan N, Weill Medical College of Cornell University, unpublished results). Such effects are consistent with the classic preconditioning effect, where a mild oxidative stress renders the tissue resistant to subsequent oxidative challenge. The term ‘preconditioning’ has been used in the context of a mild stimulus protecting a tissue from a subsequent larger stimulus of the same type; for example, low-level heat stress or NO exposure protecting from subsequent high-level heat or high levels of NO, respectively. In the context of pharmacological agents protecting against a wide array of subsequent stressful stimuli (not simply higher levels of that agent), the term ‘adaptive resistance’ has been used [69]. Thus ‘adaptive resistance’ will be used to refer to induction of protective proteins by metalloporphyrins. Note that the term ‘adaptive resistance’ is generally more appropriate than ‘preconditioning’ when refering to the ability of a pharmacological agent to induce protection against a broad range of injurious stimuli [69].

In an attempt to better understand metalloporphyrin-mediated protection in the ALS mouse model, the author's group have used a 2D gel/mass spectrometry proteomics approach to examine protein expression changes that correlate with the therapeutic effect. Preliminary results reveal significant upregulation of a number of proteins that fall into four primary categories: HSPs; metal-binding proteins; neuronal cytoskeletal (neurofilament) proteins; and enzymes associated with mitochondrial energy production. Given that SOD1 is a metalloenzyme and that mutants are less stable and more prone to misfolding than wild type, and that early mitochondrial pathology involving decreases in electron transport enzymes and

energy production is well established in the mouse model, it is easy to make logical connections between metalloporphyrin-induced changes and robust neuroprotection. Although the mechanistic relationships are not understood, it has long been known that increases in neurofilament heavy chain [70] and decreases in neurofilament light chain [71] – the precise changes detected with metalloporphyrin treatment – exert a protective effect in this mouse model. Albeit preliminary, it is noteworthy that an unbiased, global assessment of the main changes associated with metalloporphyrin treatment (via 2D gel proteomics analysis) points to those proteins that have already been implicated in the pathogenesis of motor neuron degeneration in this model.

8. Speculation as to the basis for organometallic-induced adaptive resistance

The notion that synthetic metalloporphyrins potently upregulate proteins previously associated with classical preconditioning – particularly HO-1 and other HSPs – suggests that they may be mimicking free haem (iron protoporphyrin IV) in at least one important way. Free haem may serve as a potent and universal signal to cells and tissues that haemorrhage has occurred, a catastropic event that requires mobilisation of a broad spectrum of cellular defence. As the iron in haem is redox active, free haem is a loose cannon; upregulation of HO-1 (and other haem oxygenase isoenzymes) is a logical cellular response to free haem so that the porphyrin ring can be cleaved, thereby releasing iron so that it can be safely sequestered by storage and/or transport proteins. Other protective mechanisms (e.g., metal-binding proteins, HSPs and so on) are upregulated to help the cells cope with the collateral damage of the haemorrhage. The ability of many synthetic metalloporphyrins to act as potent inducers may be related (in part) to the fact that they tend to be poor substrates for haem oxygenases [72,73] and thus they cannot be readily metabolised and cleared. The relatively long tissue residence time of some metalloporphyrins may also be related to this property and may help explain the potent adaptive resistance effects of these complexes.

Upregulation of metal-binding proteins such as metallothioneins (*de novo* metallothioneins are in the apo form or simply ‘thioneins’) is a generic response to many metals, particularly when those metals are present in potentially toxic forms (e.g., when they are free or incompletely coordinated). Whenever metals such as iron, manganese or copper possess fewer than the maximal number of coordinating ligands, they are more redox active. Although purely speculative, very low concentrations of metalloporphyrins may potently upregulate thioneins and other metal-binding proteins by acting both as ‘metal shuttles’ and ‘metal presenters’. Metal shuttling refers to the ability of a complex to cross membranes and enter subcellular compartments from which free metals would normally be excluded. Metal presenting refers to a stable metal complex that is resistant to metabolism and thereby continues to expose the cell to a form of incompletely coordinated metal. In the case of metalloporphyins (which contain redox-active metals), metal presenting may be synonymous with redox cycling and oxidant production because it is clear that they can produce oxidants under some conditions [34]. Metal-shuttling and -presenting properties may be particularly important for those metalloporphyrins that cannot be metabolised by haem oxygenases and contain central metals that are normally present in trace amounts (e.g., manganese and cobalt); that is, the defensive cellular response to a long-lived metalloporphyrin containing a foreign metal is likely to be greater than it would be to a haem, which contains iron and can be rapidly metabolised.

The relative importance of antioxidant versus adaptive resistance properties in ALS mice is currently being examined via the use of metalloporphyrin analogues where only the central metal is different; non-redox active metals do not possess catalytic antioxidant properties and, if found to be protective, must be working via other mechanisms. Ample literature precedence exists to support this concept; for example, one study found that doxorubicin-mediated apoptosis of cardiac myocytes was inhibited by pretreatment with redox-active FeTCPP and

by non-redox active cobalt tetracarboxylated phenylporphyrin but not by (redox-active) MnTCPP or (non-redox active) ZnTCPP. In this study [67], protection correlated with upregulation of HO-1 and decreased activity of caspase-3 rather than antioxidant activity per se. A systematic study [74] of several metalloporphyrins (involving variation of both the central metal and the distal functional groups) clearly demonstrated that protection of cortical neurons from three different stressors correlated with effects on calcium and not with redox activity. An earlier study [75] had reported HO-1 induction in liver cells by cobalt and iron protoporphyrin IX (haem). It should be emphasised that these were cell-based studies involving metalloporphyrin concentrations that were relatively high compared with levels observed in whole animals; however, it is entirely possible that similar effects could occur in mice at lower doses considering a treatment time of several weeks.

The authors have also seen marked survival effects (more than a twofold increase in SI) in ALS mice with the texaphyrins, another class of organometallics originally referred to as '*twisted porphyrins*' because they possess a fifth metal-coordinating ligand that distorts the symmetrical planar porphyrin geometry and also alters the redox activity of the central metal [76,77]. Manganese texaphyrin has pronounced effects on survival in the ALS mice [17] and is a modest peroxynitrite decomposition catalyst [78]. Interestingly, gadolinium texaphyrin (motexafin gadolinium [MGd]) is only slightly less efficacious than manganese texaphyrin in mice [83] and lacks any catalytic antioxidant activity. As MGd is mildly pro-oxidant [79], it seems reasonable that it is exerting a protective effect via an adaptive resistance mechanism. MGd has been given to > 600 human patients as part of a clinical trial that evaluated its ability to enhance radiation-induced tumour killing in metastatic brain cancer [84]. Results from this study suggested that MGd offered some protection (from radiation) to normal neurons. Genomic and proteomic studies are underway to determine how MGd is acting in ALS mice and preliminary data suggest involvement of some novel biochemical mechanisms [80], which can reasonably be considered to be neuroprotective in this model.

Both in human ALS and in the ALS mouse model, oxidative injury is clearly occurring and the novel antioxidant properties of the redox-active metalloporphyrins may be important for limiting oxidative modifications to biomolecules and preventing oxidant-mediated pro-apoptotic signalling. However, in a chronic neurodegenerative condition like ALS, the adaptive resistance aspects of the metalloporphyrins may also be extremely important for the long-term survival of the motor neurons. Indeed, the potency of these compounds may reside in the synergy between antioxidant properties and induction of broad-spectrum endogenous cellular defences.

9. Can therapeutic effects be extended?

Unlike many agents tested so far, the metalloporphyrins and texaphyrins largely preserve motor function during the period of extended survival despite the late administration; the ability of mice to walk, run, cage climb, eat and drink essentially remains at the level seen on the first day of symptom onset until late in the course of disease [8,17,27]. Nevertheless, the metalloporphyrins – like all agents tested in the ALS mice so far – ultimately lose their protective effect and the mice decline precipitously at end stage. Understanding why the metalloporphyrins ultimately fail and what might be done to prevent the loss of neuroprotection may be key to halting disease progression entirely. To the extent that metalloporphyrins are acting via an adaptive resistance mechanism, the dosing regimen may prove to be critical; for example, dosing to achieve the classic steady-state plasma level may not be the optimal approach, particularly considering that many of the porphyrins are largely unmetabolised [72,73] and that tissue residence times can be much greater than plasma residence [28]. Intermittent treatment with medium to high doses, continuous low dosing, dose escalation and combination with other agents are only a few of the strategies being examined to find a regimen

that provides longer-term protection. With regard to combination therapy, recent results indicate a very significant additive effect of Mn^{3+} TDE-2-ImP^{5+} and phenylbutyrate, a histone deacetylase inhibitor [27].

The relative potency of these compounds is also quite remarkable; a dose of 1 mg/kg/day of a complex with a formula weight of ~ 1000 would yield a maximum theoretical plasma or tissue concentration of ~ 1 μM. A total of 16 h after the last dose of FeTCPP in mice chronically treated with 1 mg/kg/day, spinal cord and brain levels were 0.2 and 0.35 μM, respectively, even though plasma levels were below the detection limit [26]. Although it is known that Mn^{3+} TDE-2-ImP^{5+}, like many other compounds, displays a bell-shaped dose–response curve (Crow Laboratory, University of Arkansas College of Medicine, 2003 – 2005, unpublished results), most of the work with metalloporphyrins in ALS mice to date has involved very limited dose ranges or variation in terms of dosing intervals. Thus it is not known whether the best possible survival effects have been attained. Ongoing studies will help answer questions related to both maximum efficacy and mechanisms of action.

10. Expert opinion

The decision to test iron and manganese organometallic complexes in ALS mice was originally based on their activity as catalytic antioxidants together with evidence for peroxynitrite involvement in disease pathogenesis. Consistent with this rationale, treatment with these complexes virtually eliminates oxidative markers associated with peroxynitrite and markedly extends survival, even when treatment is initiated at symptom onset. However, preliminary mechanistic studies strongly suggest a robust, broad-spectrum protein induction pattern that would be expected to make neural tissue more resistant to many types of injurious stimuli (i.e., drug-induced adaptive resistance). It is this latter effect that makes these complexes unique and may ultimately prove to be of benefit not only in other neurodegenerative conditions but also in other (non-neural) tissues and organs. The pronounced survival effects of MGd, which is not a catalytic antioxidant and is mildly pro-oxidant, are consistent with this interpretation, although it is premature to assume that its mechanism of action is the same or even similar to that of the redox-active metalloporphyrins. Indeed, there are clearly some novel aspects to the MGd effect. In any case, future studies with either class of compounds in any model system (neurodegenerative or otherwise) should include both short- (< 1 h) and long-term (> 6 h) pretreatment intervals to help distinguish between acute antioxidant effects and protein induction (adaptive resistance) effects.

It is important to consider many factors besides the sheer magnitude of the survival effect in G93A mice when deciding whether to proceed with human clinical trials in ALS [81]. Results of clinical trials based on presymptomatic studies in mice have been disappointing, thus it seems prudent to test (in humans) only those compounds that show a significant survival benefit when administered at symptom onset. The predictive power of the mouse model can only be accessed once the outcomes of a sufficient number of like-designed trials are known [81]. Variability in survival after onset exists in the G93A mice, just as it does in the human ALS population; indeed, it could be argued that this variability makes the mice a better model of human ALS. However, when testing new compounds in mice, such variability is problematic and – until the reasons for the variability are found – side by side comparisons of treated and untreated mice derived from the same pooled littermates remain the best possible means of control.

The potency of these organometallic complexes is remarkable, particularly considering that CNS concentrations are generally lower than in other tissues. Although potency can often be a predictor of increased toxic risk, it does not appear to be the case with these complexes as doses of > 100-fold the therapeutic dose are well tolerated. Nevertheless, the bell-shaped dose–

response curve observed in the ALS mice suggests that optimisation of the dosing regimen is critical for maximum efficacy. Proteomic and genomic analyses may help establish the mechanisms of action as well as provide potential biomarkers of both disease progression and therapeutic response. Understanding why these complexes work so well for so long, and the mechanisms that ultimately override the protective effect may provide critical clues to the pathogenesis and to ways of achieving longer-term protection.

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