Neurobiology of Disease 81 (2015) 20–24

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Zn^{II}(atsm) is protective in amyotrophic lateral sclerosis model mice via a copper delivery mechanism

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ARTICLE INFO

Article history:
Received 5 November 2014
Revised 28 January 2015
Accepted 3 February 2015
Available online 10 March 2015

Keywords:
Amyotrophic lateral sclerosis (ALS)
Copper/zinc-superoxide dismutase (SOD1)
Therapeutic
Copper
Zinc
Mouse model
Bisthiosemicarbazone (btsc)
Metal
Inductively coupled plasma mass spectrometry (ICP-MS)
Transmetallation

ABSTRACT

Mutations in the metalloprotein Cu,Zn-superoxide dismutase (SOD1) cause approximately 20% of familial cases of amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disease for which effective therapeutics do not yet exist. Transgenic rodent models based on over-expression of mutant SOD1 have been developed and these have provided opportunity to test new therapeutic strategies and to study the mechanisms of mutant SOD1 toxicity. Although the mechanisms of mutant SOD1 toxicity are yet to be fully elucidated, incorrect or incomplete metallation of SOD1 confers abnormal folding, aggregation and biochemical properties, and improving the metallation state of SOD1 provides a viable therapeutic option. The therapeutic effects of delivering copper (Cu) to mutant SOD1 have been demonstrated recently. The aim of the current study was to determine if delivery of zinc (Zn) to SOD1 was also therapeutic. To investigate this, SOD1G37R mice were treated with the metal complex diacetyl-bis(4-methylthiosemicarbazonato)zinc^{II} [Zn^{II}(atsm)]. Treatment resulted in an improvement in locomotor function and survival of the mice. However, biochemical analysis of spinal cord tissue collected from the mice revealed that the treatment did not increase overall Zn levels in the spinal cord nor the Zn content of SOD1. In contrast, overall levels of Cu in the spinal cord were elevated in the Zn^{II}(atsm)-treated SOD1G37R mice and the Cu content of SOD1 was also elevated. Further experiments demonstrated transmetallation of Zn^{II}(atsm) in the presence of Cu to form the Cu-analogue Cu^{II}(atsm), indicating that the observed therapeutic effects for Zn^{II}(atsm) in SOD1G37R mice may in fact be due to in vivo transmetallation and subsequent delivery of Cu.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder that involves the loss of functional motor neurons in the spinal cord resulting in progressive paralysis and death within 3–5 years of diagnosis (Pratt et al., 2012). Incomplete understanding of the pathogenesis of ALS has contributed to the absence of effective therapeutics for the disease: Riluzole is currently the only approved drug for the treatment of ALS but its efficacy is marginal with only a modest extension in survival (Bensimon et al., 1994; Miller et al., 1996). Pre-clinical development of new treatment options is needed, not only to identify compounds with potential for successful clinical translation, but also to advance our understanding of ALS pathogenesis.

The majority of ALS cases arise sporadically with no known cause. However, 10% of cases are familial (Pratt et al., 2012) and of those, approximately 20% are due to mutations in the gene for Cu,Zn superoxide dismutase (SOD1) (Andersen et al., 2003), a ubiquitous enzyme responsible for scavenging superoxide radicals (Perry et al., 2010). Transgenic rodent models that over-express mutant forms of SOD1 develop ALS like symptoms and despite legitimate caveats associated with all transgenic over-expression animal models of human disease, these mutant SOD1 animals remain the current gold-standard for pre-clinical development of new ALS therapeutics (Gurney et al., 1994; Wong et al., 1995).

The mechanisms by which SOD1 mutations cause ALS remain unclear, but accumulating data indicate a significant role for the biometals zinc (Zn) and copper (Cu) (Elliott, 2001). ALS-causing mutations alter the metal binding capacity of SOD1 (Rodriguez et al., 2002; Hayward et al., 2002) and this can result in misfolding of the protein and its aggregation (Ding and Dokholyan, 2008; Bourassa et al., 2014). Further to this, previous studies have suggested that Zn-deficient SOD1 is a significant toxic form of SOD1: Zn-deficient mutant SOD1 and Zn-deficient wild-type SOD1 are both toxic to motor neurons in culture due to their capacity to promote peroxynitrite mediated damage (Crow et al., 1997; Estevez, 1999). It has also been shown that loss of

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Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2015.02.023

Zn from SOD1 alters conformation of the homodimer, which results in the redox-active Cu^{II} ion in the active site becoming more accessible to intracellular reductants leading to reduction to Cu^{I} (Roberts et al., 2007). Under these conditions Cu^{I} promotes the formation of superoxide from molecular oxygen which is then able to react with nitric oxide to form peroxynitrite. Significantly, formation of a SOD1 heterodimer containing one Zn-deficient monomer and one fully metallated monomer stabilises the Zn-deficient form and increases its toxicity (Sahawneh et al., 2010).

Previous reports investigating the metallation status of different mutant forms of SOD1 concluded that incorrect metallation of mutant SOD1 is not involved in aggregation or toxicity due to the high levels of metal per dimer in the soluble form of the protein (Lelie et al., 2011). However, the techniques used were not able to directly detect the metallation status of SOD1 and metallation status was inferred from the amount of metal per dimer. Recently developed techniques allow us to directly detect the metallation status of SOD1 allowing a more accurate investigation of the role of metals in SOD1 toxicity (Rhoads et al., 2011; Roberts et al., 2014).

Treatment with the metal complex diacetyl-bis(4-methylthiosemicarbazonato)copper^{II} [Cu^{II}(atsm)] is therapeutic in the SOD1G93A (Soon et al., 2011) and SOD1G37R (McAllum et al., 2013) mouse models of ALS. In the SOD1G37R mice there is a large pool of metal-deficient mutant SOD1 in the spinal cord tissue and the majority of this is due to Cu-deficient SOD1 (Roberts et al., 2014). Treatment with Cu^{II}(atsm) increases Cu in the spinal cord and decreases the Cu-deficient pool of SOD1 by delivering Cu to the mutant protein, indicating that Cu-deficient SOD1 may play a role in ALS-like pathogenesis in these mice (Roberts et al., 2014). Treatment with Cu^{II}(atsm) also increased the Zn content of SOD1 and this was proposed to be due to stabilisation of the protein (Roberts et al., 2014). However, given that treatment with Cu^{II}(atsm) increased the Cu content and the Zn content of mutant SOD1 in the spinal cords of SOD1G37R mice (Roberts et al., 2014), it remained unclear whether the strong therapeutic outcomes for the Cu^{II}(atsm) treatment (McAllum et al., 2013) were due to its effects on the Cu-deficient or Zn-deficient SOD1. In an attempt to delineate the importance of Cu versus Zn delivery in the therapeutic activity of Cu^{II}(atsm), mice in the present study were treated with the Cu^{II}(atsm) analogue, Zn^{II}(atsm). Locomotor function and survival of the mice were assessed to determine therapeutic effects and the metal content of spinal cord tissue from treated mice was analysed using hyphenated HPLC–ICP-MS.

Methods

Animals

SOD1G37R mice purchased from the Jackson Laboratory were established and maintained as a colony by breeding with C57BL/6 non-transgenic mice. The genotype of these mice was determined using the REDExtract-N-Amp™ Tissue PCR kit (Sigma) to prepare DNA from tail snips taken from the mice at the age of weaning. Primers and cycle times used were those taken from the Jackson Laboratory website. Mice were housed in standard boxes with sawdust and shredded paper for bedding with 2–5 animals per box. All procedures were approved by an institutional animal ethics committee (University of Melbourne).

Preparation and administration of Zn^{II}(atsm)

Zn^{II}(atsm) was prepared as previously described (Cowley et al., 2002, 2005). For administration to mice, Zn^{II}(atsm) was prepared daily by suspending in standard suspension vehicle [SSV: 0.9% (w/v) NaCl, 0.5% (w/v) Na-carboxymethylcellulose (medium viscosity), 0.5% (w/v) benzyl alcohol and 0.4% (w/v) Tween-80] at a concentration of 7.5 mg mL^{-1}. Transgenic mice and non-transgenic littermates were treated daily by oral gavage with 30 mg kg^{-1} Zn^{II}(atsm) or an equivalent volume of vehicle. Approximately equal numbers of male and female mice were included in each treatment group. Treatment of SOD1G37R mice with Zn^{II}(atsm) was performed concurrently with treatments performed for one of our previous studies (McAllum et al., 2013). Survival and rotarod data presented herein for vehicle-treated mice has been published previously (McAllum et al., 2013) and is reproduced with permission from the publisher Informa Healthcare. None of the biochemical analyses in the present study, including data from vehicle treated mice, has been published previously.

Assessment of locomotor function

The locomotor function of the mice was assessed twice a week beginning at 12 weeks of age using the rotarod task. The rotarod was set to accelerate from 4 to 40 rpm over 180 s. The time at which the mouse failed the task was recorded as latency to fall. During each testing session, every mouse was tested twice and the highest latency to fall score was recorded. Researchers administering the task were blind to the genotypes and treatments of the mice.

Determining disease end-stage

End-stage was defined as the age at which mice had a rotarod score of less than 3 s and were unable to right themselves in less than 30 s when place on their side due to hind limb paralysis. In accordance with ethical procedures, mice were killed at disease end-stage by cervical dislocation. Researchers determining end-stage were blind to treatment group.

Tissue collection and preparation for analysis

A cohort of treated mice was reserved for tissue collection at 24 weeks of age. Mice were anesthetised with ketamine (120 mg kg^{-1}) and xylazine (16 mg kg^{-1}) prepared in phosphate buffered saline (PBS). Once fully anesthetised the mice were perfused with PBS containing protease inhibitors, phosphatase inhibitor cocktail and heparin. Spinal cords were collected, frozen on dry ice immediately and then stored at −80 °C until prepared for analysis. Prior to ICP-MS and LC–ICP-MS analyses, spinal cord tissue was prepared by homogenising in TBS containing protease inhibitors and soluble and insoluble material separated by centrifugation. Soluble material was used for both ICP-MS and LC–ICP-MS analyses and insoluble material was further processed according to published protocols (Maynard et al., 2006) before analysis by ICP-MS.

ICP-MS and LC–ICP-MS

Inductively coupled plasma mass spectrometry (ICP-MS) and hyphenated size exclusion chromatography, inductively coupled plasma mass spectrometry (LC–ICP-MS) were performed as previously described (Roberts et al., 2014; Maynard et al., 2006) with the following exceptions for LC–ICP-MS: a micro mist nebuliser was used instead of a Mira Mist; He gas flow was 3.3 mL min^{-1}; spinal cord samples were homogenised in TBS containing protease inhibitors and 80 μg total protein was injected onto the column from the TBS soluble material. Elution of SOD1 from the column was determined using purified bovine SOD1 (Sigma Aldrich). Bovine SOD1 eluted at 399 s with a Ve/Vo of 1.5.

Transmetallation in $CuCl_2$

Zn^{II}(atsm) solubilised in DMSO was mixed with increasing concentrations of $CuCl_2$ ($CuCl_2 \cdot 2H_2O$ in DMSO) ranging from 0.2 to 1 Cu molar equivalents. After thorough mixing and 1 min incubation at ambient temperature, absorbance spectra from λ = 400–600 nm were measured using a UV/Vis spectrometer.

Statistics

All statistics were performed using GraphPad Prism software. Rotarod data were analysed using a two-way repeated measures ANOVA with Bonferroni post test and survival data were analysed using the Log-rank (Mantel–Cox) test, a commonly used non-parametric test for survival analysis which assumes proportional hazard. All other data sets were analysed using the unpaired t-test to compare the mean of datasets.

Results and discussion

Locomotive function of vehicle treated SOD1G37R mice began to decline at approximately 150 days of age (Fig. 1A). This progressive decline was significantly delayed by treatment with Zn^{II}(atsm) but the magnitude of the delay was less than that seen when the SOD1G37R mice were treated with Cu^{II}(atsm) at the same dose (McAllum et al., 2013). As previously reported for the Cu^{II}(atsm) treatment, Zn^{II}(atsm) did not have any stimulatory effect on the locomotive function of non-transgenic littermates (Fig. 1B). In line with rotarod performance, survival of the SOD1G37R mice was significantly extended by treating with Zn^{II}(atsm): mean survival for the vehicle treated SOD1G37R mice was 196 days compared to 210 days for the Zn^{II}(atsm) treated mice (Fig. 1C). This equates to a 7% increase in survival for the Zn^{II}(atsm) treated SOD1G37R mice, significantly less than the 18.2% extension in survival observed for SOD1G37R mice treated Cu^{II}(atsm) at the same dose (McAllum et al., 2013). The survival effect for Zn^{II}(atsm) administered at 30 mg kg^{-1} is comparable to the 8.4% extension observed in SOD1G37R mice treated with Cu^{II}(atsm) at 10 mg kg^{-1} (McAllum et al., 2013).

Previous analyses have shown SOD1G37R mice have a significant pool of Cu-deficient SOD1 (~58 μM) in the spinal cord and that delivery of Cu by Cu^{II}(atsm) to SOD1 decreases the abundance of this species (Roberts et al., 2014). A significant pool of Zn-deficient SOD1 also exists within the spinal cords of these mice but this is present at a lower concentration (~1.2 μM) than the Cu-deficient SOD1 (Roberts et al., 2014). Given that this in vivo concentration of Zn-deficient SOD1 is higher than the concentration required to kill motor neurons in vitro (Crow et al., 1997), it is possible that the relatively small pool of Zn-deficient SOD1 contributes to motor neuron death or dysfunction in vivo. We therefore assessed whether the therapeutic activity observed for Zn^{II}(atsm) in the SOD1G37R mice involved modulation of the Zn-deficient pool of SOD1 (Fig. 1). Analysis of tissues by ICP-MS and LC–ICP-MS revealed that treating with Zn^{II}(atsm) did not significantly alter the Zn content of the spinal cords nor the Zn content of the SOD1 in the spinal cords of the SOD1G37R mice however there was a trend towards an increase in Zn in both the soluble material and insoluble material (Figs. 2A and B). Despite the absence of any significant change in Zn levels, the Zn^{II}(atsm) treatment did significantly increase the Cu content of the spinal cord from 2.32 ± 0.91 to 6.03 ± 0.63 μg g^{-1} tissue wet weight in the soluble material and from 2.21 ± 0.22 to 4.91 ± 0.35 μg g^{-1} tissue wet weight in the insoluble material (Fig. 2A). This is reflected in the Cu content of the SOD1 in the spinal cord which increased from 0.16 ± 0.01 to 0.23 ± 0.02 μg g^{-1} tissue wet weight in the Zn^{II}(atsm) treated mice (Fig. 2B). Consequently, the Cu:Zn ratio for spinal cord SOD1 also increased from 0.43 ± 0.008 in the vehicle treated SOD1G37R mice to 0.54 ± 0.015 in the Zn^{II}(atsm) treated mice (Fig. 2C). The Cu:Zn ratio in the vehicle-treated mice is consistent with that reported by others employing a similar method (Lelie et al., 2011). While this result was unexpected for mice treated with a Zn-compound, it lends support to the potential for Cu-deficient SOD1 to be a contributor to the ALS-like phenotype of mutant SOD1 mice and the therapeutic efficacy of increasing levels of bioavailable Cu in the spinal cord (Roberts et al., 2014). Presuming that the ALS-like phenotype of the SOD1G37R mice is driven solely by the presence of mutant SOD1, Zn^{II}(atsm) improves the

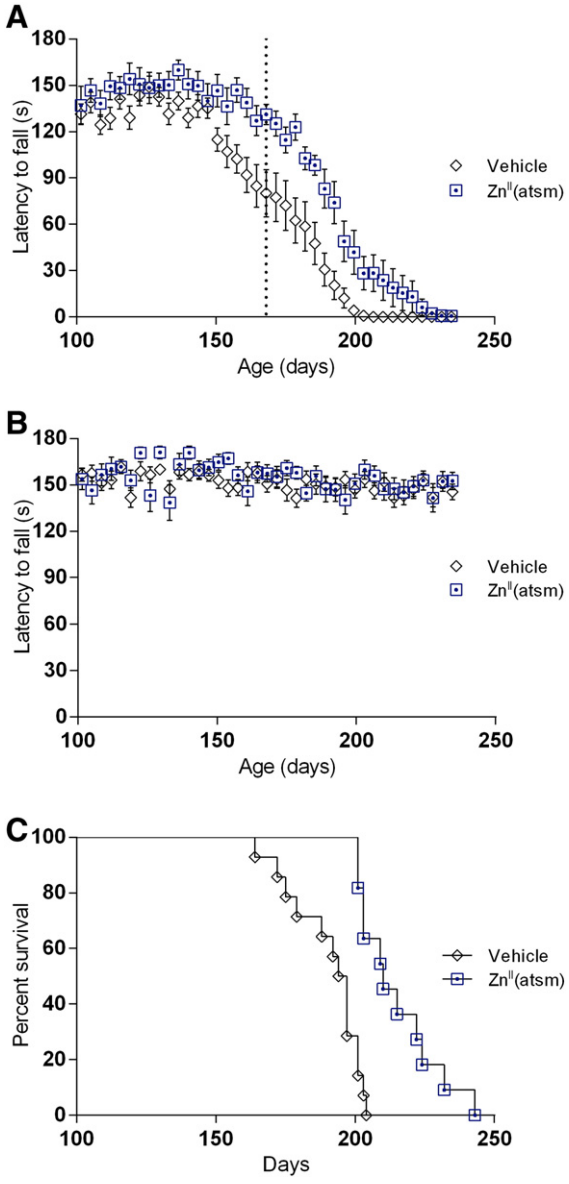

Fig. 1. The effect of Zn^{II}(atsm) treatment on locomotor function and survival in SOD1G37R mice. A. The locomotor function of SOD1G37R mice treated with 30 mg kg^{-1} Zn^{II}(atsm) or vehicle was assessed using the rotarod assay and recorded as latency to fall (n = 11 and 14 respectively; $p < 0.0001$). Due to mice reaching end-stage, the number of mice per treatment group begins to decline at 164 days for the vehicle-treated group and 201 days for the Zn^{II}(atsm)-treated group. The dotted line indicates the age at which tissue was collected from a second cohort of mice for additional analyses in Fig. 2. B. The locomotor function of non-transgenic C57BL/6 mice treated with Zn^{II}(atsm) or vehicle was assessed using the rotarod assay and recorded as latency to fall (n = 16 and 21, respectively). C. Kaplan–Meier survival curves for the same mice used in Fig. 1A. ($p < 0.0001$). Error bars in A and B represent SEM. Note, all data for vehicle treated mice have previously been published (McAllum et al., 2013) and are reproduced with permission from the publisher Informa Healthcare.

locomotive function and survival of the mice by increasing the Cu content of the mutant SOD1 (Fig. 2).

The relative chemical properties of Zn^{II}(atsm) and Cu^{II}(atsm) provide some insight to explain how treatment with Zn^{II}(atsm) can have a greater effect on the Cu content of spinal cord SOD1 than it does on the Zn content of the protein. Specifically, Zn^{II}(atsm) and similar Zn^{II}-complexes of bis(thiosemicarbazones) are less stable than their Cu equivalents and readily transmetallate in the presence of available Cu (Barnard et al., 2008; Donnelly et al., 2008) which can influence the cellular activity of these compounds in vitro and in vivo (Donnelly et al., 2008; Petering, 1974). If this occurs with Zn^{II}(atsm) in the

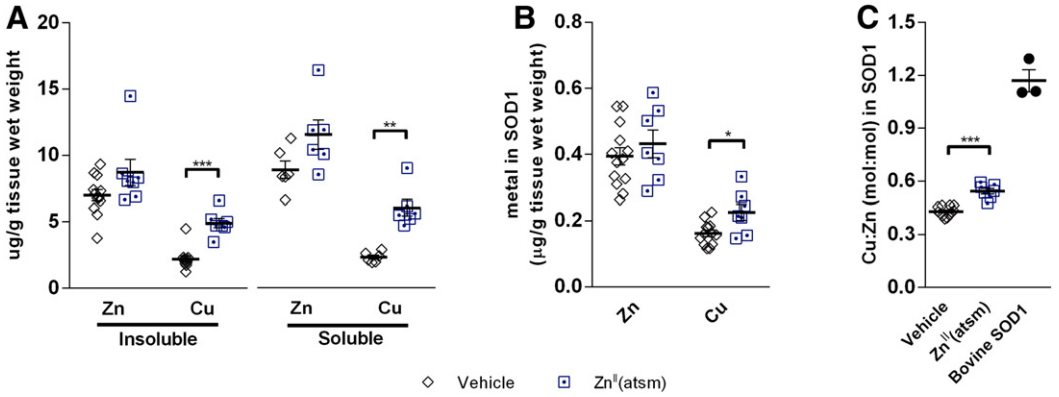

Fig. 2. The effect of Zn^{II}(atsm) treatment on the total Cu and Zn concentration and the Cu and Zn content of SOD1 in SOD1G37R mouse spinal cord tissue. A. The concentration of Cu and Zn in the soluble and insoluble material from spinal cord of vehicle and Zn^{II}(atsm) treated mice was determined by ICP-MS and presented as μg metal g^{-1} tissue wet weight. B. Cu and Zn content of the SOD1 containing size-exclusion fraction determined by LC–ICP-MS and calculated as μg $metalg^{-1}$ tissue wet weight. C. Data collected in Fig. 2B were used to calculate the molar Cu/Zn ratio of SOD1. Bovine SOD1 is shown as a control. Solid lines represent the mean and error bars, SEM. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

SOD1G37R mice, it is possible that oral administration of Zn^{II}(atsm) effectively delivers a lower dose of Cu^{II}(atsm) due to partial transmetallation once the compound enters the digestive and/or circulatory system. To test this directly Zn^{II}(atsm) was incubated with $CuCl_2$ and the distinct absorption spectra from Zn^{II}(atsm) and Cu^{II}(atsm) monitored using UV/Vis spectrometry. Increasing concentrations of $CuCl_2$ caused a shift in the absorbance spectra away from Zn^{II}(atsm) and towards Cu^{II}(atsm) (Fig. 3). This result is consistent with previous studies which have demonstrated complete transmetallation of Zn^{II}(atsm) and other Zn^{II} complexes of bis(thiosemicarbazones) when in the presence of available equimolar Cu (Holland et al., 2007; Matsumoto et al., 1992; Saji et al., 1992). Given this high susceptibility to transmetallation and the likelihood that orally administered Zn^{II}(atsm) encounters available Cu (e.g. Cu from food in the digestive system), in vivo transmetallation of Zn^{II}(atsm) appears to support the observed changes to total Cu (Fig. 2A) and SOD1-associated Cu (Figs. 2B, C) in the spinal cords of Zn^{II}(atsm) treated SOD1G37R mice.

Transmetallation affecting the therapeutic activity of Zn^{II}(atsm) in the SOD1G37R mice is analogous to results from an earlier study which examined the closely related compounds 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazone) (H_2kts) and 3-ethoxy-2-oxobutyraldehyde bis(N^4-dimethylthiosemicarbazone) (H_2ktsm) and their respective Cu- and Zn-complexes (Petering, 1974). When investigating the anti-cancer cytotoxic activity of these compounds, the metal-free ligands and their Cu and Zn complexes demonstrated clear cytotoxic activity, but metal-free ligands and Zn-complexes were only

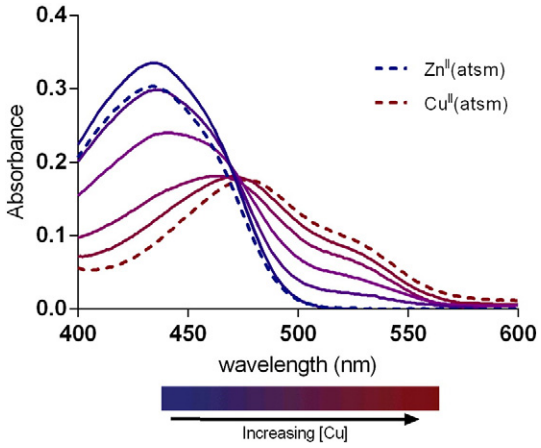

Fig. 3. Transmetallation of Zn^{II}(atsm) to form Cu^{II}(atsm). Absorbance spectra from 400 to 600 nm of Zn^{II}(atsm) mixed with increasing concentrations of $CuCl_2$ from 0.2 to 1 Cu molar equivalents. The transition of blue to red indicates the increase in $CuCl_2$ concentration and the formation of Cu^{II}(atsm).

active when administered with a Cu supplement (Petering, 1974). Further investigation found that Zn^{II}(kts) and Zn^{II}(ktsm) both readily transmetallated in the presence of available Cu to form Cu^{II}(kts) and Cu^{II}(ktsm), respectively. Transmetallation did not occur in plasma alone indicating a lack of available Cu and it was rationalised that the in vivo cytotoxic activity of the metal-free ligands and the Zn-complexes was dependent upon transmetallation utilising dietary Cu (Petering, 1974). Given the chemical similarities that these compounds share with Zn^{II}(atsm), it is plausible that the same phenomenon may be occurring in the Zn^{II}(atsm) treated SOD1G37R mice.

Data presented in this study show that orally treating ALS model SOD1G37R mice with Zn^{II}(atsm) improves their locomotive function and extends survival, albeit to a lesser extent than the same dose of Cu^{II}(atsm) as demonstrated in a previous study (Roberts et al., 2014). This may be because rectifying potential Zn deficiencies in these mice has less of an impact on their phenotype than rectifying Cu deficiencies via strategies that increase Cu bio-availability (Roberts et al., 2014), or because the therapeutic activity for Zn^{II}(atsm) in these mice is dependent upon transmetallation to Cu^{II}(atsm) and that transmetallation in these mice was incomplete. Data in Figs. 2 and 3 provide stronger support for the latter of these two possibilities, particularly the data that show treatment with Zn^{II}(atsm) improved the Cu content of mutant SOD1 in the spinal cord tissue but did not change Zn content of the protein. However, the toxicity of Zn-deficient SOD1 to motor neurons has been demonstrated (Estevez, 1999), and decreasing levels of Zn-deficient SOD1 therefore has clear therapeutic potential. The possibility still remains that Zn^{II}(atsm) itself is therapeutic. The amount of dietary available Cu would not be enough to transmetallate the entire dose of Zn^{II}(atsm) so some Zn^{II}(atsm) may still make it into the central nervous system. Once there, Zn^{II}(atsm) as a complex may be having some unknown therapeutic effect independent of Zn delivery. If true efflux of excess Zn from the cell is presumably sufficiently rapid to mask detectable changes to the total pool of Zn in the spinal cords of the Zn^{II}(atsm) treated mice. Transmetallation of Zn^{II}(atsm) has confounded our attempt to investigate the potential to treat ALS-like symptoms in SOD1G37R mice by specifically delivering bio-available Zn. Compounds less amenable to transmetallation may be required to delineate these two metals in mutant SOD1 models of ALS, in both their role in pathogenesis and their opportunity for therapeutic intervention.

Conflict of interest statement

Collaborative Medicinal Development LLC has licenced IP on this subject from the University of Melbourne, where the inventors include ARW and PSD.

Acknowledgments

Data for the vehicle treated mice presented in this study have been published previously (McAllum et al., 2013) and have been reproduced with permission from the publisher Informa Healthcare. Data for the Zn^{II}(atsm) treated mice presented in this study were collected from a single study that involved the vehicle treated and Cu^{II}(atsm) treated mice as described (McAllum et al., 2013). The research was supported by funds from the National Health and Medical Research Council (1005651 and 1061550), the Australian Research Council (DP110101368), and the University of Melbourne.

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