Therapeutic effects of Cu\textsuperscript{II}(atsm) in the SOD1-G37R mouse model of amyotrophic lateral sclerosis

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Abstract

Our objective was to assess the copper\textsuperscript{II} complex of diacetylbis(4-methylthiosemicarbazone) [Cu\textsuperscript{II}(atsm)] for its preclinical potential as a novel therapeutic for ALS. Experimental paradigms used were designed to assess Cu\textsuperscript{II}(atsm) efficacy relative to treatment with riluzole, as a function of dose administered, and when administered post symptom onset. Mice expressing human Cu/Zn superoxide dismutase harbouring the disease-causing G37R mutation (SOD1-G37R) were used and effects of Cu\textsuperscript{II}(atsm) determined by assessing mouse survival and locomotor function (rotarod assay). Cu\textsuperscript{II}(atsm) improved SOD1-G37R mouse survival and locomotor function in a dose-dependent manner. The highest dose tested improved survival by 26%. Riluzole had a modest effect on mouse survival (3.3%) but it did not improve locomotor function. Cotreatment with Cu\textsuperscript{II}(atsm) did not alter the protective activity of Cu\textsuperscript{II}(atsm) administered on its own. Commencing treatment with Cu\textsuperscript{II}(atsm) after the onset of symptoms was less effective than treatments that commenced before symptom onset but still significantly improved locomotor function and survival. Improved locomotor function and survival of SOD1-G37R mice supports the potential for Cu\textsuperscript{II}(atsm) as a novel treatment option for ALS.

Key words: Amyotrophic lateral sclerosis (ALS), diacetylbis(4-methylthiosemicarbazone)-copper\textsuperscript{II} [Cu\textsuperscript{II}(atsm)], riluzole, rotarod, therapeutic

Introduction

Riluzole is the only approved therapeutic for ALS. An initial clinical trial found riluzole at a single dose improved patient survival and slowed the rate of muscle strength deterioration (1). A follow-up trial that tested a range of doses in a larger cohort of patients confirmed the extension of patient survival but could not reproduce the effects on muscle functionality (2) and concluded that an intermediate dose provided the best benefit-to-risk ratio (2). ALS therefore remains classed as an orphan disease, necessitating a concerted effort to find disease-modifying drugs.

Efficacy of diacetylbis(4-methylthiosemicarbazonato)copper\textsuperscript{II}, Cu\textsuperscript{II}(atsm), has been shown in the low copy number SOD1-G93A mouse model of ALS (3). Cu\textsuperscript{II}(atsm) is a neutral and lipophilic complex that readily crosses the blood-brain barrier (4). Treating low copy number SOD1-G93A mice 5 days/week from a pre-symptom age with 30 mg/kg body weight Cu\textsuperscript{II}(atsm) delayed onset and progression of a locomotor deficit and extended lifespan by 14%, and treatment beginning after symptom onset extended survival by 10% (3). Biochemical analyses revealed the Cu\textsuperscript{II}(atsm) treatment decreased a range of markers of disease progression indicating broad neuroprotective activity. Consistent with this, Cu\textsuperscript{II}(atsm) also has neuroprotective and therapeutic activity in multiple mouse models of Parkinson’s disease (5). The mechanism of action for Cu\textsuperscript{II}(atsm) in animal models of neurodegenerative disease remains to be fully elucidated and this is the subject of ongoing research.

The present study was performed to examine therapeutic efficacy of Cu\textsuperscript{II}(atsm) in a more aggressive...
mouse model of ALS (SOD1-G37R) (6) using clinically relevant treatment paradigms. SOD1-G37R mice were treated seven days a week with multiple doses of Cu II (atsm) from a pre-symptom age to establish whether therapeutic efficacy is dependent on dose administered. The effects of Cu II (atsm) were directly compared with riluzole, and mice were treated with Cu II (atsm) and riluzole simultaneously to determine if there were any additive effects. Mice were also treated with Cu II (atsm) following symptom onset. The criteria used to determine symptom onset were unique to this study and established symptom onset via a non-invasive assessment of locomotor functionality. The outcomes generated support Cu II (atsm) as a novel treatment option for ALS.

Materials and methods

Animals

SOD1-G37R mice from The Jackson Laboratory (Bar Harbor, USA) were group housed with free access to food and water and a 12 h/12 h light/dark cycle. Environmental enrichment was limited to sawdust and shredded paper bedding. Tail snips taken at weaning were used to genotype the mice as per The Jackson Laboratory website. The use of mice complied with National Health and Medical Research Council guidelines and was approved by a University of Melbourne Animal Ethics Committee.

Preparation of compounds and administration to mice

Cu II (atsm) and riluzole were synthesized according to protocols described elsewhere (7–9). Both compounds were prepared in standard suspension vehicle by sonication for 60 s immediately prior to administration to mice by gavage. Treatments were randomly spread across litters with approximately equal gender distribution. Non-transgenic littermates were used as controls. Investigators administering the treatments were blinded to mouse genotype.

For studies in which treatment began before symptom onset, treatment commenced when the mice were 38–41 days old. Mice received a single daily dose of Cu II (atsm), riluzole, or an equivalent volume of vehicle. When cotreated, Cu II (atsm) was administered 1 h prior to riluzole. These treatments were all administered once daily, seven days/week. For the study in which treatment commenced after symptom onset (described below), the mice were administered Cu II (atsm) or an equivalent volume of vehicle by gavage twice daily, seven days/week.

Assessment of locomotor function – rotarod task

Locomotor function of the mice was assessed via rotarod. Mice were assessed twice a week beginning ~84 days of age (pre-symptomatic). The rotarod accelerated from 4 to 40 rpm over 180 s and the time at which each mouse failed the task recorded as ‘latency to fall’. Assessors were blinded to treatments.

Definition of symptom onset

For each mouse in the post symptom onset treatment study, a baseline rotarod score was calculated by averaging its rotarod performance from 100 to 130 days of age (pre-symptomatic). Symptom onset was then defined as the age at which an individual mouse declined to 80% of its baseline rotarod score after the age of 130 days.

Definition of disease end-stage

Disease end-stage was defined as the age at which a mouse had complete paralysis of the hind limbs, a rotarod score of less than 3 s, and was unable to right itself in less than 30 s after being placed on its side. This was in compliance with ethics guidelines stating that mice must be killed once they can no longer freely access food and water. At end-stage, mice were killed by cervical dislocation by assessors blinded to treatment.

Statistical analyses

Significance of survival data was determined using the log-rank (Mantel-Cox) test. Significance of rotarod data was determined using a two-way repeated measures ANOVA with Bonferroni post-test on the main effect of treatment. The number of mice in each study is shown in the figure legends.

Results

Regardless of treatment, SOD1-G37R mice displayed impairment in locomotor function compared with non-transgenic control mice from the beginning of the rotarod testing period. Latency to fall scores prior to 150 days of age for the SOD1-G37R mice were ~130 s compared to ~153 s for non-transgenic littermates (Figure 1A). At this early stage the impairment was not yet progressive and therefore not considered indicative of symptom onset. By contrast, vehicle treated SOD1-G37R mice displayed a pronounced progressive decline in locomotor function that began at ~150 days of age. Treatment with Cu II (atsm) delayed this progressive locomotor decline in the SOD1-G37R mice in a dose-dependent manner (Figure 1A) without showing any stimulatory effects on non-transgenic mice (Figure 1B). Consistent with the rotarod data, Cu II (atsm) also increased survival of SOD1-G37R mice in a dose-dependent manner (Figure 1C). The median survival for vehicle treated SOD1-G37R mice was 196 days. This was increased by 8.4% with 10 mg/kg Cu II (atsm), by 18.2% with 30 mg/kg Cu II (atsm), and by 26.3% with 60 mg/kg Cu II (atsm).
**Cu\textsuperscript{II}(atsm) as a therapy for ALS**

Deaths were not observed in the non-transgenic littermates and the absence of any change to mouse body weight indicated the compound was well tolerated (Figure 1D).

In contrast to treating with Cu\textsuperscript{II}(atsm), treatment with riluzole did not improve locomotor function in the SOD1-G37R mice (Figure 2A). Cotreatment with riluzole had neither an additive nor deleterious effect on the activity of Cu\textsuperscript{II}(atsm) (Figure 2A). Treatment with riluzole marginally improved median survival by 3.3% but this was not significant (Figure 2B). The increase in survival for mice treated with Cu\textsuperscript{II}(atsm) and riluzole together (17.1%) was comparable to the increase for mice treated with Cu\textsuperscript{II}(atsm) alone (18.2%).

Rotarod data collected from an untreated cohort of SOD1-G37R mice showed the decrease in rotarod score to 80% of baseline was detected at 154 days whereas a 5% decrease in body weight was not detected until 189 days (Figure 3A). A rotarod

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**Figure 1.** Effects of Cu\textsuperscript{II}(atsm) on SOD1-G37R mouse locomotor function and survival relative to the dose administered. (A) Locomotor function of SOD1-G37R assessed using rotarod. Daily gavage with vehicle or Cu\textsuperscript{II}(atsm) at the doses shown commenced at the pre-symptom age of 38–41 days. All doses tested improved locomotor performance of the SOD1-G37R mice ($p < 0.05$, $n = 14$, 10 mg/kg; $p < 0.01$, $n = 9$, 30 mg/kg; $p < 0.0001$, $n = 12$, 60 mg/kg). (B) Locomotor function of non-transgenic littermates was not affected by Cu\textsuperscript{II}(atsm) ($p > 0.5$, $n = 14$, 10 mg/kg; $p > 0.1$, $n = 17$, 30 mg/kg; $p > 0.5$, $n = 14$, 60 mg/kg). (C) Survival of the mice from the study shown in (A) was analysed using Kaplan-Meier survival curves. Cu\textsuperscript{II}(atsm) extended median survival from 196 days for vehicle treated mice by 8.4% ($p < 0.0001$, 10 mg/kg), 18.2% ($p < 0.0001$, 30 mg/kg) and 26.3% ($p < 0.0001$, 60 mg/kg). (D) Body weight of non-transgenic littermates was not affected by Cu\textsuperscript{II}(atsm) ($p > 0.1$, $n = 14$, 10 mg/kg; $p > 0.1$, $n = 17$, 30 mg/kg; $p > 0.1$, $n = 14$, 60 mg/kg). Error bars represent standard error of mean.

**Figure 2.** Cotreatment of SOD1-G37R mice with Cu\textsuperscript{II}(atsm) and riluzole. (A) The locomotor function of the mice was tested using rotarod. Daily gavage with vehicle or Cu\textsuperscript{II}(atsm) at 30 mg/kg body weight commencing at the pre-symptom age of 38–41 days delayed the progression of a locomotor deficit in SOD1-G37R mice ($p < 0.01$, $n = 14$). Cotreatment with Cu\textsuperscript{II}(atsm) at 30 mg/kg and riluzole at 20 mg/kg did not have any additive effect on locomotor function compared to treating with Cu\textsuperscript{II}(atsm) alone ($p > 0.5$, $n = 10$ for Cu\textsuperscript{II}(atsm) + riluzole treated mice). Treatment with riluzole alone (20 mg/kg body weight) did not improve the locomotor function of SOD1-G37R mice ($p > 0.5$, $n = 11$). Error bars represent standard error of mean. (B) Kaplan-Meier analysis of survival of the mice from the study shown in (A) showing riluzole treatment marginally improves median survival by 3.3% ($p = 0.0525$). Cotreatment with Cu\textsuperscript{II}(atsm) and riluzole does not have an additive improvement on survival (229 days, 17.1% compared to 231 days, 18.2% for 30 mg/kg Cu\textsuperscript{II}(atsm) alone, $p > 0.1$).
method for defining symptom onset was therefore used and SOD1-G37R mice were treated twice daily (30 mg/kg CuH(atsm) per treatment) seven days a week once symptom onset was identified. Mean age at symptom onset did not significantly differ between the different treatment groups. Treatment with CuH(atsm) commencing post symptom onset significantly attenuated the progressive decline in locomotor function (Figure 3B) and improved survival. Median survival from the time of symptom onset was increased 43% (Figure 3C) and overall lifespan increased 11.7% (Figure 3D).

Discussion

Efficacy of CuH(atsm) as a potential therapeutic for ALS has been shown in the low copy number SOD1-G93A mouse model (3). The current study extends these preliminary results using more comprehensive and relevant treatment paradigms including cotreatment with riluzole and using an assessment of symptom onset based on locomotor function. Previous studies that indicated therapeutic potential for CuH(atsm) in models of neurodegenerative disease administered a dose of 30 mg/kg (3,5) and it had not yet been established if the in vivo therapeutic benefit of CuH(atsm) was dose dependent. This study shows the therapeutic effect of CuH(atsm) is dose dependent (Figure 1). Whether higher doses may be more effective or begin to show signs of toxicity remains to be investigated.

The SOD1-G37R model used in the present study is a more aggressive model of ALS than the low copy number SOD1-G93A mice used previously (3,6). Despite this, the same dose of CuH(atsm) (30 mg/kg) increased survival and improved locomotor function in the SOD1-G37R mice to a greater extent than in the low copy number SOD1-G93A mice (3). This most likely reflects that CuH(atsm) was administered seven days/week as opposed to five days/week previously (3) and is consistent with the elimination half-life of a single dose of 30 mg/kg CuH(atsm) in the plasma being 3.3 h (5).

The present study provides the first comparison of CuH(atsm) and riluzole in ALS model mice. Riluzole administered on its own did not alter the locomotor phenotype of the mice but there was a trend towards an increase in survival (Figure 2). A higher number of mice tested may have provided a significant result, which would be consistent with
other studies that have tested riluzole in the high copy number SOD1-G93A mouse model (10,11). Cotreatment with riluzole did not have an additive therapeutic effect in the SOD1-G37R mice. Other studies have reported varying results from cotreatment with riluzole and other putative drugs (12,13). Most have reported that riluzole does not interfere with the action of putative drugs, and this appears to also be the case for CuII(atsm). This is an important factor when considering potential clinical translation of CuII(atsm) as the majority of ALS patients recruited to a clinical trial are likely to be prescribed riluzole.

In humans, diagnosis of ALS relies heavily on clinical observations. Given this, pre-clinical in vivo studies which aim to test potential therapeutics need to incorporate treatment regimes that are based on the locomotor phenotype of the animals. Attempts to achieve this include systems that score mice on the rotarod criterion that was sensitive, objective, accurate and easily reproducible. Using this method we found CuII(atsm) administered post-symptom onset improved survival post-symptom onset by 43% and overall lifespan of the mice by 11.7%.

This study used clinically relevant and comprehensive treatment paradigms to further characterize the therapeutic potential of CuII(atsm) in a second mouse model of ALS. Greater exposure to the compound improved the therapeutic effects compared to the previous study (3), including when treatment began following symptom onset. CuII(atsm) was more effective than riluzole in the SOD1-G37R mice and the two compounds were able to be coadministered. These findings, together with those from previous studies (3,5), provide support for further development of CuII(atsm) or analogous compounds as a therapeutic option for the treatment of ALS and other neurodegenerative disorders.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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