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Riluzole, ALS, SOD1, transgenic animals

Abbreviations

ALS	Amyotrophic Lateral Sclerosis
DMSO	Dimethyl Sulfoxide
FUS	Fused in Sarcoma
Ril	Riluzole (2-amino-6-trifluoromethoxy benzothiazole)
Non-tg	Non-transgenic
PND	Post Natal Day
Ril	Riluzole
SOD1	Superoxide Dismutase 1
TDP-43	Transactivation Domain Protein of 43 kDa
Tg	Transgenic
Veh	Vehicle

Abstract

Background: Riluzole is the most widespread therapeutic for treatment of the progressive degenerative disease amyotrophic lateral sclerosis (ALS). Riluzole gained FDA approval in 1995 before the development of ALS mouse models. We assessed Riluzole in three transgenic ALS mouse models: the $SOD1^{G93A}$ model (1), the $TDP\text{-}43^{A315T}$ model (2), and the recently developed FUS (1-359) model (3).

Methods: Age, sex and litter-matched mice were treated with Riluzole (22 mg/kg) in drinking water or vehicle (DMSO) from symptom onset. Lifespan was assessed and motor function tests were carried out twice weekly to determine whether Riluzole slowed disease progression.

Results: Riluzole treatment had no significant benefit on lifespan in any of the ALS mouse models tested. Riluzole had no significant impact on decline in motor performance in the FUS (1-359) and $SOD1^{G93A}$ transgenic mice as assessed by Rotarod and stride length analysis.

Conclusions: Riluzole is widely prescribed for ALS patients despite questions surrounding its efficacy. Our data suggests that if Riluzole was identified as a therapeutic candidate today it would not progress past pre-clinical assessment. This raises questions about the standards used in pre-clinical assessment of therapeutic candidates for the treatment of ALS.

Introduction

ALS is a progressive debilitating disease characterised by a progressive loss of motor neurons which leads to muscle wasting, paralysis and death. Despite investigation of over 60 molecules as potential therapeutics for ALS there are currently only two FDA-approved treatments, recent clinical trials are summarised in (4).

Riluzole was initially investigated as a therapeutic for ALS following a report showing that it can inhibit synaptic release of glutamate in hippocampal slices (5). It has subsequently been linked with additional pharmacological activities including modulation of AMPA (6), and GABA receptors (7), inhibition of persistent sodium and calcium currents (8, 9), and activation of AMP-activated protein kinase (10) in neurons, and stimulation of NGF and BDNF in astrocytes (11), reviewed in (12).

A phase I clinical trial revealed a 200 mg daily dose of Riluzole was well tolerated in healthy people however no ALS mouse models were available at the time for preclinical testing. Therefore it was accelerated into a randomized control trial where it showed beneficial effects which were more pronounced in patients with bulbar onset than those with limb onset (13). Following this trial Riluzole was approved by the FDA in December 1995 as patients treated with Riluzole showed significantly increased lifespan along with significantly less deterioration in muscle strength when compared to the placebo group. A larger follow up study investigated Riluzole doses of 50 mg, 100 mg, and 200 mg daily, and this showed that the 100 mg dose gave the best benefit-to-risk ratio due to increased serum alanine transferase levels at the 200 mg dose (14). A third clinical trial, which included older patients treated at a later disease stage, showed Riluzole gave no benefit (15). A Cochrane Review of clinical trials published in 2012 concluded that 100 mg Riluzole daily is reasonably safe and probably prolongs lifespan for around 2-3 months in ALS patients (16). However reports persist that only a subset of patients benefit from taking Riluzole which was confirmed in a small study (Sojka 1997).

In this study we aimed to assess the therapeutic effect of Riluzole on lifespan and motor function in three separate ALS mouse models. Surprisingly we found that systemic dosing of Riluzole in drinking water from symptom onset had no effect on lifespan or

motor function in any of the preclinical ALS mouse models tested, emphasizing the difficulties regarding the use of transgenic ALS mouse models in the pre-clinical assessment of therapeutic candidates for the treatment of ALS.

Methods

Animal strains

SOD1^{G93A} mice (C57B6.Cg-Tg (SOD1^{G93A}) 1Gur/J mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and originally generated in the laboratory of Professor Siddique (1). The SOD1^{G93A} transgene copy number was verified in breeding males from our colony, see Supplementary Figure 1. TDP-43^{A315T} mice on a congenic C57Bl/6 background (B6.Cg-Tg(Prnp-TARDBP*A315T)95Balo/J) were purchased from The Jackson Laboratory (Bar Harbour, Maine, USA) and originally generated in the laboratory of Dr Baloh (2). FUS (1-359) mice generated in the laboratory of Professor Buchman (3) were re-derived at the Institute of Molecular Genetics ASCR, Prague, Czech Republic, they are congenic on the C57Bl/6 background.

Animal Maintenance

Mice were housed at constant temperature (22 °C) on a 12 h light/dark cycle (07:00 h on, 19:00 h off), with *ad libitum* food and water. Experimental mice from the TDP-43^{A315T} colony were weaned at post-natal day (PND) 21 at which time they were switched on to a high fat jelly diet (DietGel Boost, Clear H20 Maine, USA). Pups from litters of the same generation were housed in groups of 3-5 per cage. Genotyping was performed using primers and conditions for SOD1^{G93A} and TDP-43^{A315T} available at www.jax.org, and for FUS (1-359) in (3). Ethical approval was received for this project from the RCSI Research Ethics Committee (REC447 & REC1122) and licences were obtained from the Health Products Regulatory Authority (HPRA: AE19127/P003 and AE19127/P004).

Assessment of lifespan and disease progression

Animals in the study were age-, sex- and litter-matched according to ALS community preclinical guidelines (17) and sample size power calculations are provided in Supplementary Data. End stage of disease in all strains was determined by loss of righting reflex when mice were placed on their back for 15 seconds according to the ALS guidelines (17). Non-transgenic littermates were culled when no transgenic mice remained in the cage. Motor function performance was assessed by Rotarod (Stoelting, IL, USA) and stride length measurements and weight was monitored. Mice were trained on motor function equipment for 2 weeks prior to the start of recording. Observers were blinded to the treatment groups when motor function data was being recorded.

Statistical analysis

Motor function data are presented as mean +/- SEM and statistical significance was assessed by one way ANOVA with post-hoc Tukey's test. Survival data were analysed by Kaplan-Meier curves with significance determined by Mantel-Cox test. Statistical analyses were performed in SPSS statistics software (IBM).

Drug preparation and dosing

Riluzole was purchased in powdered form from AKScientific (California, USA) and reconstituted in DMSO at 22 mg/ml. The stock was diluted to a final concentration of 137.5 μg/ml in drinking water. Based on the assumptions that an adult mouse weighs approximately 25g and drinks 4 ml liquid per day, this sums up to an approximate dose of 22 mg/kg/day. This dose was chosen based on previous studies as it has been shown that plasma Riluzole concentrations reach similar levels in mice as those in ALS patients treated with 50 mg Riluzole twice daily (18-21). Vehicle solutions were made using DMSO at the same dilution.

Results

We investigated the potential therapeutic effect of Riluzole in three genetically distinct mouse models of ALS: the $SOD1^{G93A}$ mice (1); the newer $TDP\text{-}43^{A315T}$ (2) and FUS (1-359) mouse models (3), Figure 1 shows a comparison of symptom onset and disease progression in these models. Mutations in SOD1 contribute to approximately 20% of familial and 2% of sporadic ALS cases (22). A mouse model containing multiple copies of a human mutant $SOD1^{G93A}$ transgene is the most established model for preclinical

testing of therapeutics (1). $SOD1^{G93A}$ mice show uniform disease progression with transgenic (Tg) animals showing symptoms from PND 90, therefore treatment was started at PND 90. Mice develop a slow progressing hind limb weakness which eventually leads to paralysis and the humane end point in our colony occurs at PND 160-180.

The TDP-43^{A315T} mouse congenic on a C57Bl/6 background develops severe gastrointestinal problems which leads to premature death at around PND 100 due to neuronal degeneration within the solar plexus, which leads to loss of innervation in the gut and severely decreased gastrointestinal motility (23, 24). Feeding a high calorie jellified diet alleviates the gastrointestinal defect and allows the mice to live long enough to develop a motor neuron disease phenotype (24, 25). This TDP-43^{A315T} model shows variation between male and female mice, with female mice living almost twice as long as male mice and showing more variable disease penetrance (23, 26), therefore we used male TDP-43^{A315T} mice raised on a high fat jelly diet. Treatment began at PND 60 due to the observation that the gastrointestinal defects manifest from PND 60 onwards and are not completely alleviated in this model (25).

The FUS (1-359) mice express a truncated fragment of the human FUS gene under the Thy-1 promoter, which leads to cytoplasmic mis-localisation and aggregation of FUS protein in neurons (3). Tg mice develop a severe motor phenotype which displays considerable variation in symptom onset and a rapid disease course (time between symptom onset and death is less than 2 weeks). This mirrors the fast disease progression often seen in FUS mutant fALS patients (27).

To determine whether Riluzole could extend lifespan we performed a lifespan study in FUS (1-359) mice. Age, sex, and litter-matched groups were treated with Riluzole (22 mg/kg; Tg n=8 male and 8 female, Non-Tg n=4 male and 4 female) in drinking water or vehicle (DMSO; Tg n=12 male and 12 female, Non-tg n=6 male and 6 female). The earliest death in our FUS (1-359) colony occurred at PND 64; therefore treatment began at PND 50. Kaplan-Meier survival analysis for Tg mice treated with Riluzole and vehicle show that there was no significant difference between treatment groups (Fig 2A, $p=0.271$). Analysis of the genders separately revealed that female Tg mice, but not male Tg mice, showed an increase in lifespan with Riluzole treatment compared to

vehicle-treated mice (Fig 2B and C), however this was not statistically significant (p=0.265).

To assess whether Riluzole treatment could delay symptom onset or reduce the rapid decline in motor function in FUS (1-359) mice we assessed motor performance and monitored weight throughout treatment. Age-, sex- and litter-matched groups of mice were treated with Riluzole (22 mg/kg) in drinking water (Tg n=16, Non-transgenic (Non-Tg) n = 8) or vehicle (DMSO; Tg n=24, Non-Tg n=12). No significant difference in motor function ability could be detected between Tg mice treated with Riluzole or vehicle by Rotarod (Fig 3A) or stride length analysis (Fig 3B). No differences were observed when analysis was performed on gender separated groups (data not shown). Interestingly, the motor neuron degeneration that occurred in the FUS (1-359) mice was not accompanied by a change in weight as seen in other ALS mouse models (Fig 3C). Non-Tg mice treated with Riluzole (22 mg/kg) or vehicle (DMSO) showed no difference in viability or behaviour and motor function was not affected (Fig 3).

We then went on to assess Riluzole (22 mg/kg) in drinking water in $SOD1^{G93A}$ mice. These mice show a more uniform onset of degeneration and a more gradual decline in motor function (see Fig 1). Age and litter-matched groups were treated from symptom onset (PND 90) with Riluzole (22 mg/kg, Tg n=4 male and 4 female, non-Tg n=2 male and 2 female) in drinking water or vehicle (DMSO, Tg n=5 male and 8 female, Non-Tg n=4 male and 2 female). Riluzole had no significant effect on lifespan in transgenic mice from the $SOD1^{G93A}$ colony when compared to vehicle (Fig. 4; p=0.427). No differences were observed in gender separated groups (data not shown).

To determine whether Riluzole could delay symptom onset or improve motor function in $SOD1^{G93A}$ mice treated from symptom onset (PND 90) were assessed by weekly motor function testing (Fig 5). Rotarod analysis revealed Tg mice showed a slow decline in motor function over 10 weeks and there was no significant difference between Tg mice treated with Riluzole (22 mg/kg) in drinking water or vehicle (DMSO, Fig 5A). Similarly Riluzole treatment had no effect on stride length or weight which both declined gradually across disease progression in Tg $SOD1^{G93A}$ mice (Fig 5B & C).

Finally we assessed the efficacy of Riluzole from PND 60 in age- and litter-matched male TDP-43^{A315T} mice raised on a high calorie jellified diet. Kaplan-Meier analysis of survival revealed no significant difference between Riluzole (average lifespan 170.8 days +/- 10.9, n=6) and vehicle (average lifespan 166.7 days +/- 10.7, n=6 p=0.79 two tailed t test) (Fig 6). Despite the high calorie jelly diet the intestinal phenotype in the TDP-43^{A315T} mice is not completely corrected (see (25)) making assessment of motor performance in these mice difficult, therefore only lifespan data was recorded.

Discussion

Despite widespread use of the SOD1^{G93A} ALS mouse model in preclinical trials many therapeutics that show promising results have failed to show positive effects in patients. This was highlighted as an issue impacting development of novel therapeutics in the recently published ALS community guidelines which recommended development of new ALS mouse models to improve the translatability of preclinical research in ALS (17). Riluzole received FDA approval in 1995 before ALS mouse models were widely available; however two later studies showed that Riluzole (in drinking water or food) can extend lifespan in SOD1^{G93A} mice (18, 28). Subsequent studies into the efficacy of Riluzole have been performed: in low copy number SOD1^{G93A} mice Riluzole in drinking water (22 mg/kg) from PND 40 delayed symptom onset (29), in high copy number SOD1^{G93A} mice Riluzole (30 mg/kg) in drinking water from PND 60 had no significant effect on lifespan in one study (30) but treatment with Riluzole (16 mg/kg) in drinking water from PND 30 showed significant lifespan extension in another (31). The different outcomes of these studies has been attributed to different treatment paradigms, low animal numbers, and lack of gender balanced groups. In 2008 the ALS Therapy Development Institute (TDI) systematically reviewed compounds which had been published as significantly increasing lifespan in SOD1^{G93A} mice (19). Unfortunately they could not replicate the published beneficial effects, including those for Riluzole (22 mg/kg in drinking water) which had no significant effect on lifespan (19). An ALS TDI update re-assessing nine compounds found that none of the initial preclinical trial results could be replicated (32).

Here we utilised Riluzole as a benchmark to assess the suitability of other preclinical mouse models. Initially we used the FUS (1-359) mouse model (3) but found no significant effect of Riluzole (22 mg/kg in drinking water) on lifespan when compared to vehicle, irrespective of gender, and no significant effect on motor performance. We next trialled Riluzole in $SOD1^{G93A}$ mice and our data support the results from the ALS TDI in that we saw no significant effect of Riluzole on lifespan or motor performance (19, 32). Finally we assessed Riluzole in TDP-43^{A315T} mice on a high calorie jellified diet; Riluzole had no significant effect on lifespan compared to vehicle. Hence we conclude that Riluzole does not extend lifespan or improve motor performance in three preclinical ALS mouse models.

During the writing of this manuscript Edaravone was granted FDA approval as a therapeutic for ALS. Interestingly, however this drug also failed to show consistent, beneficial effects on lifespan in ALS rodent models. Edaravone is a free-radical scavenger which was originally investigated for its neuroprotective effects following cerebral ischaemia (reviewed in (33)). Interestingly Edaravone (15 mg/kg daily i.p.) showed improved motor function and preserved motor neurons in the spinal cord in $SOD1^{G93A}$ female mice, however it had no significant effect on survival (34). In a further study in the $SOD1^{H46R}$ rat model Edaravone had no significant effect on survival (35). Edaravone was licenced for treatment of cerebral ischaemia in Japan in 2001, therefore it was fast-tracked into clinical trials for ALS despite the negative results in preclinical models (36-39). This highlights the challenges facing novel therapeutics for ALS where many drugs fail at preclinical trials including the only two currently licenced therapeutics.

Given the pleiotropic nature of ALS there is potential that the mouse models used here do not fully recapitulate the pathophysiology of ALS, or the precise defect targeted by Riluzole. The transgenic models used here recapitulate several important aspects of ALS pathogenesis but we have to assume that no single model generated to date captures them all. The $SOD1^{G93A}$ model recapitulates many aspects of familial ALS caused by mutations in the SOD1 gene, including aggregation of mutant SOD1 protein and impaired proteasome function, however SOD1 mutations account for

approximately 20% of fALS and only 2% of sALS cases suggesting the wider relevance of this model may be limited (22). Aberrant RNA processing has been identified as a pathological mechanism in ALS with a majority of sporadic ALS patients showing cytoplasmic TDP-43 positive inclusions in neurons (40). We did not observe a beneficial effect of Riluzole in the TDP-43^{A315T} model, however this model does not develop cytoplasmic TDP-43 inclusions (2) and despite the high calorie jelly diet the intestinal phenotype is not completely corrected (25), hence this model has limitations. Therefore we utilised the newer FUS (1-359) mice (3), which develop FUS positive neuronal inclusions that are distinct from stress granules (41) but may affect RNA metabolism via sequestration of endogenous FUS, recapitulating proteinopathy and RNA metabolism defects. The FUS model is the most wide-ranging of our three models; yet treatment with Riluzole did not extend lifespan or improve motor function.

ALS mouse models provide valuable tools to investigate the pathogenic mechanism of ALS-associated mutations and can provide important information on common pathways involved in pathogenesis which may reveal therapeutic targets. However, our study raises important questions surrounding the use of transgenic ALS mouse models in preclinical studies and the stringency by which we assess success. If Riluzole were investigated today as a novel therapeutic it would not proceed on to clinical trials yet it has documented beneficial effects in a subset of ALS patients. Conversely, many therapeutics for ALS that show significant benefit in ALS mouse models fail in clinical trials, highlighting the need for development of additional platforms (such as patient-derived, induced pluripotent stem cells) for the pre-clinical testing of novel ALS therapeutics.

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Disclosure Statement

The authors report no conflicts of interest.

Figure legends

Figure 1: Cartoon comparison of disease progression in the three ALS mouse models used in this study. Graphical representation of disease onset and duration in A) FUS (1-359), B) TDP-43^{A315T} male mice on a high calorie jellified diet and C) SOD1^{G93A} ALS mouse models.

Figure 2: Riluzole treatment does not extend lifespan in the FUS (1-359) mouse model. A) Kaplan-Meier analysis of survival in transgenic FUS (1-359) mice treated with Vehicle (blue, DMSO, n=24) or Riluzole (green, 22 mg/kg, n=16) in drinking water from PND 50. No significant effect on lifespan was recorded. B) Lifespan analysis in male transgenic FUS (1-359) mice treated with DMSO (n=12) or Riluzole (n=8) showed no significant difference in lifespan. C) Lifespan analysis in female transgenic FUS (1-359) mice showed no significant difference between DMSO (n=12) or Riluzole (n=8) treated groups.

Figure 3: Riluzole treatment does not improve motor function in the FUS (1-359) mouse model. A) Rotarod assessment of motor function was performed throughout treatment in transgenic (Tg) and non-transgenic (Non-Tg) FUS (1-359) mice treated with Riluzole (22 mg/kg) or Vehicle (DMSO) in drinking water from PND 50 onwards. No significant difference in onset or rate of decline could be detected. B) Analysis of stride lengths revealed no significant difference between Tg mice treated with vehicle (DMSO) or Riluzole (22 mg/kg). C) No significant difference in weight could be detected between Tg or Non-Tg mice, or between those treated with Riluzole (22 mg/kg) or vehicle (DMSO). Data shows mean +/- SEM, statistical significance was assessed by one way ANOVA with post-hoc Tukey's.

Figure 4: Riluzole treatment does not improve lifespan in the SOD1^{G93A} mouse model. Kaplan-Meier analysis of survival in the SOD1^{G93A} mouse model treated with Riluzole (22 mg/kg) or vehicle (DMSO) in drinking water from PND 90 onwards. There is no significant difference in survival between Riluzole treated Tg mice (green, n=8) and vehicle (DMSO) treated mice (blue, n=13, p=0.427).

Figure 5: Riluzole treatment does not improve motor function in the SOD1^{G93A} mouse model. A) Assessment of coordination and balance via rotarod testing revealed no significant difference between Tg mice treated with Riluzole (22 mg/kg, n=8) or vehicle (DMSO) treated mice (n=13). Both groups showed a gradual decrease in motor skills across disease progression. B) There was no significant difference between stride lengths measured across disease progression in Tg mice treated with Riluzole or vehicle (DMSO) control. C) There was no significant difference in weight between Tg mice treated with Riluzole and Tg mice treated with vehicle (DMSO). Data shows mean +/- SEM, statistical significance was assessed by one way ANOVA with post-hoc Tukey's.

Figure 6: Riluzole treatment does not extend lifespan in TDP-43^{A315T} mice. Kaplan-Meier analysis of mice treated with Riluzole (22 mg/kg) or vehicle (DMSO) in drinking water from PND 60 onwards. There was no significant difference in survival between Tg mice treated with Riluzole (green, n=6) or vehicle (DMSO, blue, n=5, p=0.975).