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Comparative Effect of Different Treatments with a Bacterial Polyketide in a Mouse Model of ALS (Amyotrophic Lateral Sclerosis)

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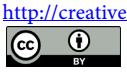

Abstract

Amyotrophic lateral sclerosis (ALS) is characterized by motor deficits that affect voluntary movements. This research aims to assess the impact of mithramycin (MTM) therapy on these deficits. Mice were subjected to behavior tests to determine whether chronic MTM therapy, initiated by the third month of life, leads to measurable improvements in motor skills. Muscle viability was evaluated using rotarod and footprint tests. The overall quality of life for the animals was assessed by monitoring their total body weight. To examine potential increases in life expectancy associated with MTM therapy in this ALS mouse model, a Kaplan-Meier survival analysis was conducted. The results indicate that treatment beginning in the first month of life with a dosage of 30 μg/kg/day yields maximum values for parameters related to muscle atrophy reduction, weight gain, and increased life expectancy. Additionally, the findings suggest an enhancement in survival when a higher dosage of 150 μg/kg/day is administered starting at three months of age. These functional improvements are consistent with a proposed neuroprotective mechanism involving the NO/ROCK/Sp1/S100A10/TASK-1 pathway, though direct molecular evidence for this pathway was not obtained in this study.

Keywords

Amyotrophic Lateral Sclerosis (ALS), Mithramycin, SOD1G93A Transgenic Mouse Model

1. Introduction

Amyotrophic lateral sclerosis (ALS) is characterized by the loss of motor neurons

and astrogliosis in the motor cortex and spinal cord. This degeneration primarily affects spinal motor neurons, resulting in paralysis and ultimately leading to the death of patients. Understanding the molecular mechanisms underlying ALS is crucial for developing new therapeutic approaches [1]. Research has shown that motor neurons with the SOD1G93A mutation exhibit higher sensitivity to excitotoxic stimuli compared to non-transgenic motor neurons [2]. This finding highlights the significance of the Sp1/S100A10/TASK-1 pathway in ALS degeneration. Additionally, it is observed that anatomical and pathological changes in motor neurons occur long before any noticeable alterations in animal behavior [3]. This suggests that early diagnosis may soon become feasible, paving the way for timely clinical interventions.

ALS, also known as Lou Gehrig's disease and first described by Charcot, is a severe and fatal degenerative disorder. It occurs sporadically around the world but was endemic on the island of Guam, where it was associated with Parkinson's disease and dementia. The pathology of ALS on Guam resulted from the consumption of seeds from the alga Cycas circinalis, which contains an endotoxin that acts on glutamate receptors, specifically the amino acid beta-methylamino-L-alanine (BMAA). BMAA, which activates NMDA receptors, also stimulates AMPA receptors that are notably present in motor neurons, making them particularly vulnerable to calcium-mediated excitotoxicity. Monkeys that consumed cycad seeds exhibited similar pathologies, showing behavioral abnormalities accompanied by degenerative changes in the motor cortex and ventral spinal cord. Research, both in vitro and in vivo, suggests that this unique vulnerability to glutamate is mediated by AMPA receptors [4].

In the brains of rats, NMDA receptor activation induces the synthesis of nitric oxide (NO) from arginine, establishing a pathway for NO in the central nervous system. NMDA receptors are also suggested to be a potential source of overexpression for mutant hSOD1G93A motor neurons in the sacral region. Excessive stimulation of NMDA receptors has been extensively studied to analyze neuromuscular junction pathology in ALS patients. Two primary mechanisms are proposed for excitotoxic motor neuron degeneration in ALS: (i) glutamate accumulation due to impaired clearance resulting from the selective loss of the astroglial glutamate transporter EAAT2/GLT1, which has been observed in both genetic mouse models and ALS patients, and (ii) an increased number of calcium-permeable AMPA receptors, as documented in the spinal motor neurons of hSOD1G93A mice [5]. Additionally, presynaptic reorganization and NO-mediated inhibition of TASK potassium channels contribute to membrane depolarization and increased excitability, aligning with the long-term effects of NO on TASK channels in hypoglossal motor neurons [1].

Although ALS can affect individuals at any age, it typically manifests in the fourth or fifth decade of life. The most common clinical features include muscle weakness, spasmodic muscle twitching, reduced reflexes, and extensor plantar responses. While symptoms often predominate in the extremities, impairment of

bulbar function (involving the muscles responsible for speech, swallowing, and chewing) can also occur, leading to tongue atrophy, dysphagia, and dysarthria. This muscle paralysis usually does not involve other cranial nerves, such as the oculomotor nerves. The gradual loss of muscle function results in paralysis, speech and motor disabilities, emotional disturbances, and ultimately respiratory failure, leading to death in most ALS patients within 2 to 5 years of disease onset. The incidence of ALS ranges from 1 to 2 cases per 100,000 individuals each year. It remains very low below age 40 but increases significantly, peaking between ages 65 and 75. The prevalence of the disease, determined by its incidence and patient survival rates, varies from 4 to 6 per 100,000 per year, with a lifetime risk of developing ALS estimated at 1 in 600 to 1 in 1,000 [6].

While most cases are classified as sporadic ALS (SALS), about 10% are inherited, known as familial ALS (FALS). Age and sex are documented risk factors for sporadic cases, with a male-to-female ratio of approximately 3 to 2. Furthermore, mutations in the SOD1 gene were the first identified genetic cause of familial ALS [6] [7]. In mouse models, the human SOD1-G93A mutation is commonly studied using transgenic animals in which the mutated gene is excised by the bacterial recombinase enzyme Cre [8]. But in this contribution, we present a transgenic model that overexpresses the human mutant SOD1 (G93A) without involving Cre-mediated excision. The percentage of ALS cases attributed to SOD1 mutations is now given as ~2% of all cases (and ~10 - 20% of familial cases). Since the discovery of SOD1, numerous other genetic causes have been identified, including expansions in C9orf72, and mutations in TARDBP, FUS, TBK1, and NEK1. These discoveries have highlighted diverse pathogenic mechanisms, including RNA toxicity, protein aggregation, and impaired autophagy. Downstream of these genetic triggers, our previous work has elucidated critical pathways in motor neuron degeneration, including nitric oxide signalling [1], Sp1-regulated p11/TASK-1 expression [2], and LPA1 receptor signalling [3], all of which may intersect with the pathogenic cascades initiated by these genetic defects.

Thus, ALS is considered an orphan disease that has not been widely adopted by the pharmaceutical industry, even though its uniform lethality is not proportional to its prevalence. In the Spanish population, the incidence of ALS is 2 per 100,000, while the prevalence stands at 1 in 10,000 [9]. Previous studies have shown that mithramycin, an inhibitor that prevents the binding of the transcription factor Sp1 to DNA [10], can reduce motor deficits in transgenic mice carrying the SOD1-G93A mutation [2]. While mutations in the SOD1 gene (superoxide dismutase enzyme) account for only about 20% of ALS cases, this enzyme is expressed in all tissues throughout life [11]-[13]. In motor neuron cells, mithramycin (MTM) has been found to decrease the overexpression of nitric oxide producing synthase (NOS1), the primary source of NO in the brain [14]. This process is considered crucial because NOS1 overexpression is the hallmark of ALS [1].

Despite these findings, there is limited research on the molecular signaling mechanisms that alter K+ homeostasis and contribute to the development of ALS,

particularly in relation to NO overproduction [7]. In mouse motor neurons treated with MTM, several changes were observed: A decrease in S100A10 retention factor levels in the endoplasmic reticulum, an increase in the expression of the TASK-1 subunit in the plasma membrane, and a reduction in apoptotic cascades. This reduction is linked to decreased mitochondrial dysfunction connected to the activation of effector caspase-3 and the release of cytochrome c [15]. Additionally, MTM inhibits the small Rho GTPase protein RhoA and its main effector, Rho kinase (ROCK), both of which promote neuropathological progression related to NO [16].

ROCK is a serine/threonine kinase that, through the phosphorylation of myosin light chain (MLC), contributes directly or indirectly to the contraction of hippocampal and cellular synapses, as well as to the formation of membrane vesicles [17] [18]. Furthermore, MTM exerts neuroprotective effects by inhibiting the binding of other transcription factors in GC-rich regions of DNA and by providing neuroprotection against glutamate-induced excitotoxicity [19].

The current investigation aims to evaluate the potential effects of MTM on the retrograde action of NO at synaptic terminals. This interaction involves cytochrome c oxidase in motor neuron cells exposed to mutated SOD1, which is associated with mitochondrial damage. Based on previous mechanistic studies [2] [10] [15] [16], we hypothesize that the beneficial effects of MTM observed in this SOD1G93A mouse model may be mediated, at least in part, through the NO/ROCK/Sp1/S100A10/TASK-1 pathway. However, the present study focuses on the functional and survival outcomes of MTM treatment; direct molecular confirmation of this pathway was not undertaken and remains a subject for future investigation.

2. Materials and Methods

2.1. Materials and Drugs

2.1.1. Drug Preparation and Administration

To evaluate the impact on neuronal survival through the inhibition of the NO/ROCK/Sp-1/S100A10/TASK-1 pathway and to prevent apoptosis, we utilized Mithramycin A (C52H76O24). Mithramycin is a potent inhibitor of RNA synthesis, effective in both mammalian and bacterial cells, in vitro and in vivo. Additionally, it inhibits enzymatic methylation and the nucleolytic degradation of DNA.

Mithramycin A (Sigma-Aldrich) was dissolved in 5% sucrose solution to achieve the desired concentrations. Three treatment regimens were applied:

- Regimen A (early, low dose): 30 μg/kg/day, starting at 1 month of age (postnatal week 4), administered via oral gavage once daily (200 μL volume). Dose was adjusted weekly based on individual body weight.
- Regimen B (late, low dose): 30 μg/kg/day, starting at 3 months of age (week 12), same route and volume.
- Regimen C (late, high dose): 150 μg/kg/day, starting at 3 months of age (week 12), same route and volume.

The vehicle control groups received 5% sucrose solution only. Treatment continued daily until the animal was perfused or died naturally.

2.1.2. Murine Model

This study used transgenic mice with a mutation in the SOD1 gene, which replaces the glycine residue with an alanine residue at position 93 of the enzyme (known as Gly93Ala or G93A). This mutation leads to increased free radical generation, contributing to muscle atrophy in the hind legs.

The SOD1G93A mice were maintained in a heterozygous state under standard conditions (temperature: μ ± 1˚C; 12-hour light-dark cycle) with unrestricted access to food. Thirty-two male mice (20 transgenic and 12 wild-type) were tested, receiving 30 μg/kg/day of the drug. Twenty-eight transgenic male mice were assayed at a dose of 150 μg/kg/day. Chronic treatment with MTM began at 12 weeks of age, when signs of motor impairment in the lower limbs appeared. Sucrose and the drug were administered orally daily until the animals were perfused or died.

2.1.3. Animals

A total of 75 male mice were used. The SOD1G93A transgenic mice (B6SJL-Tg (SOD1*G93A)1Gur/J) were obtained from The Jackson Laboratory and maintained by the Animal Experimentation and Production Service (SEPA) of the University of Cádiz. Wild-type (wt) littermates of the same genetic background served as controls. All procedures were approved by the local Ethics Committee and followed EU and Spanish regulations.

Animals were randomly assigned to experimental groups using a computer-generated randomisation sequence. The investigator performing behavioural tests (rotarod and footprint) was blinded to genotype and treatment allocation. Sample size (n = 6 - 28 per group) was determined a priori based on a power analysis ($\alpha = 0.05$, $\beta = 0.20$) using effect sizes from our previous work with MTM in this model.

2.1.4. Study Type

This is a quantitative controlled treatment experimental study consisting of two experiments. The first is a randomized study, as the treatment was assigned through a lottery mechanism.

In the first experiment, the 32 male animals were divided into three experimental groups: a) 6 wt animals (wild-type) treated with 5% sucrose in water; b) 6 wt animals treated with MTM (30 μg/kg/day); and c) 20 SOD1G93A mice treated with MTM (30 μg/kg/day). The second experiment consisted of 28 SOD1G93A transgenic mice treated with MTM (150 μg/kg/day) as a single group.

A group of SOD1G93A transgenic animals (n = 15) treated with a vehicle (5% sucrose) served as the disease control group for both experiments.

2.2. Methodology

2.2.1. Body Weight Determination

On a digital scale (CS2000, Ohaus®), with a maximum range of 2000 g and an error

level of 0.1 g, all animals were weighed once a week.

2.2.2. Survival Curve

Survival time was estimated as the time elapsed between the date of birth and death; although for motor neuron counting experiments, some animals were perfused at four months.

2.2.3. Motor Functional Tests

1. Rotarod: Ability to Walk on a Rolling Cylinder

To assess motor function, a rotarod test was performed. This test involved placing the mice on a rolling cylinder with an initial speed of 15 rpm (the rotating cylinder has a diameter of 3.4 cm) and an acceleration rate of 0.2 rpm/s until they fell off. Each week, we recorded the time (in seconds) that the animals were able to stand on the cylinder, beginning at 12 weeks of age in both the wild-type and transgenic groups. All animals were tested three times, with 10-minute rest intervals. This test allowed us to establish the onset of motor impairment and a possible therapeutic window for functional recovery.

2. Footprint test: analysis of footsteps patterns

Footprint analysis is an inexpensive method for studying the footstep patterns of mice in the ALS model. As the disease progresses from the lower to the upper limbs, parameters related to hindlimb movement were studied. This requires applying ink to the animal's hind paws. Static gait parameters were then measured from the resulting footprints. For example, step length and the distance between the hind paws [20].

For this purpose, strips of graph paper were attached to the floor of a corridor (50 cm long by 5 cm wide). The time required for each animal to traverse the corridor was quantified, using a 60-second cutoff point. This value was assigned to all animals that failed to reach the opposite end of the circuit within that time. The ink used to make the marking of the legs was Spezial-Tätowierfarbe, Hauptner-Heberholz.

The method is simple and provides reasonably sensitive information, although it has significant practical limitations. For example, the animals turn away, requiring a repeat test; and, when engaging in exploratory behavior, they sometimes stop.

2.2.4. Experimental Design and Data Analysis

We studied the data collected from the two experiments. In the first, three groups of animals were included: healthy animals treated with sucrose (wt, n = 6; 6 healthy males treated with sucrose), diseased animals treated with MTM (M^+, n = 20; 20 transgenic males treated with MTM), and healthy animals treated with MTM (M-, 6 healthy males treated with MTM). In the second, one group of animals were included: diseased animals treated with MTM (n = 28; 28 transgenic males treated with MTM). A separate cohort of SOD1G93A + vehicle animals (n = 15) was maintained under identical conditions and used as a baseline control for both dose groups in the two experiments.

Table 1 summarizes the composition of each cohort, including the number of animals enrolled, those that died naturally, those that were euthanized/perfused for histological analysis (censored), and the total analyzed. No animals were excluded from the analysis.

Table 1. Animals' cohorts and disposition.

Cohort	Enrolled (n)	Natural deaths	Censored (euthanised/perfused)	Analysed for survival
wt + vehicle	6	0 (all perfused at 21 weeks)	6	6
wt + MTM 30 (1 month)	6	0	6	6
SOD1G93A + vehicle	15	11	4 (at 4 months)	15
SOD1G93A + MTM 30 (1 month)	20	14	6 (at 4 months)	20
SOD1G93A + MTM 30 (3 months)	20[a]	14	6 (at 4 months)	20
SOD1G93A + MTM 150 (3 months)	28	20	8 (at 4 months)	28

Note: a. The SOD1G93A + MTM 30 (3 months) cohort is the same as the 20 animals in Experiment 1 (group c). The vehicle cohort (n = 15) was a separate set of animals obtained in addition to the 60 originally described, bringing the total to 75.

The actual data analysis was carried out by collecting measurements from digital records using manual (footprint) and automated (rotarod) stopwatches. Footprint measurements were also extracted from strips of graph paper and exported to a Microsoft Excel spreadsheet for the process of developing the footspan metrics.

Motor function was assessed weekly from 12 weeks of age using the rotarod and footprint tests. For the rotarod test, the latency to fall (seconds) was recorded, with a maximum cutoff of 180 s. Longer latencies indicate better motor coordination and endurance. For the footprint test, the following parameters were measured from paw prints:

- Stride length (mm): distance between consecutive footprints of the same hindpaw. Longer strides indicate better hindlimb function.
- Base width (mm): distance between the left and right hindpaws. A narrower base indicates better balance and less muscle atrophy.
- Corridor traversal time (s): time required to cross a 50 cm runway, with a cutoff of 60 s. Shorter times indicate better mobility.

To account for inter-animal variability in baseline performance, rotarod data were expressed as percentage of the maximum possible score (180 s), and footprint data were expressed as raw values (mm), as these are directly clinically interpretable. No further normalization was applied.

Body weight was recorded weekly and expressed as percentage of weight at 8 weeks of age to normalize for initial size differences. Survival was defined as the time elapsed from birth to the date of natural death. Animals that were euthanized for histological or biochemical analyses (e.g., motor neuron counting at 4 months

of age) or that perfused before reaching end-stage disease were treated as right-censored observations in the Kaplan-Meier analysis. Censoring was applied at the date of euthanasia or perfusion, and these animals were included in the denominator at risk until that time point. No animal was excluded from the survival analysis. Survival curves were constructed using the Kaplan-Meier method for each experimental group.

All data are presented as mean ± SEM unless otherwise noted. Comparisons between groups were performed using one-way ANOVA with Holm-Šidák post-hoc test for motor and body weight data. For survival analysis, Kaplan-Meier curves were generated, and groups were compared using the log-rank test. Median survival times are reported with 95% confidence intervals. Hazard ratios (HR) and their 95% CIs were calculated using Cox proportional-hazards regression. Animals that were euthanised or perfused at 4 months of age (for histological analysis) were treated as right-censored observations; censoring was applied at the date of the procedure, and these animals were included in the denominator at risk until that time. Censoring followed a prespecified schedule independent of disease severity (random selection of animals for perfusion at 4 months). All analyses were performed with SigmaPlot 11.0®.

The analysis tests specific, planned comparisons and applies the correction at each time point (not across all time points combined). All data are presented as mean ± standard error of the mean (SEM). For each time point (week), differences among experimental groups were assessed using one-way analysis of variance (ANOVA). Where the overall ANOVA was significant ($P < 0.05$), post-hoc pairwise comparisons were performed using the Holm-Šidák step-down correction to control the family-wise error rate across the multiple comparisons at that specific time point.

We acknowledge that separate ANOVAs at each time point do not explicitly model the correlation between repeated measurements from the same animal. However, we applied the Holm-Šidák correction to control the family-wise error rate at each time point. This approach is conservative enough to protect against false positives while retaining sufficient power to detect treatment effects at key symptomatic time points. Future studies with larger cohorts may benefit from linear mixed-effects models to formally test Group × Time interactions.

3. Results and Discussion

3.1. Determination of MTM Dose Levels for the Rotarod Test

The drug's effect on motor behavior in ALS model mice was estimated with MTM at doses of 30 μg/kg/day and 150 μg/kg/day in the drinking water, administered during the twelfth week of life. Previous studies from our laboratory showed that the physical activity of SOD1G93A transgenic mice receiving MTM at a dose of 30 μg/kg/day during the first month was always higher than that of these animals receiving nothing.

These ALS model animals were assessed for locomotor activity from week 8 of life until the end of their full lifespan (week 21). SOD1wt mice administered sucrose (SOD1wt + vehicle) and those receiving MTM (SOD1wt + MTM (30 μg/kg/day)) maintained walking on the rotating bar for an average of close to 180 s over the 12 weeks, the cutoff time for the test. Meanwhile, SOD1G93A mice that received no treatment were unable to remain on the rolling cylinder for more than 150 s after week 10. In contrast, SOD1G93A mice dosed with MTM (30 μg/kg/day) did not decrease this level until week 14.

The ALS model treated with MTM at one month of age with a dose of 30 μg/kg/day showed significantly better motor behavior than SOD1G93A mice treated from the third month (at both 30 μg/kg/day and 150 μg/kg/day doses). From week 14, there was a significant increase in the fall time of these animals.

The experimental groups of SOD1G93A ALS model mice treated from the third month also showed a significant increase in the mean fall time from week 14 onwards. Although SOD1G93A mice treated with doses of 30 μg/kg/day already show evidence of worsening at week 12 of age (SOD1G93A + MTM (30 μg/kg/day): 94.5 ± 22.2 s), the same as when the drug dose is 150 μg/kg/day (SOD1G93A + MTM (150 μg/kg/day): 118.6 ± 15.5).

In these groups of mutant animals treated with MTM from 90 days of age, studies carried out from week 14 (SOD1G93A + MTM (30 μg/kg/day): 123.75 ± 19.08; SOD1G93A + MTM (150 μg/kg/day): 132.19 ± 15.84), when both were under the same conditions, until week 19 of age (SOD1G93A + MTM (30 μg/kg/day): 11.5 ± 11.5 s; SOD1G93A + MTM (150 μg/kg/day): 0.0 ± 0.0 s), show the significant increase in the fall time of mice that received MTM at doses of 30 μg/kg/day with respect to those dosed with 150 μg/kg/day. μg/kg/day. However, in a mouse model of Huntington's disease, a previous study determined the optimal dose of MTM to be 150 μg/kg/day when administered daily via intraperitoneal injection [21].

3.2. Determining the Limits of Normal Motor Function Based on Footprint Measurement

Previous laboratory results demonstrate that a dose of 30 μg/kg/day administered to SOD1G93A mice in the first month of life improves step length and hindlimb span (**Figure 1**). At 14 weeks, no differences were observed between the three experimental groups. However, at 17 - 18 weeks, the performance of SOD1G93A mice decreased compared to WT mice. In turn, transgenic mice treated with MTM showed significant improvement compared to those receiving only sucrose. By examining **Figure 1**, we can see the following: (A) Representative footprints obtained at 18 weeks, indicating the length of the left and right hind legs, and the width of the base between the hind legs. Calibration bar: 5 mm. (B) Width of the base between the hind legs. (C) Length of the left hind leg. (D) Length of the right hind leg. Values are expressed as mean ± standard error. Differences between groups were analyzed by one-way ANOVA with Holm-Sidak post-hoc test ($P < 0.05$).

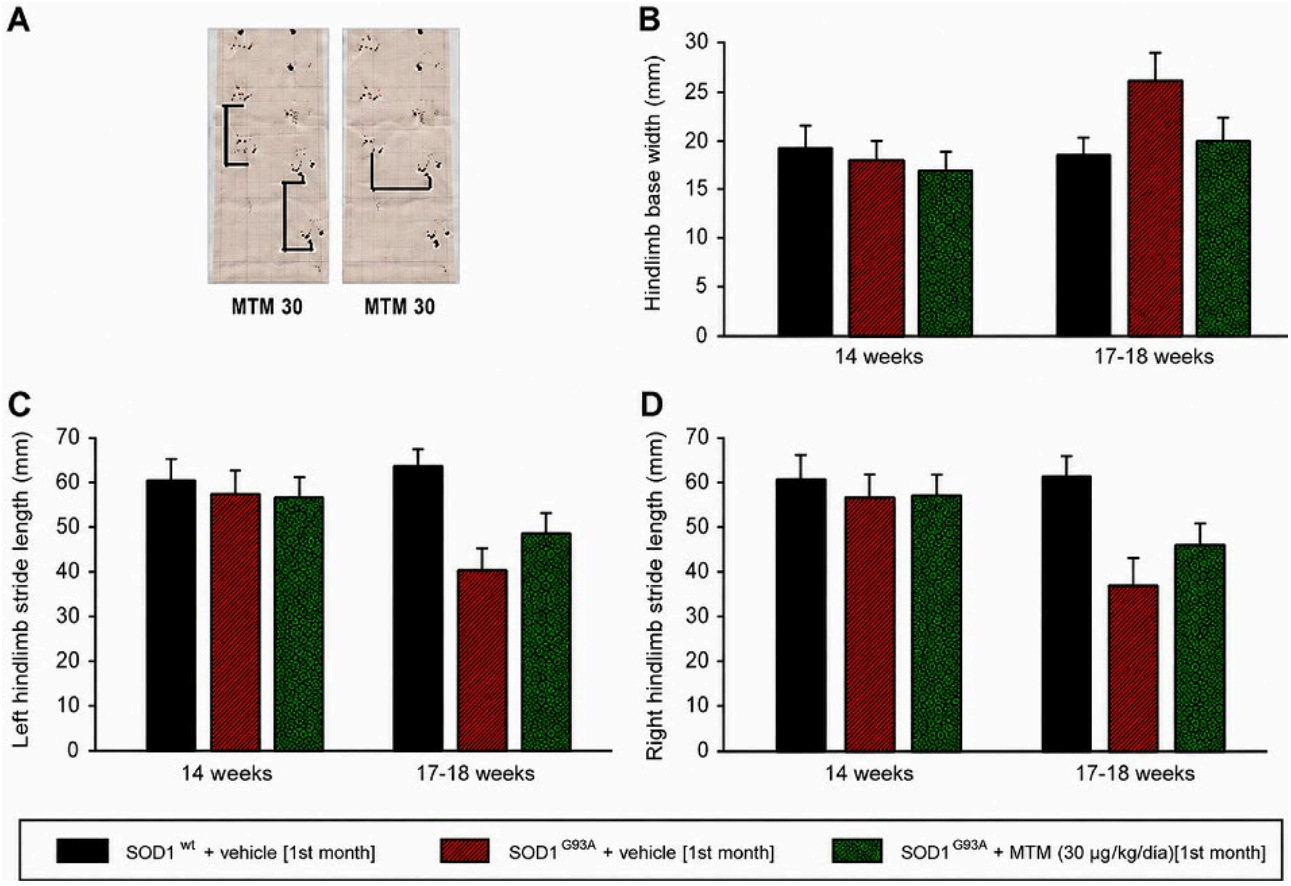

Figure 1. Effect of a 30 µg/kg/day dose of MTM administered at one month on motor behavior in SOD1G93A mice from the indicated experimental groups.

By administering doses of 30 µg/kg/day and 150 µg/kg/day to SOD1G93A mice in the third month and following their footprints using the footprint test, we were able to observe that by week 14 the three groups of SOD1G93A mice studied (those administered sucrose, MTM at a dose of 30 µg/kg/day and MTM at a dose of 150 µg/kg/day) were in similar circumstances (Figure 2). The parameters of movement of the hind limbs (step length, distance between hind limbs) and the time required to cover a 50 cm long corridor were studied. The examination of Figure 2 reveals the following details: (A) Typical footprints obtained at week 18 with the width of the base between the hind paws and the length of the step of the left and right hind paws, in the experimental groups treated with the lowest and highest doses of MTM. Calibration bar: 5 mm. (B) Width of the base between the hind paws. (C) Step length of the left hind paw. (D) Step length of the right hind paw. Their values are expressed as the mean ± standard error. $P < 0.05$ one-way ANOVA test, post-hoc Holm-Sidak test.

At weeks 17 - 18, it was observed that SOD1G93A animals treated with 30 µg/kg/day of MTM maintained a longer stride length than SOD1G93A animals treated with 150 µg/kg/day (SOD1G93A + MTM (30 µg/kg/day): 34.4 ± 4.9 mm vs. SOD1G93A + MTM (150 µg/kg/day): 21.4 ± 1.9 mm) (average data of stride

length of the right and left hind limbs) (Figure 2). Transgenic animals that received only sucrose lost more mobility in striding their left and right limbs than those treated with MTM. Differentiating between the two legs, we found that in the left hind leg (Figure 2(C)) and right hind leg (Figure 2(D)), the highest level of significance ($P < 0.05$) in step length during the degenerative phase (17 - 18 weeks) was found among SOD1G93A mice dosed with 30 μg/kg/day of MTM (SOD1G93A + MTM (30 μg/kg/day): 33.4 ± 4.6 mm (left leg), 35.4 ± 5.3 mm (right leg)). The step length results for the highest dose (150 μg/kg/day) were significantly lower (two-thirds of those obtained with 30 μg/kg/day).

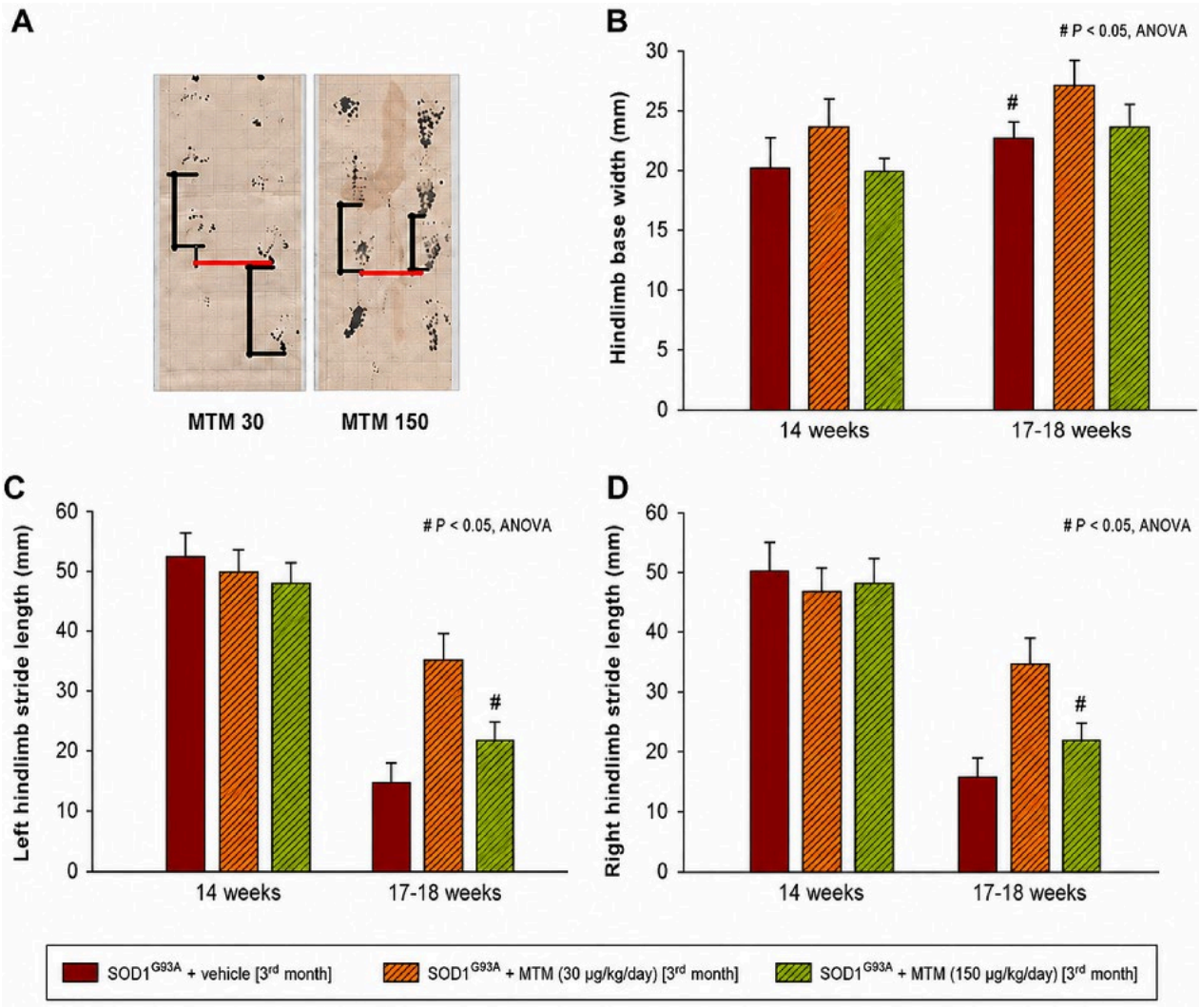

Figure 2. Effect of doses of 30 μg/kg/day and 150 μg/kg/day of MTM administered at month 3 on motor behavior in SOD1G93A mice from the indicated experimental groups.

The difference in hindlimb base measurements provides an indirect indicator of muscle atrophy in ALS-associated animals (Figure 2(B)). At 14 weeks (symptomatic phase), the atrophy profile in SOD1G93A mice was greater in mice treated with 30 μg/kg/day MTM. At the final stage (17 - 18 weeks), the greatest distance

between the hindlimb bases was also observed in SOD1G93A mice receiving 30 μg/kg/day (SOD1G93A + MTM (30 μg/kg/day): 27.6 ± 1.7 mm). The MTM dose of 150 μg/kg/day results in a shorter distance between the hind legs (SOD1G93A + MTM (150 μg/kg/day): 24.2 ± 1.7 mm).

At week 17 - 18, the stride length of SOD1G93A animals that received only sucrose was reduced by 42.7% ± 3.9% compared to the average of the other two groups that were cured (**Figure 2(C)-(D)**). Even more so compared to SOD1WT mice, the stride reduction in SOD1G93A animals treated with sucrose was 64.0% ± 3.1% on the left side and 61.4% ± 1.2% on the right. However, with the lower dose of MTM the results were much better (compared to the SOD1WT mouse, reduction of the left step by 36.5% ± 2.1% and of the right step by 32.5% ± 1.3%), and were also superior with the higher dose of MTM (left: -59.8% ± 0.8%; right: -58.4% ± 0.5%).

The evolution of the disease in SOD1G93A animals receiving the drug at a dose of 30 μg/kg/day in the first month is higher than that of other treatments administered in the third month, in the parameters of step length and width of the base of the hind legs (**Figure 3**). The analysis of **Figure 3** presents the following information: (A) Base width between both hind paws and step length in mice cured at the third month with MTM doses of 30 μg/kg/day and 150 μg/kg/day. (B) Base width between the hind paws. (C) Step length of the left hind paw. (D) Step length of the right hind paw. Two experimental groups (32, 28 mice). Values expressed as mean ± standard error. $P < 0.05$ one-way ANOVA test, post-hoc Holm-Sidak test.

If the parameter of width of the base between the hind paws improves by 30% in mice treated with MTM at the first month (with the dose of 30 μg/Kg/day) with respect to those treated at the third month (both those cured with the dose of 30 μg/Kg/day and with 150 μg/Kg/day of MTM), at 14 weeks and at 17 - 18 weeks (**Figure 3(B)**). The drug from the first month also improves the performance of the step length with respect to the third month (both at 14 weeks and at 17 - 18 weeks) (**Figures 3(C)-(D)**). Indeed, at 17 - 18 weeks, the results of the administration from the third month at both doses: left paw (30) 33.4 ± 4.6, (150) 21.2 ± 1.8; right paw (30) 35.5 ± 5.3, (150) 21.8 ± 2.0. They are lower than those obtained from the first month with the dose of 30 μg/kg/day: left paw (30) 50.2 ± 2.5; right paw (30) 46.7 ± 3.2.

As an additional measure of motor function, the animals were assessed for locomotor activity based on the time it took to cover a 50 cm corridor. During week 14, the differences were significant in the SOD1G93A experimental groups dosed at 30 and 150 μg/kg/day in the third month, compared to wt animals. Furthermore, the analysis showed that at 17-18 weeks of age, treatment at the first month in SOD1G93A animals with a dose of 30 μg/kg/day resulted in the best time (11.6 ± 3.8 s) compared to wt animals (7.0 ± 1.0 s). While in the administration during the third month to SOD1G93A animals, a difference was observed in favor of the dose of 30 μg/Kg/day (28.3 ± 6.5 s) with respect to that of 150 μg/Kg/day (33.0 ±

4.9 s) with a certain level of significance (F11,13 = 1.51, P < 0.01).

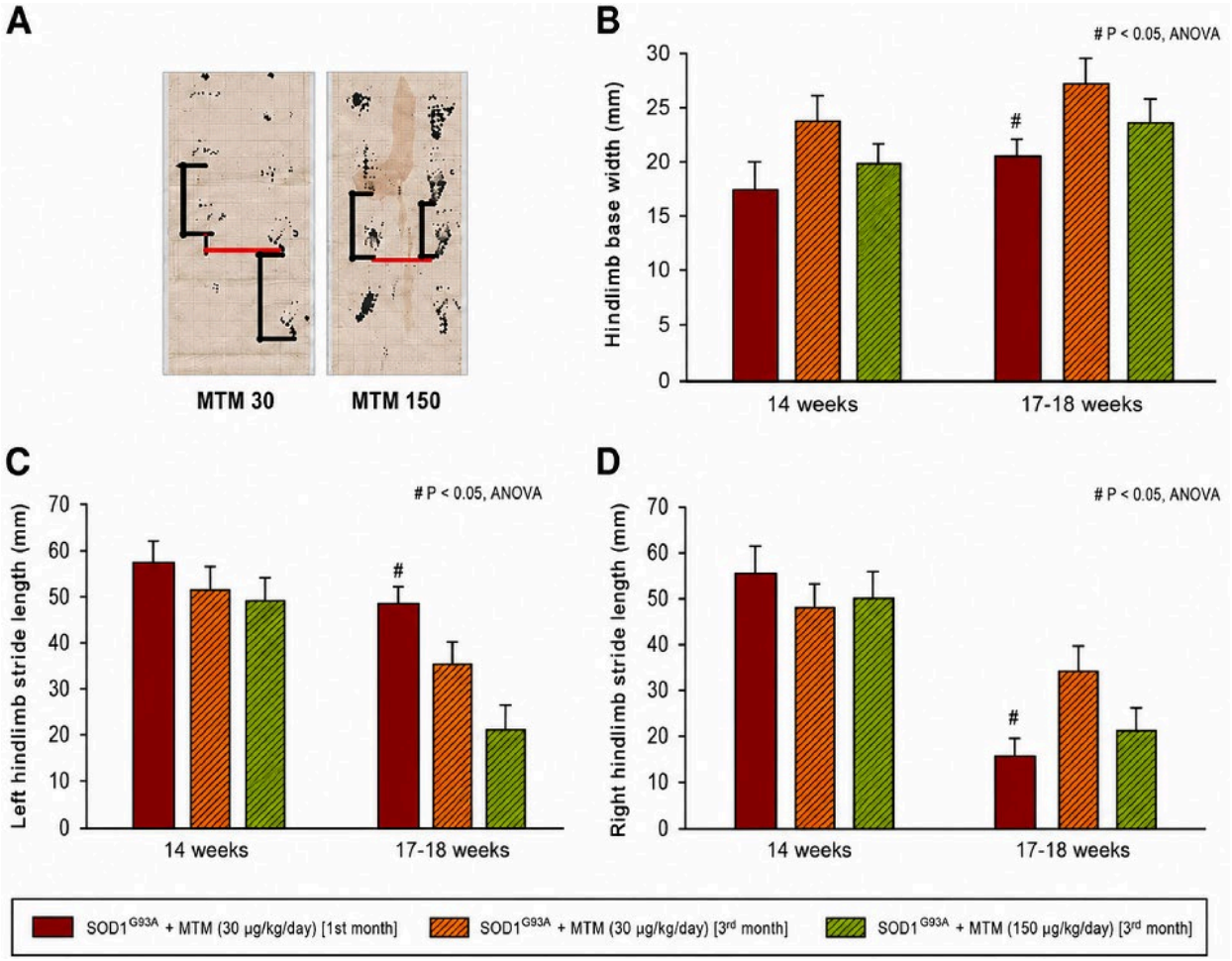

Figure 3. Use of MTM at a dose of 30 µg/kg/day from the first and third month in SOD1G93A transgenic mice.

3.3. Effect of MTM on the Assessment of Mouse Weight as a Function of Age

Previous results from our laboratory showed that the SOD1G93A ALS model mouse group showed a progressive decrease in body weight, which began to be significant starting at 11 weeks of age (−6.9% ± 0.0%) compared to non-transgenic wt mice.

Following drug administration in the third month (Figure 4), the measured body weight of the SOD1G93A ALS model mice began to experience a significant loss starting at 12 weeks, decreasing by 7.2% ± 0.26% compared to wt mice. A weight difference was also observed in SOD1G93A animals from 12 weeks of age in favor of those receiving MTM at a dose of 30 µg/kg/day (7.1% ± 0.0% more weight) compared to mice cured with MTM doses of 150 µg/kg/day (Figure 4). When comparing the data from MTM administration during the first month at a dose of 30 µg/kg/day, the mice showed greater weight than animals from all other groups, until week 15 (even greater than that of wt animals). From week 15 on-

wards, these mice showed a decrease in body weight, although always greater than that of the two MTM-treated groups from three months onwards. Figure 4 illustrates two temporal conditions for treatment: one group received an administration of 30 μg/kg/day MTM starting at 1 month of age (SOD1G93A + MTM 30 μg/kg/day, 1 month; n = 20), while another group received 150 μg/kg/day MTM starting at 3 months of age (SOD1G93A + MTM 150 μg/kg/day, 3 months; n = 28). Both groups were compared to vehicle-treated SOD1G93A controls (SOD1G93A + vehicle; n = 15) and wild-type controls (wt + vehicle; n = 6). Body weight is presented as a percentage of the weight at 8 weeks of age, which is set to 100%. The values are expressed as mean ± SEM. Statistical significance was determined with a one-way ANOVA followed by a Holm-Šidák post-hoc test, where $P < 0.01$ is considered significant.

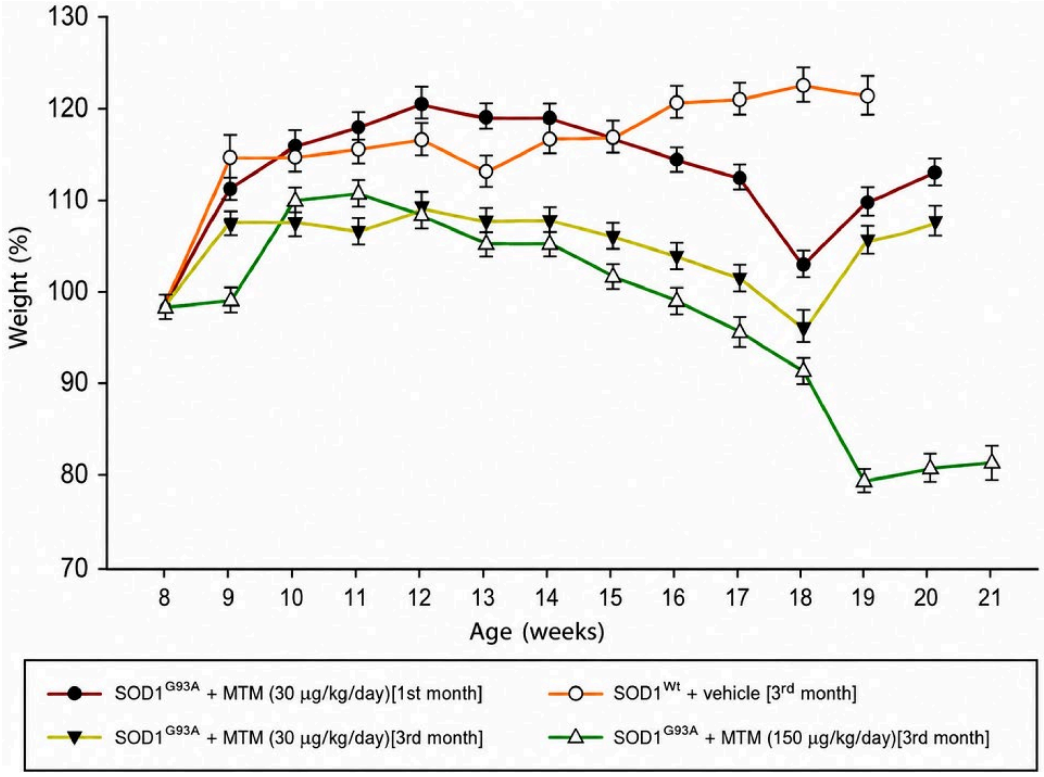

Figure 4. Evaluation of the effect of MTM on body weight evolution in wild-type (wt) and SOD1G93A ALS model mice.

3.4. Determination of the Effect of MTM on the Survival Curve in SOD1G93A Mice

Finally, we translated these positive effects derived from MTM administration - recorded by studies of locomotor behavior and animal quality of life deduced from the evolution of their body weight - into an increase in the life expectancy of the transgenic animals. To do this, we constructed Kaplan-Meyer survival curves for each group (Figure 5). Animals that were euthanized or perfused for histological analysis before reaching end-stage disease were treated as right-censored at the

date of the procedure; their data contributed to the analysis until that time point.

For SOD1G93A mice treated with MTM at 30 µg/kg/day from 1 month of age (n = 20), compared to vehicle-treated SOD1G93A controls (n = 15), the median survival was 139.8 days (95% CI: 133.5 - 146.1) vs. 130.6 days (95% CI: 125.0 - 136.2), respectively (Figure 5). The log-rank test revealed a significant difference ($\chi^2 = 6.12$, df = 1, P = 0.013). The hazard ratio (MTM vs. vehicle) was 0.58 (95% CI: 0.37 - 0.91), indicating a 42% reduction in the instantaneous risk of death.

During the study, 6 animals in the early-treated group and 4 animals in the vehicle group were censored due to euthanasia for histological analysis at 4 months of age; these animals were included in the analysis until the date of censoring. No animal was lost to follow-up.

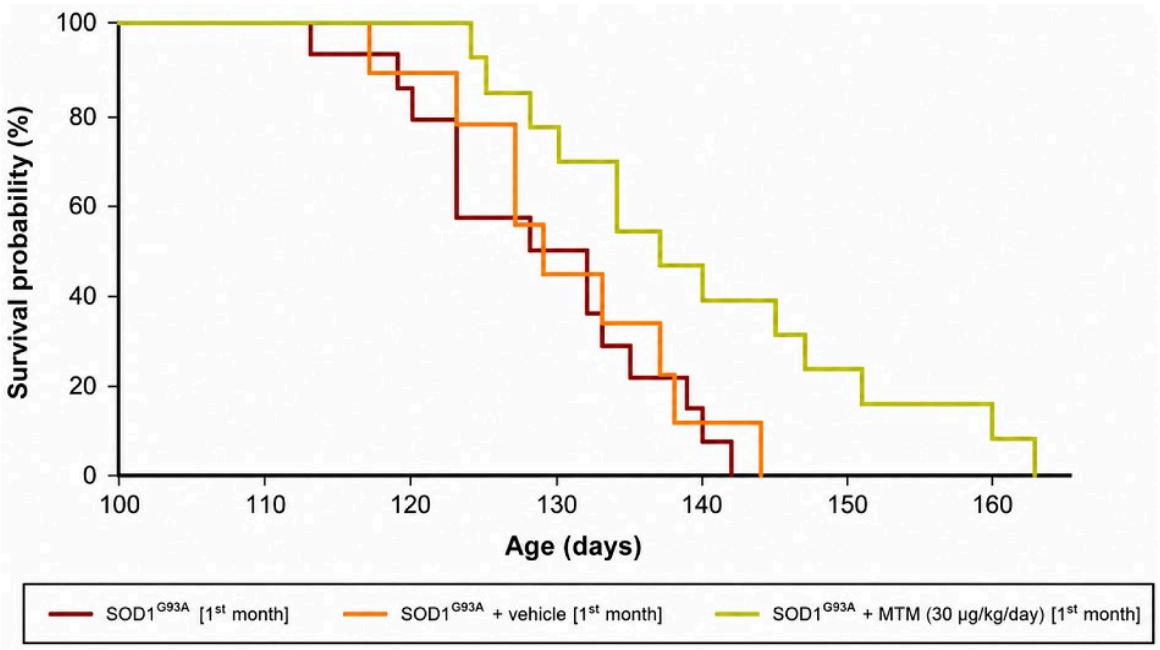

Figure 5. Effect of MTM (30 µg/kg/day from 1 month of age) on the survival curve of SOD1G93A mice.

Kaplan-Meier analysis of SOD1G93A mice treated with MTM at 150 µg/kg/day from 3 months of age (n = 28) revealed a median survival was 132.2 days (95% CI: 127.8 - 136.6), compared to 130.6 days (95% CI: 125.0 - 136.2) in the vehicle controls (Figure 6). The log-rank test showed a significant difference ($\chi^2 = 4.45$, df = 1, P = 0.035), with a hazard ratio of 0.72 (95% CI: 0.53 - 0.98).

In this experiment, 8 animals in the late-treated group and 4 animals in the vehicle group were censored due to euthanasia or perfusion for histological analyses; these were treated as censored observations at the time of the procedure. No animal was lost to follow-up.

When directly comparing the two MTM-treated groups (30 µg/kg/day from 1 month vs. 150 µg/kg/day from 3 months), the log-rank test revealed a significant difference favouring the early-treatment group ($\chi^2 = 4.98$, df = 1, P = 0.026), with a hazard ratio of 0.64 (95% CI: 0.43 - 0.95).

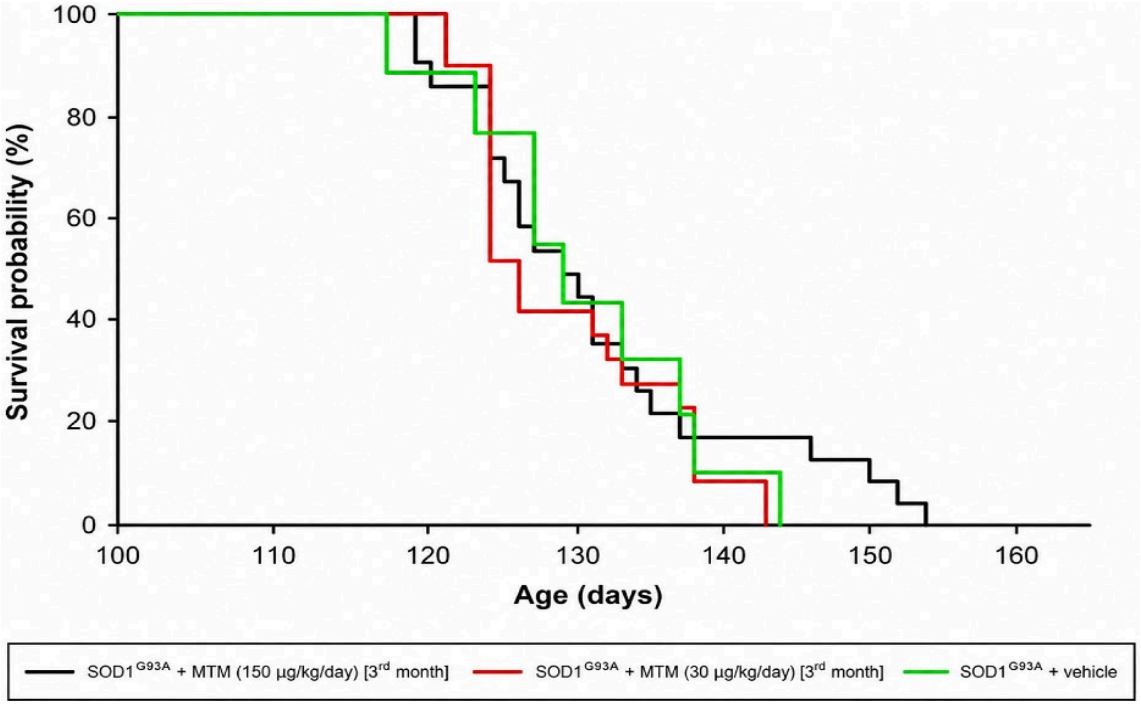

Figure 6. Effect of MTM (150 µg/kg/day from 3 months of age) on the survival curve of SOD1G93A mice.

3.5. Discussion

In this study, the combination of earlier initiation and a lower dose (30 µg/kg/day starting at 1 month) produced greater benefits than later initiation with a higher dose (150 µg/kg/day starting at 3 months). However, because both timing and dose differ, the relative contribution of each factor cannot be isolated from this design. Therefore, we cannot conclude that earlier initiation alone is responsible for the superior outcomes; the combination of earlier start and lower dose appeared more beneficial in our experimental setting. Future studies with a factorial design (e.g., early vs. late at the same dose) would be needed to isolate the effect of treatment timing.

Regarding the molecular mechanism, while the observed improvements in motor function, body weight, and survival are consistent with the proposed neuroprotective mechanism involving the NO/ROCK/Sp1/S100A10/TASK-1 pathway—as previously described *in vitro* and *in vivo* [2] [10] [15] [16]—we acknowledge that the present study did not include direct molecular measurements of these pathway components. Therefore, the involvement of this pathway in mediating the effects of MTM in our model remains a hypothesis rather than a demonstrated mechanism. Future studies incorporating biochemical and histological analyses (e.g., Western blotting for Sp1, S100A10, TASK-1, or caspase-3; immunohistochemical detection of NO or ROCK activity) will be required to confirm the molecular basis of the neuroprotective effects observed here.

4. Conclusions

1) Chronic MTM treatment improves motor performance, body weight mainte-

nance, and survival in the SOD1G93A mouse model of ALS.

2) In this study, treatment initiated at 1 month with 30 μg/kg/day produced greater benefits than treatment initiated at 3 months with 150 μg/kg/day. However, because both timing and dose differ, the individual contribution of each factor cannot be isolated.

3) Late initiation with the higher dose (150 μg/kg/day from 3 months) also confers significant improvements, though to a lesser extent.

4) Based on previous mechanistic studies, we propose that these beneficial effects may be mediated through the NO/ROCK/Sp1/S100A10/TASK-1 pathway; direct molecular confirmation is needed in future work.

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Author Contributions

The author has read and agreed to the published version of the manuscript. Study conception and design, material preparation, data collection and analysis, writing, E.W.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

References

[1] Portillo, F. and Moreno-López, B. (2020) Nitric Oxide Controls Excitatory/Inhibitory Balance in the Hypoglossal Nucleus during Early Postnatal Development. *Brain Structure and Function*, **225**, 2871-2884. https://doi.org/10.1007/s00429-020-02165-9

[2] García-Morales, V., Rodríguez-Bey, G., Gómez-Pérez, L., Domínguez-Vías, G., González-Forero, D., Portillo, F., *et al.* (2019) Sp1-Regulated Expression of P11 Contributes to Motor Neuron Degeneration by Membrane Insertion of TASK1. *Nature Communications*, **10**, Article No. 3784. https://doi.org/10.1038/s41467-019-11637-4

[3] Shefner, J.M., Musaro, A., Ngo, S.T., Lunetta, C., Steyn, F.J., Robitaille, R., *et al.* (2023) Skeletal Muscle in Amyotrophic Lateral Sclerosis. *Brain*, **146**, 4425-4436. https://doi.org/10.1093/brain/awad202

[4] Wilson, D.M., Cookson, M.R., Van Den Bosch, L., Zetterberg, H., Holtzman, D.M. and Dewachter, I. (2023) Hallmarks of Neurodegenerative Diseases. *Cell*, **186**, 693-714. https://doi.org/10.1016/j.cell.2022.12.032

[5] Gento-Caro, Á., Vilches-Herrando, E., García-Morales, V., Portillo, F., Rodríguez-Bey, G., González-Forero, D., *et al.* (2021) Interfering with Lysophosphatidic Acid Receptor Edg2/Lpa$_1$ Signalling Slows down Disease Progression in SOD1-G93A Transgenic Mice. *Neuropathology and Applied Neurobiology*, **47**, 1004-1018. https://doi.org/10.1111/nan.12699

[6] Pasinelli, P. and Brown, R.H. (2006) Molecular Biology of Amyotrophic Lateral Sclerosis: Insights from Genetics. *Nature Reviews Neuroscience*, **7**, 710-723. https://doi.org/10.1038/nrn1971

[7] Borchelt, D.R. (2006) Amyotrophic Lateral Sclerosis—Are Microglia Killing Motor Neurons? *New England Journal of Medicine*, **355**, 1611-1613. https://doi.org/10.1056/nejmcibr064358

[8] Dupuis, L., Oudart, H., René, F., de Aguilar, J.G. and Loeffler, J. (2004) Evidence for Defective Energy Homeostasis in Amyotrophic Lateral Sclerosis: Benefit of a High-Energy Diet in a Transgenic Mouse Model. *Proceedings of the National Academy of Sciences*, **101**, 11159-11164. https://doi.org/10.1073/pnas.0402026101

[9] Barceló, M.A., Povedano, M., Vázquez-Costa, J.F., Franquet, Á., Solans, M. and Saez, M. (2021) Estimation of the Prevalence and Incidence of Motor Neuron Diseases in Two Spanish Regions: Catalonia and Valencia. *Scientific Reports*, **11**, Article No. 6207. https://doi.org/10.1038/s41598-021-85395-z

[10] Sleiman, S.F., Langley, B.C., Basso, M., Berlin, J., Xia, L., Payappilly, J.B., *et al.* (2011) Mithramycin Is a Gene-Selective Sp1 Inhibitor That Identifies a Biological Intersection between Cancer and Neurodegeneration. *The Journal of Neuroscience*, **31**, 6858-6870. https://doi.org/10.1523/jneurosci.0710-11.2011

[11] Gautam, V., Sandhu, A., Rawat, K., Kumar, A. and Saha, L. (2024) Personalized Epigenetics of Neurodegenerative Diseases. In: Tollefsbol, T., Ed., *Personalized Epigenetics*, Elsevier, 565-598. https://doi.org/10.1016/b978-0-443-23802-4.00020-x

[12] Torres, A.K., Mira, R.G., Pinto, C. and Inestrosa, N.C. (2025) Studying the Mechanisms of Neurodegeneration: C. Elegans Advantages and Opportunities. *Frontiers in Cellular Neuroscience*, **19**, Article ID: 1559151. https://doi.org/10.3389/fncel.2025.1559151

[13] Singh, M., Singh, S.P., Yadav, D., Agarwal, M., Agarwal, S., Agarwal, V., *et al.* (2021) Targeted Delivery for Neurodegenerative Disorders Using Gene Therapy Vectors: Gene Next Therapeutic Goals. *Current Gene Therapy*, **21**, 23-42. https://doi.org/10.2174/1566523220999200817164907

[14] Wei, C., Zhang, W., Zhou, Q., Zhao, C., Du, Y., Yan, Q., *et al.* (2016) Mithramycin a Alleviates Cognitive Deficits and Reduces Neuropathology in a Transgenic Mouse Model of Alzheimer's Disease. *Neurochemical Research*, **41**, 1924-1938. https://doi.org/10.1007/s11064-016-1903-3

[15] Yang, K., Liu, Y. and Zhang, M. (2024) The Diverse Roles of Reactive Astrocytes in the Pathogenesis of Amyotrophic Lateral Sclerosis. *Brain Sciences*, **14**, Article 158. https://doi.org/10.3390/brainsci14020158

[16] Esteves, S., Duarte-Silva, S. and Maciel, P. (2017) Discovery of Therapeutic Approaches for Polyglutamine Diseases: A Summary of Recent Efforts. *Medicinal Research Reviews*, **37**, 860-906. https://doi.org/10.1002/med.21425

[17] Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., *et al.* (1996) Regulation of Myosin Phosphatase by Rho and Rho-Associated Kinase (Rho-Kinase). *Science*, **273**, 245-248. https://doi.org/10.1126/science.273.5272.245

[18] Martín-Cámara, O., Cores, Á., López-Alvarado, P. and Menéndez, J.C. (2021) Emerging Targets in Drug Discovery against Neurodegenerative Diseases: Control of Synapsis Disfunction by the Rhoa/Rock Pathway. *European Journal of Medicinal Chemistry*, **225**, Article 113742. https://doi.org/10.1016/j.ejmech.2021.113742

[19] Arnold, F.J., Putka, A.F., Raychaudhuri, U., Hsu, S., Bedlack, R.S., Bennett, C.L., *et al.* (2024) Revisiting Glutamate Excitotoxicity in Amyotrophic Lateral Sclerosis and Age-Related Neurodegeneration. *International Journal of Molecular Sciences*, **25**, Article 5587. https://doi.org/10.3390/ijms25115587

[20] de Medinaceli, L., Freed, W.J. and Wyatt, R.J. (1982) An Index of the Functional Con-

dition of Rat Sciatic Nerve Based on Measurements Made from Walking Tracks. *Experimental Neurology*, **77**, 634-643. https://doi.org/10.1016/0014-4886(82)90234-5

[21] Ferrante, R.J., Ryu, H., Kubilus, J.K., D'Mello, S., Sugars, K.L., Lee, J., *et al.* (2004) Chemotherapy for the Brain: The Antitumor Antibiotic Mithramycin Prolongs Survival in a Mouse Model of Huntington's Disease. *The Journal of Neuroscience*, **24**, 10335-10342. https://doi.org/10.1523/jneurosci.2599-04.2004