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CLINICAL TRIAL

The unfolded protein response in amyotrophic later sclerosis: results of a phase 2 trial

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Strong evidence suggests that endoplasmic reticulum stress plays a critical role in the pathogenesis of amyotrophic lateral sclerosis (ALS) through altered regulation of proteostasis. Robust preclinical findings demonstrated that guanabenz selectively inhibits endoplasmic reticulum stress-induced eIF2α-phosphatase, allowing misfolded protein clearance, reduces neuronal death and prolongs survival in *in vitro* and *in vivo* models. However, its safety and efficacy in patients with ALS are unknown.

To address these issues, we conducted a multicentre, randomized, double-blind trial with a futility design. Patients with ALS who had displayed an onset of symptoms within the previous 18 months were randomly assigned in a 1:1:1:1 ratio to receive 64 mg, 32 mg or 16 mg of guanabenz or placebo daily for 6 months as an add-on therapy to riluzole. The purpose of the placebo group blinding was to determine safety but not efficacy. The primary outcome was the proportion of patients progressing to higher stages of disease within 6 months as measured using the ALS Milano-Torino staging system, compared with a historical cohort of 200 patients with ALS. The secondary outcomes were the rate of decline in the total revised ALS functional rating scale score, slow vital capacity change, time to death, tracheotomy or permanent ventilation and serum light neurofilament level at 6 months.

The primary assessment of efficacy was performed using intention-to-treat analysis. The treatment arms using 64 mg and 32 mg guanabenz, both alone and combined, reached the primary hypothesis of non-futility, with the proportions of patients who progressed to higher stages of disease at 6 months being significantly lower than that expected under the hypothesis of non-futility and a significantly lower difference in the median rate of change in the total revised ALS functional rating scale score.

This effect was driven by patients with bulbar onset, none of whom (0/18) progressed to a higher stage of disease at 6 months compared with those on 16 mg guanabenz (4/8; 50%), the historical cohort alone (21/49; 43%; $P = 0.001$) or plus placebo (25/60; 42%; $P = 0.001$). The proportion of patients who experienced at least one adverse event was higher in any guanabenz arm than in the placebo arm, with higher dosing arms having a significantly higher proportion of drug-related side effects and the 64 mg arm a significantly higher drop-out rate. The number of serious adverse events did not significantly differ between the guanabenz arms and the placebo. Our findings indicate that a larger trial with a molecule targeting the unfolded protein response pathway without the alpha-2 adrenergic related side-effect profile of guanabenz is warranted.

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Abbreviations: ALS = amyotrophic lateral sclerosis; ALSFRS-R = revised ALS functional rating scale; eIF = eukaryotic translation initiation factor; ER = endoplasmic reticulum; MITOS = Milano-Torino staging; sVC = slow vital capacity

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal disease hallmarked by the non-cell-autonomous degeneration of motor neurons in the cortex, medulla and spinal cord and the inclusion of cytoplasmic misfolded proteins in degenerating neuronal and non-neuronal cells, occurring both in familial and sporadic cases.[1–6] The misfolded protein overload triggers pathological signalling and induces abnormal interactions with native membrane proteins.[7] This can lead to the diffusion of misfolded proteins in the extracellular space and cell-to-cell propagation of the disease.[8–11] Such impairment in the homeostasis and propagation of proteins is a recognized pathological pathway in ALS,[12–20] possibly driven also by disease-related genes encoding adapter proteins.[6]

Central to the synthesis and the post-translational modification of proteins is the endoplasmic reticulum (ER). One of its primary functions is to exert quality control on proteins, allowing only those that are properly folded to be packaged into vesicles and transported to their proper targets. Misfolded proteins are retained in the ER and delivered for proteasomal degradation after retrotranslocation into the cytosol. This occurs through the activation of the unfolded protein response[14,18] that regulates proteostasis,[15,21,22] namely the balance between the synthesis and degradation of proteins. If capacity and influx to the ER are impaired, as in degenerating cells, homeostasis is disrupted, leading to ER stress.[23]

The unfolded protein response is an adaptive response triggered by ER stress that reduces the load of misfolded proteins and restores homeostasis. This cellular functionality is accomplished through various transcriptional and translational controls, the induced expression of chaperones within the ER-associated protein degradation pathway and a transient decrease in the protein flux entering the ER. Specifically, the unfolded protein response has three proximal transmembrane protein sensors: inositol-requiring kinase 1 (IRE1), pancreatic ER eIF2α kinase (PERK) and activating transcription factor 6 (ATF6). Among them, PERK plays a central role in translational control.[7] During ER stress, PERK oligomerizes, autophosphorylates and phosphorylates the eukaryotic translation initiation factor (eIF2α). The phosphorylation of eIF2α leads to the attenuation of protein translation decreasing the flux of proteins entering the ER and allowing at the same time the translation of proteins involved in stress responses such as the transcription factor ATF4. Consequently, the increase in ATF4 protein expression activates a negative feedback loop through C/EBP homologous protein (CHOP) and the protein phosphatase 1 regulatory subunit 15 A (PPP1R15A; also named GADD34), which dephosphorylates eIF2α by complexing to protein phosphatase 1 (PP1c), allowing protein synthesis to resume.[7] If the stress is not resolved, the unfolded protein response induces the activation of the apoptotic pathways.

Long-term ER stress due to ER protein overload, disruption of proteostasis and accumulation of misfolded proteins is a key factor affecting cell survival in neurodegenerative disease.[24] ER stress and unfolded protein response activation have been described in patients with sporadic ALS as the increased expression of phosphorylated eIF2α, BiP (ER chaperone) and protein disulphide isomerases in the spinal cord tissue [19,20,25] as well as increased CHOP levels in motor neurons and surrounding glial cells.[26] These findings suggest that acting on this crucial hub could protect cells from degeneration.[7,27]

Guanabenz, an FDA-approved alpha-2 adrenergic receptor agonist, has been found to modulate protein synthesis by the activation of translational factors, preventing misfolded protein accumulation and ER overload.[28] *In vitro* studies have provided robust data indicating that guanabenz can spare the constitutive eIF2α phosphatase and avoid persistent eIF2α phosphorylation, which would be lethal to motor neurons.[28] In worm and zebrafish models, guanabenz counteracted neuronal toxicity through a reduction of ER stress.[29] In yeast, *Drosophila* and mouse models, guanabenz modulated ribosome folding activity and reduced the prion-like propagation of aggregates.[30] *In vivo* studies showed that guanabenz delayed disease onset, extended lifespan, improved motor performance, reduced motor neuron loss and prolonged survival in a $SOD1^{G93A}$ mouse model by attenuating ER stress due to prolonged eIF2α phosphorylation.[31–33]

Given guanabenz's close mechanism of action to pathogenic changes that are currently considered central to the pathogenesis of ALS and its availability as an approved hypertensive intervention, we report the results of a phase 2 randomized clinical trial with a futility design that evaluated the safety and efficacy of guanabenz in patients with ALS.

Materials and methods

Trial design and oversight

Protocol

This was a multicentre, randomized, double blind, placebo-controlled, phase 2 study with a futility design. The design implied that (i) the primary hypothesis and the sample size were based and estimated on the comparison between guanabenz arms and the historical cohort; (ii) the placebo arm was introduced to assess only tolerability and safety; and (iii) positive results would indicate that a phase 3 is not futile. The trial was designed following the guidelines on the clinical investigation of medicinal products for the treatment of ALS provided by the EMA and adopted by the Agenzia Italiana del Farmaco (https://www.aifa.gov.it/-/linea-guida-sui-farmaci-per-il-trattamento-della-sclerosi-laterale-amiotrofica-rilasciata-per-una-consultazione-pubblica-di-sei-mesi, accessed September 2021). The Advisory Board members, who included Prof. Orla Hardiman, Trinity College, University of Dublin, Prof. Paola Minghetti, University of Milan, Italy, Dr Graziella Filippini, IRCCS Foundation "Carlo Besta" Neurological Institute, Milan, Italy and Dr Ettore Beghi, IRCCS "Mario Negri" Pharmacological Research Institute, Milan, Italy, approved the protocol.[34]

The study protocol was approved by the Ethics Committee of IRCSS Fondazione Istituto Neurologico "Carlo Besta" of Milan on 28 October 2015 (Eudract Number 2014–005367-32) and then by the Ethics Committees of all the participating centres. The authorization of the Agenzia Italiana del Farmaco (AIFA) was obtained on 1 March 2016 (protocol number AIFA/RSC/P/20735). Patient enrolment began on December 2016. The protocol was designed adhering to the SPIRIT recommendations and Declaration of Helsinki. All of the participants provided written informed consent before screening.

Trial participants

Participants were eligible if they were aged $\geqslant$18 years, were diagnosed with probable or definite sporadic or familiar ALS according to the revised El Escorial criteria,[35] had onset of weakness $<$18 months before enrolment, had slow vital capacity (sVC) $\geqslant$70% of the predicted value in a seated position (excluding bulbar onset), were on active contraception if female of fertile age and gave written informed consent. Patients treated with riluzole were asked to remain on a stable dose of 100 mg daily for the entire study period. Patients not treated with riluzole at randomization remained off riluzole therapy for the entire study period.

Participants were excluded if they had a percutaneous endoscopic gastrostomy or equivalent device (e.g. a radiologically inserted device), were on non-invasive ventilation or had a tracheotomy, known heart, renal or liver failure, known intolerance to alpha-2-agonists, known conditions with a risk of developing cardiovascular disorders or symptomatic hypotension, severe cognitive impairment (e.g. frontotemporal dementia) or participated in a clinical trial within 3 months prior to the screening.

Randomization

Participants were randomized in blocks stratified by centre, with 1:1:1:1 allocation to the four treatment arms: (i) guanabenz 16 mg plus riluzole 100 mg; (ii) guanabenz 32 mg plus riluzole 100 mg; (iii) guanabenz 64 mg plus riluzole 100 mg; and (iv) placebo plus riluzole 100 mg. The randomization was generated by a computer-

based sequence known only to one person (I.T.) and the drug dispenser. Treatment was allocated by a web-based randomization system, available 24 h a day. The procedure incorporated eligibility checks according to protocol and was performed on request from the centres. The sequence was always available for emergency unmasking. The randomization conformed to the CONSORT 2010 guidelines.

Treatment and blinding

Guanabenz acetate was produced in accordance with Good Manufacturing Practices of the European Union for active pharmaceutical ingredients and ICH Q7A guidelines by Medichem SA, Spain. The active powder was purchased by the coordinating centre. Cosmo Pharmaceuticals performed all the procedures required by AIFA to prepare the interventional drugs (active and placebo). Both were in tablets made indistinguishable to patients and neurologists. The active drug was prepared in titration kits and boxes for the 6-month treatment. The investigational drug and placebo were dispensed to the pharmacy at each participating centre according to the allocation sequence. Treatment packs were supplied for the entire study period along with information on how to administer the treatment. The randomization unit at the coordinating centre held the treatment codes for each patient and was available 24 h a day over the entire study period to advise, in an emergency, whether a patient was receiving the active drug or the placebo.

Participants were treated for 6 months with doses of 16 mg, 32 mg or 64 mg daily. All patients started at a dose of 8 mg daily and this was titrated up every 3 days until the allocated dose was achieved. All patients took the same number of tablets. Participating centres received the investigational drug packages for the entire study within 2 weeks following patient randomization. Treatment was administered twice daily (morning and evening) for the entire trial.

End points

The primary end point was the proportion of patients who had progressed to higher stages of disease at 6 months after the start of the full allocation dose, as measured using the ALS Milano-Torino staging (MITOS) system.

The secondary end points were the rate of decline in the total ALSFRS-R score, the sVC change, the time to death, tracheostomy or permanent ventilation, the serum light neurofilament (NfL) level measured using the Simoa® HD-1 Analyzer (Quanterix) at 6 months after the start of the full allocation dose, and the proportion of withdrawals due to adverse events.

Trial procedures

After obtaining informed consent, participants underwent a screening visit to provide demographic data and undergo ECG and haematological exams, assessment according to the revised Hamilton depression rating scale[36] and blood pressure recording. After verification of eligibility, a randomization code was generated using an automated web-response system. Monthly visits were planned to record end points and adverse events. During the titration period, participants were asked to measure their blood pressure at least twice a week and were contacted weekly to record values, adverse events and symptoms of overdose (e.g. dizziness, irritability, nervousness, pinpoint pupils, slow heartbeat, unusual tiredness or weakness). Participants who withdrew from treatment for any reason (except consent withdrawal) were followed-up with monthly visits to record the ALSFRS-R score until the end of the study.

Co-treatments: supportive care

Percutaneous endoscopic gastrostomy or equivalent devices were proposed in the case of any of the following: (i) a score of 1 or 2 at item 3 of the ASLFRS-R; (ii) unintentional loss of >10% body weight in the last 3 months; or (iii) choking during the ingestion of food, fluid or medication. The ultimate decision to undergo placement of a feeding tube remained a personal decision for each patient.

Symptoms suggestive of nocturnal hypoventilation (frequent arousals, morning headaches, excessive daytime sleepiness and vivid dreams) were recorded. Non-invasive ventilation was proposed in the case of any of the following: (i) dyspnoea (score of 0 or 1 at item 10 of the ALSFRS-R); (ii) orthopnoea (score of 0 or 1 at item 11 of the ALSFRS-R); (iii) sVC <50%; or (iv) abnormal nocturnal oximetry (SaO_2 < 90% for 4% of the recorded time overnight).

Statistical analysis

The sample size was estimated by the proportion of patients progressing to higher stages of disease in 6 months as measured by the ALS-MITOS system[37,38] in a historical cohort of 200 ALS patients on riluzole.[39] In that cohort, 76.5% of patients were at stage 0, 22% were at stage 1 and 1.5% were at stage 2 at baseline, while 46.6% of patients had progressed to a higher stage of disease at the 6-month follow-up. The null hypothesis was that guanabenz reduced the proportion of patients progressing to a higher stage of disease at 6 months by >35%, compared with the historical cohort. The study investigators agreed that a pharmacological intervention achieving a reduction of more than one-third of patients (i.e. >35%) progressing to a higher stage of disease compared with the historical cohort would be clinically meaningful, particularly given the poor efficacy of riluzole and edaravone.[40,41] Accordingly, under the null hypothesis we tested whether the expected proportion of patients on guanabenz progressing to a higher stage of disease at 6 months was lower than 30% (i.e. 46.6% – [46.6% × 35%] = 30%), also calculated as a 17% (i.e. 46.6%–30%) absolute difference between the guanabenz arms and historical cohort. The alternative hypothesis was that guanabenz reduced the proportion of patients progressing to a higher stage of disease at 6 months by <35% compared with the historical cohort. If the null hypothesis was rejected, this would indicate that guanabenz was not sufficiently promising to change the progression of ALS in a phase 3 randomized controlled trial, and in that sense it was futile. The study was designed to reject the null hypothesis with an alpha of 0.1 and a power of 0.85. For this purpose, and assuming a loss to follow-up of 5%, 208 patients were calculated as the target size for randomization.

The primary analysis of efficacy was performed in the intention-to-treat population with available data at 6 months (175 of 200 enrolled in the trial and 178 of 200 in the historical cohort). Per protocol analysis was carried out after excluding non-compliers (e.g. patients who had taken <80% of the therapy). Statistics were tabulated by treatment arm. Measures of central tendency for continuous metrics were presented as mean ± standard deviation (SD) and median with interquartile range (IQR). All primary and secondary analyses were based on the comparison of the guanabenz 64 mg and 32 mg arms alone and combined versus the historical cohort alone and combined with the placebo. The historical cohort did not differ significantly from the study placebo arm with respect to sex, age, body mass index, type of onset, months from onset, baseline ALSFRS-R, progression rate, per cent on riluzole therapy and baseline ALS-MITOS. The primary end point was analysed using the chi-square test. The secondary end points of the change in the ALSFRS-R, sVC and serum NfL levels at 6 months were analysed using the Mann-Whitney test. Time to death, tracheostomy or permanent ventilation at 6 months were analysed

with the use of a Cox proportional hazards model; inferential testing was based on the log-rank test. Multivariate analyses were performed to assess the potential confounding effect of onset type (bulbar versus spinal), months from onset, ALSFRS-R, sVC and ALS-MITOS baseline values on primary and secondary outcomes. Additionally, sensitivity analyses based on multiple imputation methods using chained equations were performed for primary and secondary outcomes to account for missing data. Imputation of progression for ALS-MITOS utilized a logistic model, whereas ALSFRS-R and sVC utilized predictive mean matching. Corresponding prediction equations included type of onset (bulbar versus spinal), months from onset, ALS-FRS-R, sVC and ALS-MITOS baseline values. The truncated Hochberg procedure was used to assess significant *P*-values after adjustment for multiple dose-group comparisons with a truncation fraction of 0.5 and a corresponding cut-off of $P = 0.0375$. *P*-values for specific tests are provided directly in tables and figure or their captions. All statistical analyses were performed using STATA statistical software, version 16 (StataCorp. 2019. Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC). Two additional statisticians (J.M.N. and E.A.) independently reviewed the anonymized dataset and validated all statistical results. Neither of the independent statisticians participated in the trial design or randomization process.

Data availability

The data that support the findings of this study are openly available in open repository of the IRCCS Fondazione Istituto Neurologico "Carlo Besta" at https://doi.org/10.5281/zenodo.4554960

Results

Trial participants

A total of 205 patients were screened. Four patients were excluded because they did not meet the inclusion criteria. Eventually, 50 patients were assigned to the guanabenz 64 mg arm, 50 patients to the guanabenz 32 mg arm, 51 patients to the guanabenz 16 mg arm, and 50 patients to the placebo arm. All patients, except one in the placebo arm who was lost after randomization, started the treatment (Fig. 1).

Demographic data, disease features and progression rate at onset based on the Kimura score[42] did not differ significantly between the guanabenz trial and the historical cohort (Table 1). Two hundred patients started the treatment and 175 patients were available for the intention-to-treat analysis of the primary end point at 6 months. The attrition rate was higher than expected. (Fig. 1).

Primary end point

The guanabenz 64 mg and 32 mg arms, both alone and combined, reached the primary hypothesis of non-futility with a proportion of patients who progressed to higher stage of disease at 6 months after the start of the full allocation dose being significantly lower than that expected under the hypothesis of non-futility (Table 2). In particular, all of the 18 patients with bulbar onset allocated to the guanabenz 64 mg and 32 mg arms were at stage 0 of the ALS-MITOS at baseline and none (0%) progressed to a higher stage of disease at 6 months. All of the patients with bulbar onset in the guanabenz 16 mg arm and placebo were also at stage 0 at baseline,

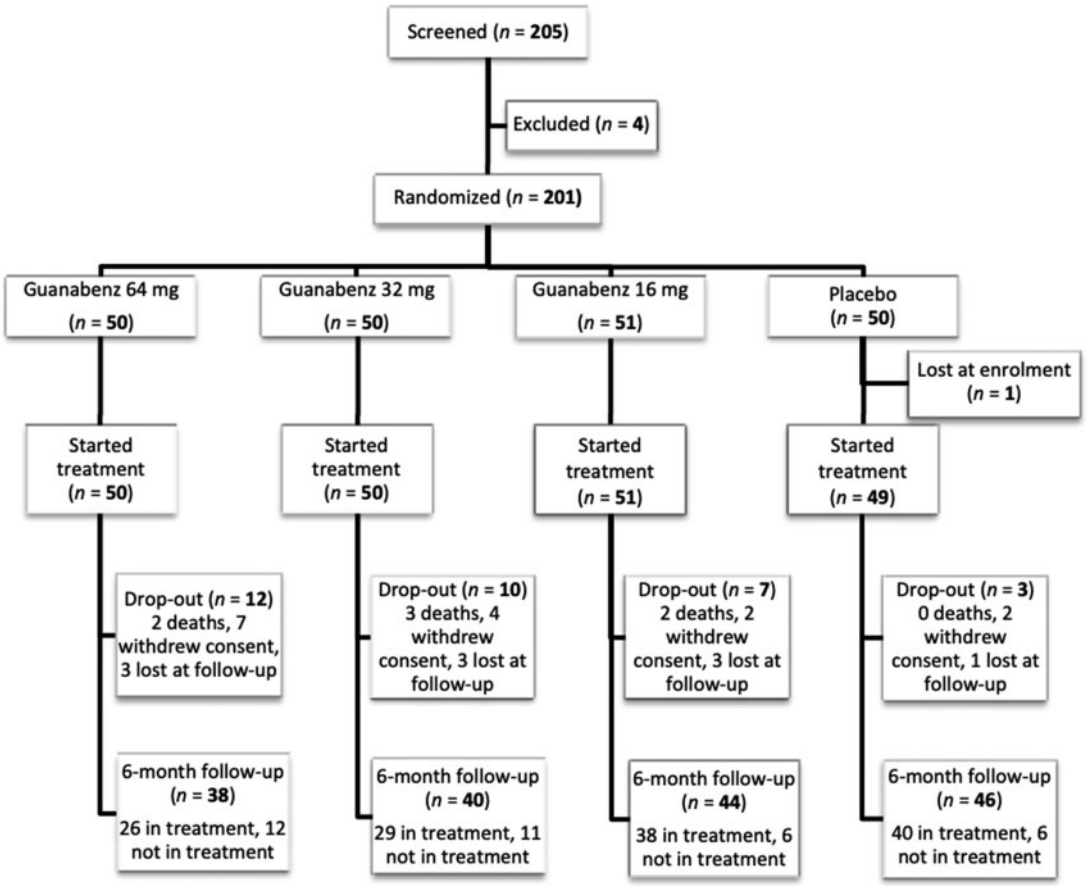

Figure 1 Screening, randomization and follow-up of ALS patients enrolled in the trial.

Table 1 Demographic and disease features of trial participants

	Guanabenz 64 mg (*n* = 50)	Guanabenz 32 mg (*n* = 50)	Guanabenz 16 mg (*n* = 51)	Placebo (*n* = 49)	Historical cohort (*n* = 200)	*P*-value[a]
Sex[b]						
Male	29 (58%)	31 (62%)	27 (53%)	29 (59%)	105 (52.5%)	0.27
Female	21 (42%)	19 (38%)	24 (47%)	20 (41%)	95 (47.5%)	
Age, years						
Mean ± SD	60 ± 10	60 ± 13	58 ± 11	61 ± 12	59 ± 10	0.64
Median (IQR)	61 (13)	62 (18)	57 (14)	61 (18)	61 (14)	
BMI						
Mean ± SD	25 ± 4	24 ± 3	25 ± 4	24 ± 3	24 ± 3	0.23
Median (IQR)	25 (4)	24 (4)	25 (4)	24 (4)	24 (4)	
Type of onset						
Bulbar	9 (18%)	12 (24%)	10 (20%)	11 (22%)	52 (26%)	0.24
Spinal	41 (82%)	38 (76%)	41 (80%)	38 (78%)	148 (74%)	
Months from onset						
Mean ± SD	12 ± 4	14 ± 4	13 ± 4	13 ± 4	13 ± 4	0.12
Median (IQR)	13 (7)	16 (7)	15 (8)	14 (5)	13 (7)	
ALSFRS-R						
Mean ± SD	38 ± 6	38 ± 5	37 ± 7	38 ± 5	38 ± 6	
Median (IQR)	40 (8)	39 (7)	38 (9)	39 (9)	39 (8)	0.69
Progression rate						
Mean ± SD	0.92 ± 0.56	0.75 ± 0.40	0.88 ± 0.61	0.85 ± 0.58	0.84 ± 0.55	
Median (IQR)	0.77 (0.70)	0.69 (0.59)	0.73 (0.76)	0.63 (0.62)	0.74 (0.62)	0.69
sVC						
Mean ± SD	91 ± 15	86 ± 15	93 ± 16	93 ± 16	86.5 ± 15	
Median (IQR)	91 (21)	86 (18)	89 (21)	93 (21)	86 (23)	0.12
Riluzole						
Yes	44 (88%)	47 (94%)	50 (98%)	48 (98%)	192 (96%)	0.48
No	6 (12%)	3 (6%)	1 (2%)	1 (2%)	8 (4%)	
ALS-MITOS						
0	36 (72%)	35 (70%)	37 (73%)	38 (78%)	153 (76.5%)	0.60
1	13 (26%)	15 (30%)	13 (26%)	11 (22%)	44 (22%)	
2	1 (2%)	0 (0%)	1 (2%)	0 (0%)	3 (1.5%)	

Progression rate was calculated using the Kimura score.[40] BMI = body mass index.
[a]*P*-value from chi-square, Mann-Whitney or *t*-test, as appropriate, of historical cohort versus guanabenz trial.
[b]The male/female ratio was 1:4 in the guanabenz trial and 1:1 in the historical cohort.

but 4 of 8 (50%) and 4 of 11 (36%), respectively, progressed to a higher stage of disease. In the historical cohort, 46 of 52 (88.5%) patients with bulbar onset were at stage 0 at baseline, and 21 of 49 (43%) progressed to a higher stage at 6 months. In patients with spinal onset, the difference between the guanabenz 64 mg and 32 mg arms and historical cohort alone or combined with placebo was not statistically significant (Table 3 and Fig. 2).

Secondary end points

The median rates of change in the ALSFRS-R total score between baseline and 6-month follow-up were –4 points (–0.67 per month) in the combined guanabenz 64 and 32 mg arms, –5 points (–0.83 per month) in the guanabenz 16 mg arm and –6 points (1 per month) in the historical cohort alone and plus placebo (difference versus the combined guanabenz 64 and 32 mg arms of 0.33 points per month) (Table 2).

Patients with bulbar onset in the combined guanabenz 64 and 32 mg arms showed a significantly slowed decline in the ALSFRS-R. The median decline at 6 months was –1 point (–0.17 per month) in the combined guanabenz 64 and 32 mg arms, –10 (–1.67 per month) in guanabenz 16 mg, –6 (–1 per month) in the historical cohort alone and –7 (–1.17 per month) combined with placebo (difference versus the combined guanabenz 64 and 32 mg arms of 1 point per month; $P = 0.0001$) (Table 3).

The decline of sVC and the time to death, tracheotomy or permanent ventilation at 6 months did not differ significantly between the groups. The median changes in serum NfL levels were 13 pg/ml (IQR 54) in the combined guanabenz 64 and 32 mg arms, 12 pg/ml (IQR 36) in guanabenz 16 mg, and 12 pg/ml (IQR 56) in placebo (Mann-Whitney test; $P = 0.88$), while they did not differ when comparing bulbar and spinal onset patients (Mann-Whitney test; $P = 0.63$ for both).

The results of the per-protocol analysis for all of the efficacy outcomes did not differ from those obtained with the intention-to-treat analysis.

Safety and tolerability

The proportion of patients who experienced at least one adverse event was higher in all of the active guanabenz treatment arms than in the placebo arm, with the 64 mg arm experiencing more events and significantly higher drop-outs than any of the other three (Table 4). Notably, 30 patients (30%) withdrew from the 64 mg and 32 mg treatment arms compared with only three (6%) from the placebo arm. The nature of adverse events experienced by patients within the active treatment arms coincided with commonly associated side effects of high-therapeutic dosing of guanabenz (e.g. hypotension, fatigue, drowsiness) and its alpha-2 adrenergic receptor activity. The number of serious adverse events did not statistically differ significantly between groups (Table 4).

Table 2 Trial primary and secondary outcomes

	Guanabenz 64 mg (n = 50)	Guanabenz 32 mg (n = 50)	Guanabenz 16 mg (n = 51)	Historical cohort (n = 200)	P-value[a]	Historical cohort plus placebo (n = 249)	P-value[b]	Placebo (n = 49)	P-value[c]
Primary outcome									
Progressed at ALS-MITOS at 6 months (%; upper level of the relative CI under null hypothesis)	10/40 (25%; 32%[d])	13/43 (30%; 37%[d]) 23/83 (28%)	20/46 (43%; 51%)	83/178 (47%)	**0.004**[e,f] **0.03**[g] **0.01**[h] **0.04**[i]	97/224 (43%)	**0.01**[e,f] 0.05[g] **0.03**[h] 0.06[i]	14/46 (30%)	0.74[e] 0.88[g] 0.86[h] 0.88[i]
Secondary outcomes									
Decline of the ALSFRS-R at 6 months Mean ± SD; median (IQR)	−5 ± 6; −4 (8)		−7 ± 6; −5 (9)	−7 ± 5; −6 (8)	**0.01**[e,f] 0.06[g] **0.02**[h] 0.13[i]	−6 ± 5; −6 (8)	**0.01**[e,f] 0.09[g] **0.01**[h] 0.22[i]	−6 ± 5; −5 (8)	0.12[e] **0.03**[g] 0.41[h] 0.12[i]
Decline of slow vital capacity at 6 months Mean ± SD; median (IQR)	−12 ± 16; −10 (21)		−17 ± 20; −12 (25)	−13 (21); −15 ± 18	0.27[e] 0.26[g] 0.49[h] 0.45[i]	−14 (22); −15 ± 18	0.22[e] 0.16[g] 0.35[h] 0.34[i]	−15 ± 20; −16 (29)	0.26[e] 0.41[g] 0.31[h] 0.34[i]
Death, tracheostomy or permanent ventilation at 6 months	6.0 ± 2.7		6.6 ± 3.8	8.4 ± 2.1	0.51 0.77	7.1 ± 1.7	0.73 0.57	2.2 ± 2.2	0.32 0.30
Estimated percentage of patients with event (cumulative hazard function ± SD)[j]									

The proportion of patients progressing of at least one stage on the ALS-MITOS scale at 6 months was expected as 30% in the guanabenz arms versus 47% in the historical cohort from the EPOS trial.[34] Based on the futility study, with alpha = 10% and power = 85%, the null hypothesis of non-futility is accepted if the upper level of the relative confidence interval (CI) is lower than 47%. Values in bold indicate significant P-values.

[a]P-value from chi-square, Fisher exact, Mann-Whitney or log-rank test, as appropriate, of guanabenz 64 mg and 32 mg combined versus historical cohort.

[b]P-value from chi-square, Fisher exact, Mann-Whitney or log-rank test, as appropriate, of guanabenz 64 mg and 32 mg combined versus historical cohort plus placebo.

[c]P-value of guanabenz 64 mg and 32 mg combined versus placebo (note that the placebo arm was not powered for efficacy comparisons).

[d]Both guanabenz 64 mg and 32 mg alone and combined reached the primary hypothesis of non-futility.

[e]Chi-square, Fisher exact, Mann-Whitney or log-rank test, as appropriate.

[f]Significant P-values after adjustment for multiple dose-group comparisons based on the truncated Hochberg procedure (cut-off of P = 0.0375).

[g]Multivariate analyses including type of onset (bulbar versus spinal), months from onset, ALS-FRS-R, sVC and ALS-MITOS baseline values as covariates.

[h]Univariate analyses following multiple imputation with prediction equations including type of onset (bulbar vs spinal), months from onset, ALS-FRS-R, sVC and ALS-MITOS baseline values.

[i]Multivariate analyses following multiple imputation.

[j]P-values testing the proportional-hazards assumption based on Schoenfeld residuals were 0.74, 0.86 and 0.37 for historical cohort alone, historical cohort plus placebo and placebo alone comparison, respectively.

Table 3 Progression of bulbar and spinal onset patients

	Guanabenz 64 mg + 32 mg (*n* = 100)	Guanabenz 16 mg (*n* = 51)	Historical cohort (*n* = 200)	*P*-value[a]	Historical cohort + placebo (*n* = 249)	*P*-value[b]	Placebo (*n* = 49)	*P*-value[c]
Progressed at ALS-MITOS at 6 months, *n* (%)								
Bulbar	0/18 (0)	4/8 (50)	21/49 (43)	**0.001**	25/60 (42)	**0.001**	4/11 (36)	**0.01**
Spinal	23/65 (35)	16/38 (42)	62/129 (48)	0.09	72/164 (44)	0.24	10/35 (29)	0.49
Decline of the ALSFRS-R at 6 months, mean ± SD; median (IQR)								
Bulbar	−2 ± 3; −1 (4)	−10 ± 8; −10 (14)	−7 ± 5; −6 (7)	**0.0003**	−7 ± 5; −7 (7)	**0.0001**	−7 ± 5; −7 (7)	**0.002**
Spinal	−6 ± 6; −4 (7)	−6 ± 6; −5 (7)	−6 ± 5.5; −6 (7)	0.36	−6 ± 5.5; −5 (8)	0.42	−6 ± 6; −5 (9)	0.83
Decline of slow vital capacity at 6 months, mean ± SD; median (IQR)								
Bulbar	−10 ± 15; −5 (19)	−19 ± 13; −20 (16)	−15 ± 15; −16 (19)	0.19	−16 ± 15; −16 (19)	0.11	−23 ± 9; −25 (12)	**0.03**
Spinal	−12 ± 16; −10 (19)	−16 ± 22; −10 (30)	−15 ± 19; −11 (24)	0.57	−15 ± 19; −11 (27)	0.60	−13 ± 22; −12 (29)	0.82
Death, tracheostomy or permanent ventilation at 6 months; estimated percentage of patients with event (cumulative hazard function ± SD)								
Bulbar	0.0 ± 0.0	0.0 ± 0.0	10.1 ± 4.5	0.18	8.3 ± 3.7	0.22	0.0 ± 0.0	NA
Spinal	7.6 ± 3.4	8.0 ± 4.6	7.8 ± 2.3	0.99	6.7 ± 2.0	0.80	2.9 ± 2.9	0.33

The proportion of ALS patients with bulbar onset on guanabenz 64 mg and 32 mg progressing to higher stage of disease was significantly lower than that on the historical cohort alone and combined with placebo. Similarly, ALS patients with bulbar onset on guanabenz 64 mg and 32 mg showed a significantly slower decline of ALSFRS-R. NA = not applicable. Values in bold indicate significant *P*-values.

[a]*P*-value from chi-square, Fisher exact, Mann-Whitney, or log-rank test, as appropriate, of guanabenz 64 mg and 32 mg combined versus historical cohort.

[b]*P*-value from chi-square, Fisher exact, Mann-Whitney or log-rank test, as appropriate, of guanabenz 64 mg and 32 mg combined versus historical cohort plus placebo.

[c]*P*-value from chi-square, Fisher exact, Mann-Whitney or log-rank test, as appropriate, of guanabenz 64 mg and 32 mg combined versus placebo (note that the placebo arm was not powered for efficacy comparisons).

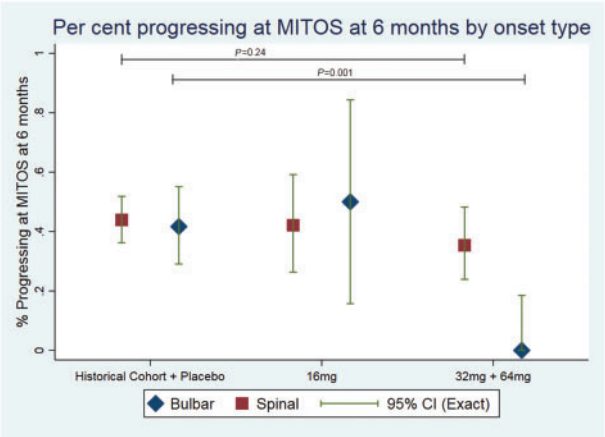

Figure 2 ALS patients with bulbar and spinal onset in the two treatment arms. The proportion of ALS patients with bulbar onset in the guanabenz 64 mg and 32 mg treatment arms progressing to a higher stage of disease (as measured by the ALS-MITOS system) was statistically significantly lower than that of bulbar patients progressing in the historical cohort plus placebo ($P = 0.001$). The proportion of patients with spinal onset in the 64 mg and 32 mg treatment arms progressing to higher stages of disease was not significantly different ($P = 0.24$) compared with the proportion progressing in the historical cohort plus placebo with spinal onset. The 95% confidence intervals (CI) were calculated using the exact binomial (Clopper-Pearson) methodology. *P*-values were calculated using chi-square or Fisher exact tests, as appropriate.

Discussion

Our study demonstrated that the treatment of ALS patients with guanabenz at dosages of 64 mg and 32 mg daily is not futile and that a phase 3 trial is warranted. Indeed, we found a significantly lower proportion of patients progressed to a higher stage of disease at 6 months than expected under the hypothesis of non-futility as measured by the ALS-MITOS system. This conclusion held even after adjusting for potential confounders. Moreover, we found a slower decline in daily living activities as measured by the total ALSFRS-R score. This result was driven by the effect on patients with bulbar onset, among which, those treated with guanabenz 64 mg and 32 mg did not show any progression to higher stages of disease in the ALS-MITOS and had a slower rate of decline in the ALSFRS-R compared with patients in the guanabenz 16 mg arm and in the historical cohort alone and combined with placebo. Notably, all bulbar onset patients enrolled in the trial were at stage 0 of the ALS-MITOS and none of those in the guanabenz 64 mg and 32 mg arms progressed to a higher stage of disease, while 50% of those in the guanabenz 16 mg arm, 43% in the historical cohort and 36% on the placebo did.

These results were obtained using a historical cohort of patients with ALS enrolled in a previous failed clinical trial carried out by the same consortium of Italian ALS centres as a comparator.[39] The use of the same diagnostic criteria and approach to fragile functions (e.g. nutrition and respiratory insufficiency management) limited the potential bias of an external comparison. Because ALS is a rare disease with an incidence of approximately two cases per 100 000 inhabitants per year and small phase 2 trials with potentially disease-modifying drugs require sufficient statistical power to address questions related to efficacy and cost-effectiveness of confirmatory phase 3 studies, the use of historical cohorts can overcome these limitations.[43,44] Several prior clinical trials have successfully adopted this methodological approach.[41,45–51]

Although the ALSFRS-R score has commonly been used to test the efficacy of therapeutic intervention in prior ALS studies,[52] we believe that the assessment of independent functions, in our trial measured by using the ALS-MITOS system, could provide more reliable clues about the disease course of ALS and its modulation by a disease-modifying drug.[53] The ALS-MITOS system measures the loss of independent functions in the four key domains included in the ALSFRS-R (i.e. walking/self-care, swallowing, communicating and breathing). This outcome was developed to overtake the intrinsic limitations of the ALSFRS-R, for which the validity in capturing disease severity is debated,[54] even though it is still the referenced outcome in the FDA guidance for clinical trials in ALS. Indeed, the ALSFRS-R is not linear, thus prone to biases; it is multidimensional, thus unfit as a single score and unable to satisfy rigorous measurement standards; it has floor-effect, thus is unable to capture late-stage clinical changes; and it does not meet the Rasch analysis requisites for a single scoring system.[38,55,39,56] The measure of function loss by domain rather than on single items could better assess ALS progression. Several previous studied showed that combined outcome measures including survival, tracheotomy, non-invasive ventilation and/or selected domains of the ALSFRS-R scale showed better performances compared with survival or mean ALSFRS-R decline alone.[39,57–59] The ALS-MITOS system exhibited a higher resolution for late disease, corresponding to functional involvement, compared with the King's scale.[53]

Survival, which in trials is comparable to tracheotomy or >23 h non-invasive ventilation, is another suitable primary outcome in ALS,[60] but it requires at least 1000 patients who are followed up for more than 3 years to have adequate power.[38] In the comparison between ALS-MITOS progression and ALSFRS-R decline over the first 6 months from baseline, the best cut-off of the ALS-MITOS to predict 6 months survival, tracheotomy or >23 h non-invasive ventilation at 12 and 18 months was the loss of one function on the ALS-MITOS and 6 to 9 points of decline on the ALSRFS-R.[38] Accordingly, as all patients enrolled in the trial at the ALS-MITOS stage 0 at baseline had bulbar onset, the corresponding predicted probability of one of the three events (e.g. survival, tracheotomy or >23 h non-invasive ventilation) for patients on guanabenz 32 mg or 64 mg was 7% at 12 months and 17% at 18 months, against the corresponding probabilities of 19%, 42%, and 70% at 12 months, and 38%, 64%, and 84% at 18 months for the ALS-MITOS stages 1, 2, and 3 at 6 months.

While we believe that the ALS-MITOS system purports a better methodology to test interventional efficacy on disease progression, we are equally encouraged by the results observed with respect to the ALSFRS-R. In the analysis of the ALSFRS-R decline, the median difference between baseline and 6 months was 0.33 points per month in patients in the combined guanabenz 32 mg and 64 mg arm, a result that was statistically significantly better than in the other arms and similar to that found in the recent trial of sodium phenylbutyrate-taurursodiol.[52] This effect was much larger in patients with bulbar onset treated with guanabenz 32 mg and 64 mg, who showed a difference of 1 point per month compared to the other arms. That differences were not seen in the comparison of bulbar patients in the 16 mg arm or across any of the spinal onset subgroups and may suggest that either threshold therapeutic dosing levels were not reached or that therapeutic benefit may, in fact, be most impactful for those patients with bulbar onset.

While this study did not show a difference in serum NfL biomarkers across treatment arms, we find this result to be unsurprising. Serum NfL is an unspecific biomarker of upper motor neuron degeneration. While ALS patients exhibit elevated levels that may correspond to disease progression, NfL levels have been found to not differ among different pathological stages and can be stable in single patients over time.[61] Additional studies have

Table 4 Adverse events

	Guanabenz 64 mg ($n = 50$)	Guanabenz 32 mg ($n = 50$)	Guanabenz 16 mg ($n = 51$)	Placebo ($n = 49$)	P-value[a]
Adverse events[b]					
⩾1 adverse event, *n* (%)	43 (86)	36 (72)	33 (65)	22 (45)	<0.001
No. of distinct events	141	128	118	51	
Withdrawals due to any adverse event, *n* (%)	15 (30)	15 (30)	8 (16)	3 (6)	0.006
Serious adverse events[c]					
⩾1 Serious adverse event, *n* (%)	4 (8)	4 (8)	6 (12)	4 (8)	0.89
Number of distinct events	5	5	7	5	
Death, *n* (%)	2 (4)	3 (6)	2 (4)	0 (0)	0.51
⩾1 Serious adverse event considered to be related to intervention, *n*. (%)	1 (2)	1 (2)	0 (0)	0 (0)	0.74
Adverse events with ⩾5% incidence in either group, *n* (%)					
Hypotension	19 (38)	14 (28)	11 (22)	1 (2)	<0.001
Dizziness	4 (8)	5 (10)	2 (4)	0 (0)	0.09
Irritability	2 (4)	6 (12)	8 (16)	5 (10)	0.27
Nervousness	5 (10)	5 (10)	11 (22)	4 (8)	0.15
Fatigue	22 (44)	21 (42)	18 (35)	8 (16)	0.02
Drowsiness	33 (66)	18 (36)	18 (35)	7 (14)	<0.001
Dry mouth	31 (62)	24 (48)	20 (39)	6 (12)	<0.001
Weakness	26 (52)	24 (48)	17 (33)	9 (18)	0.002
Headache	4 (8)	4 (8)	8 (16)	3 (6)	0.36
Nausea	5 (10)	8 (16)	8 (16)	3 (6)	0.36
Others	7 (14)	12 (24)	6 (12)	6 (12)	0.29

The safety population included all the participants who received at least one dose of guanabenz or placebo. The relatedness of adverse events or serious adverse events to the intervention was determined by the site investigator.

[a]P-value from chi-square or Fisher exact test, as appropriate.

[b,c]Adverse events[b] and serious adverse events[c] were classified according to system organ class and preferred term in the Medical Dictionary for Regulatory Activities, version 16.1.

confirmed NfL stability in ALS patients over time.[62] These analyses suggest that the potential utility of serum NfL as a dynamic biomarker of treatment effect remains uncertain.[63] In our trial, the mean rate of change in serum NfL levels did not significantly differ between the groups over the 6-month trial duration. Similarly, the plasma neurofilament H subunit level did not change in the trial of sodium phenylbutyrate-taurursodiol.[52]

The alpha-2 adrenergic activity of guanabenz was apparent in this study and led to statistically significantly higher dropout rates in the 64 mg and 32 mg dosing arms. The disproportionate dropout rate in the top dosing arms relative to the placebo may have confounded the ability to identify an even stronger signal both in the bulbar subgroup and the full study population inclusive of spinal onset patients. The ability of guanabenz to induce hypotension in the non-hypertensive patient clearly limits its practical application in further assessment in ALS. We note, however, that most of the confounding issues associated with testing the hypothesis of unfolded protein response regulation on the outcome of ALS progression can be avoided with the use of agents that similarly act to prolong eIF2α phosphorylation. Sephin1, a synthetic molecule lacking alpha-2-adrenergic receptor activity, which selectively binds to and inhibits the ER stress-induced PPP1R15A phosphatase complex, has already completed a phase 1 clinical trial under the name of IFB-088 (NCT03610334) and has demonstrated a strong effect in preventing *in vitro* motor neuron degeneration and *in vivo* ALS progression.[33,64] Use of Sephin1 (IFB-088) should strongly be considered in a confirmatory trial.

In summary, there is strong evidence to suggest that ER stress may play a critical role in the pathogenesis of ALS through altered regulation of proteostasis and that molecules acting on the functional control of the unfolded protein response pathway may be of benefit in slowing the progression of the disease.[7–18,21,65–69] The results of our phase 2 trial based on the analysis of primary and secondary functional efficacy outcomes provide indications that guanabenz at the doses of 64 mg and 32 mg slowed the progression of ALS in patients with bulbar onset. The study was not powered for subgroup analysis; therefore, this effect should be considered exploratory. The reason for the potential effect on this distinct phenotype subtype is unknown. Bulbar onset is the most homogeneous ALS phenotype both in terms of progression[40] and neuropathological features.[70] Conversely, spinal onset ALS has huge variability that may have diluted the possibility of capturing an effect in a small sample size. Overall, our findings indicate that a phase 3 trial with a molecule targeting the unfolded protein response pathway without an alpha-2 adrenergic-related side-effect profile is warranted.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

Appendix 1

PROMISE trial collaborators

Full details are provided in the Supplementary material.

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