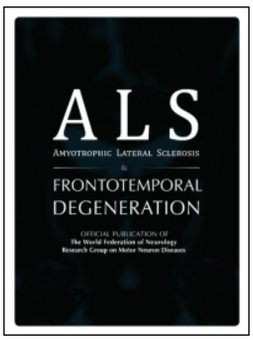

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ORIGINAL ARTICLE

Phase 2 study of sodium phenylbutyrate in ALS

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Abstract

The objective of the study was to establish the safety and pharmacodynamics of escalating dosages of sodium phenylbutyrate (NaPB) in participants with ALS. Transcription dysregulation may play a role in the pathogenesis of ALS. Sodium phenylbutyrate, a histone deacetylase inhibitor, improves transcription and post-transcriptional pathways, promoting cell survival in a mouse model of motor neuron disease. Forty research participants at eight sites enrolled in an open-label study. Study medication was increased from 9 to 21 g/day. The primary outcome measure was tolerability. Secondary outcome measures included adverse events, blood histone acetylation levels, and NaPB blood levels at each dosage. Twenty-six participants completed the 20-week treatment phase. NaPB was safe and tolerable. No study deaths or clinically relevant laboratory changes occurred with NaPB treatment. Histone acetylation was decreased by approximately 50% in blood buffy-coat specimens at screening and was significantly increased after NaPB administration. Blood levels of NaPB and the primary metabolite, phenylacetate, increased with dosage. While the majority of subjects tolerated higher dosages of NaPB, the lowest dose (9 g/day), was therapeutically efficient in improving histone acetylation levels.

Key words: *Clinical trial, amyotrophic lateral sclerosis, HDAC inhibition*

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal, degenerative disorder of motor neurons that results in progressive wasting and paralysis of voluntary muscles (1). Riluzole is the only Food and Drug Administration approved treatment for ALS and prolongs survival by approximately 10% (2). While the causative pathway of ALS is unknown, studies suggest that transcriptional dysfunction may play a role in the pathogenesis of ALS (3–8). Changes in the molecular signature of gene expression in animal models and people with ALS have been demonstrated in microarray studies (6,7,9). Transcription is regulated by complex interactions between many proteins, among them transcription factors and histones that affect the actions of RNA polymerase II on individual genes. Many of these interactions, in turn, are regulated by covalent modifications, such as acetylation, methylation and phosphorylation. Recruitment of histone deacetylases (HDACs) to DNA alters nucleosome structure locally and inhibits transcription. Transcription dysregulation perturbs cellular function and compromises protective mechanisms, leading to neuronal death.

A potential strategy in the treatment of ALS, therefore, is to modulate the aberrant transcription that may lead to motor neuron cell death using HDAC inhibition. HDAC inhibitors increase acetylation of histones, thereby promoting transcriptional activation. Sodium phenylbutyrate (NaPB) is an HDAC inhibitor currently used to treat hyperammonemia

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due to urea cycle disorders. Sodium phenylbutyrate is also under development as an anti-cancer agent because of its activity as an HDAC inhibitor causing tumor differentiation, growth arrest and apoptosis. Sodium butyrate reduces the clinical phenotype and neuronal degeneration in a number of mouse models that include multiple sclerosis (10), polyglutamine disorders (4,11), spinal muscular atrophy (SMA) and ALS (12). Sodium phenylbutyrate increases the expression of full-length survival motor neuron (SMN) protein as shown in SMA. Low SMN protein levels have been implicated in the pathogenesis of ALS. In the mutant superoxide dismutase G93A transgenic mouse model of motor neuron disease, NaPB ameliorated histone hypoacetylation, improved both transcriptional and post-transcriptional pathways, reduced levels of key components of the intrinsic apoptotic pathway, reduced SOD1-positive aggregates, and dosage-dependently prolonged survival by up to 22% (12). Since NaPB has not previously been tested in ALS, we designed a study to determine dosage, safety, and tolerability in this population.

Methods

Study design

An open-label dose escalation study was conducted at 10 clinical sites, eight Veteran's Administration (VA) centers and two non-VA hospitals under an investigator new drug application (IND #70,067). All sites had institutional regulatory board (IRB) approval and all research participants provided informed consent in accordance with the Helsinki Declaration of 1975, as revised in 1983. The duration of participation was 24 weeks. For each participant, the dosage increased over 12 weeks to a maximum anticipated dosage of 21 g/day or the maximum tolerated dosage. Participants were maintained on that dosage until week 20 (Figure 1). The primary outcome measure was tolerability. Secondary outcome measures were safety, NaPB and metabolite blood levels, riluzole levels, and histone acetylation activity levels. Data were entered into a web-based electronic data capture system. On-site monitoring occurred twice at each site with 100% reconciliation of source to electronic data.

Study medication

NaPB was purchased from Scandinavian Formulas, Inc.™, (Sellersville, PA) and dispensed as 1-g enteric-coated tablets. Research participants took three tablets t.i.d. from the baseline visit to week 2 (9 g/day), four tablets t.i.d. from week 2 to week 4 (12 g/day), five tablets t.i.d. from week 4 to week 8 (15 g/day), and six tablets t.i.d. week 8 through week 12 (18 g/day). At 12 weeks, the subject took seven tablets t.i.d. (21 g/day) through week 20, the last day of the treatment phase of the trial (Figure 1). If participants could not escalate dosage because of adverse events, they were maintained on their maximum tolerated dosage.

Participant selection criteria

Participation eligibility at the VA sites required Veteran status. Subjects had ALS diagnosed as possible, laboratory supported probable, probable, or definite according to the World Federation of Neurology El Escorial criteria (13). Inclusion criteria included ability to provide informed consent and comply with study procedures, vital capacity (VC) greater than 60% predicted value for sex, height, and age; ability to swallow tablets at screening visit and no medical conditions that could be exacerbated by sodium administration. Participants were allowed to take riluzole, if on a stable dose at least 30 days prior to the screening visit. Women of childbearing potential could be included if using adequate birth control and a screening pregnancy test was negative. Exclusion criteria included exposure to NaPB or known HDAC inhibitors within three months prior to the screening visit, exposure to

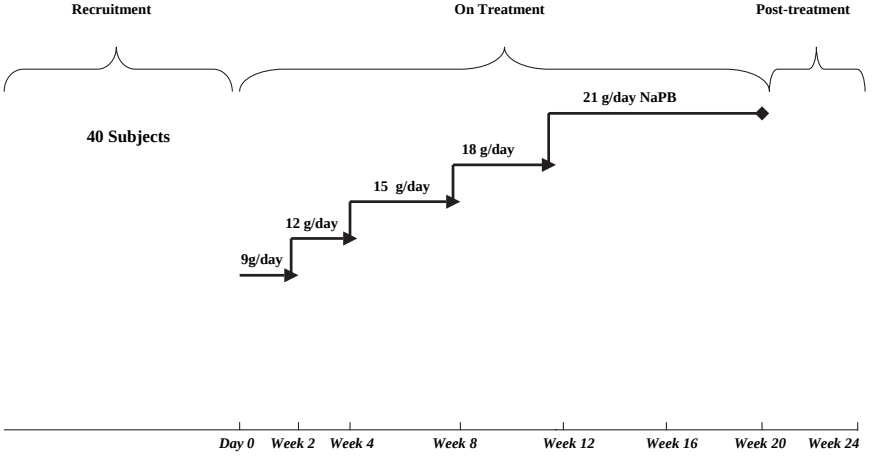

Figure 1. Dosage escalation paradigm. The dosage was increased from 9 g/day to 21 g/day over 12 weeks.

any investigational drug within 30 days of the screening visit, dependence on a mechanical ventilator (invasive and/or noninvasive) for all or part of the day, history of acute hyperammonemia, or significant cardiac conduction abnormality identified on screening EKG.

Training and study monitoring

Each site investigator and research coordinator was trained and certified on study procedures. Site evaluators were trained in the administration of VC, isometric strength of 18 muscle groups in the limbs using Microfet II™ hand held dynamometer (HHD) (Hoggan Health Industries, West Jordan, UT), grip strength using JAMAR™ grip dynamometer (Sammons Preston Roylan, Bolingbrook, IL), and the ALS functional rating scale-revised (ALSFRS-R). The ALSFRS-R is the summed score of 12 functional and respiratory items rated on a scale of 0 to 4 (14). Pre-study reliability testing yielded an average intra-rater percent difference for all muscles tested with HHD between raters across subjects of 7.6% $\pm$2.7. The average percent difference between the three VC trials across subjects tested was 4%.

Study procedures

Participants were assessed for eligibility at the screening visit. Accrued participants returned for the baseline visit (week 0) within 21 days of the screening visit. The following measures were obtained at the baseline visit: vital signs, weight, VC, isometric strength testing using HHD, grip strength, and ALSFRS-R. After baseline measures were obtained, each participant received one dose (3 g) of study medication and was monitored for untoward effects for 2–3 h. Participants took 3 g t.i.d. the following day. All research participants returned for in-person visits at weeks 2, 4, 8, 12, 16, and 20. A telephone call to each participant was conducted at weeks 6, 10, 14, and 18 to assess adverse events. Study medication was discontinued at week 20 and a post-treatment phone call occurred four weeks after last dose to address adverse events.

At each visit, vitals signs, weight, EKG, a review of adverse events and concomitant medications, assessment of drug accountability and safety laboratory tests were completed. Adverse experiences to the experimental intervention were assessed at each in-person and telephone study visit by recording all voluntary complaints of subjects and by assessment of the clinical features of ALS. Attention was directed to clinical adverse experiences associated with prior NaPB studies, as well as any evidence of unexpected worsening of the underlying ALS. Blood was collected at each visit 1–2 h (approximate Tmax) after study medication administration for measurement of NaPB and metabolites, riluzole and histone acetylation levels. The ALSFRS-R, strength measures, and VC were completed at weeks 0, 4, 8, 12, and 20.

Sample collection for NaPB, phenylacetate, riluzole, and histone acetylation

Blood was collected in one 10-ml sodium heparin green top tube and one 5-ml red top tube (both plastic). The 10-ml green top tube was inverted 8–10 times following collection and placed on crushed ice. The sample was centrifuged at 8000–9000x *g* for 20 min within 1 h of collection. After spinning, the green top tube was frozen upright on dry ice (–40°C). The 5-ml red top vacutainer was immediately frozen upright on dry ice and served as a backup sample. Urine samples were collected in a 50-ml Falcon tube and frozen immediately on dry ice (–40°C). All specimens were stored at –80°C. The buffy coat was extracted from each frozen green top tube, aliquoted, then sub-aliquoted and analyzed for histone acetylation levels within eight weeks of sample collection.

Histone acetylation, NaPB, phenylacetate and riluzole assays

To measure histone acetylation, lysates of the buffy coat containing white blood cells from the frozen sample were obtained by homogenizing each sample in 500 µl of PBS buffer containing 0.4 *M* NAB, 5% Triton X-100, 100 mM phenylmethylsulfonyl fluoride (PMSF), 3 mM DTT, leupeptin aprotinin, 1 mM sodium orthovanadate, 5 mM sodium fluoride, 3 mM PMSF, 3 mM DTT, 0.5 µg/ml leupeptin, and 10 µg/ml aprotinin. The sample was centrifuged and supernatant containing the cytosolic fraction was removed, 70 µl 0.2N HCl was added to the remaining pellet, vortexed for 1 h at 4°C, centrifuged for 8 min at 3000 rmp at 4°C, and supernatant containing the nuclear fraction assayed for protein and normalized at 0.5 µg/µl. Duplicate samples were loaded onto a nitrocellulose transfer membrane (0.2 µm) and subsequently immunoreacted for acetylated histone 3 (1:700, Upstate Biotechnology, Lake Placid, NY) and beta-actin (1:3000, Sigma-Aldrich, St. Louis, Mo). Histone acetylation values were measured by Western blot analysis (dot blot), using Image J (NIH) comparing ratios of H3 to beta-actin. Levels of NaPB, phenylacetate and riluzole were determined using liquid chromatography.

Statistical analysis

Tolerability was defined as the ability to complete 20 weeks of treatment on study medication. Since some participants did not complete the study due to

personal reasons we estimated the proportion of participants who tolerated each dosage using the method of Kaplan-Meier, assuming that a participant who did not tolerate a dosage would not have tolerated a higher dosage. In the analysis we considered all events that terminated the study whether or not the investigator considered them drug related. Non-toleration was defined as permanent drug suspension due to an adverse event or not escalating dosage due to adverse events.

Functional measures were included as a safety measure and also for exploratory measures of disease progression. Rates of change of these secondary outcomes measures (VC, HHD, ALSFRS-R) were analyzed using a placebo database that included 99 subjects from a recently completed North East ALS Consortium (NEALS) trial, as a comparison group (15). The rates of decline were compared using mixed-effects models to adjust for repeated observations on the same individuals and to control for selected baseline variables. Distributions of baseline characteristics were compared using Fisher's exact tests and *t*-tests. The treatment effects on adverse events and laboratory abnormalities were compared to placebo cohort, using Fisher's exact test and Wilcoxon test.

The dosage effects on biomarkers, including histone acetylation, NaPB, phenylacetate and riluzole levels, were assessed using a mixed-effects models with dosage modeled as an independent variable, biomarkers as response variables, and intercept and dosage as random effects. Paired *t*-tests were used to assess treatment effects on biomarkers at each dosage.

Results

Fifty-one research participants were screened and 40 were accrued (Figure 2). Baseline characteristics are found in Table I. The average study medication compliance for all subjects was 97% (SD = 3.75). No major study violations occurred.

Tolerability

Twenty-six participants completed the study as planned and 14 stopped study medication early (Figure 2, Table II). Weight at baseline visit and demographic characteristics did not affect tolerability.

The estimate of the proportion of participants who would tolerate a given dosage is shown in Table III. There were 10 participants with adverse events that directly resulted in study termination: three of these events, heart block, subarachnoid hemorrhage and joint pain, were considered by the site investigator unrelated to treatment. The subject with a subarachnoid hemorrhage was found to have an aneurysm. No evidence of a bleeding disorder was found. In addition, there were six participants who did not escalate dosage because of adverse events that did not result in study termination. Four participants stopped the study early for personal reasons, three in weeks 1–3 on 9 g/day (unable to travel, pursuing another study, lack of benefit) and one at 16 weeks on 21 g/day (illness of spouse). The last column of the table is the proportion of participants who tolerated NaPB at each dosage, estimated by Kaplan-Meier, assuming that a subject who did not tolerate a dosage would not tolerate a higher dosage.

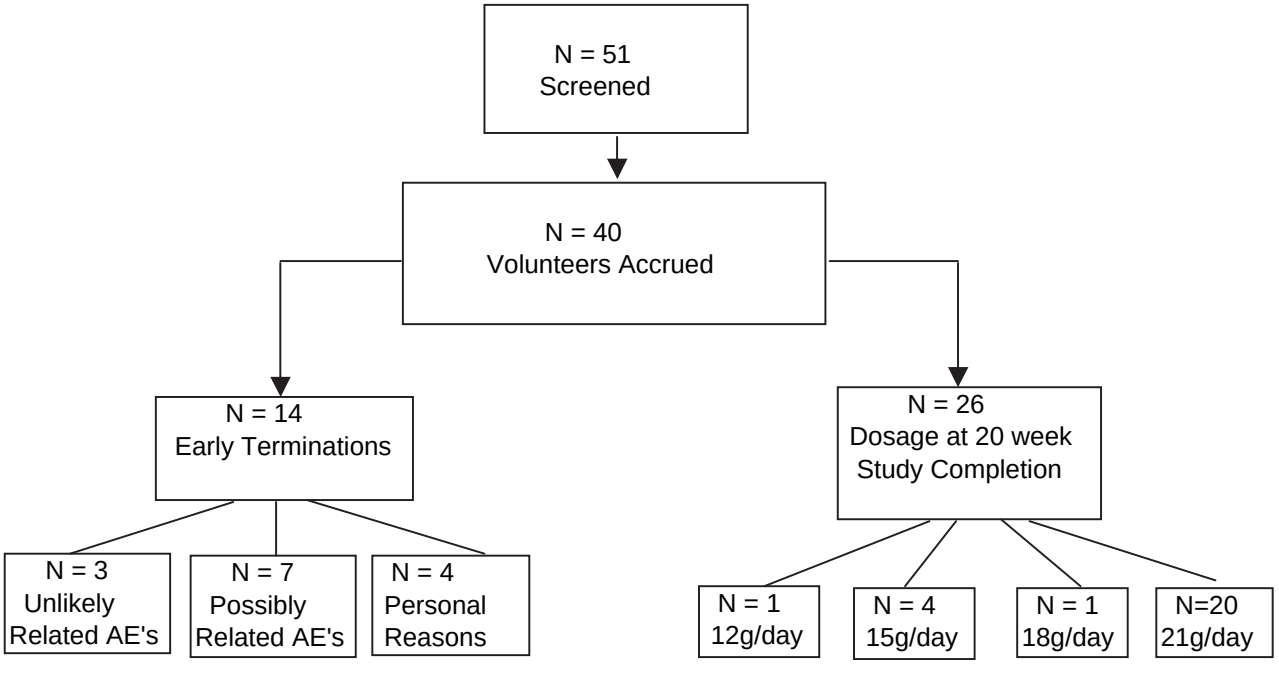

Figure 2. Participant flow chart.

Table I. Participant characteristics.

Age (years)	59.1±10.1
Males (%)	34 (85)
Veterans (%)	30 (75)
Symptom onset to screening (years)	3.2±2.6
ALSFRS-R score	36.2±7.7
Vital capacity (%)	84.1±18.1
Taking riluzole (%)	29 (73)
Bulbar onset (%)	7 (17.5)

Values are ±standard deviation.

Ten participants had dosage suspensions; study medication was re-started in all within 10 days (range 3–10 days). Reasons for temporary suspensions included nausea, vomiting, abdominal discomfort, fatigue, tonsillitis, and influenza. Four participants reduced study medication due to adverse events; the dosage was re-challenged in one after three days. Reasons for dosage reduction included edema, palpitations, nausea, dizziness, and constipation.

Safety assessments

The most common adverse events included falls, dizziness, diarrhea, edema, dry mouth, headache, nausea, and rash (Table IV). With the exception of headache, these adverse events occurred at a higher rate compared to the comparison placebo cohort. These events are expected side-effects from NaPB. There were no clinically significant changes in laboratory values, EKGs or vital signs. No deaths or unexpected and related serious adverse events occurred. Significant adverse events did not occur more frequently with subjects who were taking riluzole in addition to NaPB, compared to subjects taking NaPB alone. The NaPB-treated cohort was sicker than the comparison cohort as assessed by longer manifest disease time, lower ALSFRS-R scores, and higher bicarbonate levels. There were no differences in rate of disease progression as measured by functional outcomes (VC, grip, ALSFRS-R) compared to the placebo cohort (Table V). The results were the same after controlling the baseline covariates. The FVC declined greater with NaPb ($p=0.1$). Whether this reflects possible toxicity of NaPB on respiratory function cannot be determined from this study.

Histone acetylation, NaPB, phenylacetate, and riluzole levels

Histone acetylation was decreased at the screening visit in all participants with ALS (mean = 1.08 ng/ml, SD = 0.18), compared to a non-neurological control cohort (mean = 2.27 ng/ml and SD = 0.28). While histone acetylation levels increased with NaPB treatment (0.70 ± 0.17; $p < 0.0001$), levels did not reach acetylation levels of normal age-matched control patients (Figure 3). The largest increase in histone acetylation occurred at 9 g/day two weeks after trial start and did not increase further with dose escalation. Riluzole blood levels were unchanged with NaPB dosage ($p=0.23$). Riluzole blood levels did not change after initiation of NaPB. Blood levels of NaPB and phenylacetate at all dosages were different from the screening value. Phenylbutyrate and phenylacetate levels increased significantly with dosage ($p < 0.0001$) (Figure 4).

Discussion

Forty ALS patients participated in a dose escalation trial using NaPB from 9 g/day to 21 g/day. Twenty-six participants completed the 20-week study, 20 of which completed at 21 g/day. NaPB was safe in the participants with ALS. Tolerability for NaPB in subjects with ALS was similar to that in subjects with Huntington's disease (16), solid tumor malignancies (17), malignant gliomas (18), myelodysplastic syndromes and acute myelogenous leukemia (19), sickle cell anemia and beta thalassemias (20) where the maximum tolerated dosages of NaPB were between 15 and 30 g/day. There were no changes in safety laboratory tests or vital signs. No related, unexpected, serious adverse events occurred. The most common side-effects were those previously reported with NaPB, including gastrointestinal events and edema (17,20–23). Because dosage escalation occurred within subject, we cannot sepa-

Table II. Early study medication terminations due to adverse events.

Subjects	Weeks on study drug	Highest achieved NaPB dosage	Adverse event	Relationship to study medication*
1	1	9	Edema foot, under eyes	Possibly related
2	4	12	2nd degree heart block, Type II	Unrelated
3	5	15	Rash arm, legs, & trunk, Eyelid swelling	Possibly related
4	5	15	Increased arm weakness	Possibly related
5	7	15	Acute anemia, Gastrointestinal bleed	Possibly related
6	7	15	Headache (subarachnoid hemorrhage)	Unrelated
7	8	15	Abdominal discomfort, chills and nausea	Possibly related
8	12	15	Dizziness, shortness of breath	Possibly related
9	12	18	Lower extremity edema	Possibly related
10	14	18	Increased joint pain and stiffness	Unrelated

* Site investigator decision.

Table III. Proportion of participants tolerating each dosage.

Dosage	Participants available to start dosage	Participants terminated due to AE	Participants unable to dosage escalate (completed study at lower dosage)	Proportion who tolerated dosage and each lower dosage
9	40	1	0	97.5
12	37	5	0	84.3
15	31	2	1	76.2
18	29	2	4	59.8
21	22	0	1	57.1

rate out adverse events secondary to dosage from those due to prolonged drug exposure or disease progression. We chose to dosage escalate within subjects to have a larger number of research participants at each dosage and because of the possibility that tolerability would be improved with a slower dosage escalation within subject.

Although participants enrolled in this study were assessed using multiple measures of disease progression, the study duration, sample size and study design (open label) were insufficient to detect an efficacy signal. We chose to perform these assessments primarily as part of the safety evaluation. Previous studies (24–26) have shown that experimental agents can significantly increase the rate of disease progression, most likely because of adverse events from too high dosages (24,26). As such, the observation that rate of decline of the functional measures assessed here was in the range noted in a prior placebo cohort can be taken as evidence of safety of NAPB at the dosages employed.

Treatment with NaPB did not alter blood riluzole levels. Adverse events in participants taking riluzole and NaPB together did not occur more frequently, compared to those on NaPB alone. Since riluzole is the only approved therapy for ALS, it is important that clinical trials include data on the effects of any new therapy on riluzole levels and the safety of combination treatment.

NaPB displayed a dosage dependent increase in NaPB and phenylacetate blood levels. We did not define a limit of absorption at the tested dosages. However, the increased intolerability at higher dosages limits the utility of using higher dosages in people with ALS. Phenylbutyrate and phenylacetate have good CSF penetration based on non-human primate studies (27).

Treatment with NaPB significantly improved blood histone hypoacetylation two weeks after drug administration began, occurring at the lowest dosage of 9 g/day. Histone 3 acetylation levels did not increase further with higher dosages. This is the first study to report hypoacetylation in humans with ALS in blood buffy coat samples and parallels the findings observed in the G93A transgenic ALS mouse (12). Documentation that NaPB increases histone acetylation in ALS, although not to normal age-matched control levels, is important pharmacodynamic information and supports further clinical development of NaPB in ALS. It is not clear why acetylation levels were highest at 9 g/day. However, in a study of NaPB in Huntington's disease, the effects of NaPB on mRNA expression levels of a 12-gene biomarker set were greatest at lowest dosages (12 g) with an inverse dose response. Biomarkers are urgently needed for diagnosis, disease progression, and for potential disease-modifying therapies that are being developed and evaluated in clinical trials. Hypoacetylation, as a potential biomarker of manifest disease in ALS, may improve the power and cost-effectiveness of drug trials in this population.

The optimal dosage of NaPB for further testing in ALS is not yet known. Sodium phenylbutyrate and phenylacetate levels increased with dosage escalation and mirrored intolerability. In addition, histone acetylation levels did not elevate higher than

Table IV. Adverse events occurring in subjects taking NaPB compared to placebo cohort from celebrex study.

Adverse events	NaPB # of subjects: (% of subjects) Total sample: 40	Celebrex # of subjects: (% of subjects) Total sample: 99	*p*-value
Accidental injury	10 (25)	4 (4)	0.0002
Dizziness	9 (22.5)	2 (2)	<.0001
Diarrhea	8 (20)	8 (8)	0.039
Peripheral edema	8 (20)	5 (5)	0.006
Dry mouth	7 (17.5)	1 (1)	0.0002
Headache	7 (17.5)	13 (13)	0.449
Nausea	7 (17.5)	4 (4)	0.008
Rash	7 (17.5)	2 (2)	0.0008
Asthenia	6 (15)	5 (5)	0.045
Anxiety	5 (12.5)	4 (4)	0.068
Myasthenia (muscle fatigue)	5 (12.5)	3 (12.5)	0.031
Abdominal pain	5 (12.5)	4 (4)	0.069

Table V. Outcome measures: NaPB compared to placebo cohort from celebrex study.

Outcome measure	NaPB cohort (change/month)	Difference in slope, compared to celebrex placebo cohort	*p*-value
ALSFRS-R	−0.875±0.168	0.137±0.194	0.480
Vital capacity% predicted	−2.638±0.475	−0.882±0.543	0.106
BMI	−0.0806±0.0502	−0.0253±0.0577	0.662
Grip strength	−0.0802±0.0227	0.0190±0.0262	0.469
Weight	−0.246±0.152	−0.0948±0.175	0.588
HHD	−0.0863±0.0223	NA	NA

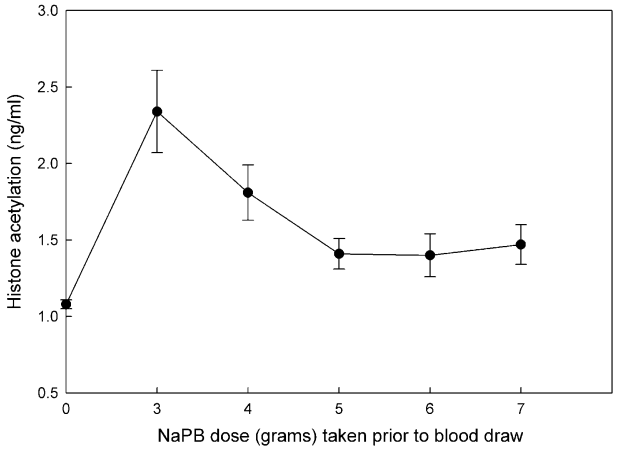

Figure 3. Histone acetylation levels with NaPB dose.
Blood histone acetylation levels are shown compared with dose taken prior to blood draw. The error bars represent standard error. * notes values significantly different from pre-treatment value.

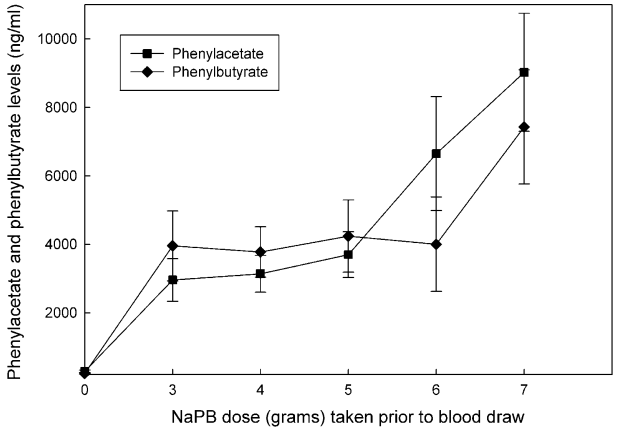

Figure 4. Phenylbutyrate and phenylacetate levels.
Blood phenylbutyrate and phenylacetate levels are shown compared with dose taken prior to blood draw. The error bars represent standard error. Levels at each dose are significantly greater than pre-treatment values.

those present at 9 g/day. As such, lower dosages are preferable to improve tolerability and may be sufficient to achieve the desired biological effect, increased histone acetylation. It is also possible that other HDAC inhibitors currently under development may have better tolerability and also increase histone acetylation to a greater degree.

We identified that 9 g/day of NaPB is well tolerated, safe and has the desired biological effect in blood. Subsequent steps in development of NaPB as a therapeutic agent for ALS include studies to assess preliminary efficacy and longer-term safety.

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Disclosure of interests: The authors have no known conflicts to report.

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