Phase II Open Label Study of Valproic Acid in Spinal Muscular Atrophy

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Abstract

Preliminary in vitro and in vivo studies with valproic acid (VPA) in cell lines and patients with spinal muscular atrophy (SMA) demonstrate increased expression of SMN, supporting the possibility of therapeutic benefit. We performed an open label trial of VPA in 42 subjects with SMA to assess safety and explore potential outcome measures to help guide design of future controlled clinical trials. Subjects included 2 SMA type I ages 2–3 years, 29 SMA type II ages 2–14 years and 11 type III ages 2–31 years, recruited from a natural history study. VPA was well-tolerated and without evident hepatotoxicity. Carnitine depletion was frequent and temporally associated with increased weakness in two subjects. Exploratory outcome measures included assessment of gross motor function via the modified Hammersmith Functional Motor Scale (MHFMS), electrophysiologic measures of innervation including maximum ulnar compound muscle action potential (CMAP) amplitudes and motor unit number estimation (MUNE), body composition and bone density via dual-energy X-ray absorptiometry (DEXA), and quantitative blood SMN mRNA levels. Clear decline in motor function occurred in several subjects in association with weight gain; mean fat mass increased without a corresponding increase in lean mass. We observed an increased mean score on the MHFMS scale in 27 subjects with SMA type II (p=0.001); however, significant improvement was almost entirely restricted to participants <5 years of age. Full length SMN levels were unchanged and Δ7SMN levels were significantly reduced for 2 of 3 treatment visits. In contrast, bone mineral density (p=0.0036) and maximum ulnar CMAP scores (p=0.0001) increased significantly.

Conclusions: While VPA appears safe and well-tolerated in this initial pilot trial, these data suggest that weight gain and carnitine depletion are likely to be significant confounding factors in clinical trials. This study highlights potential strengths and limitations of various candidate outcome measures and underscores the need for additional controlled clinical trials with VPA targeting more restricted cohorts of subjects.

Trial Registration: ClinicalTrials.gov NCT00374075

Introduction

Spinal muscular atrophy (SMA) is the most common inherited motor neuron disease, and a leading cause of infant and childhood mortality [1,2]. With an incidence of 1 in 10,000 births, it is an autosomal recessive disorder associated with severe neuromuscular weakness and premature death in the majority of patients [3–6]. More than 96% of affected individuals demonstrate a homozygous deletion/mutation involving exon 7 in SMN1 (survival motor neuron 1), resulting in the biochemical deficiency of the SMN protein, part of a complex that functions in the assembly of small nuclear ribonucleoprotein particles (snRNP) [7,8]. A genomic duplication at this locus has resulted in a nearly identical gene, SMA2 (survival motor neuron 2). SMA2 differs from SMA1 by a nucleotide substitution that promotes exon 7 exclusion. Consequently, SMN2 produces a fraction of the identical full length protein. Phenotypic variation in SMA correlates with the number of SMN2 gene copies and the level of SMN protein in cells [9–15].
The broad range of phenotypes has led to classification into clinical types including the most common severe infantile form (SMA type I), non-ambulatory variants of intermediate severity (SMA type II), and ambulatory variants (SMA types III, IV). The majority of subjects develop symptoms in infancy or early childhood.

An opportunity for therapeutic intervention has arisen from the discovery of small molecule compounds which target SMN2 gene copies present in all SMA patients to produce increased amounts of full-length SMN protein. Several compounds demonstrated to up-regulate SMN expression in SMA patient-derived cell lines, including valproic acid (VPA), sodium phenylbutyrate (NaPB) and hydroxyurea (HU), have been in clinical use for decades. These histone deacetylase (HDAC) inhibitors variably increase expression of many genes, including the SMN gene [16–20]. Preliminary in vivo data in human subjects supports up-regulation of SMN by both VPA and NaPB [21,22]. VPA up-regulates SMN2 expression at the promoter via inhibition of HDAC2, and both VPA and HU appear to alter splicing to increase full-length SMN protein [20,23]. VPA has demonstrated neuroprotective properties on glutamate-induced excitotoxicity via up-regulation of alpha-synuclein and increases neurite outgrowth in vitro[24,25]. Both VPA and NaPB have been reported to increase survival in ALS animal models [26,27]. More recently, VPA administration in an SMA mouse model resulted in apparent improved motor function, larger evoked motor potentials, less degeneration of spinal motor neurons and improved neuromuscular junction innervation in treated animals compared to age-matched controls [28,29].

Finally, two small open label trials of VPA in human subjects have reported modest strength or functional benefit in a subset of those patients [30,31]. Such observations support the potential benefit of small molecule therapeutics not only for SMA, but for other forms of motor neuron disease, such as ALS. This preliminary in vivo and in vitro evidence encouraged us to proceed with an open label study of VPA in SMA patients as a first step towards more formal efficacy studies. Our primary objectives were to determine the safety of VPA in SMA patients and to assess the utility of a number of exploratory outcome measures for future clinical trials.

Methods

The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1 and Protocol S1.

Study population

Subjects participating in a natural history study at the University of Utah were recruited for an open label study of VPA (ClinicalTrials.gov ID NCT00374075). All subjects at least 2 years of age receiving <16 hours/day ventilator support were invited to participate. Fifty-eight subjects were enrolled in the natural history study. At the start of VPA study enrollment, thirteen type I subjects were deceased, required full-time mechanical ventilation, or were less than 2 years of age. Two subjects were excluded for prior noncompliance with natural history study visits. Two subjects declined due to the burden of study visits. Three additional subjects declined due to perceived risks. Enrollment was then opened in the order of calls received to recruit 4 additional subjects. Type I subjects were not specifically excluded but enrollment was discontinued following recruitment of 40 type II and type III subjects. Two subjects had a severe phenotype (SMA type I ages 2–3 years), 29 subjects had an intermediate phenotype (SMA type II ages 2–14 years) and 11 had a mild phenotype (SMA type III ages 2–31 years). Additional details of baseline study population characteristics are shown in Tables S1 and S2. The progress of all participants through the trial is diagrammed in Figure S1.

Consent and adverse event grading

Written informed consent (subjects ≥18 years), parental consent (subjects <18 years) and assent (subjects ≥7 years) were obtained for all subjects. The study was approved by the University of Utah Institutional Review Board and General Clinical Research Center Advisory Committees. Adverse events were graded using Common Terminology Criteria for Adverse Events v3.0 (CTCAE v3.0). An independent Data and Safety Monitoring Committee provided oversight for the study.

Study design

Inclusion criteria were age ≥2 years and confirmed genetic diagnosis of SMA. Exclusion criteria were ventilator support for ≥16 hours or concomitant medications with known hepatotoxicity or potential benefit in SMA. Each subject had to complete ≥2 natural history assessments within a 3–6 month period to qualify for enrollment in the VPA trial. Treatment assessments were performed at 3 (V1), 6 (V2) and 12 (V3) months. Divalproex sodium coated particles (Depakote® sprinkle capsules, 125 mg per capsule) were administered in divided doses two to three times daily sufficient to maintain overnight trough levels of 50–100 mg/dL. Dosing was typical of that used in epilepsy patients (15–50 mg/kg/day). Laboratory testing, which included a basic chemistry profile, CBC with platelets, transaminases, carnitine profile, amylase, lipase, and trough VPA levels, was performed at baseline, 2–3 weeks following initiation, and at each treatment visit.

Primary outcome measures were laboratory safety and adverse event data. Exploratory outcome measures included change from baseline assessments of motor function, pulmonary function (subjects ≥5 years), degree of denervation via maximum ulnar compound motor action potential (CMAP) and motor unit number estimation (MUNE) values, dual-energy X-ray absorptiometry (DEXA) of body composition and bone density and quantitative assessment of SMN mRNA.

Gross motor function was assessed with the Modified Hammer-smith Functional Motor Scale for SMA (MHFMS). This scale contains the same 20 gross motor items as the original clinical scale, but was modified for use in the research setting [32,33]. A version of this scale has been successfully used in a multicenter trial with SMA subjects [34]. Each item is scored from 0–2, with a hierarchy of skills ranging from rolling to sitting to standing. Unfortunately, this scale as it stands in its present form is inadequate for testing subjects across the full range of SMA phenotypes, due to significant floor and ceiling effects for subjects with SMA types I and III, respectively (additional details available at http://www.smaoutcomes.org). The degree of denervation in the hand was estimated using maximum ulnar CMAP amplitude and MUNE negative peak area values [12,35]. In children ≥5 years, pulmonary function testing (PFT) was performed in an accredited pediatric laboratory, and included forced vital capacity (FVC), forced expiratory volume in 1 second (FEV1), and maximum expiratory and inspiratory pressures (MEP and MIP, respectively). We analyzed absolute values rather than corrected values due to concerns regarding accurate measurements of length due to contractures in many subjects. Norland DEXA XR-36 software version 3.3.1 for small subjects was used to assess whole body composition and bone mineral density and content.
SMN2 copy numbers were determined as previously described [11]. Whole blood was drawn into PAXgene tubes at baseline and each visit. Quantitative SMN mRNA levels were performed with slight modifications to the prior protocol [36]. Assays were carried out on a 7500 Real Time PCR system (Applied Biosystems). To increase efficiency on this apparatus, full-length SMN was amplified by primers 5'-GCTTTGGGAAAGTTGTATATTGTTTGATT3' (exon 6) and 5'-TGTGACGCCCTTCTTCTCCTTTCTTTTGTAT3' (exon 7), and detected by the 5'TAM-TC-TGAAACCATATAATAGCC-MGBNFQ-3' exon 6–7 junction probe. Delta-7 Δ7 SMN was detected as published [36]. Human RPLPO (large ribosomal protein) and PGK1 (phosphoglycerate kinase 1) were run as endogenous controls. Patients missing baseline data or who had only one treatment visit were excluded from this analysis. Results are reported as relative amounts of full-length (fl) or Δ7SMN transcripts normalized against the relative amount of RPLPO.

Statistical Analysis

The analysis of variance (ANOVA) test was used to evaluate the differences between means for function tests. When a factor level is empty and only two levels exist, ANOVA defaults to t-test. Linear regression analysis was used to examine the relationship between function parameters and genetic variables at baseline. The dependent variables in the linear regressions were evaluated for normality using the Shapiro-Wilk test and the paired t-test was used to test change from baseline in follow-up. If the dependent variable was not normally distributed then an appropriate transformation was used. A two-sided p-value less than 0.05 was considered statistically significant.

Results

All 42 subjects completed at least 6 months of treatment. Five subjects discontinued treatment after the V2 visit at 6 months due to drug-related side effects, as detailed below. The remaining 37 subjects continued on treatment for a full year.

Evaluation of safety and tolerability

For the most part, VPA was well tolerated and drug-related side effects mild. Grade I mood alteration was reported in three subjects. Grade II weight gain associated with functional decline led to discontinuation of VPA after V2 (6 months) in 2 subjects (both 9 year old girls, one with type II, the other with type IIIa but non-ambulatory). Poor compliance or lack of perceived benefit led to discontinuation in 3 additional subjects after V2 (2 siblings with type II, a 5 year old girl and 7 year old boy, and a poorly compliant 3 year old boy with type II who repeatedly refused oral dosing). One subject developed grade IV acidosis and carnitine depletion with respiratory failure requiring intubation and mechanical ventilation (3 year old type I subject). No deaths occurred during the study. Mean overnight (minimum 12 hour) trough VPA levels were 52 mg/dL (V1), 64 mg/dL (V2), and 59 mg/dL (V3). Abnormal laboratory values at baseline and during treatment are depicted in table 1; grade I elevations in transaminases without clinical correlate were present at baseline in 3 subjects and in 20 subjects (48%) for at least one visit. Transient grade I thrombocytopenia occurred in 2 subjects in association with illness. Grade I anemia was observed in 8 subjects at baseline and 19 subjects (45%) on treatment. Grade I leukopenia was frequent (36%) but absolute neutrophil counts remained >1.0×10^9/L in all subjects. Two subjects had low carnitine levels at baseline and a substantial proportion of the first thirteen subjects enrolled demonstrated reductions in total or free plasma carnitine within the first 3 months of treatment (Figure S2). In two subjects, this was associated with transient worsening of gross motor function which reversed with carnitine supplementation. Our Data Safety and Monitoring Board recommended supplementation of carnitine 50 mg/kg/day in all subjects subsequent to the first interim safety analysis.

Evaluation of gross motor function

Gross motor function was assessed using the MHFMS in a subgroup of 27 of 29 non-ambulatory SMA II subjects; one 14.9 year old subject with severe contractures who was no longer able to sit unsupported, and one uncooperative 2.8 year-old toddler were not evaluated with the MHFMS. Mean age of the remaining 27 subjects was 4.58 years. All subjects completed V1 and V2 visits and 25 (86%) completed all three visits. V1 was performed at 3.2 (2.3–4.3) months; V2 at 6.8 (5.5–9.9) months and V3 at 13 (8.7–15.6) months following treatment initiation. Mean MHFMS scores improved by 2.15; 2.92; and 4.63 at V1, V2 and V3 visits respectively, compared to baseline (table 2, p≤0.001). Motor function in type II subjects was then defined as stable, deteriorated or improved using a three or six point change in the MHFMS score. Each time point was examined for frequency of that change from baseline for the entire cohort (table 3, part I). We further explored the effect of age on change in motor function (table 3, part II). Of note, 8/16 (50%) children <5 years of age achieved at least a 6 point improvement after 1 year, while no children ≥5 years of age demonstrated a six point improvement. The most significant change in scores was observed in 5 SMA type II children ages 25–34 months at enrollment who gained from 8–15 points. Table 4 further documents the effect of age on change in motor function for those children who achieved at least a 5 point improvement on the MHFMS.

Gross motor function was assessed using the MHFMS in a subgroup 10 of 11 ambulatory SMA III subjects. Increase in MHFMS scores in this cohort was limited by ceiling effects since several scored maximally at baseline (table 2).

Evaluation of pulmonary function measures

Pulmonary function testing was performed in 14 subjects ≥5 years of age, comprised of 10 type II subjects and 4 type III subjects. We observed a significant improvement in MIP values at the V1 and V2 visits, and in FVC, FEV1 and MIP values at the V3 visit in type II subjects (table 5).

### Table 1. Percent of Subjects With Abnormal Lab Values Before and During Treatment All Patients (n = 42).

| Laboratory Parameter | Baseline Incidence | Treatment Incidence |
|----------------------|--------------------|---------------------|
| ALT                  | 7%                 | 19%                 |
| AST                  | 10%                | 38%                 |
| WBC                  | 12%                | 36%                 |
| HGB                  | 17%                | 45%                 |
| HCT                  | 17%                | 48%                 |
| Platelets            | 0%                 | 5%                  |
| Neutrophils          | 36%                | 57%                 |

ALT = alanine aminotransferase; AST = aspartate aminotransferase; WBC = total white blood count; HGB = hemoglobin; HCT = hematocrit.

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Evaluation of body composition and bone density

Total lean body mass was stable but fat mass increased 38% between the baseline and the one year visit in type II subjects; this is in contrast to a 14% increase in type III subjects (table 6). We observed progressive increases in total body bone mineral density (BMD, BMC) across all treatment visits in type II subjects \( (p \leq 0.0034) \) and at V2 and V3 in type III subjects \( (p \leq 0.0066) \). When change in MHFMS score was analyzed by change in fat mass, those with three or six point gains demonstrated a lower gain in fat mass than those who were stable or deteriorated \( (p = \text{NS}; \text{table 7}) \).

Evaluation of electrophysiologic measures of denervation

CMAP amplitude increased over baseline at each visit. The increase in mean values was significant for V2 and V3 in type II subjects \( (p \leq 0.0127) \) and for all visits for type III subjects \( (p \leq 0.0066) \). When change in MHFMS score was analyzed by change in fat mass, those with three or six point gains demonstrated a lower gain in fat mass than those who were stable or deteriorated \( (p = \text{NS}; \text{table 7}) \).

### Table 2. Change in MHFMS Score by Time Point.

| Time | Type II Subjects | Type III Subjects |
|------|------------------|-------------------|
|      | Avg Change  | Range  | SD  | p-value \(^1\) | Avg Change  | Range  | SD  | p-value \(^1\) |
| V1   | 2.15 N = 27   | −5/7   | 3.02 | 0.0010       | 1.0 n = 10   | −2/9   | 3.20 | NS           |
| V2   | 2.92 N = 26   | −4/13  | 3.78 | 0.0006       | 1.4 n = 10   | −4/11  | 3.92 | NS           |
| V3   | 4.65 N = 23   | −6/15  | 4.76 | 0.0001       | 1.44 n = 9   | −2/9   | 3.24 | NS           |
| Baseline | 13.7 N = 27 | 0/35 | 8.22 |              | 34.4 n = 10  | 10/40 | 9.40 |              |

\(^1\)P-Values based upon paired t-test.
V1 = 3 months; V2 = 6 months; V3 = 12 months; SD = standard deviation, NS = not significant.
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### Table 3. Deterioration vs. Improvement in MHFMS score, SMA type II.

#### Part I. Three vs. Six point change, All type II Subjects

| Criteria | Change | V1 | V2 | V3 |
|----------|--------|----|----|----|
| Three-Point | N = 27 | N = 26 | N = 23 |
| Deterioration | 2 (7%) | 1 (4%) | 1 (4%) |
| Stable | 14 (52%) | 12 (46%) | 6 (26%) |
| Improvement | 11 (41%) | 13 (50%) | 16 (70%) |

| Criteria | Change | V1 | V2 | V3 |
|----------|--------|----|----|----|
| Six-Point | N = 27 | N = 26 | N = 23 |
| Deterioration | 0 | 0 | 1 (4%) |
| Stable | 23 (85%) | 21 (81%) | 14 (61%) |
| Improvement | 4 (15%) | 5 (19%) | 8 (35%) |

### Part II. Deterioration vs. Improvement by Age, Six-Point Change

| Criteria | Change | V1 | V2 | V3 |
|----------|--------|----|----|----|
| Under 5 years | N = 18 | N = 17 | N = 16 |
| Deterioration | 0 | 0 | 0 |
| Stable | 14 (78%) | 12 (71%) | 8 (50%) |
| Improvement | 4 (22%) | 5 (29%) | 8 (50%) |

| Criteria | Change | V1 | V2 | V3 |
|----------|--------|----|----|----|
| 5+ years | N = 9 | N = 9 | N = 7 |
| Deterioration | 0 | 0 | 1 (14%) |
| Stable | 9 (100%) | 9 (100%) | 6 (86%) |
| Improvement | 0 | 0 | 0 |

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### Table 4. Deterioration vs. Improvement by Age: Three-Point Change, SMA type II.

| Criteria | Change | V1 | V2 | V3 |
|----------|--------|----|----|----|
| Under 5 years | N = 18 | N = 17 | N = 16 |
| Deterioration | 1 (6%) | 0 | 0 |
| Stable | 9 (50%) | 6 (35%) | 2 (13%) |
| Improvement | 8 (44%) | 11 (65%) | 14 (87%) |

| Criteria | Change | V1 | V2 | V3 |
|----------|--------|----|----|----|
| 5+ years | N = 9 | N = 9 | N = 7 |
| Deterioration | 1 (11%) | 1 (11%) | 1 (14%) |
| Stable | 5 (56%) | 6 (67%) | 4 (57%) |
| Improvement | 3 (33%) | 2 (22%) | 2 (29%) |

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Evaluation of body composition and bone density

Total lean body mass was stable but fat mass increased 38% between the baseline and the one year visit in type II subjects; this is in contrast to a 14% increase in type III subjects (table 6). We observed progressive increases in total body bone mineral density and content (BMD, BMC) across all treatment visits in type II subjects \( (p \leq 0.0034) \) and at V2 and V3 in type III subjects \( (p \leq 0.0066) \). When change in MHFMS score was analyzed by change in fat mass, those with three or six point gains demonstrated a lower gain in fat mass than those who were stable or deteriorated \( (p = \text{NS}; \text{table 7}) \).

Evaluation of electrophysiologic measures of denervation

CMAP amplitude increased over baseline at each visit. The increase in mean values was significant for V2 and V3 in type II subjects and for all visits for type III subjects (table 8, \( p \leq 0.0127 \)). There was also a significant difference in CMAP values by change in MHFMS. At V1, subjects showing at least a three point
improvement demonstrated higher CMAP values, indicating the best response in those with less severe denervation (table 9; \( p = 0.03 \)). Changes in MUNE negative peak area or amplitude values were not significant.

Evaluation of SMN2 copy number and quantitative measures of SMN mRNA in whole blood samples

A linear regression analysis was performed on the transformed MHFMS scores (square root transformation) to evaluate the influence of genetic variables, including SMN2 copy number and SMN mRNA levels on MHFMS (Table 10). The results indicate no statistically significant relationship between MHFMS score and SMN mRNA levels, but do indicate a positive relationship between SMN2 copy number and SMA type with MHFMS score. Subjects with 3 SMN2 copies had an average of 0.67 flSMN compared to 0.79 flSMN in subjects with 4 SMN2 copies (Table S3). D7SMN levels were on average 1.04 and 1.15 for SMN2 copies 3 and 4, respectively. These results indicate no relationship between SMN mRNA levels and SMN2 copy number. There was a trend toward an increased relative amount of D7SMN transcripts per SMA type (0.91 \( \pm \) 0.47 for type 2 vs. 1.42 \( \pm \) 1.11 for type 3; \( p = 0.053 \)). The mean relative amount of flSMN and D7SMN transcripts was 0.67 \( \pm \) 0.29 and 0.91 \( \pm \) 0.47 for type II and 0.77 \( \pm \) 0.13 and 1.42 \( \pm \) 1.11 for type III, respectively. Baseline measures point to the high degree of inter-patient variability in the relative amount of SMN transcripts, as previously reported [36,37].

There were no significant changes in the relative amount of flSMN transcripts in response to drug treatment. There was a significant decrease in D7SMN transcripts between baseline and V2 (\( p = 0.0053 \)) and V3 (\( p = 0.0429 \)) for type II subjects (table 11). Similar results were observed when using PGK1 as endogenous control to normalize for amount of input cDNA template and RT-PCR efficiency (baseline data in Table S3, treatment data not shown). Finally, there was no relationship between decreased D7SMN transcripts and any physiological or functional measure.

Discussion

In this open label study, we enrolled a relatively heterogeneous group of SMA subjects, although we focused recruitment predominantly on children with SMA types II and III. Our experience emphasizes the need to stratify subjects by SMA type, age and current gross motor abilities in future trials since the majority of outcome measures examined in this study were optimal for only a subset of subjects which was highly dependent on the
subject’s age, motor disability and the overall degree of medical fragility.

This study provides evidence that VPA can be used safely in SMA subjects 2 years of age, as long as carnitine status is closely monitored. VPA is known to alter enteral absorption, inhibit biosynthesis and secondarily deplete carnitine from muscle by direct binding and excretion in the urine [38]. Given the diminished lean body mass in SMA subjects, an increased susceptibility to carnitine depletion is not surprising. Decreased nutritional intake and depleted whole body carnitine stores related to an associated abnormality in fatty acid metabolism may be contributing factors [39,40]. Thus, treatment with VPA in the absence of close monitoring and supplemental carnitine therapy could increase risks for further muscle weakness or fatal hepatotoxicity [41]. In this study, we did not observe any serious clinical or laboratory evidence of hepatotoxicity with careful monitoring and supplementation but grade I elevations of transaminases were common. Infants and children less than two years of age, a group not included in the current study, theoretically have a greater risk of such complications.

The apparent positive effect of treatment on bone density is surprising since prolonged VPA treatment has been associated with decreased bone density in other populations [42–45]. Whether the observed increased bone density is spurious or relates to increased weight gain, weight bearing or other factors is unclear. The significant increase in fat mass in the absence of an increase in lean mass is concerning given the detrimental impact weight gain could have on gross motor function and potential long-term consequences for general health. Weight gain was not uniform across the population; rather, non-ambulatory subjects ≥5 years, or those already with such a tendency appear to be at greatest risk. Notably, children demonstrating the most significant improvement in gross motor function gained less fat mass compared to those who displayed stable or deteriorated motor function.

Maximum CMAP amplitudes showed a statistically significant increase from baseline, while MUNE values did not change. This would imply that axonal reinnervation did not account for the change, but that the observed changes may be due to reinnervation of muscle via “sprouting” from remaining motor units or a direct trophic effect on muscle. If future controlled trials confirm that increased CMAP amplitude is a consistent marker of

| Time | Type II Subjects N = 29 | | Type III Subjects N = 10 |
|------|------------------------|------|------------------------|
| | Avg Change⁠¹ | SD | p-value² | Avg Change⁠¹ | SD | p-value² |
| V1 (3 months) | | | | | | |
| Lean mass, grams | 123 | 973 | NS | −267 | 1865 | NS |
| Fat mass, grams | 1094 | 1034 | 0.0003 | 1269 | 1844 | 0.078 |
| Total BMD, g/cm² | 0.05 | 0.06 | 0.0003 | 0.02 | 0.05 | NS |
| Total BMC, grams | 33.6 | 54.4 | 0.0036 | 71.0 | 80.0 | 0.0287 |
| V2 (6 months) | | | | | | |
| Lean mass, grams | −366 | 1174 | NS | 184 | 1582 | NS |
| Fat mass, grams | 2579 | 2013 | <0.0001 | 2101 | 2213 | 0.0149 |
| Total BMD, g/cm² | 0.05 | 0.06 | <0.0001 | 0.03 | 0.03 | 0.0066 |
| Total BMC, grams | 99.5 | 68.1 | <0.0001 | 90.5 | 76.2 | 0.0045 |
| V3 (12 months) | | | | | | |
| Lean mass, grams | −212 | 1029 | NS | 322 | 960 | NS |
| Fat mass, grams | 3680 | 2470 | <0.0001 | 2596 | 3193 | 0.0406 |
| Total BMD, g/cm² | 0.07 | 0.06 | <0.0001 | 0.07 | 0.04 | 0.0011 |
| Total BMC, grams | 145 | 66.4 | <0.0001 | 124.2 | 98.1 | 0.0052 |

1Range at baseline in Table S1.
2P-values based upon paired t-test.
BMD = bone mineral density; BMC = bone mineral content; g/cm² = grams/centimeter squared; SD = standard deviation, NS = not significant.
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| Criteria | Change V1 | V2 | V3 |
|----------|-----------|---|---|
| Three-Point | | | |
| Deterioration | 2320 | 7541 | 9502 |
| N = 2 | N = 1 | N = 1 |
| Stable | 1173 | 1995 | 4459 |
| N = 14 | N = 12 | N = 6 |
| Improvement | 825 | 2318 | 2918 |
| N = 11 | N = 13 | N = 16 |
| P-value¹ | 0.73 | 0.22 | 0.09 |
| Six-Point | | | |
| Deterioration | 9502.0 |
| N = 0 | N = 0 | N = 1 |
| Stable | 1287 | 2584 | 3624 |
| N = 23 | N = 21 | N = 1 |
| Improvement | 189 | 1460 | 2839 |
| N = 4 | N = 5 | N = 8 |
| P-value¹ | 0.10 | 0.27 | 0.18 |

¹P-value based upon ANOVA.
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biologic response that correlates with improvement in motor function, then this outcome measure could prove a powerful surrogate biomarker for future clinical trials.

Changes in pulmonary function were observed; however, the significance is limited by the small number of subjects $\leq 5$ years in each cohort. Nonetheless, PFT measures are important with regard to clinical outcomes in patients with SMA, so other means of reliably determining pulmonary function in younger children is an important goal.

We had hypothesized that VPA might increase flSMN and Δ7SMN mRNA levels in blood samples in light of prior data demonstrating increased mRNA and protein levels in patient-derived cells [16–20]. When analyzed as a group, we observed a significant decrease in the relative amount of Δ7SMN mRNA in SMA type II subjects while flSMN transcripts were unchanged. However, flSMN and Δ7SMN mRNA levels fluctuated throughout drug treatment, with patients showing increased, decreased or unaltered relative amounts of both transcripts. This variability contrasts with the fairly stable SMN mRNA levels observed in untreated patients [21,36,37]. Consistent with previously published data, these results are suggestive of a VPA response and SMN mRNA could help discriminate between non-responders and responders [21]. Furthermore, the implication of decreased relative Δ7SMN mRNA levels in the absence of changes in flSMN would be to increase the ratio of flSMN to SMN Δ7, the major discriminator between SMA subjects and unaffected controls/carriers when using relative measures of SMN mRNA, suggesting that these changes could be important. Finally, in order for SMN mRNA levels in blood to inform us about VPA effects in spinal cord, there must be a significant relationship between SMN mRNA and a clinical outcome measure. We did not observe any relationship between changes in Δ7SMN and any of the outcome measures in this pilot open label study. One reason for this may be that blood may not be the appropriate tissue to screen for biomarkers of SMA. Alternatively, extensive variability in SMN mRNA levels during treatment trials may mask significant relationships in a sub-set of SMA patients. Fluctuations in SMN mRNA levels may be amplified by fluctuations in transcript levels produced by endogenous controls used normalize for the amount of input template and efficacy of RT-PCR. VPA is a non-specific drug target and may itself affect expression of genes used as endogenous controls. We observed similar

| Table 8. Change in Maximum Ulnar CMAP Amplitude and MUNE Negative Peak Area from Baseline by Time Point. |
| --- |
| **Time** | **Type II Subjects (n = 29)** | **Type III Subjects (n = 10)** |
| | **Avg Change** | **Range** | **SD** | **p-value** | **Avg Change** | **Range** | **SD** | **p-value** |
| | | | | | | | | | |
| V1 (3 months) | | | | | | | | | |
| CMAP (mV) | 0.12 | −0.98/1.26 | 0.38 | 0.0999 | 1.03 | −0.10/2.50 | 0.95 | 0.0074 |
| MUNE (µVms) | −3.03 | −95.0/10.0 | 18.6 | NS | −0.50 | −38.0/53.0 | 28.2 | NS |
| V2 (6 months) | | | | | | | | | |
| CMAP (mV) | 0.23 | −0.96/1.12 | 0.39 | 0.0041 | 1.20 | 0.23/3.56 | 1.13 | 0.0085 |
| MUNE (µVms) | −5.59 | −117.0/7.0 | 22.2 | NS | −16.4 | −93.0/17.0 | 33.8 | NS |
| V3 (12 months) | | | | | | | | | |
| CMAP (mV) | 0.35 | −0.52/1.58 | 0.54 | 0.0031 | 1.44 | −0.24/3.75 | 1.35 | 0.0127 |
| MUNE (µVms) | −8.58 | −126.0/10.0 | 26.3 | NS | −17.7 | −93.0/17.0 | 51.0 | NS |

1P-values based upon paired t-test.
SD = standard deviation, NS = not significant.
doi:10.1371/journal.pone.0005268.t008

| Table 9. Average Maximum Ulnar CMAP vs. Three-point and Six-point Change in MHFMS Score. |
| --- |
| **Criteria** | **Change** | **CMAP (mV)** | **CMAP (mV)** | **CMAP (mV)** |
| | **V1** | **V2** | **V3** |
| 3-Point | | | | |
| Deterioration | 0.46 | 0.47 | 0.55 |
| N = 2 | N = 1 | N = 1 |
| Stable | 1.59 | 1.70 | 1.22 |
| N = 14 | N = 12 | N = 6 |
| Improvement | 2.05 | 2.11 | 2.00 |
| N = 11 | N = 13 | N = 16 |
| P-value1 | 0.03 | 0.07 | 0.29 |
| 6-Point | | | | |
| Deterioration | | 0.55 |
| N = 0 | N = 0 | N = 1 |
| Stable | 1.52 | 1.68 | 1.33 |
| N = 23 | N = 21 | N = 14 |
| Improvement | 2.67 | 2.58 | 2.61 |
| N = 4 | N = 5 | N = 8 |
| P-value1 | 0.29 | 0.24 | 0.24 |

1P-value based upon ANOVA.
Maximum ulnar CMAP (compound muscle action potential amplitude).
doi:10.1371/journal.pone.0005268.t009

| Table 10. Linear Regression of Square Root of MHFMS Total Score Versus Genetic Variables. |
| --- |
| **Term** | **Estimate** | **Std Error** | **t Ratio** | **Prob>|t|** |
| | | | | |
| Intercept | −0.77 | 1.07 | −0.71 | 0.4818 |
| flSMN | −0.19 | 0.82 | −0.23 | 0.8177 |
| Δ7SMN | −0.68 | 0.45 | −1.50 | 0.1455 |
| SMN2 | 0.52 | 0.25 | 2.05 | 0.0497 |
| Type | 2.35 | 0.48 | 4.93 | <.0001 |

1P-values based upon ANOVA.
doi:10.1371/journal.pone.0005268.t010

Maximum ulnar CMAP (compound muscle action potential amplitude).
results with two endogenous controls (RPLPO and PGK1) and do not have any evidence to suggest that these genes are affected by VPA treatment. Nonetheless, there is a need for an absolute quantification method to measure SMN mRNA in order to remove variability introduced by the use of endogenous controls to normalize data.

In conclusion, this study provides good evidence that VPA can be used safely in SMA subjects over 2 years of age in the setting of close monitoring of carnitine status. This being said, further studies of VPA in infants and young children are needed to better assess safety in this more vulnerable cohort. This study provides evidence in support of improvement in gross motor function in younger non-ambulatory type II children. This finding was unexpected and it is unclear whether this improvement reflects a therapeutic drug effect, maturation or increased cooperation on improved scores in the youngest subjects. These data suggest that further studies with VPA are warranted, although clearly such findings must be replicated in randomized, controlled efficacy studies. The data presented here emphasizes the benefit of a trial design with a less heterogeneous population of younger SMA subjects, particularly in light of significantly increased fat mass and lack of apparent benefit of VPA treatment in older non-ambulatory subjects. Furthermore, our experience indicates that the MHFMS scale alone is not adequate to measure motor function in ambulatory SMA subjects, prompting us to explore other measures to assess functional change in this population. Future clinical trials targeted to this cohort should generate additional informative data as to the usefulness of PFTs, timed test modules, and biomarker data including electrophysiologic measures of denervation, which appear to have promise in this cohort.

Supporting Information

Protocol S1  Clinical trial protocol summary
Found at: doi:10.1371/journal.pone.0005268.s001 (0.11 MB DOC)

Figure S1  CONSORT Flowchart
Found at: doi:10.1371/journal.pone.0005268.s002 (4.21 MB TIF)

Figure S2  Free carnitine levels at enrollment and subsequent follow-up for the first thirteen subjects
Found at: doi:10.1371/journal.pone.0005268.s003 (0.31 MB TIF)

Checklist S1  CONSORT Checklist
Found at: doi:10.1371/journal.pone.0005268.s004 (0.06 MB DOC)

Table S1  Found at: doi:10.1371/journal.pone.0005268.s005 (0.08 MB DOC)

Table S2  Found at: doi:10.1371/journal.pone.0005268.s006 (0.05 MB DOC)

Table S3  Found at: doi:10.1371/journal.pone.0005268.s007 (0.04 MB DOC)

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Author Contributions

Conceived and designed the experiments: KJS CBS SPR SLS GA TC JTK KJK GD MBB MKS LRS. Performed the experiments: KJS TWP JW BE LRS. Analyzed the data: KJS CBS BL GMC. Contributed reagents/materials/analysis tools: KJS TWP LRS. Wrote the paper: KJS SPR BL SLS JW GA TC JTK KJK GD MBB MKS GMC.

Table 11. Change in Quantitative SMN mRNA levels by Time Point.

| Time  | Type II Subjects (n = 25) |  | Type III Subjects (n = 11) |  |
|-------|--------------------------|--------------------------|---------------------------|--------------------------|
|       | Avg Change | Range | SD | p-value¹ | Avg Change | Range | SD | p-value¹ |
| V1    |  flSMN | 0.10 | −0.19/2.00 | 0.42 | NS | −0.02 | −0.17/0.04 | 0.06 | NS |
|       |  A7SMN | 0.42 | −1.17/10.91 | 2.26 | NS | −0.47 | −2.68/0.65 | 0.98 | NS |
| V2    |  flSMN | −0.01 | −0.38/0.52 | 0.17 | NS | −0.04 | −0.25/0.12 | 0.11 | NS |
|       |  A7SMN | −0.35 | −1.82/0.60 | 0.57 | 0.0053 | −0.46 | −3.17/1.25 | 1.26 | NS |
| V3    |  flSMN | −0.01 | −0.34/0.33 | 0.16 | NS | −0.30 | −0.14/0.10 | 0.06 | NS |
|       |  A7SMN | −0.32 | −1.61/1.01 | 0.64 | 0.0429 | −0.30 | −3.25/1.63 | 1.34 | NS |
| Baseline values | flSMN | 0.67 | 0.05/1.27 | 0.29 |  | 0.77 | 0.63/1.05 | 0.13 |  |
|       |  A7SMN | 0.91 | 0.05/2.14 | 0.47 |  | 1.42 | 0.41/4.29 | 1.11 |  |

¹P-value based upon ANOVA.

References

1. Brahe C, Bertini E (1996) Spinal muscular atrophies: recent insights and impact on molecular diagnosis. J Mol Med 74: 555–562.
2. Roberts DF, Chavez J, Court SD (1970) The genetic component in child mortality. Arch Dis Child 45: 33–38.
3. Pearn J (1978) Incidence, prevalence, and gene frequency studies of chronic childhood spinal muscular atrophy. J Med Genet 15: 409–413.
4. Czeizel A, Hamula J (1989) A hungarian study on Werdnig-Hoffmann disease. J Med Genet 26: 761–763.
5. Emery AE (1991) Population frequencies of inherited neuromuscular diseases—a world survey. Neuromusc Disord 1: 19–29.

6. Merlino L, Stagmi SB, Marri E, Granata C (1992) Epidemiology of neuromuscular disorders in the under-20 population in Bologna Province, Italy. Neuromusc Disord 2: 197–200.

7. Leebrecht S, Burgin L, Rebuliet S, Clermont O, Burle P, et al. (1995) Identification and Characterization of a Spinal Muscular Atrophy-determining Gene. Cell 80: 155–165.

8. Wan L, Battle DJ, Yong J, Guhit AK, Kolb SJ, et al. (1995) The Survival of Motor Neurons Protein Determines the Capacity for snRNP Assembly: Biochemical Deficiency in Spinal Muscular Atrophy. Mol Cell Biol 25(13): 5543–51.

9. Lorson CL, Hahnen E, Androphy EJ, Wirthe B (1998) A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. Proc Natl Acad Sci USA 96: 6307–6311.

10. Feldkotter M, Schwarzen Y, Wirthe R, Wobisch T, Wirthe B. Quantitative Analyses of SMN1 and SMN2 Based on Real-Time LightCycler PCR: Fast and Highly Reliable Carrier Testing and Prediction of Severity of Spinal Muscular Atrophy (2002) Am J Hum Genet. 70(2): 358–368.

11. Mailman MD, Heinz JW, Paul AG, Seydor PJ, Sedra MS, et al. (2002) Molecular Analysis of Spinal Muscular Atrophy and Modification of the Phenotype by SMN2. Genet Med 4: 20–26.

12. Swoboda KJ, Prior TW, Scott CB, McNaught TP, Wride MG, et al. (2005) Natural history of denervation in SMA: relation to age, SMN2 copy number, and function. Ann Neurol 57: 794–712.

13. Wirthe B, Brichla I, Schrank B, Lochnhiller B, Rieck S, et al. (2006) Mildly affected patients with spinal muscular atrophy are partially protected by an increased SMN2 copy number. Hum Genet 119: 422–428.

14. Prior TW, Swoboda KJ, Scott CB, Hejmanowski AQ (2004) Homozygous SMN1 deletions in unaffected family members and modification of the phenotype by SMN2. Am J Med Genet A 130: 307–310.

15. Leebrecht S, Burle P, Liu Q, Bertraudy S, Clermont O, et al. (1997) Correlation between severity and SMN protein level in spinal muscular atrophy. Nat Genet 16: 265–269.

16. Chang JG, Hiebl Li H, Yong J, Wang NM, Tsai CH, et al. (2001) Treatment of spinal muscular atrophy by sodium butyrate. Proc Natl Acad Sci U S A 98: 9008–9013.

17. Brichla I, Hofmann Y, Hahnen E, Siebelhuzb B, Aaschle H, et al. (2003) Valproic acid increases the SMN2 protein level: a well-known drug as a potential therapy for spinal muscular atrophy. Hum Mol Genet 12: 2481–2489.

18. Sumner CJ, Huyhn TN, Markowski JS, Perhec JS, Hill B, et al. (2003) Valproic acid increases SMN levels in spinal muscular atrophy patient cells. Ann Neurol 54: 647–654.

19. Andreassi C, Angelozi C, Tiziano FD, Vitali T, De Vincenzi E, et al. (2004) Phenybutyrate increases SMN expression in vitro relevance for treatment of spinal muscular atrophy. Eur J Hum Genet 12: 59–65.

20. Grzeschik SM, Ganta M, Prior TW, Heavlin WD, Wang CH (2005) Hydroxyurea enhances SMN2 gene expression in spinal muscular atrophy cells. Ann Neurol 58: 194–202.

21. Brichla I, Holzger L, Hahng K, Klockgether T, Wirthe B (2006) In Vivo Activation of SMN in Spinal Muscular Atrophy Carriers and Patients Treated with Valproate. Ann Neurol 59: 970–973.

22. Brahe C, Vitali T, Tiziano FD, Angelozi C, Pinto AM, et al. (2005) Phenybutyrate increases SMN gene expression in spinal muscular atrophy patients. Eur J Hum Genet 13: 256–259.

23. Kernosch E, Rossi ML, Wooding NS, Huyhn TN, Avila AM, et al. (2005) The role of histone acetylation in SMN gene expression. Hum Mol Genet 14: 1171–1182.

24. Leng Y, Chuan DM (2006) Endogenous S-Symelin is Induced by Valproic Acid through Histone Deacetylase Inhibition and Participates in Neuroprotection against Glutamate-Induced Excitotoxicity. J Neurosci 26(8): 7502–7512.

25. van Bergeijk J, Haastert K, Grothe G, Claus P (2006) Valproic acid promotes neurite outgrowth in PC12 cells independent from regulation of the survival of motoneuron protein. Chem Biol Drug Des 67: 244–247.

26. Sugih F, Yamamoto Y, Miyaguchi K, Zhou Z, Sumi H, et al. (2004) Benefit of valproic acid in suppressing disease progression of ALS model mice. Eur J Neurosci 20: 3179–3183.

27. Petri S, Kier M, Kiptian K, Chen J, Chalingasani NY, et al. (2006) Additive neuroprotective effects of a histone deacetylase inhibitor and a catalytic antioxidant in a transgenic mouse model of amyotrophic lateral sclerosis. Neurobiol Dis 22: 49–49.

28. Tsai LK, Tsai MS, Lin TB, Hwu WL, Li H (2006) Establishing a standardized therapeutic testing protocol for spinal muscular atrophy. Neurobiol Dis 24: 286–295.

29. Tsai LK, Tsai MS, Ting CH, Li H (2008) Multiple therapeutic effects of valproic acid in spinal muscular atrophy model mice. J Mol Med 86: 1243–1254.

30. Weihl CC, Connolly AM, Pestronk A (2006) Valproate may improve strength and function in patients with type III/IV spinal muscular atrophy. Neurology 67: 500–501.

31. Tsai LK, Yang CC, Hwu WL, Li H (2007) Valproic acid treatment in six patients with spinal muscular atrophy. Eur J Neurol 14: e8–e9.

32. Main M, Karrow E, Mercuri E, Muntoni F (2003) The Hammersmith functional motor scale for children with spinal muscular atrophy: a scale to test ability and monitor progress in children with limited ambulation. Eur J Paediatr Neurol 7: 155–159.

33. Krostrell KJ, Maczukf JA, Crawford TO, Scott C, Swoboda KJ (2006) A modified Hammersmith functional motor scale for use in multi-center research on spinal muscular atrophy. Neuromusc Disord 16: 417–426.

34. Mercuri E, Messina S, Batti R, Bernardinielli A, Bolfi P, et al. (2006) Reliability of the Hammersmith functional motor scale for spinal muscular atrophy in a Multicentric Study. Neuromusc Disord 16: 93–98.

35. Bromberg MI, Swoboda KJ (2002) Motor unit number estimation in infants and children with spinal muscular atrophy. Muscle Nerve 25: 445–447.

36. Simard LR, Belanger M-C, Morrisette S, Wride M, Prior TW, et al. (2007) Preclinical validation of a multiplex real-time RT-PCR assay to quantify SMN transcripts in whole blood from SMA patients. Neurology 68: 451–456.

37. Sumner CJ, Kolb SJ, Harmon GG, Jefferys NO, Schadt K, et al. (2006) SMN mRNA and protein levels in peripheral blood. Neurology 66: 1067–1073.

38. Melleg B, Pap M, Morava E, Molnar D, Dani M, et al. (1994) Carnitine-dependent changes of metabolic fuel consumption during long-term treatment with valproic acid. J Pediatr 125: 317–321.

39. Ten I, Sloane AE, Donner EJ, Ledosay DC, Millington DS, et al. (1995) Fatty acid oxidation abnormalities in childhood-onset spinal muscular atrophy: primary or secondary defect(s)? Pediatr Neurol 12: 21–30.

40. Crawford TO, Shadly JT, Harko O, Rosier-Johnston A, Kellef RI (1999) Abnormal fatty acid metabolism in childhood spinal muscular atrophy. Ann Neurol 45: 337–343.

41. Couter DL (1984) Carnitine deficiency: a possible mechanism for valproate hepatotoxicity. Lancet 1(8378): 689.

42. Kumandas S, Koklu E, Gu'mus H, Koklu S, Kurtoglu S, et al. (2006) Effect of carnitine and valproic acid on bone mineral density, IGF-I and IGFBP-3. J Pediatr Endocrinol Metab 19: 529–534.

43. Boluk A, Guzelipek M, Savlı H, Temel I, In Ozıık H, et al. (2004) The effect of valproic acid oxidation abnormalities in childhood-onset spinal muscular atrophy: primary or secondary defect(s)? Pediatr Neurol 12: 21–30.

44. Ecevit C, Aydogan A, Kavakli T, Altinoz S (2004) Effect of carbamazepine and valproic acid on bone mineral density, IGF-I and IGFBP-3. J Pediatr Endocrinol Metab 19: 529–534.

45. Leet AI, Mesfin A, Pichard C, Launay F, Brintzenhofeszoc K, et al. (2006) Effect of carbamazepine and valproic acid on bone mineral density, IGF-I and IGFBP-3. J Pediatr Endocrinol Metab 19: 529–534.