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SHORT REPORT

Phenylbutyrate increases *SMN* gene expression in spinal muscular atrophy patients

Christina Brahe*[,1], Tiziana Vitali[1], Francesco D Tiziano[1], Carla Angelozzi[1], Anna Maria Pinto[1], Federica Borgo[1], Umberto Moscato[2], Enrico Bertini[3], Eugenio Mercuri[4] and Giovanni Neri[1]

[1]*Istituto di Genetica Medica, Università Cattolica S Cuore, Rome, Italy;* [2]*Istituto di Igiene, Università Cattolica S Cuore, Rome, Italy;* [3]*Ospedale Bambino Gesù, Rome, Italy;* [4]*Istituto di Neurologia, Università Cattolica S Cuore, Rome, Italy*

Spinal muscular atrophy (SMA) is caused by insufficient levels of survival motor neuron (SMN) protein. Recently, we found that sodium 4-phenylbutyrate (PB), a well-tolerated FDA approved drug, enhances *SMN* gene expression *in vitro*. We provide here the first evidence that oral administration of PB (*tri*Butyrate®) significantly increases *SMN* expression in leukocytes of SMA patients. This finding provides a strong rationale to further investigate the effects of PB as also supported by preliminary clinical data.
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Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive motor neuron disorder characterized by degeneration of anterior horn cells of the spinal cord. SMA is caused by loss of the functional survival motor neuron gene (*SMN1*).[1] However, all patients have one or more copies of the *SMN2* gene, nearly identical to *SMN1*.[2,3] Both genes encode the SMN protein but, due to alternative splicing, the level of functional protein produced by *SMN2* is insufficient to protect from disease. The phenotype can range from very severe to mild (type I to III) and the clinical severity inversely correlates with the amount of functional SMN protein.[4] No cure for SMA is available at present. Increasing *SMN2* gene expression could be of invaluable therapeutic importance. Recently, we have shown that treatment of fibroblast cultures from SMA patients with sodium 4-phenylbutyrate (PB), a chromatin hyperacetylating agent, leads to a significant increase in *SMN2* gene expression.[5] Moreover, in a preliminary clinical study, we observed an improvement of motor function in SMA children following treatment with PB (*tri*Butyrate®).[6] We now performed further molecular studies which provide evidence that PB is effective in enhancing SMN expression in peripheral blood leukocytes.

*Correspondence: Christina Brahe, Istituto di Genetica Medica, Università Cattolica S Cuore, Largo F Vito, 1, 00168 Rome, Italy.
Tel.: +39 06 35500877; Fax: +39 06 305 0031;
E-mail: cbrahe@rm.unicatt.it
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Subjects and methods

Six SMA patients (P1–P6) and three parents (M2, M3, F6) were enrolled for the present pilot trial. Four patients had SMA type II (P1–P4). P1 is a 2.5-year-old boy who had lost the ability to sit unaided and to control the upright position of the head. Patients P2 (5 years), P3 and P4 (9 years) are all able to sit independently. Two patients had SMA type III (P5, P6). P5 is a 38-year-old male who is now only able to walk with aid and P6 is a 15-year-old female who still is able to walk independently. All patients had three copies of the *SMN2* gene.[5] The trial was approved by the Ethical Committee of the Catholic University. A written informed consent was obtained from all patients/parents. *Tri*Butyrate® was orally administered at 500 mg/kg/d (maximum dose 19 g/d), divided into six doses (every 4 h) for 7 days. Blood samples were taken

from patients and parents during the morning of day 0 (T0, baseline) and days 1–4 (T1–T4) and 7 (T7) of treatment, usually 1–3.5 h after drug administration, and from five healthy untreated controls on five consecutive days (T0–T4). Total RNA was extracted by Trizol from leukocytes immediately after hypotonic lysis of samples and all blood samples were processed equally.

Real-time PCR

SMN full-length (*SMN-fl*) transcripts and transcripts lacking exon 7 (Δ7) were measured by real-time RT-PCR using ABI-PRISM 7700 Sequence Detector System (Applied Biosystems) as described elsewhere.[5] Transcripts were amplified at least twice in triplicate or quadruplicate. *SMN* transcript levels were calculated by comparing *SMN versus* glyceraldehyde-3-phosphate dehydrogenase transcripts, whose expression is apparently not affected by PB.[5] The relative amounts of *SMN-fl* and Δ7 transcripts in samples obtained from treated patients/parents and untreated controls were normalized *versus* those of T0.

Myometry

Muscle strength was assessed using a hand-held dynamometer (Citec, CIT Technics BV).[7] Patients were tested independently four times by two raters, and the highest measure of the maximal voluntary isometric contraction was selected. Inter-rater reliability was assessed by calculating the intraclass correlation coefficient (ICC)[8] using SPSS 10.0 for Windows.

Statistical analysis

Significance of real-time PCR data was assessed by ANOVA and by testing the null hypothesis of no effect of treatment with Student's *t* for independent variables and Fisher's tests (Winstat 4.01 and SPSS 10.0.1 for Windows software). For myometry data, χ^2 test and nonparamentric Friedman test for multiple samples were used. *P*-values <0.05 were accepted as significant.

Results

SMN expression studies

SMN mRNA analysis showed for all patients a marked increase in relative *SMN-fl* transcript levels in one or more blood samples obtained during PB administration compared to baseline (Table 1, Figure 1a). The mean increase in patients transcript levels ranged from 0.4-fold for P6 up to 2.4-fold observed in P4, and from 0.9- to 1.7-fold in the parents. The relative amount of *SMN-fl* mRNA during treatment varied considerably, both among the different subjects and the different blood samples of the same subject. Despite the day-to-day variations, data were found statistically significant (*t*-test and *F*-ratio values >1, $P\leq0.03$), thus the null hypothesis of no effect of PB was rejected. To investigate whether *SMN-fl* transcript levels are subject to physiologic variation, *SMN* expression was studied in five healthy untreated controls during 5 days. A slight-to-moderate variation in *SMN-fl* levels of T1–T4 *versus* T0 was observed in the controls with mean variation between 4.2 and 16.5% (Figure 1b). Variation in *SMN-fl* levels in treated patients/parents *versus* controls was statistically significant.

To investigate whether PB influences inclusion/exclusion of exon 7, we studied *SMN-fl* and Δ7 transcripts in some blood samples. We found a slight reduction of Δ7 transcripts in all blood samples analyzed suggesting an effect of PB on both promoter activation and alternative splicing (Figure 1c).

To estimate the percentage of the *SMN-fl* transcript levels in our patients before treatment respective to that of unaffected subjects, their baseline *SMN* mRNA levels were compared to a reference internal standard (RIS), calculated as average amount of *SMN-fl* mRNA of the controls at T0. The relative *SMN-fl* transcript levels were 22–28 and 47–48% of control levels in the type II and type III patients, respectively (Figure 1c).

Clinical observations

All subjects tolerated the drug well except for P6 who on day 2 complained of dizziness and tinnitus, which resolved immediately after reducing dosage from 18 to 12 g/d. Full blood counts and liver function tests, performed for all subjects at days 0 and 7, did not show relevant changes.

The first patient studied (P5) reported a reduction of hand tremor at day 3 of treatment and subsequently complete absence of tremors lasting for 4 days after the end of the trial. The second patient (P1), a young child with severe type II phenotype, showed a slight improvement in head and trunk control. These subjective improvements prompted us to perform myometry in the other four patients on days 0 and 7 (Table 2). Muscle strength was found significantly increased in the group of patients ($P=0.041$). When analyzed separately, improvement of muscle strength was most evident for P4, less pronounced in P2 and P3, whereas no changes in muscle strength were found for P6.

Discussion

We report here that *SMN*2 gene expression can be increased in leukocytes of SMA patients by oral administration of PB. The small amounts of blood samples obtained from the patients did not allow to perform protein studies. PB is an FDA approved drug which has been used for several years for the treatment of young patients with urea cycle disorders and is well tolerated.[9] Recently, evidence was given that PB can cross the blood–brain barrier.[10]

A drawback to the use of PB is its short-half life (0.8–1 h). Previous pharmacokinetic studies have shown rapid changes in serum levels of PB in treated subjects with

Table 1 *SMN-fl* transcripts variation in PB treated patients and parents relative to T0

Patients/parents (sex)	*T1 mean (±SD)*	*T2 mean (±SD)*	*T3 mean (±SD)*	*T4 mean (±SD)*	*T7 mean (±SD)*
P1 (M)	2.80 (0.7)	ND	−0.15 (0.1)	−0.18 (0.2)	3.33 (0.5)
P2 (M)	0.73 (0.0)	0.27 (0.0)	0.21 (0.1)	1.29 (0.3)	ND
P3 (M)	4.32 (0.2)	0.63 (0.1)	0.82 (0.2)	1.90 (0.5)	0.56 (0.2)
P4 (F)	0.53 (0.1)	1.84 (0.6)	0.07 (0.2)	1.20 (0.4)	8.27 (1.9)
P5 (M)	−0.01 (0.0)	0.35 (0.3)	ND	−0.03 (0.0)	1.55 (0.1)
P6 (F)	0.54 (0.2)	0.25 (0.1)	1.31 (0.5)	0.21 (0.0)	−0.15 (0.4)
M2 (F)	0.68 (0.0)	0.14 (0.0)	1.16 (0.0)	1.56 (0.2)	ND
M3 (F)	0.65 (0.1)	−0.02 (0.0)	−0.37 (0.0)	3.63 (0.6)	1.90 (0.2)
F6 (M)	1.17 (1.0)	0.96 (0.1)	1.97 (0.7)	3.87 (0.9)	0.71 (0.3)

P1–P6: patients; M2, M3: mothers of P2 and 3, respectively; F6: father of P6.
F of Fisher (case *versus* control, T1–T4): $P<0.0001$; ANOVA (patients/parents T1–T7 *versus* T0): $P=0.0047$; T of Student (patients/parents T1–T7 *versus* T0): $P<0.03$ except for T2.

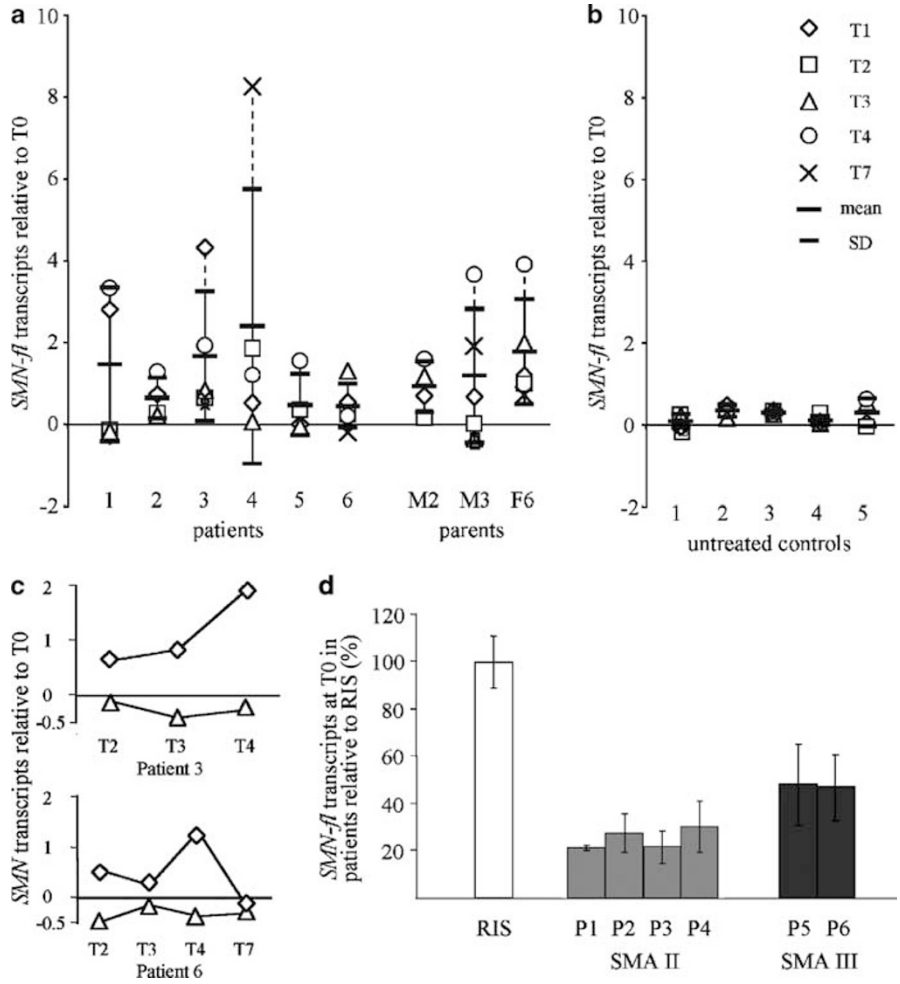

Figure 1 SMN expression studies in PB-treated and-untreated subjects. (**a**) Variation of *SMN -fl* transcript levels in leukocytes of patients and parents at the single days (T1–T4, T7) of PB treatment relative to baseline levels. Mean values and SD are indicated by long and short horizontal bars, respectively. M2, M3, F6: mothers of P2, P3 and father of P6, respectively. (**b**) Variation of *SMN* mRNA in healthy untreated controls during 4 days relative to mean level at T0. (**c**) *SMN-fl* (◇) and Δ7 (Δ) transcript studies. (**d**) Percent of *SMN-fl* transcript levels in patients before treatment relative to that of unaffected individuals. RIS (Reference Internal Standard) indicates the average amount of *SMN-fl* mRNA in five unaffected individuals at T0. Error bars indicate SD.

Table 2 Outcome of myometry

	P2		*P3*		*P4*		*P6*	
	T0	*T7*	*T0*	*T7*	*T0*	*T7*	*T0*	*T7*
Hand grip	18 (0.6)	18 (0.8)	6 (1)	7 (0.81)	3 (0.8)	4 (1)	57 (0.82)	52 (0.82)
Elbow flexion	38 (0.86)	47 (0.8)	19 (0.81)	35 (0.93)	3 (1)	15 (0.93)	105(0.77)	86 (0.91)
Three-point pinch	20 (1)	23 (0.95)	6 (0.83)	9 (0.77)	4 (0.8)	2 (0.8)	30 (0.96)	37 (0.94)
Total arm megascore	76	**88**	31	41	10	**21**	192	175
Knee extension	6 (0.83)	8 (0.87)	6 (1)	7 (0.87)	5 (0.8)	11(0.72)	21 (0.85)	21 (0.8)
Knee flexion	12 (0.91)	16 (0.94)	9 (0.82)	16 (0.92)	8 (0.62)	12 (0.91)	51 (0.86)	55 (0.92)
Foot dorsiflexion	14 (0.85)	15 (0.6)	2 (0.6)	9 (0.81)	4 (0.7)	11 (0.81)	12 (0.75)	11 (0.9)
Total leg megascore	32	39	17	**32**	17	**34**	84	88

Measures are expressed in Newtons; inter-rater correlation coefficients (ICC) are shown in parentheses. Arm and leg megascores are the Σ of upper and lower limb measures, respectively. T0 = baseline; T7 = day 7. In bold: statistically significant single values $P < 0.001$ (χ^2 test, T7 *versus* T0). All measures in grouped patients: $P = 0.041$ (nonparametric Friedman test for multiple samples).

sickle cell disease.[11] The variability in *SMN* gene expression following PB administration observed in our patients may be related to the varying levels of plasmatic PB concentrations at different intervals between drug administration and blood sampling.

When we investigated on the extent of reduction in *SMN-fl* levels in our patients before treatment compared to unaffected individuals, we found that the type II and III patients had approximately 25 and 50%, respectively, of *SMN-fl* levels of controls. If we consider that in all patients a more than 100% increase in *SMN-fl* transcripts was detected in at least one blood sample during treatment we may speculate that *SMN-fl* levels in leukocytes of SMA type II patients could transiently exceed the baseline levels of type III patients and that the latter could achieve levels similar to that of controls.

The observation of an increase in muscle strength after 1 week of treatment was unexpected and may be the result of a placebo effect. However, a similar trend was found also in our recent open pilot study.[10] Regarding our present data, it may be not by chance that the patient (P4) with the highest mean increase in *SMN* transcript levels showed also the more obvious improvement in muscle strength and the case with the lowest mean increase (P6 who has taken a 33% reduced dosage) had no change in muscle strength. Further data on the effect of PB on muscle strength will be provided by the ongoing placebo-controlled double-blind clinical trial.

Our observation that PB increases SMN expression was made on peripheral blood leukocytes. Although we do not have direct information on the effects on muscle or motor neuron cells, our preliminary clinical data suggest that PB improves motor function in SMA patients.[6]

In conclusion, while an effect of PB on SMN protein expression has still to be determined, we provide here the first evidence that SMN transcript levels can be enhanced in SMA patients by drug administration.

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