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ARTICLE

Treatment of spinal muscular atrophy cells with drugs that upregulate SMN expression reveals inter- and intra-patient variability

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Spinal muscular atrophy (SMA) is a genetic neuromuscular disorder caused by mutations in the *SMN1* gene. The homologous copy (*SMN2*) is always present in SMA patients. *SMN1* gene transcripts are usually full-length (FL), but exon 7 is spliced out in a high proportion of *SMN2* transcripts (delta7) (Δ7). Advances in drug therapy for SMA have shown that an increase in *SMN* mRNA and protein levels can be achieved *in vitro*. We performed a systematic analysis of *SMN* expression in primary fibroblasts and EBV-transformed lymphoblasts from seven SMA patients with varying clinical severity and different *SMN1* genotypes to determine expression differences in two accessible tissues (skin and blood). The basal expression of *SMN* mRNA FL and Δ7 in fibroblasts and lymphoblasts was analyzed by quantitative real-time PCR. The FL-*SMN* and FL/Δ7 *SMN* ratios were higher in control cells than in patients. Furthermore, we investigated the response of these cell lines to hydroxyurea, valproate and phenylbutyrate, drugs previously reported to upregulate *SMN2*. The response to treatments with these compounds was heterogeneous. We found both intra-patient and inter-patient variability even within haploidentical siblings, suggesting that tissue and individual factors may affect the response to these compounds. To optimize the stratification of patients in clinical trials, *in vitro* studies should be performed before enrolment so as to define each patient as a responder or non-responder to the compound under investigation.

INTRODUCTION

Spinal muscular atrophy (SMA) is caused by degeneration of the spinal cord α-motor neurons, leading to muscle denervation and atrophy. SMA is the second most common inherited lethal disorder of childhood. It is classified into four types (I–IV), depending on age at onset and disease severity. Type I SMA (Werdnig–Hoffmann disease) is the most severe form of the disease, with clinical onset usually occurring before 6 months of age, whereas type II SMA is the intermediate form with onset between 6 and 18 months. Type III SMA (Kugelberg–Welander disease) is a relatively mild form of the disease, with onset usually occurring in childhood or adolescence. Type IV SMA appears in adult life and clinical manifestations may be minimal. Mutations or deletions in the survival motor neuron gene, *SMN1*, have been found to be the cause of SMA.[1,2] In humans, two highly homologous copies of the survival motor neuron genes (the telomeric *SMN1* and the centromeric *SMN2*) are located in a duplicated region of chromosome 5q13.[2] Over 95% of 5q-linked SMA patients have homozygous deletions for *SMN1* or gene conversion at exons 7 or 8, whereas the remaining subjects have missense, nonsense or splice site mutations.[1] A 4-bp deletion in exon 3 (c.399_402del AGAG) – exclusive to the Spanish population as far as we know – constitutes one of the most frequently found subtle mutations in SMA. It has been associated with a large spectrum of phenotypes, from severe type I to non-symptomatic patients.[3–5]

The *SMN1* gene contains a C nucleotide at position six of exon 7 (Ex7+6) and produces predominantly full-length transcripts (FL-*SMN*). The *SMN2* gene, however, contains a T nucleotide at this position, leading to a differentially spliced form that lacks exon 7 (Δ7-*SMN*).[2,6–8] The resulting putative shorter protein has a different C-terminus. It is less stable and cannot oligomerize or self-associate, as occurs with the complete protein.[9,10]

Patients with the type I form usually have a lower number of *SMN2* copies than the chronic types II–IV. However, the correlation is not absolute and patients with three *SMN2* copies may suffer type I, II or III SMA.[11,12]

As the differences between *SMN1* and *SMN2* are related to the complete transcript and the amount of protein, drugs capable of increasing FL-*SMN* expression and SMN protein may have therapeutic effects for SMA patients.[13] Histone deacetylases inhibitors (HDACi), for example, increase acetylation of histones and other proteins[14] and this hyperacetylation relaxes the tertiary structure of chromatin, facilitating access of the transcriptional machinery to target genes. *In vitro* experiments with phenylbutyrate (PBA) and valproic acid (VPA) – two well-known HDACi – have shown an increase in *SMN* mRNA and protein levels in SMA fibroblasts.[15–17] Similar studies with hydroxyurea (HU) in EBV-immortalized SMA lymphoblasts[18] have shown an increase in the FL-*SMN*/Δ7-*SMN* (FL/Δ7 ratio). Pilot trials with these drugs have been performed in SMA patients and results

were promising,[19–21] leading to the development of placebo-controlled clinical trials. PBA has been investigated in a double-blind placebo-controlled trial in 107 children with type II SMA. VPA–carnitine has been administered to 42 type II SMA children in a multi-centre phase II trial.[22] A double-blind placebo-controlled trial with HU in 28 type II SMA and 29 type III SMA patients has recently been completed.[23] Results from these three clinical trials have not revealed a clear benefit of these drugs for the patients.

To detect possible differences in individual responses in a group of SMA patients with diverse genotypes and phenotypes, we analyzed SMN mRNA and protein levels in response to HU, VPA and PBA in two cell types (fibroblasts and lymphoblasts). In particular, we aimed to compare the responses of four sisters, born to consanguineous parents, who are homozygous for a frameshift mutation in the SMN1 gene and have the same SMN2 copy number but discordant phenotypes.

MATERIALS AND METHODS
Patients and cell cultures
A total of 10 individuals (3 controls and 7 SMA) were included in this study. SMA was diagnosed using the criteria outlined by the International SMA Consortium,[24] and confirmed by detection of molecular alterations in the SMN1 gene. SMN1 genotype and SMN2 copy number were determined as previously described.[1,11] Informed consent was obtained from all subjects or their parents. Human fibroblasts and EBV-immortalized lymphoblasts were cultured according to standard protocols. RNA analysis was performed at 8 and 24 h after feeding for PBA and after 24 and 48 h for VPA and HU. Table 1 shows patients' characteristics. Four sisters (patients 4–7) were homozygous for a frameshift mutation in exon 3 with four copies of SMN2. They were haploidentical for the SMA locus sharing the region comprised between markers rs4613646 and rs13162495 (12 Mb) (Also-Rallo et al, unpublished data). One of the sisters, patient 7, is able to walk, whereas her three sisters have been wheelchair-confined since early infancy.

Drugs
In accordance with previously described optimal concentrations, the final concentration of the drugs used in fibroblasts and lymphoblasts was 1 mM for VPA and HU and 2 mM for PBA.[15,16,18] All compounds were freshly prepared immediately before each use. Cells were treated for 24 and 48 h (with daily addition) for HU and VPA and for 8 and 24 h for PBA. All treatments were performed in triplicate.

Real-time RT-PCR
RNA concentration was determined using a spectrophotometer, and 1 μg RNA was used for first-strand cDNA synthesis. FL-SMN and Δ7-SMN mRNA were determined using GAPDH as an endogenous control in the ABI PRISM 7000 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). All primers, probes and PCR conditions were performed as previously described.[15] Using the ΔΔCt method, we analyzed the relative amount of FL-SMN, taking the FL-SMN of each untreated sample as a calibrator (further explained in Figure 3 legend). We also studied the FL/Δ7 ratio using the FL-SMN value for each sample as a calibrator.

PCR to determine the origin of SMN transcripts
Given that the c.399_402del AGAG is a 4-bp deletion that creates a stop codon after 34 bp in the SMN1 gene; FL-SMN levels observed in patients with this mutation should originate only from the SMN2 gene. To confirm this hypothesis, we studied the origin of SMN transcripts amplifying cDNA by using SMN exon 6 (541C618) forward[25] and exon 8 (541C1120) reverse primers[2] as previously described.[26] The PCR product obtained (approx 1 Kb) was digested with the DdeI restriction enzyme – which cleaves the amplicon from SMN2 only – and separated on 2% agarose gel.

Cell viability
To determine the sensitivity of fibroblasts and EBV-transformed lymphoblasts to the drugs, cell viability was checked using the XTT method (Roche, Mannheim, Germany). In this assay, metabolically active cells induce the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye. Cell viability can be determined by quantifying the amount of formazan. The trypan blue exclusion assay was also performed.[27] Viable (trypan blue-excluding) and dead (trypan blue-stained) cells were counted using a Newbauer chamber (Marienfeld, Germany). The experiments were performed using four different cell lines in each experiment and averaged.

Western blot
Fibroblasts and EBV-transformed lymphoblastoid cells from SMA patients and control individuals were harvested in RIPA buffer and protease inhibitors to prepare protein extracts (Sigma-Aldrich, St Louis, MO, USA). Subsequently, all samples were quantified by the BCA method (Pierce, Rockford, IL, USA). Western blotting analyses were performed following standard protocols and signal intensity was measured using Chemilmager 5500 (Alpha Innotech, Santa Clara, CA, USA). Further explanation is given in Figure 3 legend. Experiments were performed in triplicate.

Antibodies
Antibodies used in the present study were: monoclonal antibody anti-SMN (BD Transduction Laboratories, Lexington, KY, USA; 1/5000), anti-GAPDH (Millipore, Temecula, CA, USA; 1/10 000), anti-β-tubulin (Sigma-Aldrich; 1/300) anti-β-actin (Sigma-Aldrich; 1/5000) and horseradish peroxidase-conjugated rabbit anti-mouse IgG (DakoCytomation, Copenhagen, Denmark; 1/1000).

Statistical analysis
The unpaired t-test was performed to analyze differences between control and SMA baseline FL-SMN transcripts, FL/Δ7 ratios and protein levels. The paired t-test was used to investigate intra-patient differences between fibroblasts and lymphoblasts.

RESULTS
Origin of the FL-SMN transcripts
Figure 1 shows that virtually all FL-SMN transcripts of patients harboring the c. 399_402del AGAG mutation originated from SMN2. Similar results were observed in lymphoblasts (data not shown).

Relative quantitative analysis of SMN mRNA and protein in control and SMA cell lines
As illustrated in Figures 2a and b, some SMA patients showed diminished FL-SMN mRNA levels in comparison with controls. Others showed similar or even slightly higher values of FL-SMN.

Table 1 Subjects analyzed in this study

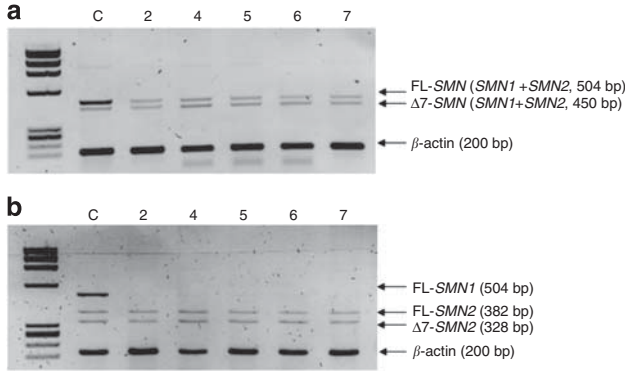| Sample	Gender	SMN1 genotype	SMN2 copies	Phenotype
C1	Male	+/+	2	Control
C2	Female	+/+	2	Control
C3	Female	+/+	2	Control
1	Male	Del/Del	2	I (Died <2 years)
2	Female	SM/Del	3	II (Sitter)
3	Male	Hyb/Del	3	III (Sitter)
4	Female	SM/SM	4	III (Sitter)
5	Female	SM/SM	4	III (Sitter)
6	Female	SM/SM	4	III (Sitter)
7	Female	SM/SM	4	IV (Walker)

Abbreviations: Del, deletion; Hyb, hybrid; SM, subtle mutation c.399_402del AGAG.
Patients 4–7 are sisters of consanguineous parents. I–IV refer to the SMA type.

Sample 1 (type I patient) showed the lowest values in comparison with the remaining SMA samples.

The FL/Δ7 ratio was significantly higher in control fibroblasts and lymphoblasts than in patients ($P < 0.001$) (Figures 2c and d). The

FL/Δ7 ratio in SMA fibroblasts ranged from 1.13 to 1.73, whereas in SMA lymphoblasts, it ranged from 1.79 to 2.6. These differences reached statistical significance ($P=0.028$).
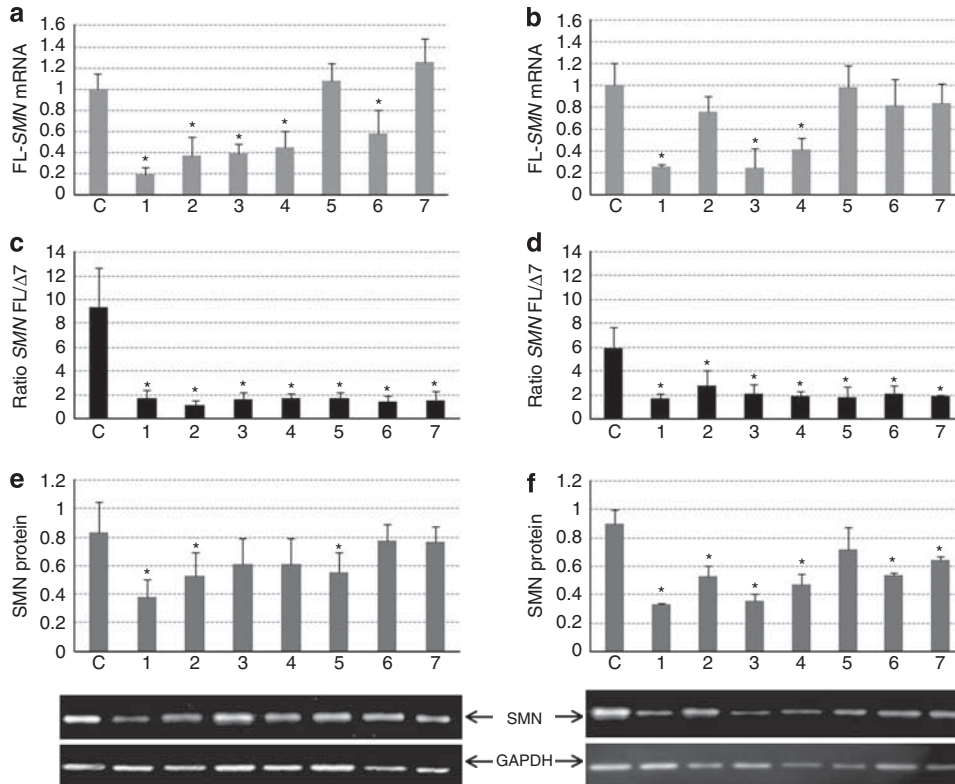
Regarding SMN protein expression, we observed that sample 1 (type I patient) showed the lowest value in comparison with the remaining SMA samples (Figures 2e and f).

Relative quantitative analysis of SMN mRNA and protein in response to drugs

Fibroblasts. When we analyzed the effect of HU and VPA on the FL-SMN transcripts, we observed the maximum increases at 48 h. For PBA, the maximum increases were at 24 h. However, not all cell lines showed changes (Figure 3a). For protein analyses, the highest levels for each cell were mainly reached at 24 h (Figure 3c). Time-fold increases were higher in mRNA than in protein. Table 2 summarizes results that allow each patient to be classified as a responder, a partial responder or a non-responder to each drug, based on their changes in FL-SMN mRNA and protein (positive or absent). All samples considered responders also showed an increase in their FL/Δ7 ratios (data not shown) but we did not consider these data as criteria to define the response of a cell line.[28] If a discrepancy between mRNA and protein was noted, we considered the patient a partial responder. We found two responders (patients 1 and 7) for HU, four (patients 1, 2, 4 and 6) for VPA and two (patients 1 and 4) for PBA. We detected patients responding to all drugs (patient 1), to two drugs (patient 4), to one drug (patients 2, 6 and 7) and to none of the three compounds under study (patient 3) (summarized in Table 2). We classified this last

Figure 1 Origin of the FL-SMN transcripts in patients with the c.399_402del AGAG mutation. (**a**) RT-PCR analysis of total RNA extracted from fibroblasts. The 504-bp fragment includes exons 6–8 of the SMN gene. The 450-bp fragment includes exons 6–8 lacking exon 7 and the 200-bp fragment corresponds to the β-actin fragment. (**b**) DdeI digestion of the SMN RT-PCR amplified fragment to determine the origin of the various transcripts. Note that no SMN transcripts originate from the SMN1 gene in these patients. All patients are identified using the same numbers as in Table 1.

Figure 2 Baseline levels of SMN mRNA and protein. (**a**) Histogram showing the FL-SMN mRNA in fibroblasts and (**b**) lymphoblasts relative to control levels determined by the ΔΔCt method. (**c**) Histogram of FL/Δ7 mRNA ratio in fibroblasts and (**d**) lymphoblasts. (**e**) Histogram of SMN protein levels in fibroblasts and (**f**) in lymphoblasts relative to GAPDH as depicted by the respective immunoblots (results with β-actin and β-tubulin were similar, data not shown). C=mean of C1, C2 and C3; 1–7=SMA patients. All patients are identified using the same numbers as in Table 1.
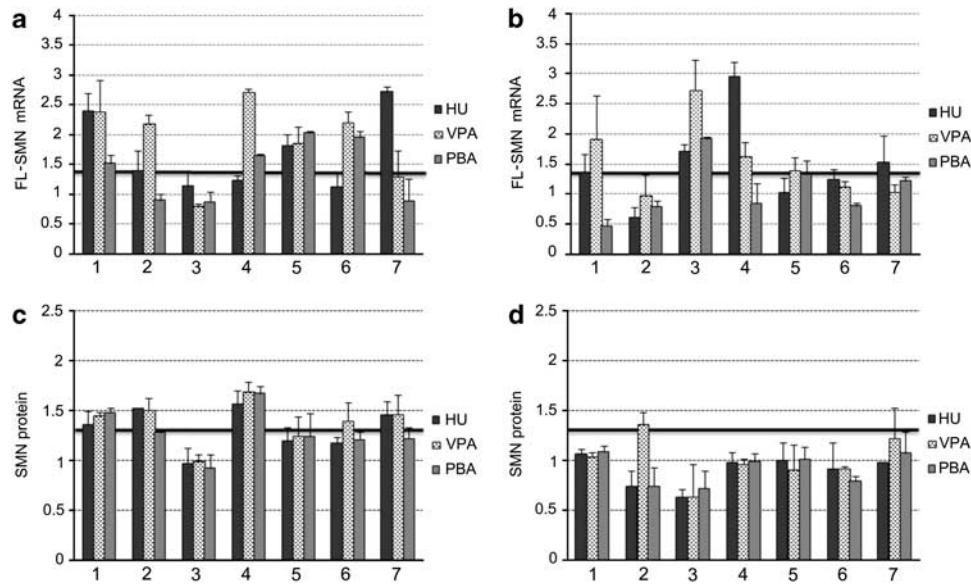
Figure 3 Histograms showing the effect of treatment of seven SMA cells with hydroxyurea (HU), valproic acid (VPA) and phenylbutyrate (PBA). (**a**) Levels of FL-*SMN* transcript in fibroblasts and (**b**) lymphoblasts determined by the $\Delta\Delta Ct$ method. We observed variations in *GAPDH C*t values (≤ 0.5) after different biological replicates, resulting in up to 1.4-fold differences. Thus, an increase of mRNA FL-*SMN* was considered positive when the value obtained was more than 1.4-fold its baseline level. (**c**) Histogram showing the effect of the same compounds on levels of SMN protein detected by immunoblot in fibroblasts and (**d**) lymphoblasts. Levels of mRNA and protein are depicted according to the maximum increase observed and are expressed as *n*-fold their baseline set to 1 (continuous line). The highest values of protein were reached at 24 h, with the exception of patient 2, whose peak values for HU and VPA treatment were reached at 48 h, and patient 1, whose peak values for PBA were reached at 8 h. Owing to the standard deviation observed in basal protein levels ($\leq 30\%$) (Figures 2e and f), values higher than 1.3-fold were considered positive. The thick line represents the threshold for positive response. All patients are identified using the same numbers as in Table 1.

Table 2 Positive (+) or no response (−) of FL-*SMN* mRNA and SMN protein in fibroblasts after treatment with HU, VPA and PBA

	HU		VPA		PBA	
Samples	FL-SMN	Prot	FL-SMN	Prot	FL-SMN	Prot
1	**+**	**+**	**+**	**+**	**+**	**+**
2	−	+	**+**	**+**	−	−
3	−	−	−	−	−	−
4	−	+	**+**	**+**	**+**	**+**
5	+	−	+	−	+	−
6	−	−	**+**	**+**	+	−
7	**+**	**+**	−	+	−	−

Positive categorical responders (positive concordance between mRNA and protein) are in bold and partial responders (discordance between mRNA and protein) are in normal font. Note the differences between the four sisters (samples 4–7).

patient as a 'complete non-responder'. Interestingly, with a dose increase of one order of magnitude (from 1 to 10 mM), fibroblasts from this patient showed a response to VPA (Figure 4), even though they remained unresponsive to HU (10 mM) and to the repetitive administration of PBA (every 4 h).

We did not observe a correlation between the response to the compound and the number of *SMN2* copies. For example, patient 1, who had two *SMN2* copies, reached increases that were similar to those for patients 4–6, who had 4 *SMN2* copies. Focusing on the haploidentical sisters (samples 4–7, Table 1) we also observed heterogeneity in their pattern of response. Although patient 7 (non-affected sister) was only a responder for HU, two of her more affected sisters (patients 4 and 6) were responders to VPA. Patient 4 was also a PBA responder, whereas patient 5 (the remaining affected sister) was classified as a partial responder for all compounds (Table 2).

Lymphoblasts. The effect of the different drugs on lymphoblasts is shown in Figures 3b and d. In samples 1, 3, 4 and 7, basal FL-*SMN* levels increased after some treatments, whereas basal protein levels remained unchanged; these patients were, therefore, classified as partial responders in lymphoblasts (Table 3). The remaining samples showed no response in mRNA and protein, regardless of the compound investigated. All samples under treatment with HU showed a certain increase in FL/$\Delta 7$ ratios (data not shown). Interestingly, sample 3 – a complete non-responder in fibroblasts – showed lymphoblasts with increments of up to 2.5-fold in FL-*SMN* after VPA treatment, and increments of up to two-fold in FL-*SMN* after HU and PBA treatment (Figures 3b and d, and Table 3).

Cell viability. When SMA cells were cultured in a drug-free medium, the number of cells growing as a monolayer (fibroblasts) or in suspension (lymphoblasts) increased at 24 and 48 h, as observed with trypan blue stain (data not shown). Cell viability was inhibited under experimental doses of the different compounds. As depicted in Figure 5, which illustrates the cell's metabolic state, at doses of 1 mM for HU and VPA and 2 mM for PBA, cell viability was decreased by 15–20% in fibroblasts and by 20–30% in lymphoblasts after 48 h of treatment. A dose increment of HU by one order of magnitude resulted in a 20% decrease in cell viability in fibroblasts and a 40% decrease in lymphoblasts. The effect of VPA on cell viability was stronger, decreasing by 50% in fibroblasts and 75% in lymphoblasts. We observed no differences between control and SMA cells and no association with the response to the drug treatment (data not shown).

DISCUSSION

We performed a systematic analysis to determine the level of responses in fibroblasts and lymphoblasts to compounds (HU, VPA, and PBA)
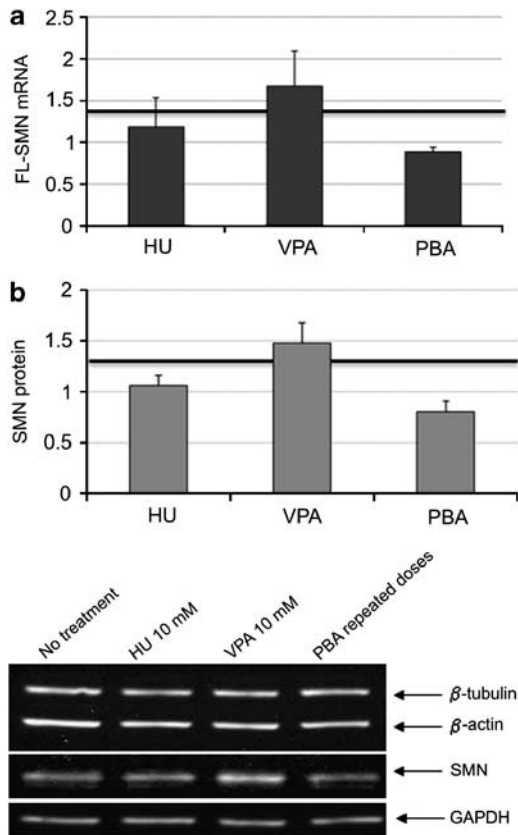
Figure 4 Histograms showing the effect of the increase in dose (10 mM) of HU and VPA and the repetitive administration of PBA (2 mM) every 4 h in fibroblasts of the non-responder patient 3. (**a**) FL-*SMN* transcripts expressed as *n*-fold their baseline (set to 1). (**b**) Protein levels relative to β-actin and β-tubulin and GAPDH. Note that the increase of SMN expression was achieved only with VPA. The thick line represents the threshold for positive response.

Table 3 Positive (+) or no response (−) of FL-*SMN* mRNA and SMN protein in lymphoblasts after treatment with HU, VPA and PBA

	HU		VPA		PBA	
Samples	FL-SMN	Prot	FL-SMN	Prot	FL-SMN	Prot
1	−	−	+	−	−	−
2	−	−	−	+	−	−
3	+	−	+	−	+	−
4	+	−	+	−	−	−
5	−	−	−	−	−	−
6	−	−	−	−	−	−
7	+	−	−	−	−	−

No categorical responders (positive in mRNA and protein) were observed, only non-responders (negative in mRNA and protein) or partial responders (discordance between mRNA and protein).

that upregulate *SMN2* in a group of SMA patients with different genotypes and phenotypes, in particular, in four haploidentical SMA sisters. We found intra- and inter-patient variability even among the sisters, suggesting that tissue and individual factors may affect the response to these compounds.

Levels of FL-*SMN* in the type I sample were lower than levels from the type II and III samples, in agreement with previous studies.[29–32]

Control samples (with two *SMN1* and two *SMN2* copies) showed an FL/Δ7 ratio of around 6–9, whereas SMA samples showed values ranging from 1.13 to 2.6. Interestingly, in the SMA patients, we found that lymphoblast ratios were slightly higher than those for fibroblasts. These findings may be due to regulation of *SMN2* expression in these cells, although the effect of the EBV immortalization on *SMN2* regulation is unknown. The FL/Δ7 ratio in the SMA cells under study was similar, even though our patients had diverse genotypes and phenotypes. Previous reports have also shown a lack of correlation of FL/Δ7 ratios in blood cells,[20,28,32] fibroblasts[16] and lymphoblasts.[18] In the four haploidentical sisters, we found no differences in the FL/Δ7 ratios, either in fibroblasts or in lymphoblasts. In fibroblasts from eight discordant families, Helmken *et al*[33] found no differences in FL/Δ7 ratios between affected and unaffected siblings, although they did find ratio differences in lymphoblasts in one family. The FL/Δ7 ratio, therefore, clearly differentiates SMA patients from controls but it is not useful to classify patients according to their severity.

The baseline expression of SMN protein in western blots was lower in SMA samples than in controls. The lowest level was found in the type I patient, in agreement with previous reports.[28–30] In our remaining patients, there were no noticeable differences even between the discordant sisters, again in concordance with findings in previous studies.[4,29]

Our most significant finding was the intra-and inter-patient variability found in cells exposed to different compounds.

Intra-patient variability was detected after comparison of FL-*SMN* and protein levels in some patients. Table 2 summarizes patients with a homogeneous response to the drug (either responder or non-responder) and patients whose response was discrepant (positive mRNA result and no substantial changes in protein or *vice versa*). We considered these discrepant patients as partial responders. Sumner *et al*[32] reported similar discrepancies in SMA blood samples. They suggested that SMN protein levels might plateau when there are more than two *SMN2* copies, as in most of our patients. Patient 3 was considered a categorical non-responder for all three compounds. However, the dose increase by one order of magnitude (which is beyond the therapeutic range for patients) led to a positive response for VPA but not for HU as illustrated in Figure 4. Given that PBA is very toxic at higher doses, we treated the cells of this patient with repetitive PBA doses,[15] but mRNA and protein levels remained unchanged.

Intra-patient variability was also observed when a patient responded to one drug but not to another. Two of the compounds, VPA and PBA, belong to the same group of HDAC inhibitors (aliphatic acids) and one might presume that their effect on the *SMN2* gene would be similar. However, our cells responded differently. The mechanism by which HDACi activate *SMN2* expression remains unknown. It is common knowledge that HDACi not only deacetylate histones but also other proteins that are transcriptional regulators.[34] Two to ten percent of genes are believed to be regulated by histone acetylation and deacetylation,[35] but we do not know whether these compounds upregulate *SMN2* by increasing histone acetylation at the *SMN* promoter, or by activating the acetylation state of a critical transcription factor.[17] Kernochan *et al*[36] observed that even though acetylated H3 and H4 histones predominated in the transcriptional origin of the *SMN* gene, VPA treatment led to acetylation in far-upstream regions where there are usually fewer acetylated H3 and H4 histones. Further research will help to clarify the mechanism of action and individual response to these compounds. The third drug investigated, HU, used in sickle cell disease, induces fetal hemoglobin gene expression by stimulating the production of transcription or splicing
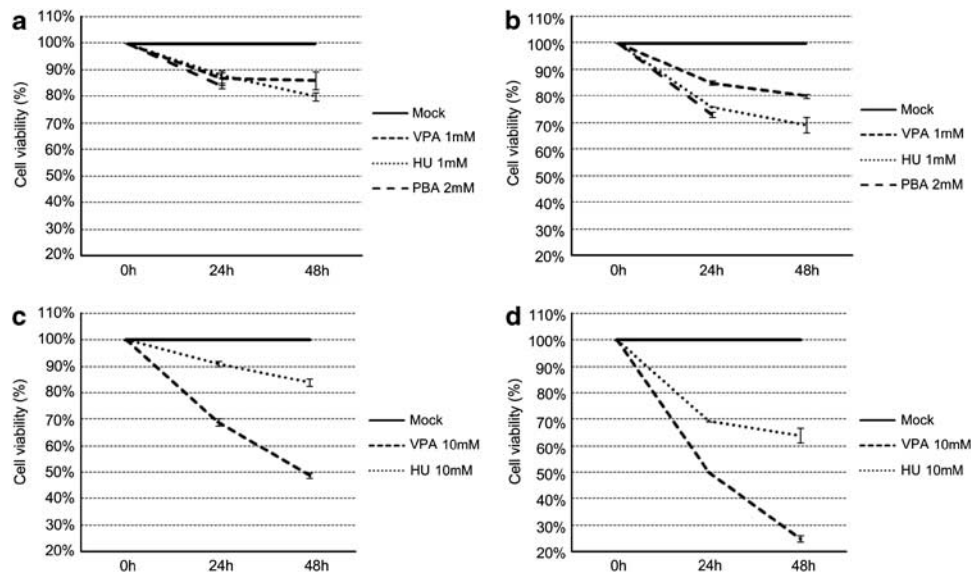
Figure 5 Impact of the different compounds on cell viability determined by XTT assay. (**a**) Fibroblasts and (**b**) lymphoblasts treated with HU (1 mM) VPA (1 mM) and PBA (2 mM); A decrease of around 20–30% in cell metabolism was observed in both cell lines (lymphoblasts tended to be more sensitive than fibroblasts); (**c**) fibroblasts and (**d**) lymphoblasts treated with a dose increase of one order of magnitude. The decrease was around 20–50% in fibroblasts and 40–80% in lymphoblasts. Each value is given as mean of four cell lines in three experiments ± SEM.

factors. Grzeschik et al[18] proposed that this mechanism could explain the increase in FL/Δ7 ratios observed in SMA lymphoblasts. It has recently been suggested that HU enhances *SMN2* expression in lymphoblasts through the release of nitric oxide but its mechanism of action on *SMN2* genes is as yet unknown.[37]

In the present study, the three compounds tended to show a different intra-patient response depending on the tissues under investigation. This can be partially explained by the fact that fibroblasts are primary cultures that grow as monolayers, whereas lymphoblasts are EBV-immortalized cells that grow in suspension. Inhibition of cell proliferation has been described in relation to PBA,[38,39] VPA[40] and HU.[41] In our study, the three compounds inhibited cell growth without noticeable differences between controls and SMA lines. Furthermore, our analysis showed that lymphoblasts were more sensitive than fibroblasts to this effect. Taking the number of viable cells into account may help to normalize RNA or protein values in drug experiments. Lymphoblasts in patient 3 increased their mRNA levels after administration of drugs, whereas their basal protein levels decreased, illustrating the lack of correlation between RNA and protein and the variable range of response in these cells. In this respect, Dayangac-Erden et al[42] pointed out that EBV-immortalized lymphoblasts are probably not an appropriate cell model to study the upregulation of *SMN2*.

Inter-patient variability was also notable (Table 2). Four patients were responders to VPA, two to HU and two to PBA. Explanations for this variability in our patients include the molecular characteristics of their *SMN2* genes and their individual metabolisms. The number of *SMN2* copies has been considered irrelevant for the *in vitro* positive response.[15,17] However, their orientation in the SMA locus, their methylation activity,[43] and the presence of mutations affecting their expression or splicing[28,44] could influence the final response. Individual metabolism appears to be associated with the differential response to VPA, as previously suggested by Brichta et al.[20] These investigators studied the level of *SMN* expression in blood after the oral administration of VPA and found positive, negative and

non-responder patients. It is reasonable to hypothesize that HU and PBA could also have a wide range of response.

SMA patients' cell lines described in previous papers studying *SMN2* upregulation had homozygous absence or deletion of the *SMN1* gene, whereas most of our patients had a frameshift mutation in *SMN1*. Our results indicate that pharmacological upregulation of SMN2 may also be a suitable option for patients with point mutations. Indeed, we were able to replicate the results of colleagues who analyzed these compounds separately. However, when we studied a patient concomitantly for the three drugs under analysis, we found a wide range of response. Further testing seems crucial to determine each patient's putative response to these drugs to decide subsequently whether he or she may benefit from a treatment. To date, clinical trials with these compounds have not shown categorical improvements in outcomes and most of these studies conclude the need for homogeneous cohorts of patients.[15,20–22,45] Selecting patients according to their *in vitro* response may be a good approach.

Interestingly, our four SMA sisters born to consanguineous parents showed a heterogeneous response to drugs in our experiments (Table 2). We were unable to determine a specific or differential pattern of *SMN* response based on phenotype. This finding illustrates how variability is present even in patients from the same family, supporting our view that response to each compound should be investigated individually. The variability observed suggests that the positive effects of the drugs are not linked to a direct effect on the *SMN2* gene and that their targets are different. This is supported by the fact that the four sisters, with different responsiveness, share the same haplotype that includes the *SMN2* gene.

In conclusion, we observed a variable intra-and inter-patient *in vitro* response in SMA cells exposed to compounds that upregulate *SMN2* expression, illustrating the complexity of drug effects. As drug response is dictated by factors beyond *SMN* genotype, the effect cannot be predicted based on *SMN1* mutation or *SMN2* copy number alone. To optimize patient stratification in future clinical trials, we suggest primary fibroblasts could be tested before enrolment to

determine those patients who may respond to the compound under investigation. Garbes et al[46] have shown that a patient's responsiveness to VPA in whole blood correlated with results in treated fibroblasts. However, in view of the variability that we observed in the cell lines studied a short oral delivery *in vivo* could be an alternative to better classify the patients. The recent availability of an absolute quantification method for *SMN* transcripts may help to further categorize patients according to their responder status.[47] Moreover, in another report, induced pluripotent stem cells generated from skin fibroblasts from a child with SMA were differentiated into motor neurons.[48] This new model of motor neurons is a promising resource to study SMA disease mechanisms and to further predict the response to drugs that upregulate *SMN2* expression.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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