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RESEARCH PAPER

Triptolide increases transcript and protein levels of survival motor neurons in human SMA fibroblasts and improves survival in SMA-like mice

Ya-Yun Hsu[1], Yuh-Jyh Jong[1,4], Hsin-Hung Tsai[1], Yu-Ting Tseng[2], Li-Mei An[3] and Yi-Ching Lo[1,2,3]

[1]*Graduate Institute of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan,* [2]*Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan,*[3]*Department of Pharmacology, Kaohsiung Medical University, Kaohsiung, Taiwan, and* [4]*Departments of Pediatrics and Clinical Laboratory, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan*

Correspondence
Yi-Ching Lo, Department of Pharmacology, School of Medicine, College of Medicine, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung 80708, Taiwan.
E-mail: yichlo@kmu.edu.tw

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BACKGROUND AND PURPOSE

Spinal muscular atrophy (SMA) is a progressive neuromuscular disease. Since disease severity is related to the amount of survival motor neuron (SMN) protein, up-regulated functional SMN protein levels from the *SMN2* gene are considered a major SMA drug-discovery strategy. In this study, we investigated the possible effects of triptolide, a diterpene triepoxide purified from *Tripterygium wilfordii* Hook. F., as a new compound for increasing SMN protein.

EXPERIMENTAL APPROACH

The effects and mechanisms of triptolide on the production of SMA protein were determined by cell-based assays using the motor neuronal cell line NSC34 and skin fibroblasts from SMA patients. Wild-type (*Smn*$^{+/+}$*SMN2*$^{-/-}$, C57BL/6) and SMA-like (*Smn*$^{-/-}$ *SMN2*) mice were injected with triptolide (0.01 or 0.1 mg·kg^{-1}·day^{-1}, i.p.) and their survival rate and level of change in SMN protein in neurons and muscle tissue measured.

KEY RESULTS

In NSC34 cells and human SMA fibroblasts, pM concentrations of triptolide significantly increased SMN protein expression and the levels of SMN complex component (Gemin2 and Gemin3). In human SMA fibroblasts, triptolide increased SMN-containing nuclear gems and the ratio of full-length transcripts (*FL-SMN2*) to *SMN2* transcripts lacking exon 7 (*SMN2Δ7*). Furthermore, in SMA-like mice, triptolide significantly increased SMN protein levels in the brain, spinal cord and *gastrocnemius* muscle. Furthermore, triptolide treatment increased survival and reduced weight loss in SMA-like mice.

CONCLUSION AND IMPLICATIONS

Triptolide enhanced SMN protein production by promoting *SMN2* activation, exon 7 inclusion and increasing nuclear gems, and increased survival in SMA mice, which suggests triptolide might be a potential candidate for SMA therapy.

Abbreviations

FL-SMN, full-length survival motor neuron; *FL-SMN2*, full-length transcript; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SMA, spinal muscular atrophy; SMN, survival motor neuron; *SMN2Δ7*, *SMN2* transcript lacking exon 7; snRNPs, small nuclear ribonucleoproteins

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disease characterized by degeneration of α-motor neurons in the anterior horn of the spinal cord, leading to muscular paralysis with muscular atrophy. Traditionally, SMA is divided into three types based on the age of onset, severity and clinical motor function test. The classification from most severe to mild type is: type I (Werdnig–Hoffmann disease), type II (intermediate) and type III (Kugelberg–Welander disease). There are also two additional types, type IV (adult-onset with very mild symptoms) and type 0 (prenatal onset combine with severe symptoms leads to early neonatal death) (Dubowitz, 1999; Russman, 2007; Lunn and Wang, 2008).

SMA is caused by homozygous disruption and functional loss of the *survival motor neuron 1* (*SMN1*) gene by deletion or mutation. In humans, there is one telomeric copy of the *SMN1* gene and several centromeric copies of the *SMN2* gene. The *SMN1* and *SMN2* gene encode 90% and 10% full-length survival motor neuron (FL-SMN) protein, respectively. Normally, sufficient FL-SMN protein can maintain the survival of motor neurons. However, more than 98% of patients with SMA have functional loss of the *SMN1* gene, but always retain at least one copy of *SMN2* gene (Monani, 2005). The FL-SMN protein is ubiquitously expressed and is localized to both the cytoplasm and nucleus. In the nucleus, SMN forms a stable multi-protein complex by a tight protein–protein interaction associate with Gemin2-8, called gems (Liu and Dreyfuss, 1996), which play an essential role in the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs) (Meister *et al.*, 2002; Eggert *et al.*, 2006; Neuenkirchen *et al.*, 2008; Talbot and Davies, 2008). In the components of SMN complex, both of Gemin2 and Gemin3 interact directly and stably with SMN (Todd *et al.*, 2010). The relationship between SMN and Gemin2 is greater than any other components of the SMN complex (Ogawa *et al.*, 2007). Down-regulation of the SMN complex interaction might result in a lower level of SMN protein and degeneration of motor neurons (Friesen *et al.*, 2001; Gubitz *et al.*, 2004; Yong *et al.*, 2004; Morris, 2008; Chari *et al.*, 2009).

To date, there are no effective treatments to slow or reverse neurodegeneration in SMA (Chen *et al.*, 2010). The therapeutic strategies for SMA patients have been focused on increasing functional FL-SMN levels by increasing *SMN2* transcription, modulating splicing and increasing SMN stability (Pruss *et al.*, 2010). Triptolide (PG490 or designated as T_{10}) (Figure 1) is a diterpene triepoxide purified from the Chinese herb *Tripterygium wilfordii Hook. F.*, a traditional Chinese medicine with a potent immunosuppressive effect, that is used to treat rheumatoid arthritis (Chen, 2001). Triptolide has reported anti-tumour effects by acting on apoptotic and autophagic pathways (Mujumdar *et al.*, 2010) via the regulation of histone methylation and down-regulation of histone methyltransferases SUV39H1 and EZH2 (Zhao *et al.*, 2010). In db/db diabetic mice, triptolide exerts several protective effects, preventing diabetic nephropathy, and the inhibitory effects of triptolide on renal inflammation and oxidative stress are more effective than those of valsartan (Gao *et al.*, 2010). In addition, triptolide crosses the blood-brain barrier easily due to its small molecular size and lipophilic property

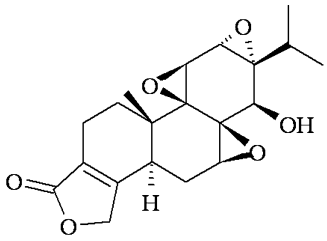

Figure 1
The chemical structure of triptolide, the biologically active diterpene triepoxide derivatives from the Chinese herb *Tripterygium wilfordii* Hook. F.

(Wang *et al.*, 2008). Triptolide protects neurons and glial cells in the nervous system by a variety of mechanisms, including attenuating microglia activation (Zhou *et al.*, 2005), increasing synthesis and release of nerve growth factor in astrocytes (Xue *et al.*, 2007) and has been shown to promote spinal cord repair by down-regulating astrogliosis and inflammation in an animal model of spinal cord injury (Su *et al.*, 2010). From our preliminary chemical compound screening test in NSC34 cells, a spinal cord motor neuron-like cell line, we found that triptolide might possess potential effects on the regulation of SMN protein. In this study, we first determined the effects and mechanisms of action of triptolide on the regulation of SMN protein in human SMA fibroblasts. Then we explored the ability of triptolide to increase survival by measuring SMN protein expression in brain, spinal and muscle tissues of *SMN2* transgenic mice injected with triptolide.

Methods

Cell culture

NSC34, a mouse neuroblastoma N18TG2 and mouse embryonic spinal cord motor neurons cell line was cultured as previously described (Rizzardini *et al.*, 2006). Briefly, cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin). Cultured cells were incubated at 37°C containing 5% CO_2 and humidified air until confluence. The medium was changed every 3 days, and confluent cells were passed every 3–5 days with trypsin solution. The following experiments were performed in an 80% confluent state.

Human SMA fibroblast cell lines were established from SMA patients' skin biopsies with informed consent and prepared as previously described (Yuo *et al.*, 2008). The informed consent was approved by the institutional review board of the Kaohsiung Medical University Chung-Ho Memorial Hospital (KMUH-IRB-990122). Fibroblasts were maintained in DMEM/F12 medium supplemented with 10% FBS, 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin. Cells were maintained at 37°C containing 5% CO_2 in a humidified atmosphere.

Cell viability assay

Cell viability was measured with a quantitative colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-

zolium bromide (MTT), showing the mitochondrial activity of living cells. Cells were plated in 96-well plates and incubated with various concentrations of triptolide. After 24 h, cells were incubated with MTT at a final concentration of 0.1 mg·mL^{-1} at 37°C for 3 h. The reaction was terminated by addition of 100 µL of dimethyl sulfoxide (DMSO). The amount of MTT formazan product was determined by measuring the absorbance at 560 nm using a microplate reader.

Apoptosis assay with Annexin V

Cells undergoing apoptosis were detected with Annexin V-FITC/propidium iodide (PI) (Rizzardini *et al*.) double staining. Briefly, cells attached to plastic dishes were harvested by 0.25% trypsin and washed twice with cold PBS. The cell pellets were suspended in 1 × binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1 × 10^6 cells·mL^{-1}. Then cells were incubated with Annexin V-FITC and PI for 15 min (22–25°C) in the dark. The stained cells were immediately analysed by a Coulter CyFlow® Cytometer (Partec, Germany). Annexin V-positive cells were considered as apoptotic cells.

Animals and triptolide treatment

SMA-like (*Smn*$^{-/-}$ *SMN2*) mice were generated as previously described (Hsieh-Li *et al*., 2000). All animals used for this study and all protocols involving the use of animals were approved by the Animal Care and Use Committee at Kaohsiung Medical University (approval ID: 98096). Briefly, heterozygous mouse *Smn* knockout-human *SMN2* transgenic mice (*Smn*$^{+/-}$*SMN2*$^{+/-}$) were backcrossed with C57BL/6 mice (purchased from the National Laboratory Animal Breeding and Research Center, Taiwan). The *Smn*$^{+/-}$*SMN2*$^{+/-}$ mice, which could generate SMA-like mice were obtained after more than six generations of purifying the genetic background (Tsai *et al*., 2008). Mice were genotyped by PCR analyses of tail DNA. Animals were allowed food, water *ad libitum* and housed under constant temperature and controlled illumination (lights on between 07h30min and 19h30min) conditions.

Age-matched animals were divided into five experimental groups: wild-type (WT, *Smn*$^{+/+}$*SMN2*$^{-/-}$) group; SMA type III mice group; vehicle-treated SMA type III mice group (received 5% DMSO in saline solution); SMA type III mice injected with 0.01 or 0.1 mg·kg^{-1}·day^{-1} of triptolide (dissolved in 5% DMSO/saline) i.p. Survival and body weight were monitored daily during the experiment.

Mouse brain, spinal cord and the *gastrocnemius* muscle were harvested after the animals had been killed. All tissues were homogenized in radio immuno precipitation assay buffer immediately, and supernatants were collected after centrifugation. Protein amounts were determined before Western blotting analysis.

Survival analysis and weight progression

Five-day-old SMA mice received 0.1 mg·kg^{-1}·day^{-1} triptolide using 30 gauge 1 inch long needles via an i.p. injection. The date of birth was designated as postnatal day (P) 1. Control animals received equal volumes of vehicle alone. Daily weights were measured starting from P1.

Western blotting analysis

Cells or tissues were homogenized in cell lysis buffer (Thermo Scientific, Waltham, MA, USA). Protein concentration was determined by Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA, USA). Each lane was loaded with 20 µg protein, then separated by SDS-PAGE and transferred to polyvinyl difluoride membrane by immunoblotting. Non-specific binding was blocked with Tris-buffered saline Tween 20 (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) containing 5% BSA for 1 h at room temperature and then incubated overnight at 4°C with one of the following specific primary antibodies: mouse monoclonal anti-SMN (1:5000), rabbit polyclonal anti-Gemin2 H-100 (1:500), rabbit polyclonal anti-Gemin3 H-145 (1:500), rabbit polyclonal anti-SUV39H1 (1:1000), rabbit polyclonal anti-EZH2 (1:1000), mouse monoclonal anti-β-actin (1:10000). Membranes were incubated with secondary antibodies at room temperature for 1 h and then determination of enhanced chemiluminescence by exposure to BioMaxMR film (Kodak, Rochester, NY, USA). For detection expression of nuclear proteins, nuclear extracts were isolated using the Nuclear Extraction Kit (Panomics) according to the user manual.

Quantitative analysis of messenger RNA

For the quantitative real-time PCR (qPCR) experiment, mRNA was isolated and purified from fibroblasts, after the indicated treatments, homogenized with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and then extracted by acid guanidinium thiocyanate–phenol–chloroform extraction. Reverse transcriptase was performed using Reverse Transcription System kit (Promega, Madison, WI, USA) following the manufacturer's recommended procedure, 1 µg of total RNA was reverse transcribed to cDNAs. *FL-SMN2 and SMN2Δ7* transcript levels were determined quantitatively using qPCR. qPCR was performed on the ABI PRISM 7500 Sequence Detector System (Applied Biosystems, Foster City, CA, USA) using Power SYBR Green PCR Master Mix (Applied Biosystems). Primers were chosen to bind in *SMN* exon 7 (5′-GAAGGTGCTCACATTCCTTAAAT-3′) and *SMN* exon 8 (5′-ATCAAGAAGAGTTACCCATTCCA-3′) for amplification of *FL-SMN2* transcripts, and in *SMN* exon 5 (5′-CCACCACCCCA CTTACTATCA-3′) and at the *SMN* exon6/ exon8 border (5′-GCTCTATGCCAGCATTTCCATA-3′) in order to amplify truncated *SMN2Δ7* transcripts (Riessland *et al*., 2006). The relative amount of transcripts was calculated by using the threshold cycle (Ct) method, according to the manufacturer's instructions, comparing *SMN* versus *GAPDH* transcripts. The results were normalized in each culture as a ratio between relative amounts of transcripts in treated versus untreated sample. qPCR reactions of each sample were performed in triplicate and experiments were repeated at least three times.

Immunofluorescence staining and nuclear gems counting

Immunofluorescence staining followed a general procedure. Briefly, human SMA fibroblasts were grown on glass chamber slides. After treatment, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS. After being blocked with 2% BSA in PBS, cells were incubated with mouse monoclonal anti-SMN antibody and rabbit polyclonal anti-Gemin2 H-100 at 4°C overnight. Alexa Fluor 555 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG secondary antibody were incubated for 1 h. 4′,6-diamidino-

2-phenylindole nucleic acid staining ensured the localization of gems. Slides were mounted and sealed before observation with a confocal laser scanning microscopy (FluoView1000; Olympus, Center Valley, PA, USA). Total stained gems per 100 cells were analysed.

Data analysis

Survival was analysed by Kaplan–Meier curves and log-rank test followed by Bonferroni *post hoc* test for multiple comparisons. The weight curve was evaluated by ANOVA followed by Tukey's *post hoc* test for multiple comparisons. The statistical analyses of protein levels and real-time PCR data were performed with ANOVA followed by a Dunnett's test for all pair's comparisons. Data are expressed as mean ± SEM. Probability values (P) less than 0.05 were considered to be significant in all experiments. All data were analysed with the Statistical Package for Social Sciences (SPSS, Version 14.0, Chicago, IL, USA).

Materials

Triptolide and MTT were obtained from Sigma-Aldrich (St. Louis, MO, USA). DMEM, Ham's F-12 Medium, FBS, penicillin, amphotericin B and streptomycin were obtained from Invitrogen. All materials for SDS-PAGE were obtained from Bio-Rad. Rabbit antibodies against Gemin2, Gemin3 and SUV39H1 and all secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit antibody against EZH2 was obtained from Cell Signaling Technology (Beverly, MA, USA). Mouse antibody against SMN was obtained from BD Bioscience (San Jose, CA, USA). Mouse antibody against β-actin was obtained from Sigma-Aldrich. All other chemicals were purchased from Sigma Chemical Co.

Results

Triptolide at pM concentrations increases protein levels of SMN, Gemin2 and Gemin3 in a motor neuron-like cell line NSC34

First, we examined the effects of triptolide (0.01–1 pM) on the regulation of SMN protein by using NSC34 cells. Cells were treated with triptolide for 24 h. Results indicated that triptolide significantly up-regulated SMN protein levels compared with vehicle (0.001% DMSO in distilled water)-treated cells (Figure 2A). Then, we investigated the effects of triptolide on the expression of SMN complex components, Gemin2 and Gemin3. As shown in Figure 2B, triptolide significantly increased the expression of Gemin2 and Gemin3.

Triptolide increases SMN protein levels in human SMA fibroblasts at pM concentrations

We further examined the effect of triptolide on the regulation of SMN protein expression in human SMA fibroblasts by treating cells from three different types of SMA patients with triptolide (0.01–1 pM) for 24 h. Results indicated that triptolide significantly up-regulated SMN protein levels compared with vehicle-treated cells in all three types of SMA fibroblasts (Figure 3).

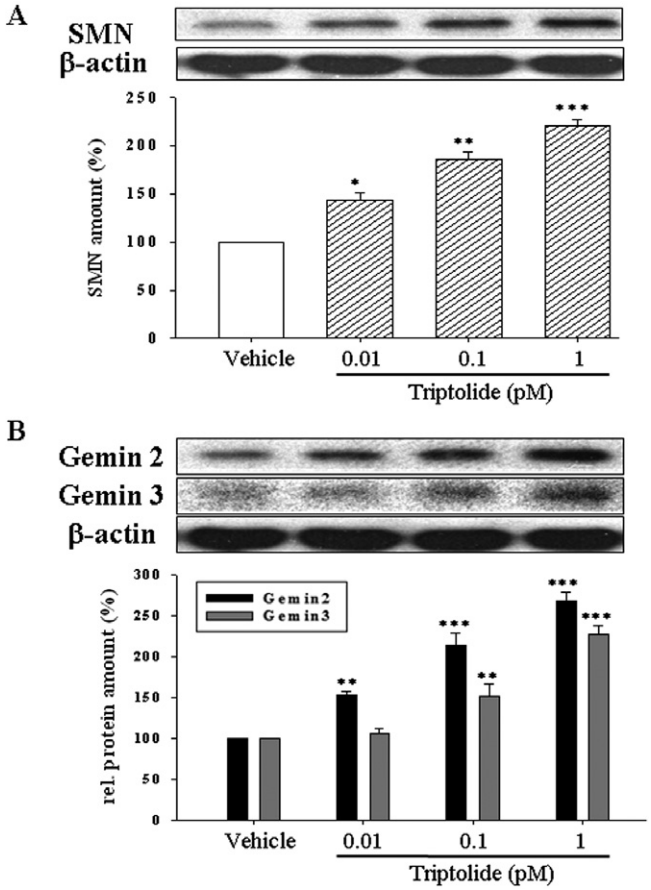

Figure 2

Effects of triptolide on (A) SMN protein levels and (B) SMN complex components in NSC34 cells. Cells were treated with vehicle (0.001% DMSO in distilled water) and triptolide (0.01, 0.1 and 1 pM) for 24 h. Protein extractions were then subjected to SDS-PAGE and protein expression of SMN, Gemin2, Gemin3 and β-actin were analysed by Western blotting. Changes in SMN, Gemin2 and Gemin3 levels normalized to β-actin were quantified and represented as percentages of vehicle group. Columns represent the mean ± SEM from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. vehicle group. ANOVA followed by Dunnett's test.

Triptolide increases expression of Gemin2 and Gemin3 and number of SMN-containing nuclear gems in SMA fibroblasts

Next, we investigated the effects of triptolide on the expression of SMN complex components in the three different types of SMA fibroblasts. As shown in Figure 4, triptolide significantly increased the expression of Gemin2 and Gemin3 in all three types of SMA fibroblast. The number of nuclear gems correlated with SMN protein levels and was markedly reduced in patients with SMA. To further ascertain that the increased amount of SMN did indeed represent functional protein, the intracellular localization of SMN in triptolide-treated cells was analysed using confocal microscopy. As shown in Figure 5, treatment with triptolide (1 pM) significantly increased the number of nuclear gems compared with vehicle-treated SMA fibroblasts.

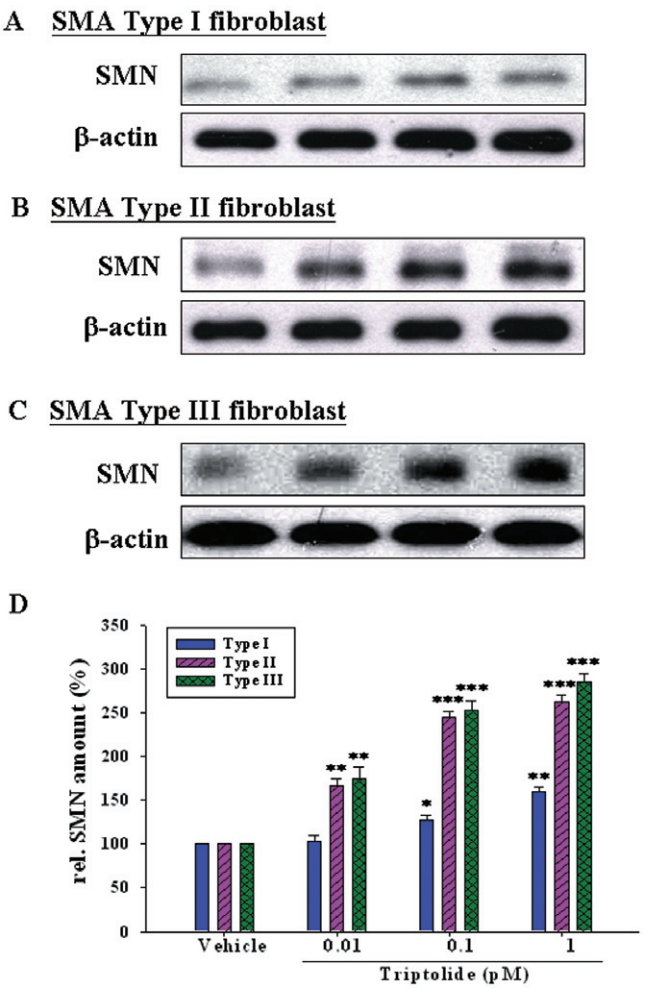

Figure 3

Up-regulation of SMN protein levels by triptolide in human SMA fibroblasts derived from different types of SMA patients. Fibroblasts were treated with triptolide (0.01, 0.1 and 1 pM) for 24 h, respectively. Proteins were extracted and subjected to SDS-PAGE. Then protein expression of SMN and β-actin were measured by Western blotting. Changes in SMN protein levels normalized to β-actin were quantified and represented as percentages of vehicle group. Columns represent the mean ± SEM from three independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. vehicle group. ANOVA followed by Dunnett's test.

Triptolide increases FL-SMN2 *transcripts by in human SMA fibroblasts*

To determine whether triptolide-induced increase in SMN protein resulted in an increased amount of exon 7-containing SMN protein, we examined the effects of triptolide on *SMN2* transcription using qPCR with primer sets that distinguish between the two variants (*FL-SMN2* and *SMN2Δ7*). *FL-SMN2* transcripts increased to a greater degree than did *SMN2Δ7* transcripts. The ratio of *FL-SMN2* to *SMN2Δ7* significantly increased up to 1.6-fold after triptolide treatment in SMA type III fibroblasts (Figure 6A and B), but not in SMA type I fibroblasts (Figure 6C and D). These results suggest that triptolide increases SMN protein level, partly, by activation of the *SMN2* transcript. They also suggest that triptolide, at least in SMA type III fibroblasts, is able to promote the inclusion of exon 7.

Cytotoxic effects are not detectable at SMN2 activating concentration

Triptolide has reported anti-tumour effects via the inhibition of histone methyltransferase SUV39H1 and EZH2 (Zhao *et al.*, 2010). Here, we examined whether triptolide affects the protein expressions of SUV39H1 and EZH2 in SMA fibroblasts. As shown in Supporting Information Figure S1, triptolide (1pM) treatment slightly down-regulated the expression of SUV39H1 and EZH2. Therefore, we evaluated the cytotoxicity of triptolide at a concentration that activates *SMN2* using the MTT assay and Annexin V/PI staining. Human SMA fibroblasts were exposed to triptolide (0.01–100 pM) for 24 h. As shown in Figure 7A neither the vehicle nor triptolide (0.01, 0.1, 1, 10, 100 pM) were cytotoxic or increased apoptotic death (Figure 7B).

Triptolide increases SMN protein levels in neuronal and muscular tissues of SMA-like mice

Since our *in vitro* data showed that triptolide could significantly increase SMN protein levels in human SMA fibroblasts, we further investigated the *in vivo* effect of triptolide in type III SMA-like mice. Mice received daily injections of triptolide (0.01 and 0.1 $mg \cdot kg^{-1} \cdot day^{-1}$, i.p.) for 1 week. There was no significant change in body weight or death rate of mice in all the groups during this *in vivo* experiment (data not shown). In order to examine the SMN levels, various tissues including the brain, spinal cord and *gastrocnemius* muscle were obtained from WT ($n = 5$), vehicle- and triptolide-treated (0.01 and 0.1 $mg \cdot kg^{-1} \cdot day^{-1}$) type III SMA-like mice group ($n = 4$ per group), respectively. As shown in Figure 8, all tissues from triptolide-treated SMA mice group show a significant elevation of SMN protein levels, especially at the higher dose (0.1 $mg \cdot kg^{-1} \cdot day^{-1}$). Most importantly, these *in vivo* data indicate that triptolide increased SMN protein levels in the two major tissues, spinal cord and muscle, that are involved in the pathology of SMA.

Triptolide treatment improves survival and attenuates weight loss of SMA-like mice

Previous studies have shown that P5 SMA mice show clear manifestations of this disease (Le *et al.*, 2005; Avila *et al.*, 2007), and P5 to P13 is the minimal window for drug treatment to benefit these SMA mice (Narver *et al.*, 2008). In the present study, SMA mice were treated daily with triptolide (0.1 $mg \cdot kg^{-1} \cdot day^{-1}$) starting at P5 and continued until P18. Triptolide-treated mice and untreated controls (vehicle injected) were monitored for survival and weight. As shown in Figure 9A, triptolide increased overall survival of the mice by 3.63 days (triptolide-treated, 11.86 ± 1.25 days; vehicle-treated, 8.23 ± 1.45 days), which represented a gain of 44.16 % over their lifetime. One of the first clinical symptoms of the disease is body weight reduction. The SMA mice are significantly underweight at 5 days of age (P5) (Le *et al.*, 2005). Vehicle-treated SMA mice showed significant weight differences by 5 days of age compared with WT mice (1.90 ± 0.37 g vs. 2.65 ± 0.70 g; Figure 9B). However,

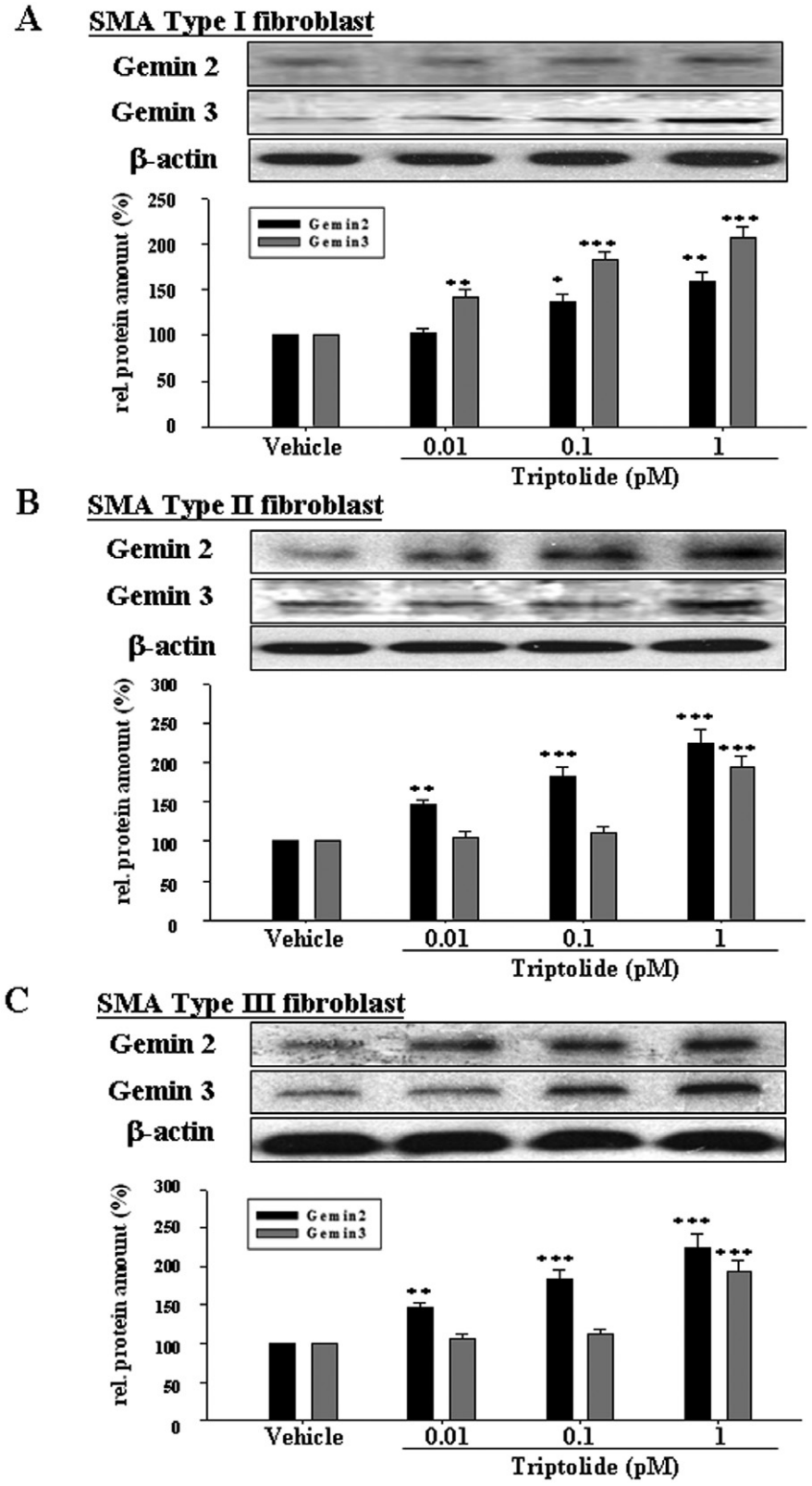

Figure 4

Effects of triptolide on SMN complex components Gemin2 and Gemin3 protein expression in human SMA fibroblasts derived from different types of SMA patients. Fibroblasts were treated with triptolide (0.01, 0.1, 1 pM) for 24 h. Protein expression of Gemin2, Gemin3 and β-actin were analysed by Western blotting. Changes in Gemin2 and Gemin3 protein levels normalized to β-actin were quantified. Columns represent the mean ± SEM from three independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. vehicle group. ANOVA followed by Dunnett's test.

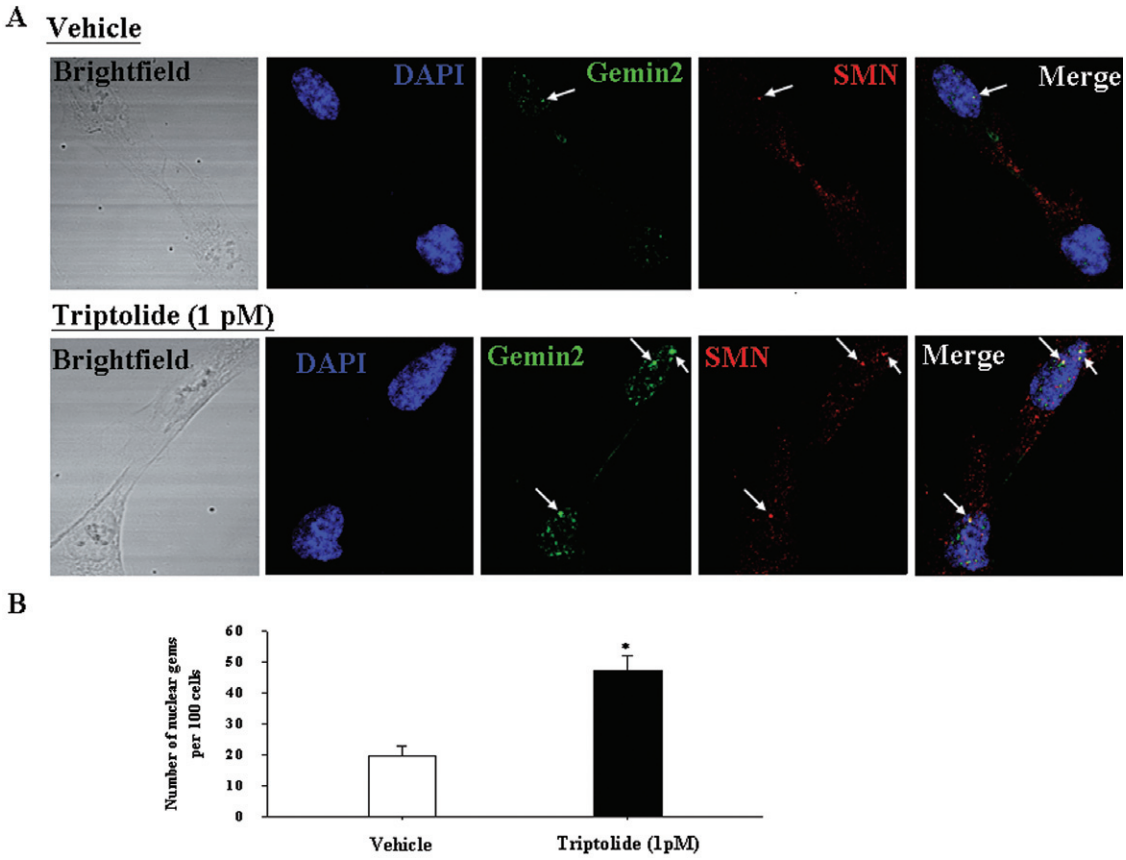

Figure 5

Effect of triptolide on (A) SMN-containing gems in the nucleus and (B) the number of nuclear gems in human SMA type III fibroblast cell lines. Fibroblasts were treated with triptolide (1 pM) for 24 h. The total number of SMN-containing nuclear gems/100 cells was observed by confocal microscopy with anti-SMN- and anti-Gemin2-specific antibodies. Alexa Fluor 555 goat anti-mouse IgG (red) and Alexa Fluor 488 goat anti-rabbit IgG (green) were used as secondary antibodies. 4′,6-diamidino-2-phenylindole (DAPI) (blue) was used for nuclei staining. Columns represent the mean ± SEM from three independent experiments. *$P < 0.05$ vs. vehicle group. ANOVA followed by Dunnett's test.

triptolide-treated SMA mice showed a mild increase in body weight compared to vehicle-treated SMA mice. The growth curve was significantly different at 10 to 12 days of age.

Discussion and conclusions

SMA is a progressive neuromuscular disease caused by deletions or mutations in the *SMN1* leading to a lack of functional SMN protein. Because disease severity is related to SMN protein levels, increasing functional SMN protein level in the form of the *SMN2* gene has been a major SMA-drug discovery strategy. In this study, we investigated the effects of triptolide on SMN protein production by cell-based assays using neuronal cell lines and cells from SMA patients, and *in vivo* using SMA-like mice. The present results show triptolide increases SMN protein levels by (i) increasing the number of gems and so increasing protein stability, and (ii) increasing *SMN2* transcription. The increase in SMN protein occurred after treatment with 0.01 pM triptolide, whereas the increase in SMN transcript was observed only after a higher dose treatment. These results indicate that at lower doses (0.01 pM) triptolide only exerts effects on SMN protein stability probably by increasing SMN complex formation. Furthermore, triptolide significantly extended the lifespan of SMA mice.

The SMN complex plays an essential role in biogenesis of spliceosomal snRNPs and pre-mRNA splicing. The *SMN* mutations might reduce SMN complex formation and SMN protein stability by attenuating SMN oligomerization or protein–protein interactions (Burnett *et al.*, 2009). In cytoplasmic and nuclear gems, SMN forms a stable multi-protein complex associated with Gemins. It has been reported that lack of Gemin2 leads to a decrease in SMN protein oligomerization (Ogawa *et al.*, 2007), dissociation of Gemin3 and a lethal phenotype of mice embryos (Ogawa *et al.*, 2009). In addition, knockdown of Gemin2 or Gemin3 leads to not only to a decrease in the biogenesis of snRNPs but also loss of spinal cord motor neurons and results in neuromuscular dysfunction (Ogawa *et al.*, 2009). In this study, we demonstrated that triptolide up-regulates not only SMN protein levels but also SMN complex components, such as Gemin2 and Gemin3 in mouse motor neuron NSC34 cells and human SMA fibroblasts. Moreover, triptolide treatment also increased the number of nuclear gems. Therefore, triptolide enhances SMN protein by increasing the formation of SMA complexes and the stability of this protein.

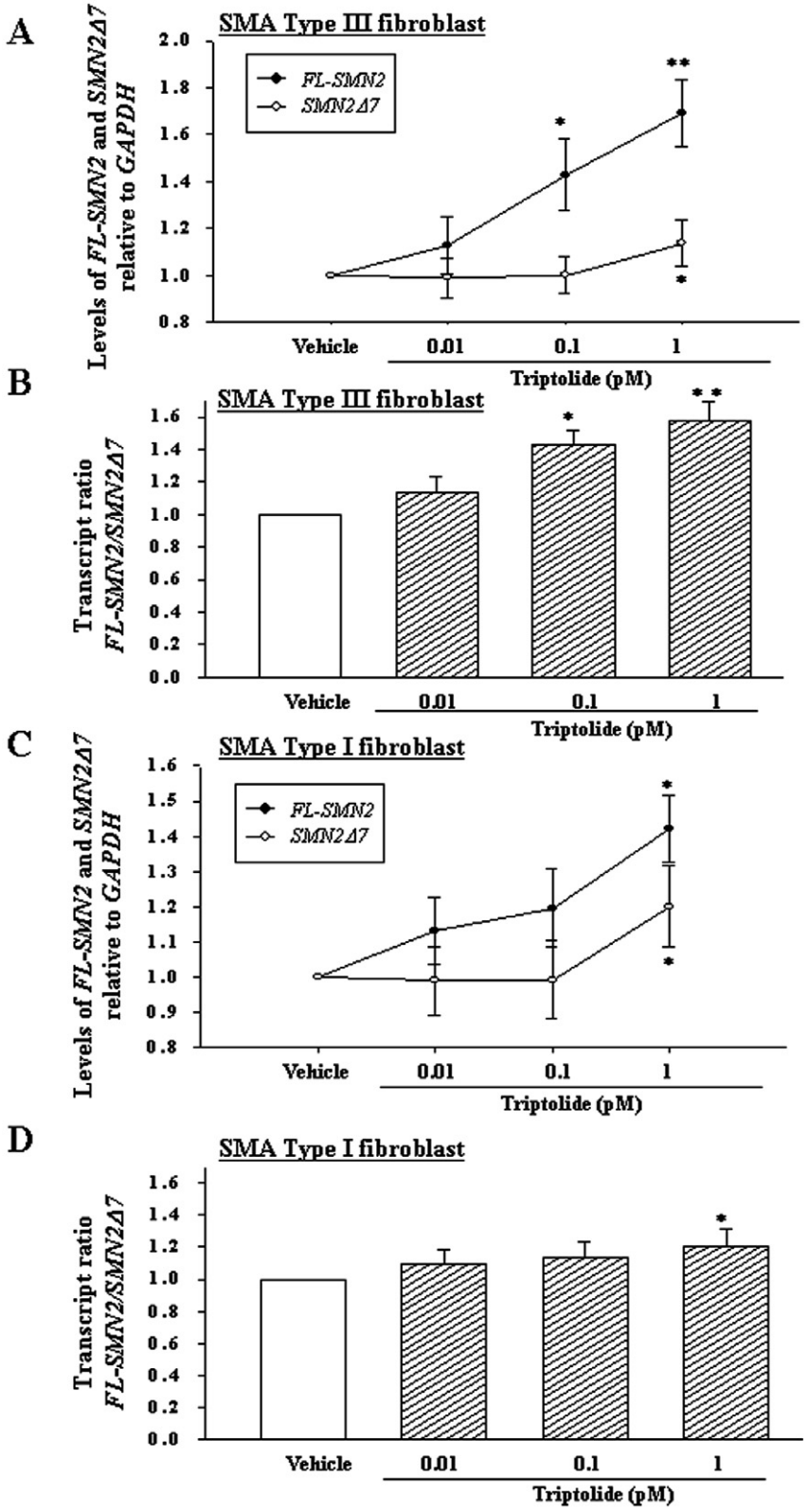

Figure 6

Quantitative analysis of *SMN* transcripts. *FL-SMN2* and *SMN2Δ7* transcripts levels were determined by quantitative real time-PCR in (A, B) SMA type III and (C, D) type I fibroblasts. Cells were treated with triptolide (0.01, 0.1 and 1 pM) for 24 h. The *FL-SMN2/SMN2Δ7* ratio normalized to *GAPDH* was quantified. All data are expressed as the mean ± SEM in arbitrary units relative to *GAPDH* from three independent experiments. *$P < 0.05$ and **$P < 0.01$ vs. vehicle group; ANOVA followed by Dunnett's test.

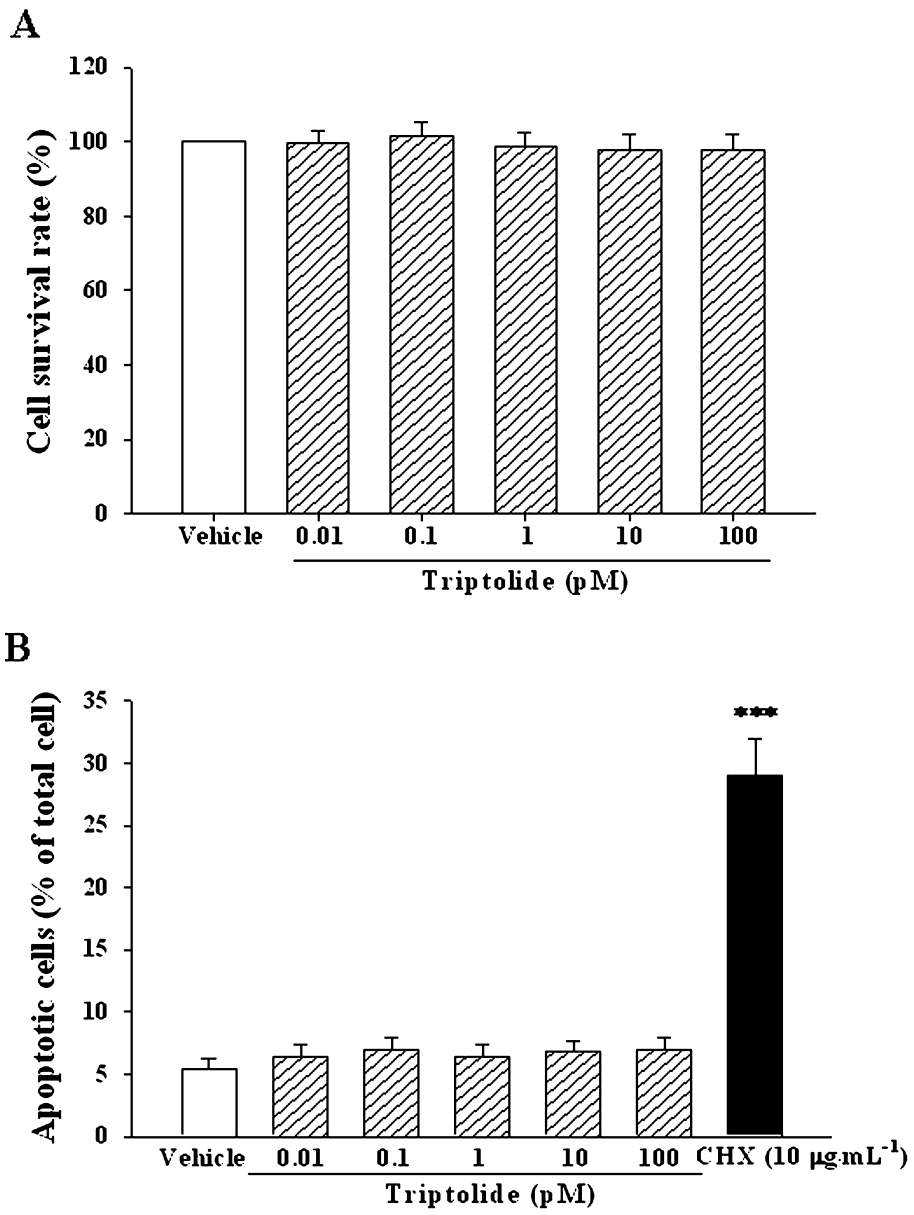

Figure 7

(A) Lack of cytotoxic effect of triptolide on human SMA type III fibroblasts. SMA fibroblasts were treated with vehicle and triptolide (0.01, 0.1, 1, 10 and 100 pM) for 24 h. Cell viability was determined by the MTT assay. Changes in survival rate are presented as percentages of the vehicle group. (B) Apoptosis was analysed by flow cytometry using Annexin V/PI staining. The apoptosis of each group is shown as an apoptosis index, evaluated by counting the percentage of apoptotic cells (Annexin V-positive cells). Cycloheximide 10 $\mu g \cdot mL^{-1}$ (CHX) was used as a positive control for apoptosis induction. Columns represent the mean ± SEM from three independent experiments. ***$P < 0.001$ vs. vehicle group. ANOVA followed by Dunnett's test.

To date, increasing functional FL-SMN levels and providing neuroprotection are two main directions for SMA drug discovery (Pruss *et al.*, 2010). Triptolide has been noticed for its novel neuroprotective effects. Growing evidence has shown that triptolide can protect neurons in the CNS and promote axon growth of dopaminergic nerves (Zhou *et al.*, 2003; 2005). Triptolide also promotes spinal cord repair through down-regulation of astrogliosis and inflammation in an animal model of spinal cord injury (Su *et al.*, 2010). Moreover, triptolide can cross the blood-brain barrier easily due to its small molecular size and lipophilic property (Wang *et al.*, 2008). Therefore, triptolide shows high potential as a new pharmaceutical drug for neuroprotection (Chen *et al.*, 2007). Here, we demonstrated that triptolide increases *FL-SMN2* levels in SMA fibroblasts. The lack of exon 7 in *SMN2* transcripts results in the production of unstable and dysfunctional SMN proteins and leads to insufficient amounts of protein to maintain the survival of motor neurons and con-

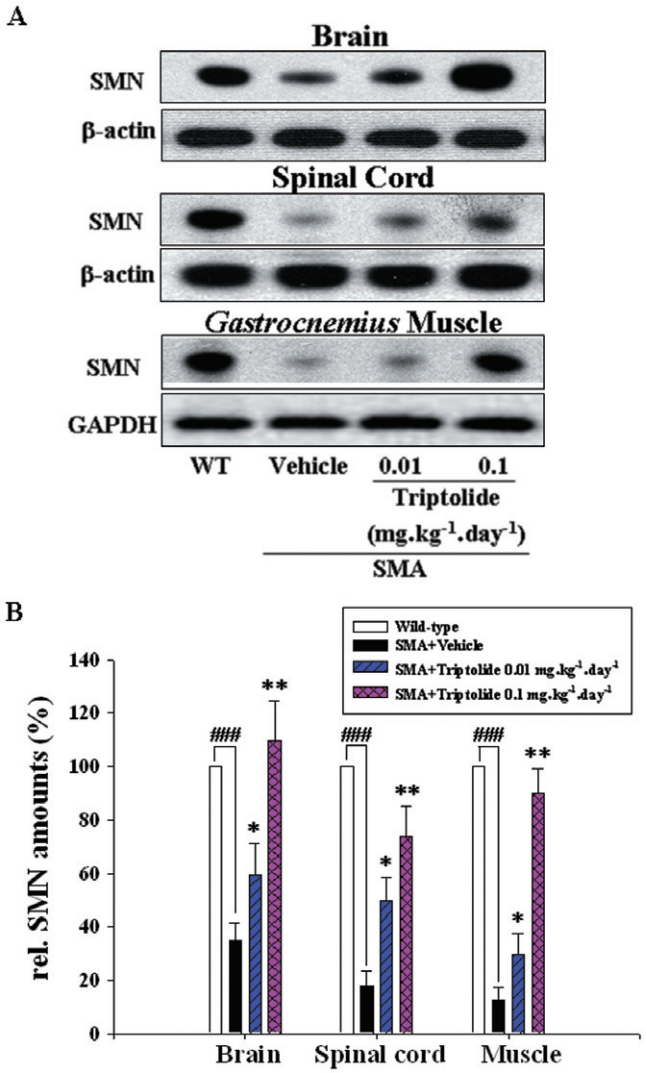

Figure 8

Up-regulation of SMN protein levels in neuronal and muscular tissues of triptolide-treated SMA-like mice. Mice (*Smn*$^{-/-}$*SMN2*) were injected with vehicle or triptolide (0.01 or 0.1 mg·kg^{-1}·day^{-1}i.p.) for 1 week. Brain, spinal cord and *gastrocnemius* muscle were isolated from wild-type (WT, *Smn*$^{+/+}$*SMN2*$^{-/-}$) and vehicle- or triptolide-treated groups. (A) Total protein from multiple tissues were extracted and the SMN protein levels were determined by Western blotting. (B) Quantification of SMN protein in the brain, spinal cord and *gastrocnemius* muscle showed a significant elevation after triptolide treatment. β-actin (for brain and spinal cord) or GADPH (for *gastrocnemius* muscle) were used as loading controls. *$P < 0.05$ and **$P < 0.01$ vs. vehicle group; $^{\#\#\#}P < 0.001$ vs. WT group; ANOVA followed by Dunnett's test.

tributes to disease severity (Lefebvre *et al.*, 1997; Hsieh-Li *et al.*, 2000; Jablonka *et al.*, 2000; Feldkotter *et al.*, 2002). Therefore, increasing the ratio of *FL-SMN2* to *SMN2Δ7* has been shown to be a promising target for SMA therapy. Here, we showed that pM concentrations of triptolide increase *SMN2* expression at both the transcript and protein levels in SMA fibroblast cell lines. An increased ratio of *FL-SMN2* to *SMN2Δ7* induced by triptolide was also observed in this study. Treatment with triptolide increased not only the levels of *FL-SMN2* transcript but also those of *SMN2Δ7* transcripts. Therefore, it is likely that triptolide increases SMN levels mainly through activation of *SMN2* transcription. Furthermore, the present results suggest that triptolide, at least in SMA type III fibroblasts, is able to promote the inclusion of exon 7.

In a previous study it was shown that at concentrations of 40–160 nM triptolide down-regulates the expression of histone methyltransferase SUV39H1 and EZH2 and is cytotoxic (Zhao *et al.*, 2010). When we evaluated the cytotoxicity of triptolide, we noticed that 1 pM of triptolide slightly down-regulated histone methyltransferase SUV39H1 and EZH2 in human SMA fibroblasts (Supporting Information Figure S1). However, no cytotoxic effects were detectable below this concentration. Notably, pM concentrations of triptolide (0.01–1 pM) increased SMN protein levels in human SMA fibroblasts without showing any signs of cytotoxicity.

In a mouse model of a severe type of SMA, the relationship between SMN levels and nervous system development suggest that the nervous system might be considered as a new therapeutic region for SMA treatment (Wishart *et al.*, 2010). To corroborate our data obtained in human SMA fibroblasts, we further examined whether triptolide is able to regulate the expression of SMN in a mouse model of SMA. It was found that i.p. injections of triptolide increased SMN levels in brain tissues of SMA mice up to threefold compared with vehicle-treated SMA mice. Furthermore, a significant increase in SMN protein levels in the spinal cord and *gastrocnemius* muscle was also observed. These data imply that the beneficial effects of triptolide reach the most important tissues involved in the pathology of SMA. Furthermore, triptolide treatment ameliorated the weight loss and increased the survival of the SMA-like mice.

In conclusion, our results demonstrate that triptolide can effectively increase SMN protein production both *in vitro* and *in vivo*. Triptolide increases SMN protein level by enhancing the stability of the SMN protein and increasing *SMN2* transcription. As a new modulator of SMN expression, triptolide might be a promising candidate for SMA therapy.

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Conflict of interest

The authors declare no conflicts of interest.

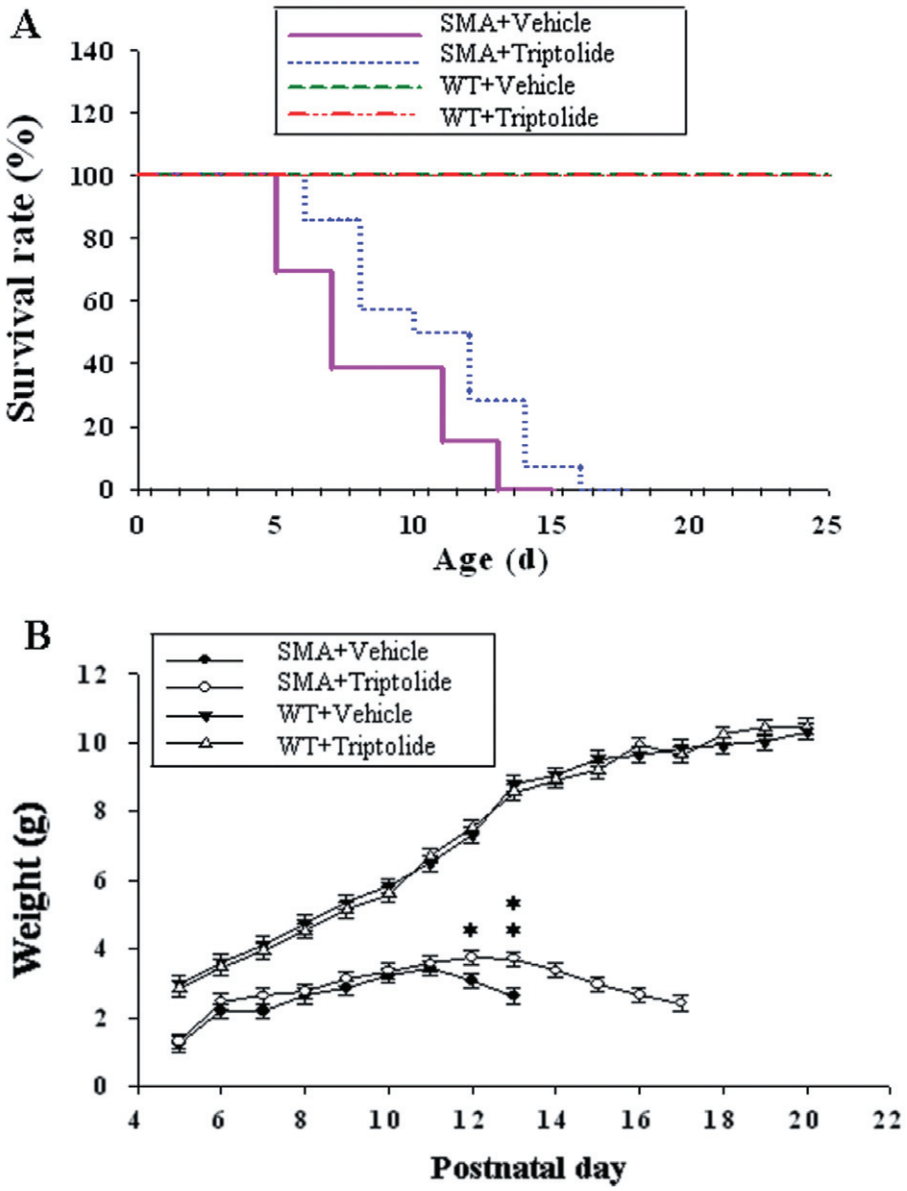

Figure 9

Triptolide increases survival rate and attenuates weight loss of SMA-like mice. SMA-like mice (*Smn*$^{-/-}$*SMN2*) and wild-type mice (WT, *Smn*$^{+/+}$*SMN2*$^{-/-}$) were treated with daily i.p. injections of triptolide (0.1 mg·kg^{-1}) or vehicle on days P5–P18. (A) Kaplan–Meier survival curves of mice treated with triptolide ($n = 15$) or vehicle ($n = 13$). $P < 0.001$, log-rank test. (B) Weights of SMA mice treated with triptolide ($n = 15$) or vehicle ($n = 13$), and WT mice treated with triptolide ($n = 20$) or vehicle ($n = 20$). *$P < 0.05$ and**$P < 0.01$ vs. SMA mice treated with vehicle group; ANOVA followed by Tukey's test.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 The expression of histone methyltransferase (A) SUV39H1 and (B) EZH2 in triptolide-treated human SMA type III fibroblasts. Cells were treated with triptolide (1 pM) for 24 h. Cell lysates were subjected to SDS-PAGE and protein expressions of SUV39H1, EZH2 and β-actin were analysed by Western blotting. Changes in SUV39H1 and EZH2 protein levels normalized to β-actin were quantified and represented as percentages of vehicle group. Columns represent the mean ± SEM from three independent experiments. *$P < 0.05$ and **$P < 0.01$ vs. vehicle group. ANOVA followed by Dunnett's test.

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