

Celecoxib increases SMN and survival in a severe spinal muscular atrophy mouse model via p38 pathway activation

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The loss of functional Survival Motor Neuron (SMN) protein due to mutations or deletion in the *SMN1* gene causes autosomal recessive neurodegenerative spinal muscle atrophy (SMA). A potential treatment strategy for SMA is to upregulate the amount of SMN protein originating from the highly homologous *SMN2* gene, compensating in part for the absence of the functional *SMN1* gene. We have previously shown that *in vitro* activation of the p38 pathway stabilizes and increases SMN mRNA levels leading to increased SMN protein levels. In this report, we explore the impact of the p38 activating, FDA-approved, blood brain barrier permeating compound celecoxib on SMN levels *in vitro* and in a mouse model of SMA. We demonstrate a significant induction of SMN protein levels in human and mouse neuronal cells upon treatment with celecoxib. We show that activation of the p38 pathway by low doses celecoxib increases SMN protein in a HuR protein-dependent manner. Furthermore, celecoxib treatment induces SMN expression in brain and spinal cord samples of wild-type mice *in vivo*. Critically, celecoxib treatment increased SMN levels, improved motor function and enhanced survival in a severe SMA mouse model. Our results identify low dose celecoxib as a potential new member of the SMA therapeutic armamentarium.

INTRODUCTION

Childhood spinal muscular atrophy (SMA) is one of the most common genetic causes of infant death globally. The autosomal recessive neurodegenerative disease is characterized by the loss of motor neurons from the anterior horn of the spinal cord leading to muscle weakness, muscle atrophy and respiratory insufficiency (1). The estimated heterozygote frequency is 1/50 with an incidence of 1:11 000 in newborns (2). SMA is broadly classified into four major categories based on age of onset as well as clinical severity. SMA type I is the most severe and frequent form of the disease accounting for more than half of the known diagnosed cases of SMA; children with Type I SMA usually succumb by the age of five.

The loss of the *SMN1* gene due to homozygous deletion or mutations is documented in 95% of the SMA patients (3). All patients harbour a nearly identical twin centromeric copy of the *SMN1* gene, *SMN2* (3). The C to T transition at position 6

of *SMN2* exon 7 leads to exclusion of this exon in ~90% of the transcripts. However, the centromeric copy gene still produces 5–10% of functional full-length Survival Motor Neuron (SMN) transcript (4,5). All SMA patients have one or more copies of the *SMN2* gene; in general, the higher the *SMN2* copy number, the milder the SMA.

Post-transcriptional regulation of the *SMN* genes is mediated, at least in part, by the presence of AU-rich elements (ARE) in the 3'-untranslated region (UTR) (6) which act as a signal for mRNA degradation. The p38 MAPK pathway is known to play an important role in post-transcriptional regulation observed in ARE-containing mRNAs, regulating the abundance and/or activity of RNA-binding proteins that control mRNA stability (7–11). The RNA-binding protein HuR (a ubiquitously expressed member of the ELAV family of proteins) is a known ARE-binding protein (AREBP) which in some cases antagonizes the degradation of these mRNA by stabilizing them (12–15). Activation of the

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p38 pathway has been shown to lead to a cytoplasmic accumulation of HuR protein with its increased binding to target mRNAs, resulting in their stabilization (6,16). Our group has previously shown that the p38 MAPK activation induces SMN expression in this fashion; triggering HuR-mediated stabilization of SMN mRNA and increases the pool of transcripts available for translation thus increasing functional SMN protein levels (6).

In our previous work, the known p38 MAPK activator anisomycin was used; however, this drug cannot cross the blood brain barrier (BBB), thus limiting its ability to act *in vivo* on the central nervous system in particular the motor neurons, where the lack of SMN contributes to SMA pathophysiology. A search of the literature identified the FDA-approved selective COX-2 (cyclo-oxygenase 2) inhibitor non-steroidal anti-inflammatory drug celecoxib as an alternative to anisomycin which besides showing p38 activation in some systems also crosses the BBB, making it a good candidate for further exploration as an SMA therapeutic (17–19).

We show here a celecoxib conferred increase in SMN protein in both neuronal cells and wild-type (WT) mice. We present *in vitro* evidence that celecoxib activates the p38 pathway leading to increase SMN mRNA and a subsequent increase in SMN protein. Importantly, we show that treatment with celecoxib increases SMN levels, improves motor function and survival in a severe SMA mouse model. Our results confirm earlier work proposing p38 MAPK pathway activators as potential therapeutic compounds for the treatment of SMA, identifying celecoxib as one such promising agent.

RESULTS

Celecoxib treatment upregulates SMN protein *in vitro*

To investigate a potential role of celecoxib in the regulation of the *SMN* gene expression *in vitro*, NT2, MN-1 cells along with

SMA I patient fibroblasts were treated with celecoxib (5 nM) for 24 h and subsequently harvested for western blot analysis. SMN protein levels were found to be increased in all cell lines upon treatment with celecoxib (Fig. 1a–c). These results show that low dose (nM range) of celecoxib increases SMN protein levels in both human and mouse neuronal cell lines as well as in SMA patient fibroblasts.

Celecoxib activates the p38 MAPK and increases SMN protein levels in a time-dependent manner *in vitro*

Since the p38 pathway has been implicated in the regulation of the *SMN* gene, and given that celecoxib has been shown to be an activator of this pathway, we wished to confirm that at concentrations we have shown induce SMN, celecoxib also activates the p38 MAPK pathway. NT2 cells were thus treated with celecoxib at 5 nM and then harvested for western blot analysis at the indicated time intervals revealing a time-dependent increase in the ratio of phosphorylated-p38/total p38 protein (up to 8 h after celecoxib treatment). This confirms that celecoxib activates the p38 MAPK pathway in NT2 cells (Fig. 2a and b). We also found that activation of the p38 MAPK pathway by celecoxib treatment was followed by a time-dependent increase in SMN protein level in NT2 cells (Fig. 2a and c).

Activation of p38 pathway and HuR protein are required for celecoxib-conferred SMN protein increase

To further investigate a potential role for p38 in *SMN* gene regulation by celecoxib, NT2 cells were pre-treated with the p38 inhibiting agent SB-239063 for 2 h followed by the treatment with celecoxib for 24 h. Western blot analysis revealed that p38 inhibition effectively blocked the celecoxib-mediated increase in SMN protein (Fig. 3a and b). We have shown previously that upon p38 pathway activation, HuR protein binds to

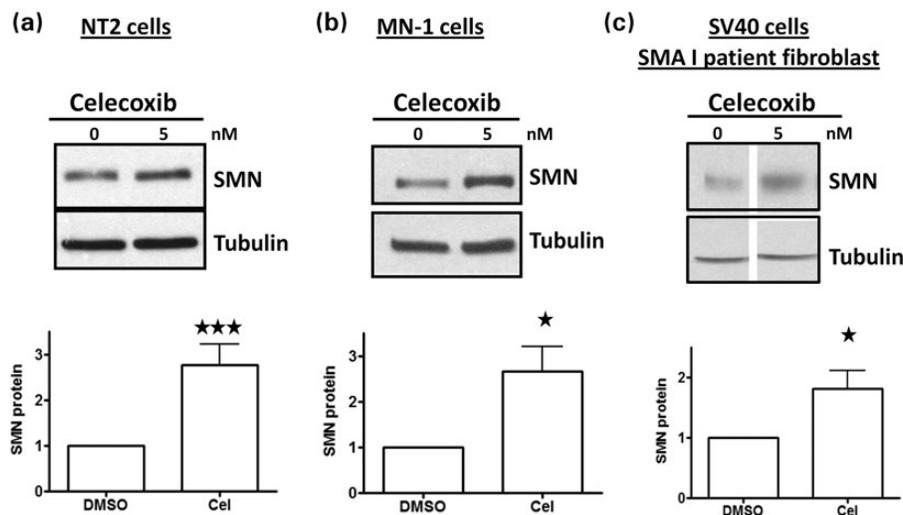


Figure 1. Celecoxib (Cel) treatment upregulates SMN protein *in vitro*. NT2, MN-1 and SMA I patient fibroblasts were treated with celecoxib (5 nM) and then harvested at 24 h for western blot analyses. (a) Representative western blots showing the effect of celecoxib on SMN protein in NT2 cells. Densitometric quantification of SMN protein relative to Tubulin [the ratio at control treatment was set as 1; mean + SEM (bars) of six independent experiments] are shown below for NT2 cells. (b) Representative western blot showing effect of celecoxib on SMN protein in MN-1 cells. Densitometric quantification of SMN protein relative to Tubulin [the ratio at control treatment was set as 1; mean + SEM (bars) of three independent experiments] are shown below for MN-1 cells. (c) Representative western blots showing the effect of celecoxib on SMN protein in SMA I patient fibroblasts (all lanes were run on the same gel but were non-contiguous). Densitometric quantification of SMN protein relative to Tubulin [the ratio at control treatment was set as 1; mean + SEM (bars) of three independent experiments] are shown below for SMA I patient fibroblasts. * $P < 0.05$; *** $P < 0.001$.

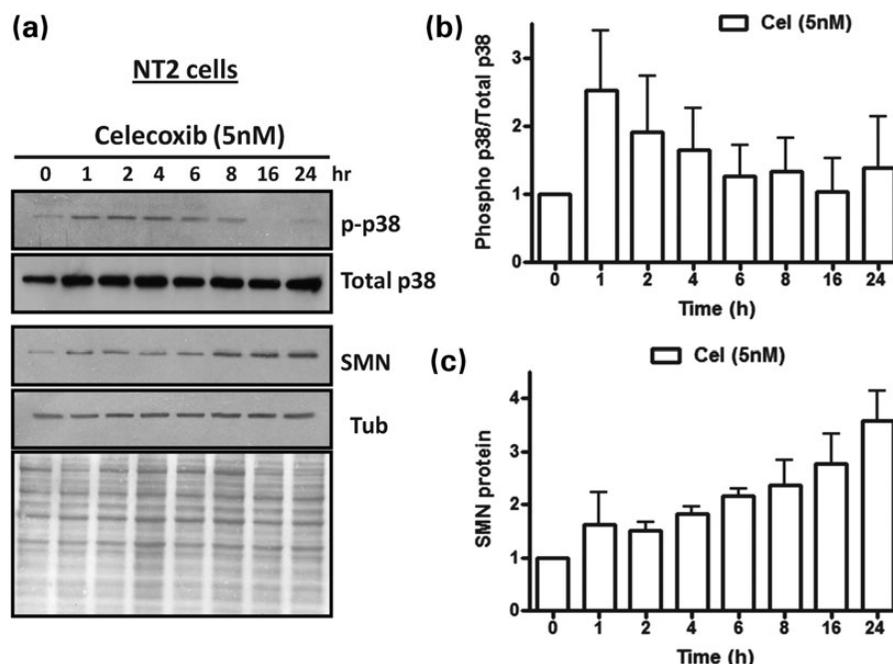


Figure 2. Celecoxib treatment activates p38 MAPK pathway. (a) Representative western blot showing activation of p38 MAPK pathway upon celecoxib (5 nM) treatment in NT2 cells. NT2 cells were treated with celecoxib at indicated times and then harvested for western blot analysis. Activation of p38 pathway by celecoxib is followed by increase in SMN protein. (b) Densitometric quantification of phospho-p38 relative to total-p38 [the ratio at control treatment was set as 1; mean + SEM (bars) of three independent experiments] are shown for NT2 cells. (c) Densitometric quantification of SMN protein relative to Tubulin [the ratio at control treatment was set as 1; mean + SEM (bars) of three independent experiments] are shown for NT2 cells.

3'-UTR of SMN transcript which leads to increase in SMN levels. To further elaborate the role of HuR protein in celecoxib-induced increase in SMN protein, NT2 cells were transfected with HuR-specific siRNA or control siRNA for 48 h, and then treated with celecoxib for 24 h. siRNA-mediated attenuation of HuR expression blocked the celecoxib-mediated increase in SMN protein expression (Fig. 3c and d). These observations strongly implicate the p38 pathway and HuR protein in the celecoxib-induced *in vitro* increase in SMN levels.

Celecoxib treatment upregulates SMN protein levels in WT mice

In order to both confirm that celecoxib-mediated SMN induction extends to the *in vivo* setting, and to begin to explore celecoxib treatment in animals, CD-1 mice were given daily intraperitoneal (IP) celecoxib injections for 5 days over a range of doses. Brain and spinal cord samples were subsequently isolated for western blot analysis. Celecoxib treatment (20 µg/kg) increased SMN protein levels in brain (Fig. 4a and b) and spinal cord samples (Fig. 4c and d) in CD-1 mice.

Celecoxib treatment upregulates SMN protein levels in a SMA mice model

In order to explore the *in vivo* impact of celecoxib-induced SMN upregulation, SMAΔ7 mice [*mSmn*^{-/-}; *hSMN2*^{+/+}, *hSMNΔ7*^{+/+} (20)] were given 20 µg/kg celecoxib IP injections every day from P1 until P6. Mice were euthanized 24 h after their last treatment. Brain, spinal cord, muscle and heart samples were then harvested for western blot analysis. Importantly, celecoxib

treatment was observed to increase *SMN2*-derived full-length SMN protein levels significantly in both brain (Fig. 5a and b) and spinal cord samples (Fig. 5c and d) when compared with vehicle-treated animals. No induction was observed in skeletal muscles and a lesser degree of induction in SMN protein was observed in the hearts of SMA mice following celecoxib treatment when compared with vehicle-treated mice (Supplementary Material, Fig. S2).

Celecoxib treatment upregulates SMN expression within motor neurons in a SMA mice model

To check the expression of SMN protein within motor neurons after treatment with celecoxib, SMAΔ7 mice were given daily 20 µg/kg celecoxib IP dose from P1 till P6. Mice were euthanized 24 h after the last treatment. Spinal cord samples were harvested for immunohistochemistry analysis. Motor neurons were labelled with HB9 antibody (motor neuron marker). Celecoxib treatment increased SMN protein levels significantly in motor neurons of spinal cord samples when compared with vehicle-treated mice (Fig. 6A–E).

Celecoxib treatment improves disease phenotype in a SMA mice model

We next examined the impact of celecoxib-induced increase in SMN levels on disease phenotype in SMAΔ7 mice. The SMAΔ7 mice have severely impaired righting time and muscle weakness detectable by P5. They are also significantly underweight compared with heterozygous and WT littermates with a median survival of 13 days. SMAΔ7 mice were given

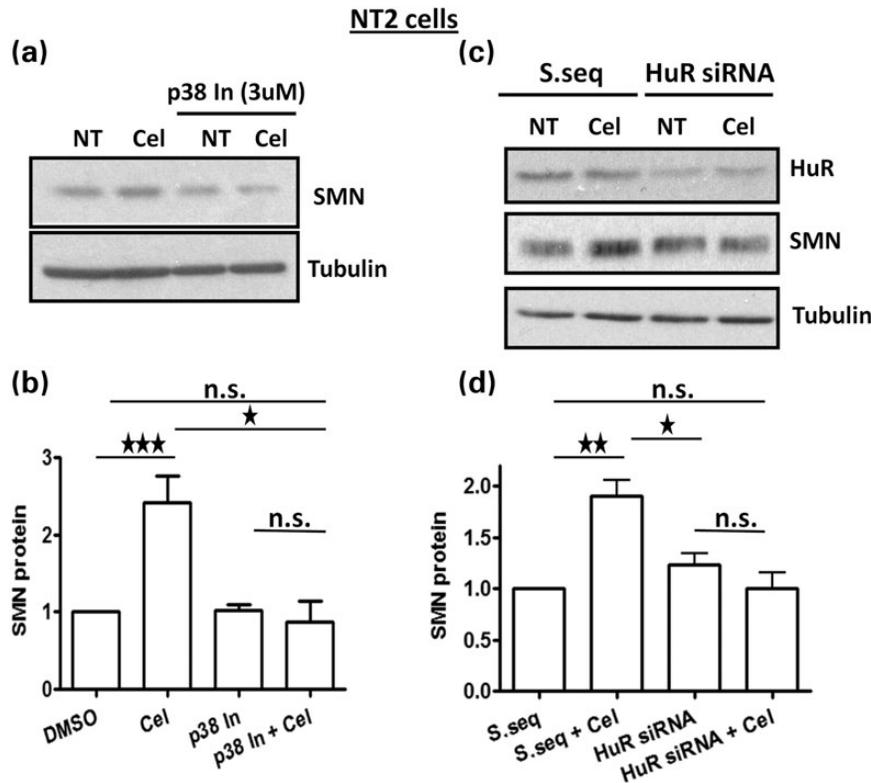


Figure 3. Celecoxib increases SMN expression via p38 MAPK pathway. (a) Representative western blots showing the effect of p38 inhibition on celecoxib-induced increase in SMN protein. p38 inhibitor (SB-239063) blocked the celecoxib-induced increase in SMN protein in NT2 cells. NT2 cells were treated with SB-239063 (p38 In; 3 μ M) for 2 h followed by treatment with celecoxib (5 nM) for 24 h and then harvested for western blot analysis. (b) Densitometric quantification of SMN protein relative to Tubulin [the ratio at control treatment was set as 1; mean + SEM (bars) of three independent experiments] are shown for NT2 cells. (c) Representative western blots showing both HuR knockdown and its effect on celecoxib-induced increase in SMN protein. The siRNA knockdown of HuR protein attenuates celecoxib-induced increase in SMN protein. NT2 cells were transfected with HuR siRNA (100 nM) or control sequence (S.seq; 100 nM) for 48 h and then treated with celecoxib (5 nM) for 24 h. Cells were harvested for western blot analysis. (d) Quantification of SMN protein relative to Tubulin [the ratio at control treatment was set as 1; mean + SEM (bars) of three independent experiments] are shown for NT2 cells. * P < 0.05; ** P < 0.01.

daily celecoxib or vehicle IP injections starting at P1 and their weight, motor function and longevity were assessed daily. SMA Δ 7 mice treated with celecoxib showed a dramatic improvement of motor function (as assessed by righting time), and significant extension of survival (median survival of 18 days) when compared with vehicle-treated SMA Δ 7 mice (median survival of 13 days, Fig. 7a and b). However, SMA Δ 7 mice treated with celecoxib showed no improvement in weight loss when compared with vehicle-treated SMA Δ 7 mice (Supplementary Material, Fig. S3).

DISCUSSION

SMA is a frequently severe neurodegenerative disease which mostly affects children, many of whom die in the first years of life. Currently, there is no effective therapy for SMA in clinical use. Increasing SMN levels through the *SMN2* gene can partially rescue the disease phenotype (21). One strategy for SMA therapy is to increase levels of SMN protein from the *SMN2* gene through stabilization of the full-length SMN transcript which ultimately leads to an increase in SMN mRNA and protein levels (6). The p38 pathway regulates a number of cell processes including post-transcriptional regulation of a certain class of transcripts with an ARE sequence in their 3'-UTR (7–11). We have

previously documented that anisomycin, a known p38 activator, increases SMN levels through mRNA stabilization *in vitro* (6). However, a number of issues need to be addressed in order to recapitulate our *in vitro* results with anisomycin in a SMA mouse model. The transient increase in SMN levels observed with anisomycin treatment, along with its inability to cross BBB, limits its use as a therapeutic for SMA, a motor neuron disease. An alternative to anisomycin is p38-activating, FDA-approved COX-2 inhibitor celecoxib which can cross the BBB thus making it an ideal candidate to test as a therapeutic for the treatment of SMA. In this study, we have demonstrated that celecoxib induces SMN expression *in vitro* in a p38 and HuR protein-dependent fashion.

We document here a celecoxib-mediated increase in SMN protein levels in human neuronal NT2 cell line and murine motor neuron MN-1 cells as well as in SMA I patient fibroblasts. It was shown in previous studies that low dose celecoxib activates the p38 pathway (17–19) an effect not seen at higher doses (18,22–24). Here, we treated neuronal cells with a low concentration (nanomolar range) of celecoxib observing a rapid increase in phospho p38 levels followed by an increase in SMN protein levels. The p38 pathway regulates the stability of COX-2, TNF α , p21 and SMN transcripts which are known to contain ARE in their 3'-UTR region (6,8,10,16). To confirm the role of

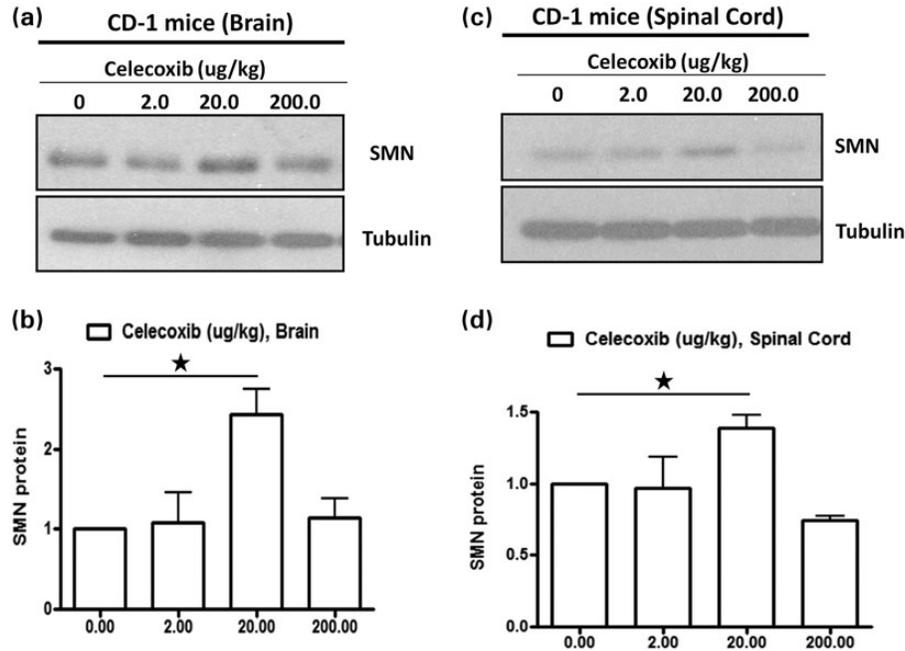


Figure 4. Celecoxib upregulates Smn protein in WT mice. Four-week-old CD-1 WT mice were treated daily with celecoxib (2.0, 20.0, 200.0 $\mu\text{g}/\text{kg}$) for 5 days, and then sacrificed. Brain and spinal cord tissues were harvested for western blot analysis. (a) Representative western blot showing the effect of celecoxib on Smn protein in brain samples of CD-1 mice treated with saline (control, lane-1) or celecoxib (lanes 2, 3 and 4, respectively) ($n = 3$). (b) Densitometric quantification of SMN relative to Tubulin [mean + SEM (bars)] is shown for brain samples. (c) Representative western blot showing the effect of celecoxib on SMN protein in spinal cord samples of CD-1 mice treated with Saline (control, lane-1) or celecoxib (lanes 2, 3 and 4) ($n = 3$). (d) Densitometric quantification of SMN relative to Tubulin [mean + SEM (bars)] is shown for spinal cord samples. * $P < 0.05$.

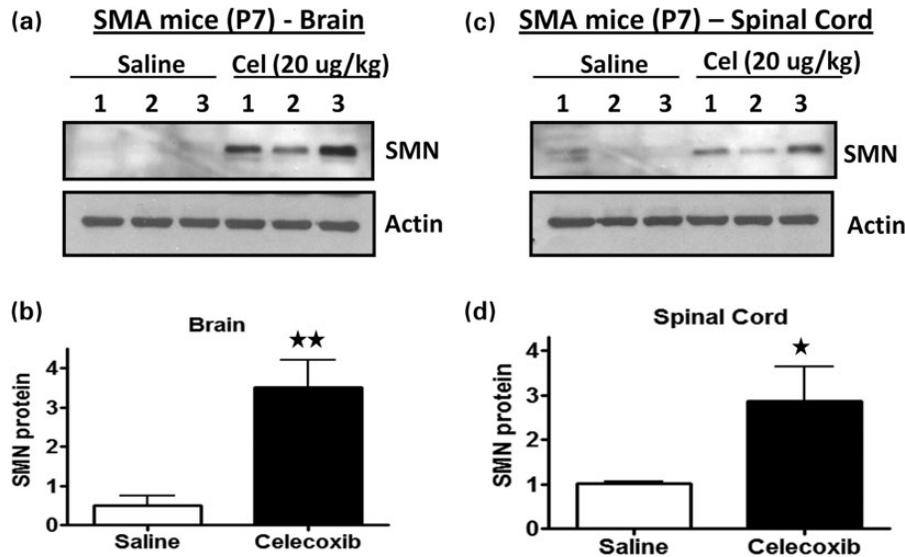


Figure 5. Celecoxib upregulates SMN protein in the SMA mouse model. SMA Δ 7 mice were treated daily with saline or celecoxib (20 $\mu\text{g}/\text{kg}$) from P1 for 6 days, and then sacrificed at P7. Brain and spinal cord were harvested for western blot analysis. (a) Representative western blots showing effect of celecoxib on SMN protein in brain samples of SMA Δ 7 mice treated with Saline (control, lanes 1, 2 and 3) or celecoxib (treatment lanes 1, 2 and 3, respectively) (each lane represents individual animal). (b) Densitometric quantification of SMN relative to actin [mean + SEM (bars)] is shown for brain samples. (c) Representative western blots showing effect of celecoxib on SMN protein in spinal cord samples of SMA Δ 7 mice treated with Saline (control, lanes 1, 2 and 3) or celecoxib (treatment lanes 1, 2 and 3, respectively) (each lane represents individual animal). (d) Densitometric quantification of SMN relative to actin [mean + SEM (bars)] is shown for spinal cord samples. * $P < 0.05$; ** $P < 0.01$.

p38 activation in celecoxib-mediated increase in SMN protein, cells were pre-treated with a p38 inhibitor before celecoxib treatment. This resulted in an attenuation of SMN induction consistent with a role for celecoxib-based activation of p38.

We have shown in an earlier report that *in vitro* p38 activation-conferred SMN induction is through transcript stabilization via binding of AREBP HuR to the 3'-UTR region of SMN transcripts (6). A second report showed a similar mechanism for the

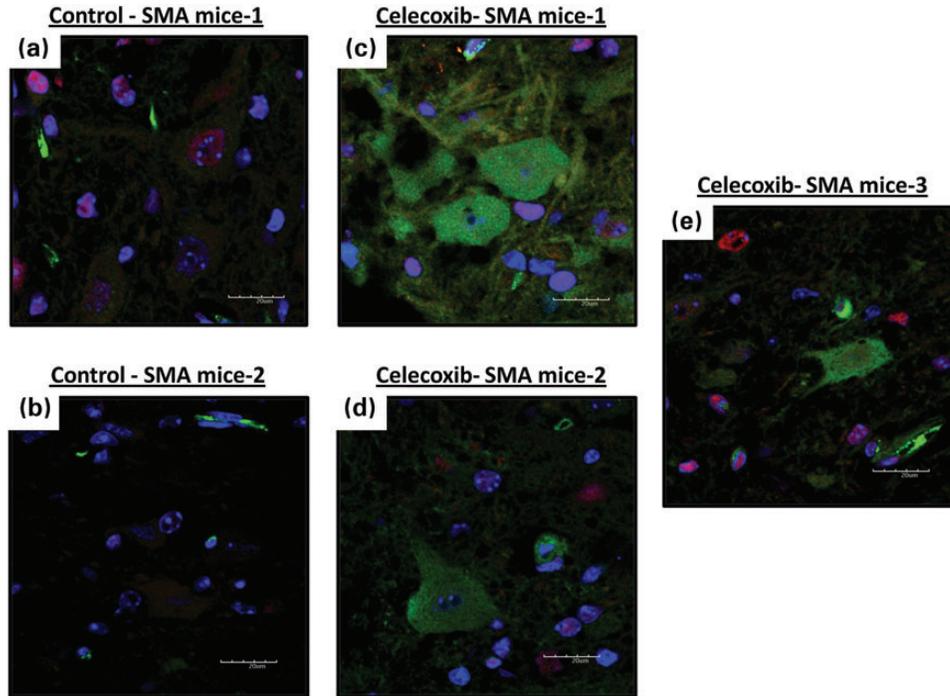


Figure 6. Celecoxib upregulates SMN protein expression in motor neurons of the SMA mice model. SMAΔ7 mice were treated daily with saline or celecoxib (20 μg/kg) from P1 for 6 days, and then sacrificed at P7. Spinal cords were harvested for immunohistochemistry analysis. Representative merged confocal images [SMN/alexa488 (green) + HB9/alexa 568 (red; motor neuron marker) + Hoechst (blue)] for spinal cord samples are shown. Representative confocal images showing effect of celecoxib on SMN protein expression in spinal cord motor neurons samples of SMAΔ7 mice treated with Saline (control, **a** and **b**) or celecoxib (**c–e**), respectively ($n = 3$). Scale bars: 20 μm.

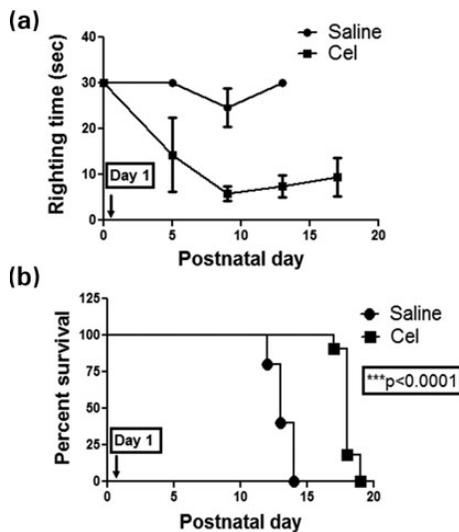


Figure 7. Celecoxib ameliorates disease phenotype and increases survival of the SMA mouse model. SMAΔ7 mice were treated daily with intraperitoneal injections of celecoxib (20.0 μg/kg) from P1 onward. (a) Righting times of SMAΔ7 mice treated with celecoxib (black filled square) or saline (black filled circle) ($n = 5$) [mean ± SEM (bars)]. (b) Kaplan–Meier survival curves of SMAΔ7 mice treated with celecoxib (black filled square) or vehicle (black filled circle) ($n = 10$); $P < 0.0001$.

regulation of p21 mRNA by the p38 pathway and HuR protein (16). To explore whether a similar role exists for HuR protein in the *in vitro* celecoxib-mediated increase in SMN, NT2 cells

were pre-treated with HuR siRNA followed by celecoxib treatment. Celecoxib-mediated SMN induction was blocked when cells were pre-treated with HuR siRNA, suggesting that HuR is required for the celecoxib-mediated increase of SMN protein.

Treatment of WT mice with different doses of celecoxib revealed an induction of SMN protein in the brain and spinal cord samples at 20 μg/kg celecoxib dose compared with saline-treated control mice. We have previously documented a difference in the responsiveness of mouse *Smn* (low) and human *SMN2* (high) to STAT5 kinase activation (25) and thus wondered if the same distinction might exist in the case of p38 activation. To further explore this possible effect, SMA Δ7 mice were treated with celecoxib for 5 days postnatally. Celecoxib treatment resulted in a significant and sustained increase in SMN protein levels in CNS tissues (brain and spinal cord) and, to a lesser degree or no increase, in muscle tissues (heart and skeletal muscle) compared with saline-treated SMA mice. It has been reported that the p38 transcripts are expressed less in SMA I muscle compared with normal muscle (26). This could be one of the factors resulting in the absence of SMN induction in muscle tissues upon celecoxib treatment compared with saline-treated SMA mice.

The recent observations of modest increases in mouse SMA longevity conferred by robust motor neuron SMN repletion (27,28) (such as that seen here with low dose celecoxib) have made clear that, for murine SMA at least, motor neuron SMN is necessary but not sufficient for long-term survival. Although therapies which confer profound longevity extension in the mouse such as ASO and gene therapy clearly target the

secondary site(s) of SMN repletion needed for murine SMA survival, it is not clear what these sites are. It would appear that the skeletal muscle is not that secondary site as SMN repletion does not have an enormous impact on the disease phenotype of SMA mouse (29). Recently, however, clinical, electrophysiologic and anatomical evidence of significant dysautonomia conferred cardiac failure in severe mouse SMA has implicated the autonomic nervous system (ANS) as the likely second system requiring increased SMN for mouse SMA survival. While dysautonomia has been observed in human SMA, it is not characteristically as severe as that documented in the mouse SMA model. We are currently conducting investigation of mouse ANS with and without celecoxib; we anticipate seeing modest ANS SMN induction at best. Because of this critical species difference resulting in therapeutic failure in mice, there exists the risk of discounting credible SMA therapeutics which may work in human based exclusively on mouse SMA studies.

There are a number of factors which we need to take into account when using SMA mice as a disease model, particularly when comparing results between laboratories (e.g. health of mice at birth, competition within cage between littermates, housing conditions). Therefore, to account for this variability, we used the ratio of median survival of treated to non-treated animals to assess drug response on survival. With celecoxib, we have achieved a ratio of 18d/13d or 1.38, a number that compares favourably with the 1.2 (19d/16d) observed with TSA (albeit P1 celecoxib initiation versus P5 TSA initiation) and 1.3 (12.9d/9.9d) seen with SAHA (30,31).

In addition to HDAC inhibitors, antisense oligonucleotides (ASOs), have shown promise for the treatment of SMA by preventing alternative splicing of the SMN2 transcript and ultimately resulting in more full-length SMN transcript (32,33). A major hurdle with these compounds is their failure to cross the BBB. However, a recent study shows a marked improvement in motor function along with increase in survival in SMA mice with systemic delivery of ASO which results into increase in SMN levels largely in peripheral tissues (32); it is unlikely, however, that the systemic effect extends to human given the lack of ASO BBB penetration. The other encouraging treatment of SMA is gene therapy using sc-AAV9-SMN resulting in a marked increase in longevity of SMA mice along with a significant improvement in motor function (34). Similar results were seen by other groups who used similar gene therapy approaches (35–37). While there is a phase II SMA therapy trial currently underway for intra-theal ASO, there are a number of issues which need to be addressed before wide spread clinical introduction of gene therapy for SMA (clinical safety, species barrier, quantity of virus, overall cost, immune response) (38,39).

Off-target drug effects, either harmful or useful, are commonly observed occurrences. In the current study, celecoxib is used as an alternative to the p38 activating compound anisomycin. Celecoxib was the first in class of selective COX-2 inhibitors to be FDA approved for the treatment of rheumatoid arthritis and osteoarthritis. Although celecoxib causes a lower incidence of gastrointestinal ulceration and other complications than do other non-steroidal anti-inflammatory drugs (40,41), the effects are nonetheless real. A recent study demonstrated possible damage of hepatic and renal tissue when rats were treated with 10 and 50 mg/kg celecoxib doses (42). However, the doses of celecoxib (20 µg/kg) which we have used for treatment

in this study are two orders of magnitude lower than doses currently used for osteoarthritis (1–2 mg/kg) in humans, and much lower than levels shown by toxicology reports to be harmful (40), making it likely that this would be a safe intervention for the vulnerable demographic comprised by infants and children diagnosed with SMA.

Our results demonstrate clear amelioration of the SMA disease phenotype in mouse model using BBB penetrant, FDA-approved celecoxib raising the prospect of its use in clinical trial studies. As many recent studies have shown that early timing of SMN gene therapy is critical for maximum benefit in the SMA mouse model, diagnosing newborn pre-symptomatic SMA infants and their early treatment will be critical. It may even be beneficial for SMA type II and III patients as a recent study showed that increasing SMN levels even post-symptomatically ameliorates SMA disease phenotype (43). It may be of value to combine the effect of celecoxib with SMN2 transcriptional activator prolactin (25) and/or neuroprotective compounds such as Y-27632 and fasudil (Rho kinase inhibitors) (44,45).

Presently, there is no effective treatment for SMA. This study provides evidence of low dose celecoxib activation of p38 resulting in significant neuronal SMN protein induction and thus a low-cost, clinically practical potential SMA therapy that could be trialled in the immediate future.

MATERIALS AND METHODS

Animals

All protocols were approved by Animal Care and Veterinary Services (ACVS) and Ethics board of University of Ottawa. All experiments were carried out in accordance with the Canadian Institute of Health Research (CIHR) Guidebook and ACVS legislation. CD-1 mice were obtained from Charles River Laboratory. The original breeding pair of heterozygous SMA Δ 7 mice (*mSmn*+/-, *hSMN2*+/, *hSMN Δ 7*+/) on the FVB background were provided by the Jackson Laboratory. The animals were maintained in an air-conditioned ventilated animal facility. Survival, righting time and weight were monitored daily as described by Avila *et al.* (30).

Celecoxib administration

Celecoxib was diluted in DMSO and administered through IP injection using a 30-gauge needle. Control animals received equal volumes of vehicle alone. SMA Δ 7 mice were genotyped at P0 and celecoxib treatment was started from P1. Animals were sacrificed within 24 h of final celecoxib dose.

Reagents

Celecoxib was purchased from Toronto Research Chemicals. p38 inhibitor SB239063 was purchased from Sigma. Non-silencing siRNA control and HuR siRNA were purchased from Qiagen and Dharmacon, respectively. The antibodies used in this study were SMN/Smn (BD Transduction Laboratories), Actin (Abcam), Tubulin (Abcam), Phospho-p38 (Cell signalling) and Total p38 (Cell signalling).

Primer sequences

For genotyping

Genotyping was performed as previously described by Avila *et al.* (30) using the following primers

mSmn *WT*. Forward: 5'-TCTGTGTTTCGTGCGTGGTGACTTT-3';
Reverse 1877: 5'-CCCACCACCTAAGAAAGCCTCAAT-3'.

Lac Z. Forward: 5'-CCAACTTAATCGCCTTGCAGCACA-3';
Reverse: 5'-AAGCGAGTGGCAACATGGAAATCG 3'.

Human SMN2 transgene. Forward: 5'-CAAACACCTGGTATGGTCAGTC-3';

Reverse: 5'-GCACCACTGCACAACAGCCTG-3'.

Product sizes:

mSMN: 372 bp;

Lac Z: 626 bp;

SMN2 transgene: 250 bp.

Cell culture and drug treatment conditions

Human neuron-committed teratocarcinoma (NT2) or mouse motor neuron-derived (MN-1) cells were maintained in standard conditions (37°C in a 5% CO₂ humidified atmosphere) in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 1% antibiotics (100 units/ml penicillin–streptomycin) and 2 mM glutamate.

NT2 or MN-1 cells were seeded in 12-well plates (2.5 × 10⁵ cells/well) and treated 24 h later with celecoxib (5, 50 and 500 nM) for 24 h. For time course experiment, NT2 cells were seeded in 12-well plates (2.5 × 10⁵ cells/well) and treated 24 h later with celecoxib (5 nM) for up to 24 h. For p38 inhibitor treatment, NT2 were seeded in 12-well plates (2.5 × 10⁵ cells/well) and pre-treated with p38 inhibitor SB239063 for 2 h followed by celecoxib treatment (5 nM) for 24 h.

Transfection

For siRNA transfections, NT2 cells were seeded in 12-well plates (2.0 × 10⁵ cells/well) and transfected on the following day in serum-free DMEM with HuR siRNA (100 nM) or non-silencing control siRNA (100 nM), using LipofectAMINE 2000 transfection reagent for 48 h.

Western blot analysis

Cells were washed two times with 1 ml PBS (1 ×) and lysed in 75 μl RIPA buffer containing 10 mg/ml each of aprotinin, PMSF and leupeptin (all from Sigma), 5 mM β-glycerolphosphate, 50 mM NaF and 0.2 μM sodium orthovanadate for 30 min at 4°C, followed by centrifugation at 13 000g for 15 min; supernatants were then collected and kept frozen at –20°C. Tissue samples were homogenized in 1 ml RIPA (10 mg/ml each of aprotinin, PMSF and leupeptin) and then sonicated for 15 s. Total protein concentrations were determined by Bradford protein assay using a Bio-Rad protein assay kit. For western blot analysis, protein samples were separated by 10% SDS–PAGE. Proteins were subsequently transferred onto nitrocellulose membrane and incubated in blocking solution (PBS,

5% non-fat milk, 0.2% Tween-20) for 1 h at room temperature followed by overnight incubation with primary antibody at 4°C at the dilution prescribed by the manufacturer. Membranes were washed with PBS-T (PBS, and 0.2% Tween-20) three times followed by incubation with secondary antibody (anti-mouse or rabbit, Cell Signalling) for 1 h at room temperature. Antibody complexes were visualized by autoradiography using the ECL Plus and ECL western blotting detection systems (GE Healthcare). Quantification was performed by scanning the autoradiographs and signal intensities were determined by densitometric analysis using the Image J program.

Immunofluorescence staining and confocal microscopy

Spinal cords were briefly rinsed in PBS, fixed for 6 h in 4% paraformaldehyde in PBS and then transferred for cryopreservation into 30% sucrose/PBS prior to the making of the cryostat blocks. Ten micrometer sections were obtained with a cryostat, collected onto positively charged slides and air-dried for 1 h at room temperature. The slides were then incubated for 30 min with 0.2% Triton X-100/PBS, briefly rinsed with PBS and then incubated with 10% normal goat serum in PBS. Blocking solution was discarded and the slides were then incubated overnight at 4°C with the mouse anti-SMN antibody diluted in PBS at 1:1000 (BD Transduction Laboratories™) and the rabbit anti-HB9 neuronal marker diluted in PBS at 1:500 (Abcam). After incubation with the primary antibodies, the slides were rinsed three times for 10 min with PBS and then incubated for 1 h at room temperature with goat anti-mouse Alexa Fluor® 488 and goat anti-rabbit Alexa Fluor® 568 (Invitrogen™) diluted at 1:1000 in PBS. The slides were then rinsed three times for 10 min with PBS, counterstained for 5 min with Hoechst 33342 (Invitrogen™), diluted at 10 μg/ml in PBS and mounted with Dako Fluorescent Mounting Medium. Confocal microscopy was performed with an Olympus FluoView™ FV1000 confocal microscope. Confocal microscope settings remained constant for each of the channels imaged. Channels were acquired in a sequential mode, the lasers output was set at 5% and the confocal aperture was set at 176 μm.

Statistical methods

GraphPad Prism software package (version 5.04 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com) was used for the Kaplan–Meier survival analysis. The log-rank test was used and survival curves were considered significantly different at $P < 0.0001$.

Data in figures (histograms, points on graphs) are mean values with the standard error mean (SEM) shown as error bars. The Student's two-tail *t*-test was used and were considered significantly different at $P < 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *HMG* online.

AUTHORS' CONTRIBUTION

F.F. designed and executed the experiments, analysed the data and wrote the manuscript; D.M., J.H., F.S. and S.O. assisted with the experiments and analysis; M.H. and A.M. assisted with analysis and writing.

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Conflict of Interest statement. None declared.

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