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# VPAC2 receptor agonist BAY 55-9837 increases SMN protein levels and moderates disease phenotype in severe spinal muscular atrophy mouse models

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## Abstract

**Background:** Spinal Muscular Atrophy (SMA) is one of the most common inherited causes of infant death and is caused by the loss of functional survival motor neuron (SMN) protein due to mutations or deletion in the *SMN1* gene. One of the treatment strategies for SMA is to induce the expression of the protein from the homologous *SMN2* gene, a rescuing paralog for SMA.

**Methods and results:** Here we demonstrate the promise of pharmacological modulation of *SMN2* gene by BAY 55-9837, an agonist of the vasoactive intestinal peptide receptor 2 (VPAC2), a member of G protein coupled receptor family. Treatment with BAY 55-9837 lead to induction of SMN protein levels via activation of MAPK14 or p38 pathway *in vitro*. Importantly, BAY 55-9837 also ameliorated disease phenotype in severe SMA mouse models.

**Conclusion:** Our findings suggest the VPAC2 pathway is a potential SMA therapeutic target.

**Keywords:** p38 pathway, SMN, SMA, VPAC2 receptor agonist, Therapeutics

## Background

Spinal muscular atrophy (SMA), is an untreatable recessive neuromuscular disorder; with an incidence of 1:11000, it is a leading genetic cause of pediatric death [1]. The loss of lower motor neurons from the ventral horn of spinal cord is the major pathological feature of the disease and results in generalized weakness, progressive muscle loss and respiratory failure [2]. SMA is caused by the pathologic reduction in survival of motor neuron (SMN) protein levels due to deletions and mutations in *SMN1* gene [3]. Although the complete loss of SMN protein is embryonically lethal, the presence of the paralogous *SMN2*, a result of a recent duplication event, which produces a limited full length SMN mRNA (~10%) precludes this outcome in humans [4,5]. Thus all SMA patients have 2 or more copies of *SMN2* gene

which in part compensates for the loss of *SMN1* gene. The inverse correlation between the severity of the disease phenotype and copy number of *SMN2*, both confirms the gene's disease modifying function and has made the induction of *SMN2* a common SMA therapeutic goal. In this regard we have showed the post transcriptional stabilization of SMN mRNA through activation of p38 pathway leads increased SMN levels [6]. We have recently reported that the activation of the p38 pathway through celecoxib upregulates SMN protein levels and can ameliorate disease phenotype in SMA mouse model [7]. In this regard, Vasoactive intestinal peptide receptor 2 (VPAC2), a member of G protein coupled receptor family when activated has been reported to activate p38 pathway *in vivo* [8,9].

We thus decided to assess the blood brain barrier (BBB) penetrant VPAC2 receptor agonist BAY 55-9837 for its potential SMA therapeutic utility. We show here that BAY 55-9837 conferred an increase in SMN protein levels via p38 activation in human neuronal cells. Importantly, we

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show that treatment with BAY 55-9837 also increases brain and spinal cord SMN protein levels as well as improving disease phenotype and survival in a severe SMA mouse model. Our results provide further evidence that p38 MAPK pathway activators act as potential therapeutic compounds for the treatment of SMA and identify the VPAC pathway as one means of achieving such activation.

## 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 $\Delta$ 7 (*mSmn*<sup>+/-</sup>, *hSMN2*<sup>+/+</sup>, *hSMN $\Delta$ 7*<sup>+/+</sup>; stock# 005025), Taiwanese mice (*Smn1*<sup>tm1Hung</sup> Tg(SMN2)2Hung/I; stock# 005058) and heterozygous *Smn* knock-out mice (*Smn*<sup>+/-</sup>) 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 Aviva et al [10].

### BAY 55-9837 administration

BAY 55-9837 was diluted in PBS/dH<sub>2</sub>O and administered through IP injection using a 30-gauge needle (0.2 mg/kg dose). Control animals received equal volumes of vehicle alone. SMA $\Delta$ 7 and Taiw/Jax SMA mice were genotyped at P0 and BAY 55-9837 treatment was started from P1. Animals were sacrificed within twenty four hours of the final dose.

### Reagents

BAY 55-9837 was purchased from Tocris Bioscience. p38 inhibitor SB239063 was purchased from Sigma. 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).

### Cell culture and drug treatment conditions

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

NT2 or MN-1 cells were seeded in 6 well plates (5 × 10<sup>5</sup> cells/well) and treated 24 h later with BAY 55-9837 (0.25 μM) for 24h. For time course experiment, NT2 cells were seeded in 6 well plates (5 × 10<sup>5</sup> cells/well) and treated 24h later with BAY 55-9837 (0.25 μM) for up to

24h. For p38 inhibitor treatment, NT2 were seeded in 6 well plates (5 × 10<sup>5</sup> cells/well) and pre-treated with p38 inhibitor SB239063 for 2 h followed by BAY 55-9837 treatment (0.25 μM) for 24 h.

### Western blot analysis

Cells were washed 2 times with 1 ml PBS (1X) and lysed in 150 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 13 000 × g for 30 min; supernatants were then collected and kept frozen at -20°C. Tissue samples were homogenized in 0.5 ml RIPA (10 mg/ml each of aprotinin, PMSF and leupeptin) and then sonicated for 15 seconds. 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 11% 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) 3 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 ImageJ program.

### Primer sequences

#### For genotyping

Genotyping was performed as previously described by Aviva et al [10] for SMA $\Delta$ 7 mice using the following primers

*mSmn* WT Forward: 5'-TCTGTGTTCGTGCGTGGTG ACTTT-3'.

Reverse 1877: 5'-CCCACCACCTAAGAAAGCCTCA AT-3'.

**Lac Z** Forward: 5'-CCAACTTAATCGCCTTGCAGCA CA-3'.

Reverse: 5'-AAGCGAGTGGCAACATGGAAATCG 3'.

**Human SMN2 transgene** Forward: 5'-CAAACACCT GGTATGGTCAGTC-3'.

Reverse: 5'-GCACCACTGCACAACAGCCTG-3'.

Product sizes:  
*mSMN*: 372 bp

Lac Z: 626 bp

*SMN2* transgene: 250 bp

Genotyping for Taiwanese SMA mice was performed as previously described by Riessland *et al* [11] using recommended primers.

### Statistical methods

GraphPad Prism software package was used for the Kaplan–Meier survival analysis. The log-rank test was used and survival curves were considered significantly different at  $P < 0.05$ .

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 to test for statistical differences between samples and were considered significantly different at  $P < 0.05$ .

### Results and discussion

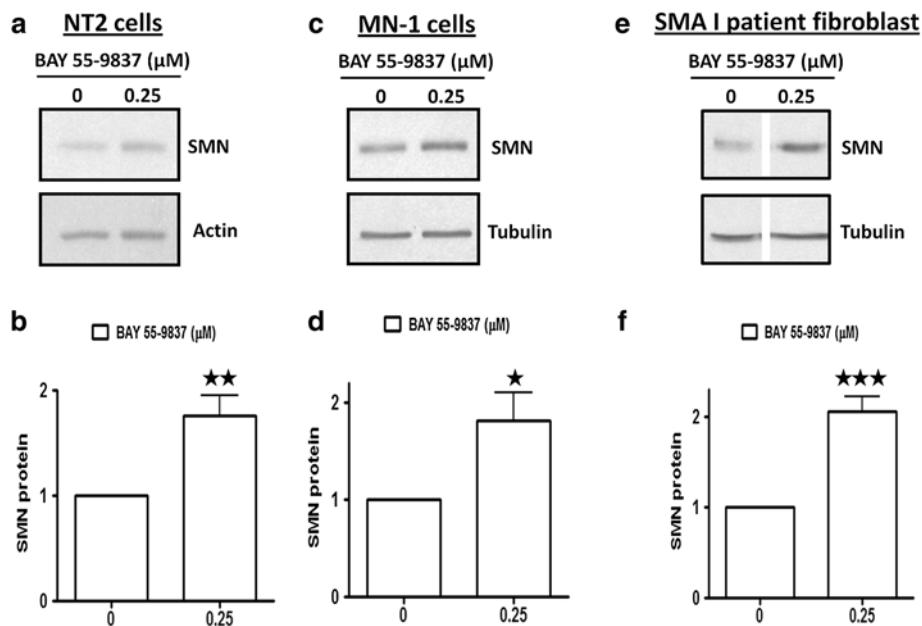
SMA is a frequently severe neurodegenerative disease which most frequently affects children; many of them do not survive beyond the first few years of life. Although there is no effective therapy for SMA, one translational approach is to induce the paralogous gene *SMN2*. This results in the production of more SMN protein, which

can partially compensate for the loss of *SMN1* gene and to moderate the disease phenotype.

**BAY 55-9837 treatment upregulates SMN protein *in vitro***  
VPAC2 receptor activation has been reported to activate the p38 kinase pathway [8,9] which, in turn, we have shown to stabilize SMN transcript and increase SMN protein level [6]. In order to assess the potential of VPAC-2 receptor activation in the regulation of *SMN* gene expression; human NT2, mouse MN-1 cells and SMA I patient fibroblasts were treated with VPAC2 receptor agonist BAY 55-9837 (25  $\mu$ M) for 24 h and subsequently harvested for western blot analysis. SMN protein levels were found to be increased by  $\sim 2$  fold in all cell lines upon treatment with BAY 55-9837 (Figure 1a-f). These results were encouraging in that the increase in SMN protein levels was observed in both neuronal cell lines and patient fibroblasts suggesting that the induction was not specific to a given cell line.

### BAY 55-9837 conferred increase in SMN protein levels is mediated by p38 MAPK activation

The p38 MAPK pathway regulates a number of cellular process including post-transcriptional stabilization of a distinct class of mRNAs that contain AU rich elements (ARE) mapping to their 3' UTRs [12-16]. This class of



**Figure 1** BAY 55-9837 treatment upregulates SMN protein *in vitro*. NT2, MN-1 and SMA I patient fibroblasts were treated with BAY 55-9837 (0.25  $\mu$ M) and then harvested at 24 hours for western blot analyses. **(a)** Representative western blots showing the effect of BAY 55-9837 on SMN protein in NT2 cells. **(b)** Densitometric quantification of SMN protein relative to Actin (the ratio at control treatment was set as 1; mean + SEM (bars) of six independent experiments) are shown for NT2 cells. **(c)** Representative western blot showing effect of BAY 55-9837 on SMN protein in MN-1 cells. **(d)** 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 MN-1 cells. **(e)** Representative western blots showing the effect of BAY 55-9837 on SMN protein in SMA I patient fibroblasts (all lanes were run on the same gel but were non-contiguous). **(f)** Densitometric quantification of SMN protein relative to Tubulin (the ratio at control treatment was set as 1; mean + SEM (bars) of five independent experiments) are shown for SMA I patient fibroblasts. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , t-test.

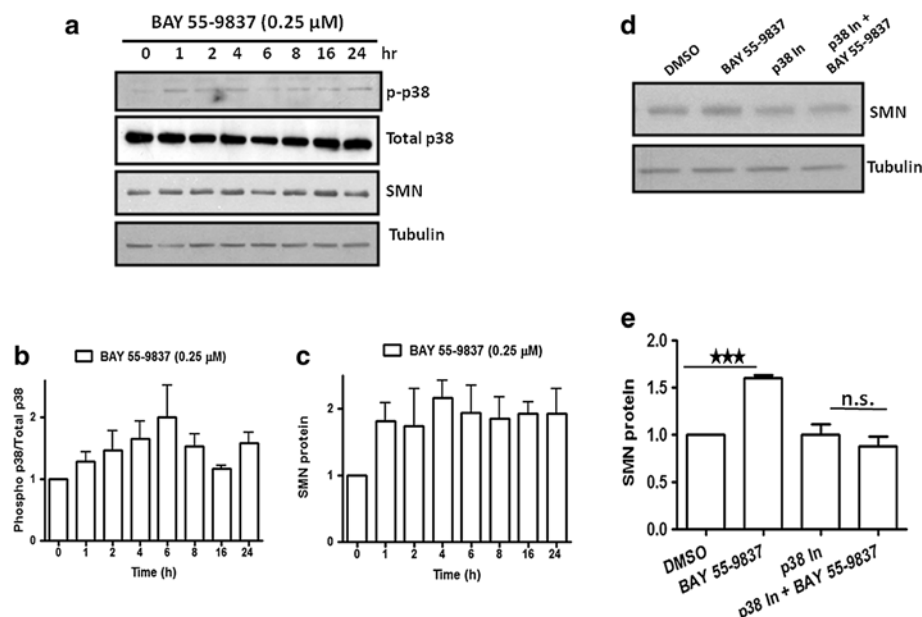
mRNA includes that encoded by *SMN2* [14]; we have previously reported that p38 MAPK increases *SMN* protein expression by virtue of the binding of HUR protein to *SMN2* 3'UTR [6]. The VPAC2 receptor agonist Ro 25-1553 has been previously shown to activate the p38 MAPK pathway [9]; we wished to confirm that BAY 55-9837 could elicit the same p38 activation and that this was underlying the observed *SMN* protein induction. NT2 cells were therefore treated with BAY 55-9837 and then harvested; western blot analysis at the indicated time intervals revealed within one hour an increase in the ratio of phosphorylated/ total p38 protein (up to 24 hrs after BAY 55-9837 treatment) consistent with p38 activation (Figure 2a-b). p38 MAPK activation was concurrent with the increase in *SMN* protein levels in NT2 cells (Figure 2a & c). To confirm the role of p38 in the observed *SMN* protein induction, NT2 cells were pre-treated with the p38 inhibiting agent SB-239063 [17] for 2 h prior to treatment with BAY 55-9837 for 24 h. Western blot analysis revealed that p38 inhibition effectively blocked the BAY 55-9837-mediated increase in *SMN* protein (Figure 2d & e). These results demonstrate that activation of p38 pathway presumable through

binding of VPAC2 receptor agonist to its receptor confers the increase in *SMN* protein levels observed upon BAY 55-9837 treatment. This result is consistent with our previous observation of increased *SMN* protein levels conferred by the p38 MAPK activating small compounds anisomycin and celecoxib [6,7].

#### BAY 55-9837 treatment upregulates *SMN* protein levels *in vivo*

In order confirm that BAY 55-9837-mediated *SMN* protein induction extends to the *in vivo* setting, a dose finding study was initiated. CD-1 mice were given daily intraperitoneal (IP) BAY 55-9837 injections for 5 days 0.02, 0.2 and 2 mg/kg and brain and spinal cord samples then isolated for western blot analysis. Increased *SMN* protein levels were observed both in brain (Additional file 1: Figure S1a & b) and spinal cord samples (Additional file 1: Figure S1c & d) following BAY 55-9837 treatment with the greatest induction (~2 fold), seen at 0.2 mg/kg dose in CD-1 mice.

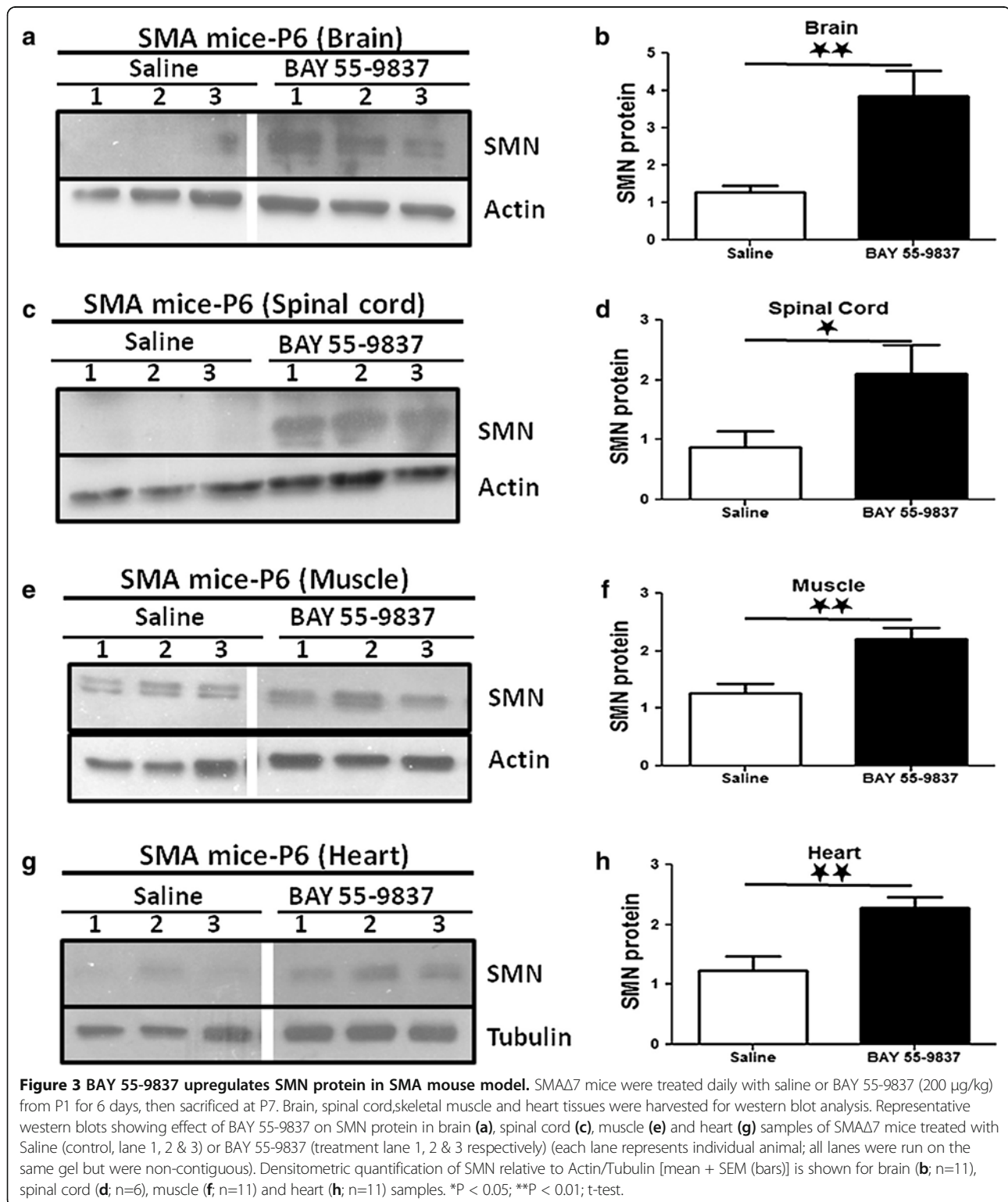
We next explored the impact of BAY 55-9837-induced *SMN* upregulation in a severe mouse model of the disease (*SMAΔ7* mouse; *mSmn*<sup>-/-</sup>;*hSMN2*<sup>+/+</sup>, *hSMNΔ7*<sup>+/+</sup> [18]). *SMAΔ7* mice were given 0.2 mg/kg BAY 55-9837



**Figure 2 BAY 55-9837 treatment increases *SMN* expression via p38 MAPK pathway.** (a) Representative western blot showing activation of p38 MAPK pathway upon BAY 55-9837 treatment in NT2 cells. NT2 cells were treated with BAY 55-9837 at indicated times and then harvested for western blot analysis. Activation of p38 pathway by BAY 55-9837 leads to an 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. (d) Representative western blots showing the effect of p38 inhibition on BAY 55-9837-induced increase in *SMN* protein. p38 inhibitor (SB-239580) blocked the BAY 55-9837-induced increase in *SMN* protein in NT2 cells. NT2 cells were treated with SB-239580 (p38 In; 3 μM) for 2 h followed by treatment with BAY 55-9837 for 24 h and then harvested for western blot analysis. (e) 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 showing the effect of p38 inhibition on BAY 55-9837-induced increase in *SMN* protein. \*P < 0.05; \*\*\*P < 0.01, t-test.

IP injections twice daily from P1 until P6. Mice were euthanized 24 hours after their last treatment and brain, spinal cord, muscle and heart samples then harvested for western blot analysis. Mice treated with BAY 55-9837

demonstrated an approximate doubling in *SMN2*-derived full length SMN protein levels in all tissues except brain where an approximate quadrupling of SMN protein was observed when compared with vehicle treated animals



(Figure 3). In keeping with these results, VPAC2 receptors are expressed in CNS as well as in peripheral tissues [19-25]. The most modest (although still significant) induction of SMN protein was seen in muscle tissues compared to saline treated SMA mice, a possible result of the comparatively low amount of p38 transcript in SMA I muscle compared with normal muscle [26].

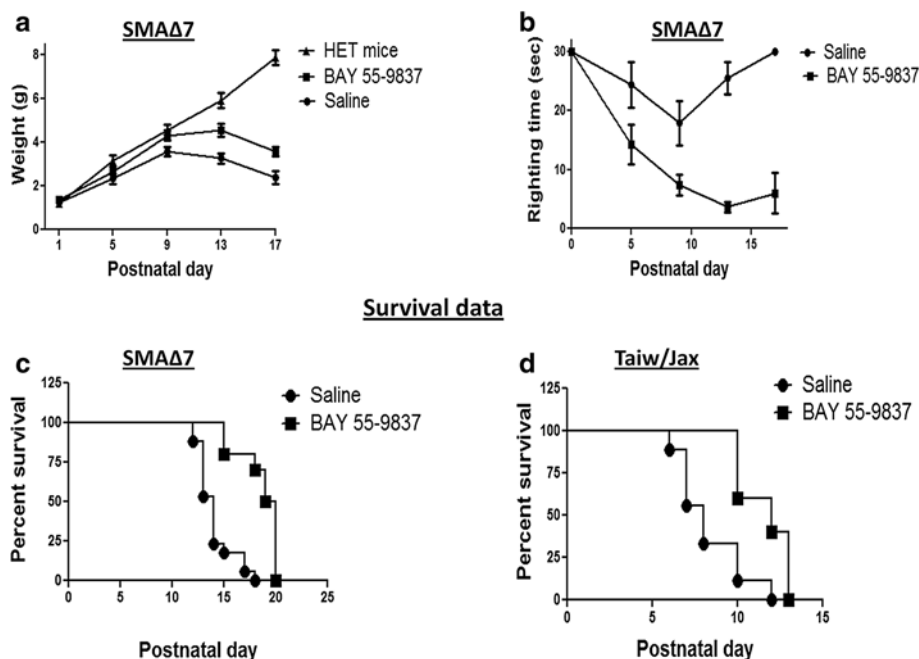
#### BAY 55-9837 treatment improves disease phenotype in SMA mice model

We next examined the effect of BAY 55-9837 treatment on SMA $\Delta$ 7 mouse disease phenotype. The SMA $\Delta$ 7 mice are significantly underweight and have reduced motor activity compared to heterozygous and WT littermates. SMA $\Delta$ 7 mice were given twice daily BAY 55-9837 or vehicle IP injections starting at P1; their weight and motor function were assessed daily. SMA $\Delta$ 7 mice treated with BAY 55-9837 showed significant improvement in weight gain and motor function (as assessed by righting time), as compared to vehicle-treated SMA $\Delta$ 7 mice (Figure 4a & b).

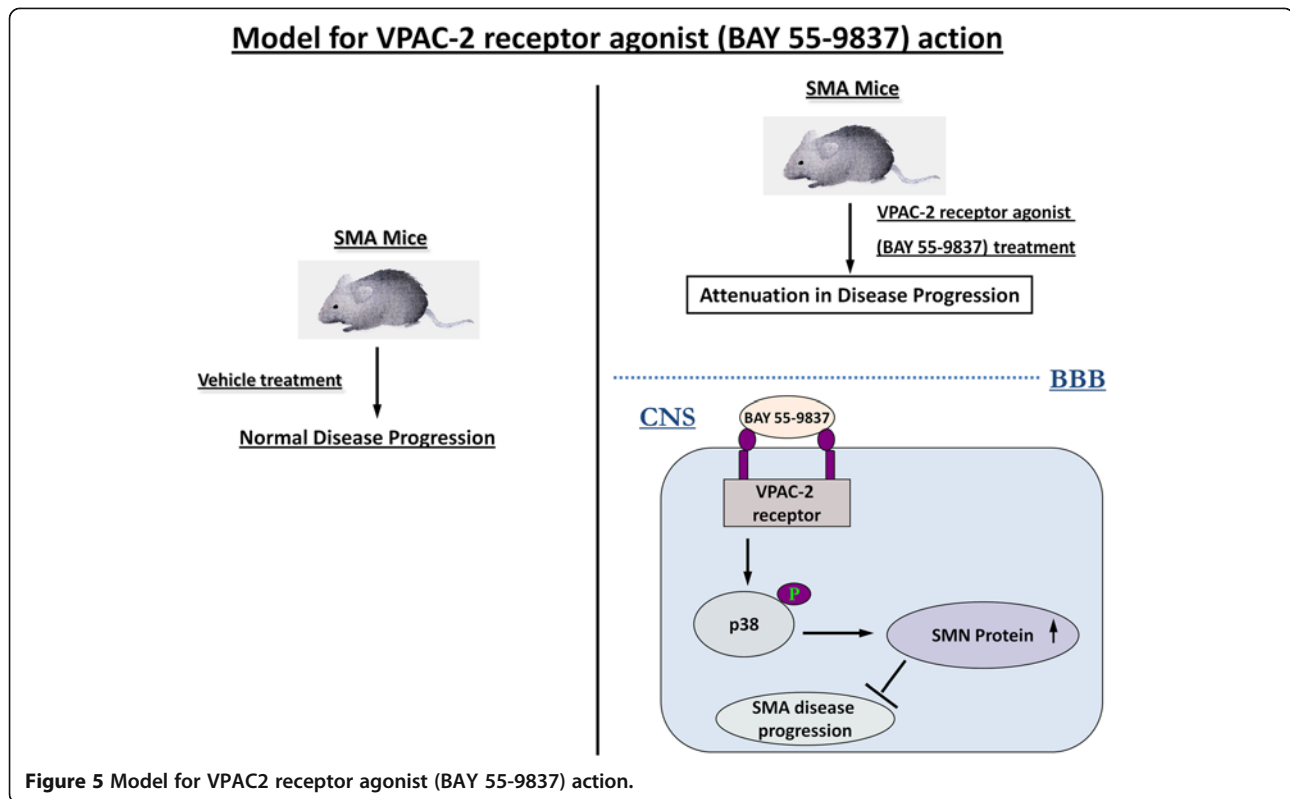
We also examined the impact of BAY 55-9837 on survival in two different severe SMA mouse models (SMA $\Delta$ 7 [18] and Taiw/Jax-SMA{Cross between Taiwanese mice

(*Smn1<sup>tm1Hung</sup> Tg(SMN2)2Hung/*; stock# 005058) and heterozygous *Smn* knock-out mice (*Smn<sup>+/-</sup>*) [11]}. Significant extension of survival was observed in both mouse models upon treatment with BAY 55-9837 (median survival of 19.5 d for SMA $\Delta$ 7 mice from 14d and 12 days from 8d for Taiw/Jax SMA mice) as compared with vehicle-treated (Figure 4c & d). To account for the treatment effect variability between various laboratories and mouse models, the ratio of median survival of treated to non treated animals was used to assess drug response on survival. With BAY 55-9837 we have achieved a ratio of 1.39 (19.5d/14d for SMA $\Delta$ 7) & 1.5 (12d/8d for Taiw/Jax-SMA) for the two different models. These numbers compare favourably with the other small compounds previously used for SMA treatment such as TSA (1.2; 19d/16d albeit P5 TSA initiation in SMA $\Delta$ 7) [10], SAHA (1.3; 12.9d/9.9d in Taiw/Jax-SMA) [11], celecoxib (1.38; 18d/13d) [7] and Prolactin (1.5; 21d/14d) [27].

SMA is primarily considered as a motor neuron disease and consequently treatment strategies focus on drugs which can cross the blood brain barrier (BBB) to target tissues within central nervous system (CNS). However several recent studies challenge this notion and suggest that SMN has function above and beyond motor



**Figure 4** BAY 55-9837 ameliorates disease phenotype and increases survival of SMA mouse models. SMA $\Delta$ 7 mice were treated daily with intraperitoneal injections of BAY 55-9837 (200.0  $\mu$ g/kg) from P1 onward. **(a)** Weights of SMA $\Delta$ 7 mice treated with BAY 55-9837 (black filled square, n =10) or saline (black filled circle, n =10); weights for heterozygous mice treated with saline (black filled triangle, n =5) are also shown for comparison [mean  $\pm$  SEM (bars)]. **(b)** Righting times of SMA $\Delta$ 7 mice treated with BAY 55-9837 (black filled square, n = 10) or saline (black filled circle, n =10) [mean  $\pm$  SEM (bars)]. **(c)** Kaplan-Meier survival curves of SMA $\Delta$ 7 mice treated with BAY 55-9837 (black filled square, n = 10; median survival 19.5 days) or saline (black filled circle, n =17; median survival 14.0 days). **(d)** Kaplan-Meier survival curves of Taiwanese-SMA mice treated with BAY 55-9837 (black filled square, n = 5; median survival 12.0 days) or saline (black filled circle, n =10; median survival 8.0 days); \*P < 0.05; \*\*\*P < 0.0001, log-rank test.



neurons and reclassify SMA as a multi-system disorder (including cardiovascular, peripheral necrosis, pancreatic and liver defects) [28-36]. In this regard the widespread presence of the VPAC2 receptor augurs well for this pathway as a therapeutic SMA target [25].

### Conclusion

Re-purposing drugs for distinct disease indications is becoming a more common practice given the approximately 7000 orphan genetic disorders that are estimated to exist. This approach is both cost-effective as well as shortening the path to treatment for significant (and currently untreatable) disorders such as SMA. In the current study, BAY 55-9837 initially developed for the treatment of diabetes [37,38] has been used as a p38 activating compound for the treatment of murine SMA. Our results demonstrate that VPAC2 receptor agonist BAY 55-9837 increases SMN protein levels and attenuates disease progression in two distinct severe SMA mouse models (Figure 5) providing a proof of concept and support for other VPAC2 agonists/p38 activating compounds to be tested as effective SMA therapies. Although the literature on the safety profile of BAY 55-9837 is divided (e.g. 38 and 39), in our experiments we did not observe any adverse effects. Nevertheless further work to obtain comprehensive safety profile for BAY 55-9837 will be beneficial [39]. This study provides a good supportive evidence as well as functional insight

how p38 pathway can be targeted for its potential application towards development of therapeutics for SMA.

### Additional file

**Additional file 1: Figure S1.** BAY 55-9837 upregulates Smn protein in wild type mice. 4 weeks old CD-1 wild type mice were treated daily with BAY 55-9837 (2.0, 20.0, 200.0 µg/kg) for 5 days, then sacrificed. Brain and spinal cord tissues were harvested for western blot analysis. (a) Representative western blot showing the effect of BAY 55-9837 on Smn protein in brain samples of CD-1 mice treated with saline (control, lane-1) or BAY 55-9837 (lane 2, 3 & 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 BAY 55-9837 on SMN protein in spinal cord samples of CD-1 mice treated with Saline (control, lane-1) or BAY 55-9837 (lane 2, 3 & 4) (n = 3). (d) Densitometric quantification of SMN relative to Tubulin [mean + SEM (bars)] is shown for spinal cord samples. \*P < 0.05, t- test.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

FF designed and executed the experiments, analyzed the data, and wrote the manuscript; JH, and DM assisted with the experiments and analysis of the data; FS and KM assisted with the experiments; MH and AM assisted with analysis and writing. All authors read and approved the final manuscript.

### Acknowledgments

We want to thank Nathalie Earl for technical help with mice treatment. We would also like to thank Animal Care and Veterinary Service staff at the University of Ottawa. This work was supported by operating grants from the Canadian Gene Cure Foundation and CIHR Institute of Genetics (to F. Farooq), and from Tori's Buddies, CML Healthcare, FightSMA, the SMA Foundation, the

Canadian Gene Cure Foundation, Physicians Services Incorporated, Ilsa Mae SMA Research Fund and the Canadian Institutes of Health Research (to A. MacKenzie).

Received: 13 November 2013 Accepted: 3 January 2014  
Published: 9 January 2014

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doi:10.1186/1750-1172-9-4

**Cite this article as:** Hadwen *et al.*: VPAC2 receptor agonist BAY 55-9837 increases SMN protein levels and moderates disease phenotype in severe spinal muscular atrophy mouse models. *Orphanet Journal of Rare Diseases* 2014 **9**:4.

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