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## Effect of the butyrate prodrug pivaloyloxymethyl butyrate (AN9) on a mouse model for spinal muscular atrophy

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### Abstract

Spinal muscular atrophy (SMA) is an early-onset motor neuron disease that leads to loss of muscle function. Butyrate (BA)-based compounds markedly improve the survival and motor phenotype of SMA mice. In this study, we examine the protective effects of the BA prodrug pivaloyloxymethyl butyrate (AN9) on the survival of SMN<sup>-/-</sup> SMA mice. Oral administration of AN9 beginning at PND04 almost doubled the average lifespan of SMN<sup>-/-</sup> SMA mice. AN9 treatment also increased the growth rate of SMN<sup>-/-</sup> SMA mice when compared to vehicle-treated SMN<sup>-/-</sup> SMA mice. In conclusion, BA prodrugs like AN9 have ameliorative effects on SMN<sup>-/-</sup> SMA mice.

### Keywords

motor neuron disease; spinal muscular atrophy; preclinical drug trial; neonatal mouse; AN9

### INTRODUCTION

Spinal muscular atrophy (SMA) is an early-onset neurological disease characterized by loss of anterior horn  $\alpha$  motor neurons in the spinal cord [1]. Limb and trunk muscles atrophy as a result of motor neuron degeneration. SMA is an autosomal recessive genetic disorder with an incidence of 1:6,000 – 10,000 live births [2;3]; in fact, it is a leading genetic cause of infant death worldwide. SMA results from the loss or mutation of the *SMN1* (survival motor neuron 1) gene but retention of the near-perfectly duplicate *SMN2* gene [4]. *SMN2* copy

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### AUTHORS CONFLICTS OF INTEREST

The authors have no conflict of interest to report.

number modifies the severity of SMA disease in humans as well as in transgenic mouse models for SMA (reviewed in [5]). Because of its ability to modulate disease severity, *SMN2* is an ideal molecular target for the development of therapies to treat SMA.

Compounds based on the short chain fatty acid butyric acid (BA) have shown efficacy in mouse models for SMA. Continuous administration of BA via the water supply to SMA mice (*SMN2;mSmn<sup>+/+</sup>*) results in a moderate increase in survival of these mice [6]. However, oral administration of BA directly to *SMN<sup>-/-</sup>* SMA neonates via modified gavage does not improve survival [7]. *SMN<sup>-/-</sup>* SMA mice treated with the BA analogue 4-phenylbutyrate (4PBA) or one of two BA prodrugs—glyceryl tributyrates (BA3G) or VX563—show marked improvement in survival and motor function [7]. BA is present in the forebrains of neonatal mice treated with BA3G or VX563 but not with BA [7]. The lack of efficacy of BA in *SMN<sup>-/-</sup>* SMA mice is most likely due to the poor plasma pharmacokinetics of this compound in rodents [8]. BA, therefore, can provide neuroprotective benefits in SMA mice as long as it can reach detectable levels in the central nervous system (CNS).

Pivaloyloxymethyl butyrate (AN9, Pivanex) is a BA prodrug with a different chemistry from BA3G and VX563 that is metabolized by intracellular esterases to release BA along with formaldehyde and pivalic acid (2,2-dimethylpropionic acid) [9]. It possesses superior pharmacokinetics to BA and exhibits anti-tumor activity *in vitro* and *in vivo* [9–13]. In this study, we show the protective effects of AN9 on the survival of *SMN<sup>-/-</sup>* SMA mice.

## MATERIALS AND METHODS

### Animals and Ethical Statement

*SMN<sup>-/-</sup>* SMA mice (*SMN2<sup>+/+</sup>; SMN<sup>-/-</sup>; mSmn<sup>-/-</sup>*) were generated from male and female carrier mice (*SMN2<sup>+/+</sup>; SMN<sup>+/+</sup>; mSmn<sup>+/-</sup>*) [14]. The breeder mice were provided with *ad libitum* water and Harlan-Teklad 22/5 Rodent Diet chow [15–17]. All experiments were conducted in accordance with the protocols described in the National Institutes of Health *Guide for the Care and Use of Animals* and were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

### Drug Administration

AN9 (Pivanex) was provided by Titan Pharmaceuticals (South San Francisco, CA). Working solutions were prepared by mixing neat compound with an emulsion of 4% ethanol and Intralipid-20 (Sigma-Aldrich, St. Louis, MO) at a concentration of 0.1 mL AN9 per mL emulsion. Carrier and SMA littermate mice were dosed twice daily (b.i.d.; at 09.00 and 17.00 daily) with AN9 (200 mg/kg/d) or vehicle (4% ethanol in Intralipid-20) via oral administration as described previously [18]. Treatment began at postnatal day 4 (PND04) and continued for the lifetime of each SMA mouse. The body mass of each mouse was determined each day during treatment. The treatment cohorts were not stratified based on sex because there is no significant difference in lifespan between male and female *SMN<sup>-/-</sup>* SMA mice [19].

## Butyrate Measurements

Tissue levels of butyrate were measured in neonatal mice receiving a single dose of AN9 or vehicle by high-performance liquid chromatography (HPLC) as described previously [7].

## Statistical Analysis

Data are expressed as means  $\pm$  standard errors. Kaplan-Meier curves were generated from the survival and onset of body mass loss data and tested using the Mantel-Cox log rank test. All statistical analyses were performed with SPSS v.22.0.

## RESULTS

SMN<sup>-7</sup> SMA mice ( $n = 8$ /group) were dosed daily with either AN9 (200 mg/kg/d, b.i.d.) or vehicle (4% ethanol in Intralipid-20) beginning at PND04. Lifespan and onset of body mass loss were used as indices of the *in vivo* efficacy of AN9 in this mouse model [14;19]. Oral administration of AN9 improved the mean lifespan of treated SMN<sup>-7</sup> SMA mice by 84.6% (Figure 1A;  $21.0 \pm 4.9$  d for AN9 vs.  $11.4 \pm 0.8$  d for vehicle;  $p = 0.039$ ;  $\chi^2 = 4.264$ ). AN9 treatment also delayed the onset of body mass loss in SMN<sup>-7</sup> SMA mice by 94.9% (Figure 1B;  $19.2 \pm 5.2$  d for AN9 vs.  $9.9 \pm 0.5$  d for vehicle;  $p = 0.031$ ;  $\chi^2 = 4.641$ ). AN9 ranked as follows when compared with other BA-based compounds tested in the SMN<sup>-7</sup> SMA mouse model [7]: VX563 > AN9 > 4PBA > BA3G > BA.

We have previously shown that BA-based drug efficacy in SMN<sup>-7</sup> SMA mice is related to the levels of BA detected in the CNS [7]. Forebrain BA levels, one of the products from the metabolism of AN9 [9], were measured in neonatal mice ( $n = 3$ /group) treated with a single dose of AN9 (200 mg/kg/d) or vehicle. Tissue BA levels were measured using HPLC. In the AN9-treated mice, BA levels in the forebrain were  $34.14 \pm 9.61$  pmol/mg protein while vehicle-treated mice had undetectable BA levels ( $< 12.00$  mol/mg protein) in the CNS.

The body masses of SMN<sup>-7</sup> SMA mice treated with AN9 (Figure 2A; closed circles) were similar to those for vehicle-treated SMN<sup>-7</sup> SMA mice (Figure 2A; closed triangles) until PND11 after which time the drug-treated mice had higher body masses than age-matched, vehicle-treated SMN<sup>-7</sup> SMA mice. After PND18, the mean body masses of AN9-treated SMN<sup>-7</sup> mice were similar to drug-treated, non-SMA mice (Figure 2A; open circles). After this timepoint, non-SMA mice treated with AN9 exhibited lower body masses than age-matched mice receiving vehicle (Figure 2A; open triangles).

The growth rate—i.e. the change in body mass between PND14 and PND04—was diminished in SMN<sup>-7</sup> SMA mice relative to non-SMA mice (Figure 2B;  $p < 0.001$ ), which is consistent with previous studies [14;19]. AN9 increased the growth rate of SMN<sup>-7</sup> SMA mice by 87% when compared against vehicle-treated SMN<sup>-7</sup> SMA mice (Figure 2B;  $p = 0.053$ ). In fact, the growth rate of AN9-treated, SMN<sup>-7</sup> SMA mice was similar to that for drug-treated non-SMA mice; however, the growth rate of non-SMA mice treated with AN9 was significantly diminished when compared against vehicle-treated non-SMA mice (Figure 2B;  $p = 0.016$ ). While AN9 does improve the growth rate of SMN<sup>-7</sup> SMA mice, this compound may exhibit slightly toxic properties *in vivo*, at least in neonatal mice.

## DISCUSSION

We show in this study that early administration of the BA prodrug AN9 significantly improves the survival of SMN<sup>-/-</sup> SMA mice. AN9 treatment also marked delays the end stage of disease as defined by the onset of body mass loss [14;19]. Oral administration of two other BA prodrugs, BA3G and VX563, significantly ameliorate the degenerative phenotype in SMN<sup>-/-</sup> SMA mice [7]. AN9 is a more potent BA prodrug in these mice since its therapeutic dose is 25- to 30-fold lower than the other BA prodrugs previously tested. We can detect exogenous BA levels in the CNS of neonatal mice treated with AN9 (this study), BA3G or VX563 [7] but not with BA. BA can, therefore, provide therapeutic benefit to SMN<sup>-/-</sup> SMA mice if levels of the drug can be detected in the CNS.

AN9 administration also increases the growth rate, which is measured by compared the body mass of a mouse at PND14 to that at PND04, of SMN<sup>-/-</sup> SMA mice when compared to vehicle-treated SMN<sup>-/-</sup> SMA mice. At first glance, the growth rate of AN9-treated SMN<sup>-/-</sup> SMA mice approaches that observed for drug-treated non-SMA mice; however, the growth rate of AN9-treated non-SMA mice is significant lower than that for vehicle-treated non-SMA mice. This latter observation would suggest that AN9 may have an off-target effect on neonatal mice. Neither BA3G nor VX563 exhibit a similar phenomenon in non-SMA mice [7]. This off-target effect could be due to the other compounds released—i.e. formaldehyde and pivalic acid [9]—from the metabolism of AN9.

How do BA-based compounds exert their protective effects on SMA mice? BA and its analogue 4PBA increase the expression of SMN in cultured fibroblasts and lymphoblasts obtained from SMA patients [6;20]. Surprisingly, neither 4PBA nor the BA prodrug VX563 alters the levels of SMN mRNA or protein in the spinal cords of treated SMN<sup>-/-</sup> SMA mice [7]. BA and 4PBA are weak inhibitors of histone deacetylase (HDAC) activity [21–24]; HDAC activity is also reduced in SMN<sup>-/-</sup> SMA mouse spinal cord extracts treated with 4PBA or VX563 [7]. AN9 also possesses HDAC inhibitor activity [25]. In addition to reducing HDAC activity, 4PBA and VX563 restore the phosphorylation states of Akt and one its targets, GSK3 $\beta$ , that are reduced in SMN<sup>-/-</sup> SMA mice [7]. Future studies will examine how BA-based compounds like 4PBA, VX563 and AN9 regulate the activities of HDACs and Akt signaling to protect SMA motor neurons from degeneration.

In summary, we show that the BA prodrug AN9 markedly improves the survival and growth rate of SMN<sup>-/-</sup> SMA mice. This study along with other work [7] demonstrate that treatment with the short chain fatty acid BA can have significantly beneficial effects on a mouse model for SMA so long as a sufficient amount of drug reaches the motor neurons in the spinal cord. BA prodrugs show promise as *SMN2*-independent therapeutic agents for SMA in that they protect the vulnerable motor neurons from cell death. These prodrugs could be used in concert with *SMN2* inducers [26] to maximize therapeutic benefit for SMA.

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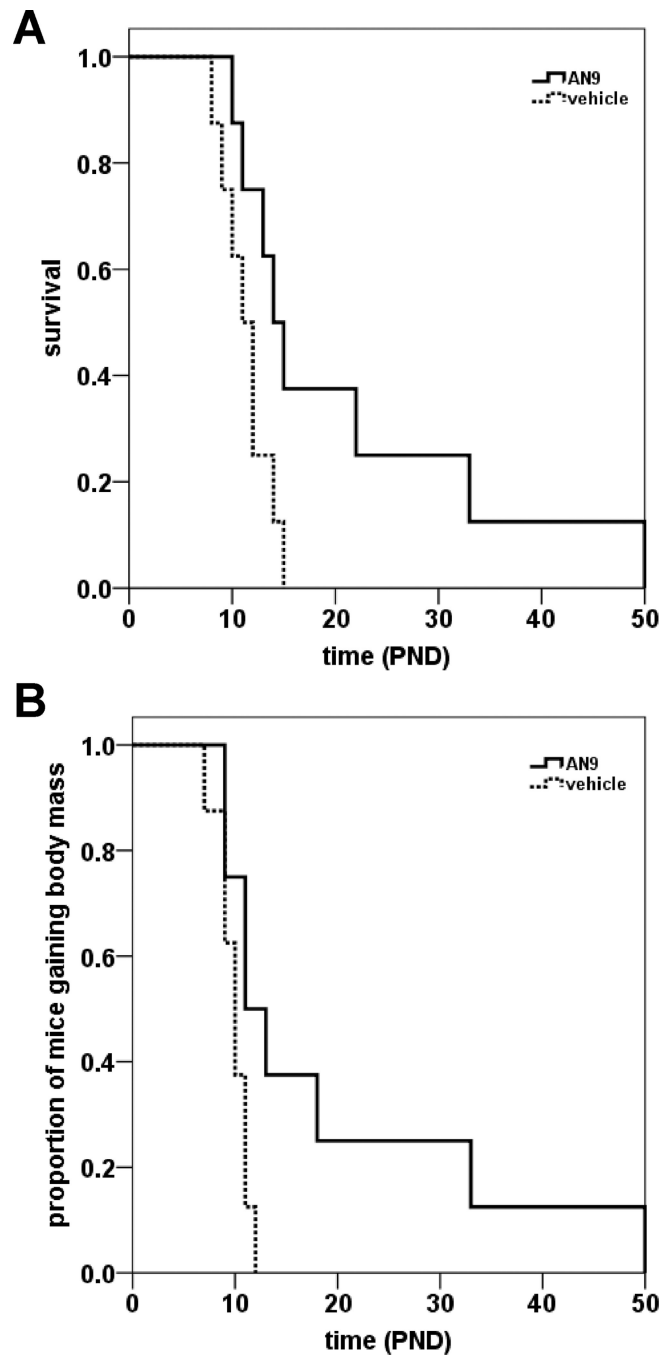
## ABBREVIATIONS

<b>BA</b>	butyrate
<b>BA3G</b>	glyceryl tributyrat
<b>CNS</b>	central nervous system
<b>4PBA</b>	4-phenylbutyrate
<b>PND</b>	postnatal day
<b>SMA</b>	spinal muscular atrophy
<b>SMN</b>	survival motor neuron

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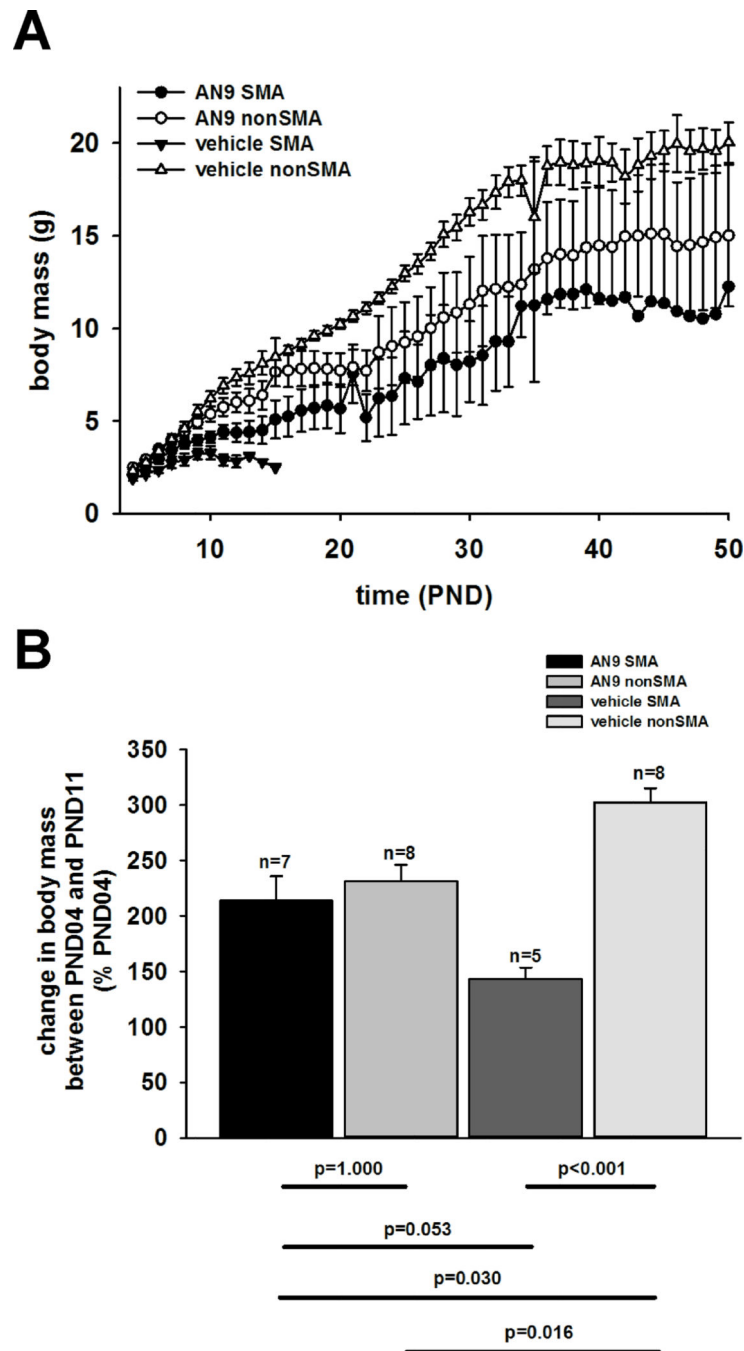
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**Figure 1. The effect of AN9 on survival of SMN-7 SMA mice**  
 SMN-7 SMA mice were treated daily with AN9 (200 mg/kg/d, b.i.d.) or vehicle (4% ethanol in Intralipid-20) starting at PND04 and monitored for change in lifespan (**A**) and onset of loss of body mass (**B**). AN9 increased the average lifespan of SMN-7 SMA mice by 85% (**A**;  $p = 0.039$ ) and delayed the onset of body mass loss by 95% (**B**;  $p = 0.031$ ).





**Figure 2. The effect of AN9 on the growth of SMN-7 SMA mice**

(A) Body mass curves of SMN-7 SMA mice (solid shapes) or non-SMA littermates (either carrier or normal; open shapes) treated daily with either AN9 (circles) or vehicle (triangles). (B) Growth rates—measured as changes in body mass between PND11 and PND04—of SMN-7 SMA mice and non-SMA littermates treated with either AN9 or vehicle. The statistical significances for pairs of experimental groups are provided below the bar graph.