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Combination of valproic acid and morpholino splice-switching oligonucleotide produces improved outcomes in spinal muscular atrophy patient-derived fibroblasts

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ABSTRACT

Spinal muscular atrophy (SMA), the leading genetic cause of infant mortality worldwide, is characterised by the homozygous loss of the survival motor neuron 1 (*SMN1*) gene. The consequent degeneration of spinal motor neurons and progressive atrophy of voluntary muscle groups results in paralysis and eventually premature infantile death. Humans possess a second nearly identical copy of *SMN1*, known as *SMN2*. However, *SMN2* produces only 10–20% functional SMN protein due to aberrant splicing of its pre-mRNA that leads to the exclusion of exon 7. This level of SMN is insufficient to rescue the phenotype. Recently developed splice-switching antisense oligonuclotides (SSO) have shown great promise in correcting the aberrant splicing of *SMN2* towards producing functional SMN protein. Several FDA approved drugs are being repurposed for SMA treatment including valproic acid (VPA), a histone deacetylase inhibitor, which has been shown to increase overall *SMN2* expression. In this study, we have characterised the effects of single and combined treatment of VPA and a SSO based on phosphorodiamidate morpholino oligomer (PMO) chemistry. We conjugated both VPA and PMO to a single cell-penetrating peptide (Apolipoprotein E (ApoE)) for their simultaneous intracellular delivery. Treatment of SMA Type I patient-derived fibroblasts with the conjugates showed no additive increase in the level of full-length *SMN2* mRNA expression over both 4 and 16 h treatments indicating that conjugation of VPA to ApoE-PMO has limited benefit. However, treatment with a combination of VPA and ApoE-PMO induced more favourable splice switching activity than either agent alone, promoting exon 7 inclusion in *SMN2* transcripts. Our results suggest that combination therapy of VPA and ApoE-PMO is superior in upregulating *SMN2* production *in vitro*, as compared to singular treatment of each compound at both transcriptional and protein levels. This study provides the first indication of a novel dual therapy approach for the potential treatment of SMA.

1. Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disease that affects between 1/6000–1/10,000 children (Frugier et al., 2002; Smith et al., 2007; Tisdale and Pellizzoni, 2015). Type I SMA, also known as Werdnig-Hoffman's disease, is the most common form accounting for approximately 50% of all cases (Lunn and Wang, 2008). Onset is infantile, typically before 3 months of age, and children are usually unable to sit upright or become ambulant. Patients experience proximal muscle weakness, hypotonia and respiratory deficits, with death usually resulting before the age of two years (Frugier et al., 2002).

The exact pathogenesis of SMA is unknown, however homozygous loss of function of the survival motor neuron 1 (*SMN1*) gene has been identified as the main disease-causing mechanism in 95% of cases (Bussaglia et al., 1995; Lefebvre et al., 1995, 1997). Loss of *SMN1* results in deficiency of survival motor neuron (SMN) protein. SMN is expressed by all cells, however, motor neurons in the anterior horn of the spinal cord are more susceptible to SMN deficiency which results in their selective death (Moultrie et al.,

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2016). The impaired axonal transport of mRNAs in SMN deficient motor neurons due to their short axonal length and limited growth has been suggested as the causative mechanism for their selective degeneration (Wertz and Sahin, 2016) (Burghes and Beattie, 2009; Fallini et al., 2014; Rossoll et al., 2003). SMN is also essential for small nuclear ribonucleoprotein (snRNP) assembly and accurate spliceosome function (Poulos et al., 2011) and it has also been implicated in RNA processing through its interactions with RNA binding proteins such as FMRP, IMP1 and HuD (Saal et al., 2014). SMN protein has also been shown to cause systemic defects in multiple organs including liver, lung heart, testis and vascular systems (Szunyogova et al., 2016). Thus SMN replacement or upregulation provides a promising therapeutic approach to SMA.

One potential strategy towards SMN upregulation involves targeting the survival motor neuron 2 (*SMN2*) gene, an almost identical version of *SMN1* that undergoes aberrant splicing to produce non-viable SMN protein (Lefebvre et al., 1995; Ruggiu et al., 2012). The aberrant splicing of *SMN2* is due to a C to T transition at position +6 of exon 7 which results in exon 7 exclusion in almost 90% of *SMN2* mRNA transcripts. The 10% of *SMN2* transcripts that are spliced correctly to produce full length SMN (FL-SMN) is insufficient to compensate for the loss of *SMN1*. Therefore, clinical severity of SMA is inversely correlated with the number of *SMN2* copies (Butchbach, 2016; Kolb and Kissel, 2015; Monani and De Vivo, 2014).

Modifying *SMN2* splicing and expression has been identified as one of the most promising therapeutic approaches for potential treatment of SMA (Sendtner, 2010; Tsai, 2012). One such class of molecules that act in this manner are the histone deacetylase inhibitors (HDACis), which have been shown to increase overall *SMN2* transcription and expression both *in vivo* and *in vitro* (Kernochan et al., 2005). HDACis act to induce hyperacetylation of histone in the promoter region of *SMN2*, increasing accessibility of the chromatin to transcription factors and machinery (Kernochan et al., 2005; Mohseni et al., 2013). Several HDACis such as valproic acid (VPA) (Brichta et al., 2003; Sumner et al., 2003), phenylbutyrate (Andreassi et al., 2004), suberoylanilide hydroxamic acid (SAHA) (Hahnen et al., 2006; Riessland et al., 2010) and M344 (Riessland et al., 2006) have been shown to increase *SMN2* expression. In this class of compounds, only VPA and phenylbutyrate have reached clinical trials generating mixed results in terms of clinical efficacy (Mohseni et al., 2013).

Modification of *SMN2* splicing to induce expression of FL-SMN has also been achieved with splice-switching oligonucleotides (SSOs). SSOs represent a particularly promising avenue for SMA therapy as all patients possess at least one copy of *SMN2* that can be targeted with SSOs to correct aberrant splicing in favour of FL-*SMN2* production. Recently, FDA has approved an SSO, Spinraza™ (nusinersen), to treat SMA (Biogen, 2016; BIOGEN and Pharmaceuticals™, 2016). SSOs act via a steric blocking mechanism by binding the intronic splice silencer (ISS-N1) motif found within intron 7 of *SMN2* (Porensky and Burghes, 2013). The binding of SSOs to the ISS-N1 prevents the binding of inhibitory splice element hnRNP-A1 at this site (Hua et al., 2008). This leads to the retention of exon 7 and restoration of full-length *SMN2* as a result of correction of the *SMN2* splicing pattern.

Although the above therapeutic strategies have demonstrated efficacy in increasing the level of SMN protein, in this study, for the first time, we investigated the effect of combination of VPA and SSO. We have shown that combination therapy of VPA and SSO is superior in upregulating SMN production *in vitro* as compared to the singular treatment of each compound at both transcriptional and protein levels.

2. Materials and methods

2.1. Materials

9-Fluorenylmethoxycarbonyl (Fmoc) protected L-α-amino acids and coupling reagents HBTU were from GL Biochem (Shanghai, China). Fmoc-Rink Amide ProTide resin was purchased from John Morris (Melbourne, Australia). Di-isoproplyethylamine (DIEA), piperidine, acetonitrile and dimethylformamide (DMF) were from Merck (Melbourne, Australia). *N*,*N*′-Diisopropylcarbodiimide (DIC), Triisopropylsilane (TIS) and NH_4HCO_3 were obtained from Sigma-Aldrich (Castle Hill, Australia), Trifluoroacetic acid (TFA) was from AusPep (Melbourne, Australia). High Capacity cDNA RT Kit was from Applied Biosystems (Melbourne, Australia), Power SYBR Green PCR Master Mix form Life Technology (Melbourne, Australia) and primers from Sigma-Aldrich (Melbourne, Australia). PMO was purchased from GeneTools, LLC. (Philomath, USA). Valproic acid (VPA) was from Stemgent (Lexington, USA). All other reagents were obtained from Sigma-Aldrich.

2.2. Peptide synthesis and conjugation to PMO

Peptides were synthesised and purified as previously described (Shabanpoor et al., 2015). VPA was coupled to the *N*-terminus either through a non-hydrolysable amide bond by activating the carboxyl group of VPA using HBTU (5 eq) in the presence of DIEA (10 eq). However, for the hydrolysable VPA, ester conjugation was achieved using a hydroxymethyl benzoic acid (HMBA) linker. VPA (4 eq.) was coupled to HMBA (4 eq.) using DIC/DMAP (4eq./1 eq.) at room temperature overnight.

The ISS-N1 targeting 20-mer PMO (5′-ATTCACTTTCATAATGCTGG-3′) was purchased from Gene Tools LLC (Philomath, USA). The unmodified PMO was functionalised by coupling 3-maleimido-propanoic acid to the free secondary amine group at the 3'end of PMO as previously described (Shabanpoor and Gait, 2013). This maleimide-functionalised PMO was dissolved in H_2O and the peptides (2-fold excess over PMO) with a cysteine residue at their C-terminus dissolved in PBS (pH 7.4) was added. The pH of the reaction was adjusted to 7–8 using NH_4HCO_3. The reaction was monitored by MALDI-TOF mass spectrometry and the absence of PMO indicated the completion of reaction. The peptide-PMO conjugates (P-PMO) were purified as described above. The molar absorption at 265 nm in 0.1 M HCl solution was measured and used to calculate the molar concentration of P-PMOs for their subsequent use in the cell assays.

2.3. Human SMA fibroblast culture and transfection

SMA Type I patient-derived fibroblasts (GM03813, Coriell Cell Repositories) were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin (Pen-Strep) and 1% L-glutamine (L-Glu) at 37 °C. Cells used in this study were from passage 15–25. VPA (100 mM) was dissolved in Milli-Q H_2O and peptide-PMO conjugates were diluted to appropriate concentrations in Opti-MEM/1% Pen-Strep reduced serum medium. The SMA fibroblasts were treated for either 4 h or the previously determined optimal time of 16 h (Brichta et al., 2003).

2.4. Quantitative real-time PCR

Total RNA was extracted using Trizol (TRIreagent, Sigma, UK) according to the manufacturer's instructions (Life Technologies, AUS). Using an ABI High Capacity cDNA Reverse Transcription Kit, 1 μg of purified RNA was used to synthesise single-strand cDNA.