REGULATION OF SMN EXPRESSION BY DcpS INHIBITION IN SPINAL MUSCULAR ATROPHY

by

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Spinal muscular atrophy (SMA) is a neurodegenerative disorder that is characterized by the loss of motor neurons in the anterior horn of the spinal cord and brainstem, resulting in progressive muscle weakness and atrophy. SMA is caused by the loss or mutations of the survival motor neuron 1 (SMN1) gene, which encodes the SMN protein. SMA patients retain at least one copy of the survival motor neuron 2 (SMN2) gene. SMN2 differs from SMN1 by 11 nucleotides; only one of which is functionally relevant and it is a C-to-T inversion in exon 7. This single nucleotide transition causes SMN2 to predominantly produce an incorrectly spliced mRNA that excludes exon 7. SMN2 transcripts that lack exon 7 are termed SMNΔ7 and encode for a truncated form of the SMN protein that is not fully functional. There is currently no effective treatment available for SMA, however one of the potential therapeutic approaches is to develop small molecules that promote an increase in the transcription of SMN2 gene. Ultrahigh-throughput screening identified a series of C5-substituted 2,4-diaminoquinazoline compounds (2,4-DAQs) that increase SMN2 gene expression. In this study, we investigated the effects of four 2,4-DAQs—D156844, D158872, D157161, and D157495—as potential therapeutics for SMA. We show that the compounds increase SMN2 promoter activity in the motor neuron-like, NSC-34 reporter assay. D157495 had the lowest EC50, proving that it is the most effective of
the four compounds at inducing SMN2 transcription. However, when fibroblasts derived from SMA type II patients were treated with varying concentrations of 2,4-DAQs, the compounds had no significant effects on SMN2 mRNA or SMN protein levels in these cell lines. The 2,4-DAQs also showed no significant differences in the levels of Smn mRNA and protein in the motor neuron-like NSC-34 cell line.

Using protein microarrays, these compounds were found to bind to and inhibit the activity of the mRNA scavenger decapping enzyme, DcpS. This protein is known to be a modulator of RNA metabolism and functions to hydrolyze the resulting cap structure following mRNA decay. DcpS was previously found to modulate the expression levels of several RNAs in SH-SY5Y retinoblastoma cells and HEK293T cells. These include, a member of the basic helix-loop-helix family of transcription factors (ATOH7) and two long non-coding RNAs (DRNT1 and DRNT2). We were able to validate these findings using fibroblasts derived from SMA type II patients as well as non-SMA fibroblasts. Treatment of these fibroblast cell lines with all four compounds resulted in an increase in the expression levels of ATOH7, DRNT1, and DRNT2. Treatment of the motor neuron-like NSC-34 cells also resulted in an increase in Atoh7. Additionally, we found that the basal level of ATOH7 is significantly decreased in SMA type II patients when compared to non-SMA fibroblasts.

Collectively, our data demonstrate that the 2,4-DAQs do not increase SMN expression in patient-derived fibroblasts however, these compounds proved to be effective at increasing SMN2 promoter activation in the NSC-34 cell-based promoter assay. The property of these compounds of inhibiting DcpS may indirectly influence SMN
expression by modulating the levels of ATOH7, DRNT1, and DRNT2. Our findings suggest that the 2,4-DAQs have beneficial effects that are independent of SMN expression and the DcpS-inducible transcripts could be the actual molecular targets of these compounds.
1.1 Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) is an autosomal recessive disorder. SMA is classified as one of the most common genetic causes of infant death worldwide due to its high incidence rate—approximately 1 in 6000-10,000 babies born are affected (Pearn, 1978; Cuscó et al., 2002) and 1 in 25-50 people are genetic carriers (Ben-Shachar et al., 2011; Su et al., 2011; Sugarman et al., 2012). The major pathological hallmark of SMA is the loss of α motor neurons in the lower brainstem and the anterior horn of the spinal cord (Crawford and Pardo, 1996). This results in progressive weakness of limb and trunk muscles and paralysis. As shown in table 1.1, SMA is classified into five clinical grades based on the age of onset of clinical symptoms and disease severity (Munsat and Davies, 1992; Russman, 2007). SMA type 0, with prenatal onset is a very severe form of the disease. SMA type I is another severe form of SMA and it is also referred to as Werdnig-Hoffmann disease. SMA type I patients typically show generalized muscle weakness and hypotonia within 6 months after birth and are never able to sit unaided. Patients with this type do not
survive beyond their first 2 years. SMA type II is of intermediate severity and affected patients develop clinical symptoms before 18 months of age. These patients generally have a longer life expectancy—approximately 30 years of age (Russman, 2007). SMA type II patients achieve the ability to sit however; they cannot stand or walk independently. Type III is a less severe form of SMA and it is referred to as Kugelberg-Welander disease or juvenile SMA. Clinical symptoms of SMA type III manifest before 18 months of age. These patients can survive long into adulthood; they also achieve the ability to sit and walk on their own but often become wheelchair bound. Type IV is the adult form of SMA and symptoms begin between 18 and 21 years of age. SMA type IV patients are comparatively mildly affected and have a normal life expectancy.

<table>
<thead>
<tr>
<th>Type</th>
<th>Age of onset</th>
<th>Clinical symptoms</th>
<th>Life Expectancy</th>
<th>SMN2 Copy Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Prenatal</td>
<td>Requires respiratory support at birth, unable to sit, stand or walk</td>
<td>&lt;6 months</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>&lt;6 months</td>
<td>Unable to sit, stand or walk</td>
<td>&lt;2 years</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>6-18 months</td>
<td>Unable to stand or walk</td>
<td>10-40 years</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>&gt;18 months</td>
<td>Requires assistance to walk</td>
<td>Adult</td>
<td>3-4</td>
</tr>
<tr>
<td>4</td>
<td>&gt;5 years</td>
<td>Symptoms are benign</td>
<td>Adult</td>
<td>&gt;4</td>
</tr>
</tbody>
</table>

Table 1.1. **Classification criteria for spinal muscular atrophy.** Adapted from Butchbach, 2016. SMA patients are characterized into subgroups based on clinical severity of the disease and age of onset.
1.2 Genetic Bases of SMA

In 1995, Lefebvre et al. identified the SMA disease-determining gene as survival motor neuron (SMN) gene. The SMN gene is located on chromosome 5q13. This region of the human genome contains a 500-kb inverted duplication, resulting in two versions of the SMN gene—the telomeric SMN1 gene and the centromeric SMN2 gene. It has been shown that SMN1 is missing in approximately 95% of type I SMA patients (Lefebvre et al., 1995; Lorson et al., 1999; Monani et al., 1999).

Although SMN1 and SMN2 are almost identical, they differ in the processing of their primary transcripts. SMN1 contains a ‘C’ nucleotide at position 6 of exon 7 whereas SMN2 contains a ‘T’ nucleotide at this position (DiDonato et al., 2001; Lorson et al., 1999; Monani et al., 1999). The C in SMN1 has been shown to form an exonic splicing enhancer (ESE) element that is bound by the SR protein ASF/SF2 to promote incorporation of exon 7 (Cartegni & Krainer, 2002). The T at that same position in SMN2 exon 7 disrupts the ESE motif and creates an exonic splicing silencer (ESS) that is recognized by the ribonucleoprotein hnRNPA1, causing the exclusion of exon 7. Thus, SMN1 produces predominantly full-length transcripts that contain nine exons (1, 2a, 2b, 3 to 8). These transcripts are termed FL-SMN and code for the 294-amino acid protein, SMN. SMN2 produces low levels of FL-SMN transcripts however, the C-to-T inversion results in the exclusion of exon 7 from the majority of SMN2 transcripts, as shown in figure 1.1. The transcripts generated from SMN2 that lack exon 7 are referred to as SMNΔ7 (Cartegni et al., 2006).
Alternatively spliced transcripts code for a truncated form of the SMN protein of only 282 amino acids (Chang et al., 2004). This shortening of SMN protein reduces its stability therefore, the protein is not fully functional and degrades rapidly. Only a small amount of SMN2 transcripts splice correctly to include exon 7 and produce FL-SMN (Cartegni et al., 2006). Consequently, SMN2 produces low levels of the fully functional SMN protein.

![Diagram of SMN1 and SMN2 genes](image)

**Figure 1.1. Illustration of the SMN1 and SMN2 genes.** Adapted from Butchbach and Burghes 2004. The C-to-T inversion in exon 7 of SMN2 causes it to splice incorrectly and to exclude exon 7 in the majority of its transcripts. Due to the nucleotide transition, these transcripts produce a truncated form of the SMN protein that is not fully functional.
A unique feature of SMA is that all patients carry at least one copy of \textit{SMN2} and studies have shown that \textit{SMN2} is present in variable copy number between individuals affected with different types of SMA (Butchbach, 2016). Patients with high \textit{SMN2} copy number have a less severe form of SMA and are able to produce an increased amount of full length SMN transcripts, resulting in an increase in the fully functional SMN protein (Cuscó et al., 2006; Wirth et al, 2006). Hence, SMA type III patients have on average, more \textit{SMN2} copies than SMA type II or type I patients (Table 1.1). Moreover, studies in SMA mouse models have shown the similar inverse correlation between \textit{SMN2} copy number and the severity of the disease. Mice carry only one \textit{Smn} gene, which is equivalent to the human \textit{SMN1}, and lack another copy comparable to the human \textit{SMN2} (DiDonato et al. 1997; Viollet et al. 1997). In 1997, Schrank et al. showed that when the single murine \textit{Smn} gene is completely knocked out (\textit{Smn} \textsuperscript{-/-}), major cell death occurs at an early embryonic stage. To overcome the embryonic lethality, different copy numbers of a transgene containing the human \textit{SMN2} have been expressed in m\textit{Smn} knock-out mice and the ones expressing one copy of \textit{SMN2} died shortly after birth while the ones with two \textit{SMN2} copies showed reduced number of motor neurons by day 5 and only survived to 6 to 8 days after birth (Hsieh-Li et al., 2000; Monani et al., 2000). Mice expressing three copies of \textit{SMN2} had a normal lifespan and only showed a mild form of SMA (Michaud et al., 2010). However, when eight copies of \textit{SMN2} are introduced in the Smn\textsuperscript{-/-} mice, they survived without developing any SMA-like symptoms (Monani et al., 2000). These findings
support the hypothesis that SMN2 influences disease severity in SMA and increasing its copy number ameliorate or even rescues the SMA phenotype.

1.3 The SMN Protein

The SMN protein is expressed in all cells of the body and has a molecular weight of 38 kDa. SMN is present in both the nucleus and the cytoplasm (Lefebvre et al, 1997; Liu et al., 1997; Meister et al., 2000). Immunostaining of several cell types have shown that in the nucleus, SMN is found within discrete structures known as Gemini of Cajal (Coiled) bodies or gems (Liu & Dreyfuss, 1996). Fibroblasts from SMA patients display low level of SMN expression and almost complete absence of SMN-containing gems (Coovert et al., 1997). As suggested by their name, gems are similar in size and number to Cajal bodies and often associate with them. Cajal bodies were identified in 1903 (Ogg and Lamond, 2002) and were shown to contain high levels of small nuclear ribonucleoproteins (snRNPs) and small nucleolar ribonucleoproteins (snoRNPs).

Although their precise functions are not yet clarified, previous studies have shown that Cajal bodies are mainly implicated in the biogenesis, trafficking and regeneration of snRNPs and snoRNPs. In all cell types and tissues, SMN forms a complex along with Gemin proteins 2-8 and unr-interacting protein (unrip). The SMN complex is involved in the assembly of snRNPs, which in turn are translocated to the nucleus and act as components of the spliceosome complex to perform pre-mRNA
splicing (Kolb et al., 2007; Pellizzoni et al., 2007). Altogether, these studies provide sufficient evidence for the role of the SMN protein in mRNA processing through the assembly of snRNPs, however, deficiency in SMN protein predominantly affects α motor neurons. This phenomenon remains a mystery given the fact that in the pathogenesis of SMA, there is a relative depletion of SMN throughout the body.

Numerous immunocytochemical studies have localized the SMN protein in dendrites and axons. This suggests that SMN has additional functions in the axon of neurons. In 2003, Zhang and colleagues have shown that SMN is localized in granules that are found in neurites and growth cones of cultured neuronal cells. These granules display rapid, bidirectional movements controlled by both microtubules and microfilaments (Rossoll et al., 2003; Zhang et al., 2003). SMN also associates with several mRNA-binding proteins proving that SMN plays a role in the transport and processing of RNA along the axons. SMN interacts with the mRNA-binding protein HuD that is known to be restricted to neurons and regulates the stability of mRNAs that are important for neural development and plasticity (Pascale et al., 2004; Hinman & Lou, 2008). Both SMN and HuD interact with cpg15 mRNA in axons. Cpg15, originally termed candidate plasticity-related gene 15, functions in axon branching and the formation of neuromuscular junction (Atken et al., 2011). SMN also binds hnRNP-R, which regulates the localization of β-actin mRNA along the axons (Rossoll et al., 2003; Todd et al., 2010; Glinka et al., 2010). Although a definitive function for the
SMN protein remains unclear, these findings provide evidence for the implication of SMN in the processing and transport of mRNAs along axons.

1.4 C5-substituted 2,4-diaminoquinazoline Compounds as Therapeutics for SMA

There is currently no approved treatment for SMA however; different treatment strategies are being investigated. Knowing that there is a direct inverse correlation between SMN2 copy number and SMA phenotype, researchers have been exploring multiple mechanisms that induce SMN expression from SMN2. Using this therapeutic strategy, a variety of compounds have been found to increase SMN protein levels either by increasing the inclusion of exon 7 in the SMN2 transcripts, activating SMN2 gene expression, or stabilizing the SMN protein that is generated from SMN2 (Cherry et al., 2014). Other approaches that do not target the SMN2 gene have also been investigated and these include stem cell therapy, neuroprotection, and gene therapy.

A high-throughput screening of over 500,000 compounds in a cell-based reporter assay identified two series of compounds as potential hits for SMA therapy (Jarecki et al., 2005). These include the C5-substituted 2,4-diaminoquinazolines (2,4-DAQs), which are a series of compounds that increase SMN2 gene expression in a cell-based assay that was established by inserting a 3.4 kb of the SMN2 gene promoter linked to a bacterial β-lactamase gene into the motor neuron-like cell line, NSC-34. These compounds were shown to increase SMN2 mRNA levels in SMA patient-
derived fibroblasts, SMN protein levels as well as the number of gems (Jarecki et al., 2005). However, these compounds were toxic, or poorly metabolized; they also had poor blood-brain barrier penetration and required high doses to achieve a sufficient increase in *SMN2* expression. To overcome these obstacles, the 2,4-DAQs were further optimized and resulted in a class of quinazoline derivatives that are permeable across the blood-brain barrier, orally bioavailable, have good pharmacokinetics profile and are generally more potent at activating *SMN2* promoter activity (Thurmond et al., 2008). One of these derivatives, D156844 was shown to be very potent at activating *SMN2* gene expression. In fibroblasts derived from SMA patients, these compounds increased SMN protein levels and the number of gems to levels that are comparable to SMA carrier fibroblasts. This compound was also tested in a severe SMA mouse model, *SMNΔ7* SMA mouse, and was shown to increase the mean lifespan of these mice and prevent additional loss of motor neuron when administered prior to the onset of degeneration of motor neurons (Butchbach et al., 2010). Le et al. (2005) developed the *SMNΔ7* SMA mouse model by crossing expressing *SMN2* that lack exon 7 with mice carrying *SMN2* and *mSmn* knockout allele. *SMNΔ7* SMA mice display a slightly less severe SMA-like phenotype that resembles type II SMA in humans and live for around 14 days (Le et al., 2005; Butchbach et al., 2007). These findings were used as basis to further optimize the 2,4-DAQs and develop the quinazoline derivative, D157495 also known as RG3039. This compound has been the first small-molecule that was selected for phase I clinical trial for SMA therapy. RG3039 and was
extensively studied in three independent SMA mouse models—Taiwanese 5058 Hemi SMA mice, 2B/- SMA mice, and SMNΔ7 mice (Gagliotti et al., 2013; Van Meerbeke et al., 2013). The Smn^{2B/-} mice are a product of combining the 2B allele with the Smn^{-/-} allele in an FVB/N background (Schrank et al., 1997) while the 5058 Hemi SMA mice have four copies of the human \textit{SMN2} gene on an \textit{Smn}{\textit{Δ7}} background (Hsieh-Li et al., 2000). RG3039 increases motor function and improves survival in all three SMA mouse models.

D156844 was used in a protein microarray to determine its molecular target and Singh et al., 2008 identified that this 2,4-DAQ inhibits the scavenger mRNA decapping enzyme, DcpS. This enzyme is a member of the Histidine Triad (HIT) family of nucleotide hydrolases and is implicated in mRNA degradation (Liu et al., 2002). After complete 3’ to 5’ degradation of the mRNA in eukaryotes by exonucleases, DcpS hydrolyzes the residual cap structure, 7-methylguanosine nucleoside triphosphate (m7GpppN), to release 5’ diphosphate-oligonucleotide mRNA and 7-methylguanosine monophosphate (m7GMP) as products (Liu et al., 2002, Wang & Kiledjian, 2001). DcpS is also implicated in the 5’ to 3’ mRNA decay pathway where it is found to dephosphorylate the m7GDP decapping product to m7GMP (van Dijk et al., 2003). Based on crystal structures and NMR data, Singh et al. (2008) revealed that these compounds bind to DcpS at a site that promotes the enzyme to acquire an inactive conformation so that it cannot easily perform its decapping function. Cell-based reporter assays revealed that DcpS has additional functions in
pre-mRNA splicing and in transcription. These studies have shown that DcpS knockdown displaces its interaction with the cap-binding proteins, Cbp20 and eIF4E (Liu et al., 2004; Shen et al., 2008). This results in a dysfunction in translation and the normal processing of mRNAs. By disrupting this essential function of DcpS, the 2,4 DAQs can accumulate the levels of the cap binding proteins in cells as well as the cap products m\(^7\)GDP and m\(^7\)GpppN to return to earlier steps in the mRNA decay process (Singh et al., 2008). Hence, the 2,4 DAQs inhibit DcpS to ultimately reduce mRNA turnover. A recent study by Zhou et al., 2015 also showed that the decapping inhibition of DcpS can affect global levels of RNAs by treating HEK293T cells and SH-SY5Y cells with RG3039. DcpS was found to alter the levels of four RNAs—HS370762, BC011766, ATOH7, and RAB26—in the two cell lines. HS370762 and BC011766 are two long noncoding RNAs and their levels in these cells were shown to be dependent on the decapping property of DcpS. Thus, HS370762 and BC011766 have been respectively renamed DcpS-responsive noncoding transcripts (DRNT) 1 and 2 (Zhou et al., 2015).

### 1.5 Hypothesis and Aims

The aim of this project is to assess the relationship between DcpS and SMN expression through the quinazoline derivatives—D156844, D158872, D157161, and D157495. Previously, treatment of the SMA type II fibroblast cell line—GM03813—with increasing concentration of each of the quinazoline compounds was shown to
increase gems to levels observed in the carrier cell line, GM03814 (manuscript in preparation). Since gems are found in association with the SMN protein in the nucleus, we hypothesize that treatment of SMA and non-SMA fibroblasts as well as the motor neuron-like cell line—NSC-34—with varying concentrations of the quinazoline derivatives will translate into an increase in SMN2 gene expression, resulting in an overall increase in SMN protein level. Using the SMN2 β-lactamase (BLA) reporter gene NSC-34 clonal cell lines, we also explored whether these compounds have a direct effect on the activation of the SMN2 promoter or promote the inclusion of exon 7 into the SMN2 gene. Additionally, we investigated the effect of these compounds on SMN2 mRNA stability and the splicing patterns of FL-SMN and SMNA7. Considering these compounds were developed to be used in human patients, we looked at their effects on the expression of DcpS-inducible transcripts—ATOH7, DRNT1, DRTN2, MAOB, and PAQR8—in SMA and non-SMA fibroblasts. The expression of Atoh7 was further studied in NSC-34 cells. Short hairpin RNA-mediated knockdown of DcpS in the GM03813 fibroblasts resulted in an increase in gems. Thus, we looked at the effect of knocking-down DcpS in the presence of these compounds or DMSO on SMN2 mRNA, SMN protein, and the expression of ATOH7.
2.1 Drug Compounds

The C5-substituted 2,4-diaminoquinazoline derivatives (D156844, D157495, D157161 and D158872) were obtained from Repligen and were all dissolved in DMSO.

2.2 Cell Culture

Fibroblasts derived from type II SMA (GM03813, GM22592 and AIDHC-SP22) and non-SMA (GM03814, AIDHC-NMC1, AIDHC-SC1 and AIDHC-SC2) individuals were grown in DMEM containing 10% EquaFETAL (Atlas Biologicals), 2mM L-glutamine (Life Technologies) and 1% penicillin-streptomycin (Life technologies). GM03813, GM03814 and GM22592 fibroblast lines were obtained from Coriell Cell Repositories (Camden, NJ). All other fibroblast lines were generated at Nemours/Alfred I. duPont Hospital for Children. The SMA fibroblasts are homozygous for deletion of exon 7 in the \textit{SMN2} gene and have three copies of \textit{SMN2}
(Stabley et al., 2015). As shown in Stabley et al., 2015, the fibroblast line GM03814 has one copy of \textit{SMN1} and five copies of \textit{SMN2}. The non-SMA fibroblasts possess two copies of each, \textit{SMN1} and \textit{SMN2}; these cell lines were used as references to compare changes in \textit{SMN} mRNA and protein expression in SMA fibroblasts in response to treatment.

The mouse motor neuron cell line NSC-34 (Cashman et al., 1992) and the NSC-34-based reporter lines—clones 5.3 (Andreassi et al., 2001) and 11 (Jarecki et al., 2005) were grown in DMEM, 5% fetal bovine serum and antibiotics. In all instances, the cells were maintained at approximately 80-90% confluency, 37°C, and 5% carbon dioxide in a humidified chamber.

2.3 Drug Treatment of Fibroblasts

All cells were plated 24 hours prior treatment with drug compounds and harvested following five days of treatment. Plating density was dependent on the assays being conducted. Medium was changed daily and fresh drug compounds or DMSO was added at a 1:1000 dilution approximately every 24 hours during the five-day treatment period.

2.4 \(\beta\)-Lactamase Reporter Assays

The NSC-34 clone 11 cell line (Vertex Pharmaceuticals, Jarecki et al., 2005) was used on the \textit{SMN2} promoter assay and the NSC-34 clone 5.3 (Vertex Pharmaceuticals,
Andreassi et al., 2001) was used on the SMN2 splicing assay. The cells were seeded at 5 x 10⁴ cells/dish onto a black-walled, clear bottom 96-well tissue culture plates (Santa Cruz Biotechnology). For these experiments, the compounds were tested in quadruplicate. Drug compounds were added to the medium using a 96-well pin tool and plates were incubated for 19 hours. At the end of the incubation, 20µL of 6X CCF2-AM dye (GeneBlazer In Vivo Detection Kit, Life Technologies) was added to each of the assay wells and plates were incubated at room temperature for 2 hours before the plates are read on a plate reader (Victor X4, Perkin Elmer). Two readouts were obtained for each sample: the CCF2-AM substrate at 530 nm (green channel) and the product at 460 nm (blue channel). The readouts obtained for the negative control, which contains serum-free media and antibiotics but no compounds, were subtracted from the ratio of blue to green signal for each sample. The resulting value was plotted against compound concentration for both assays and used to generate a dose response curve for the SMN2 promoter assay.

2.5 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Fibroblasts were plated onto 6-well plates at a density of 3.2 X 10⁴ cells/well and treated as described above. The total RNA was extracted from cell lines using the RNAeasy Mini columns (Qiagen), as described by the manufacturer’s recommendations. First-strand complementary DNA was carried out using the iScript
cDNA synthesis kit (Bio-Rad) according to manufacturer’s directions. Quantitative PCR was performed in a 384 well plate on a 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Target transcripts were amplified by real time polymerase chain reaction using the SYBR Green PCR Master Mix (QIAGEN) in 10µL total volume and the following cycling conditions: a 10-minute initial denaturation step at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The following primers (Integrated DNA Technologies) were used: ATOH7, (F) 5’-AAAGCTGTCCAAAGTACGAGAC-3’, (R) 5’-CGAAGTGCTCACAGTGAGG-3’; DRNT1, (F) 5’-CACCTAGACTCATCAGTTAGATCCACC-3’, (R) 5’-GAGACCTGATGGCTACAACGTGACA-3’; DRNT2, (F) 5’-TGGGAAAGCGATGGATGACAGAGA-GAGACCTGATGGCTACAACGTGACA-3’, (R) 5’-GGTGAACGGGACACAAATTGCCAGAA-3’; MAOB, (F) 5’-ACTCTGGACTGAATGTGGTTG-3’, (R) 5’-GATACGATTCTGGGTGGTGC-3’; PAQR8, (F) 5’-AACGTCTGGACCCTTTACTG-3’, (R) 5’-CAGGTGAGGTAAATGTGATTGAC-3’; SMNex6Fq, 5’-CCATATGTCCAGTTCTCATCATTGA-3’, SMNex78Rq, 5’-ATGCCAGCATTTCTCCTAATTTA-3’; and SMNex68Rq, 5’-ATGCCAGCATTTCCAATAGC-3’; Smn (F) 5’-ACGGTGCATTACCCAGCTA-3’, Smn (R) 5’-ACGCTGTCCAAAGTACGAGAC-3’; mAtoh7 (F) 5’-CAGCTTTGGACCTTGCTGTGCTTT-3’; mAtoh7 (R) 5’-
TCTACCTGGAGCCTAGCAC-3’. Data for each transcript analyzed in human fibroblasts were normalized to the geometric mean of three reference genes, \( \beta\)-actin (\( ACTB \)), glyceraldehyde 3-phosphate dehydrogenase (\( GAPD \)), and ribosomal protein \( L0 \) (\( RPLP0 \)), to minimize the variability in the expression of a single housekeeping gene (Vandesompele et al., 2002). Transcripts analyzed in the motor neuron-like cell line were normalized to the geometric mean of two reference transcripts; murine phosphoglycerate kinase 1 (\( mPgk1 \)) and murine ribosomal protein L13a (\( mRpl13a \)). The relative transcript levels were calculated using the \( 2^{-\Delta\Delta C_t} \) method (Schmittgen and Livak, 2008) where \( \Delta C_t \) is the difference between the \( C_t \) for the target transcript and the \( C_t \) for the geometric mean of the three reference genes and \( \Delta\Delta C_t \) is defined as the difference between the \( \Delta C_t \) for the SMA sample and the \( \Delta C_t \) for the control sample. All samples were assayed in triplicate.

2.6 Messenger RNA Stability Assay

mRNA stability was assayed as described (Heier et al., 2007). Fibroblasts were plated onto 6-well plates at a density of \( 3.2 \times 10^4 \) cells/well and treated with compounds or DMSO as previously described. Twenty-four hours the five-day treatment, cells were treated with 5 \( \mu \)g/mL actinomycin D (ActD; Sigma-Aldrich). Cells were harvested by scraping at 0, 2, 6, 12, and 24 hours after ActD treatment. RNA isolation and first strand complementary DNA synthesis were performed as already described. The thermocycling profile was 50\(^\circ\)C for 2 minutes, 95\(^\circ\)C for 10
minutes, followed by 30 cycles at 95°C for 15 seconds and 60°C for 1 minute, and a final cycle at 72°C for 5 minutes. RT-PCRs were performed using primers specific for sequences within SMN exons 6 and 8: SMN exon 6 (F), 5’-

CCCATATGTCCAGATTCTCTTGAT-3’; SMN exon 8 (R), 5’-

CTACAACACCCTTCTCACAG-3’. CollagenIII (Col3A) was used as control because of its high expression in fibroblasts (Heier et al., 2007): COL3A (F), 5’-

GCTCTGCTTCATCCCACTATT-3’; COL3A (R), 5’-

GGAATACCAGGGTCACCATT-3’. The PCR products were electrophoresed through a 2% agarose gel. Data were recorded on AlphaImager (Cell Biosciences) and band intensities were quantified using ImageJ.

2.7 **Immunoblot**

To make protein extracts, cells were plated at a density of 4.0 X10^5 cells/dish and treated with test compounds as previously described. The resultant cell pellets were lysed in 50µL cell lysis buffer containing the following: phosphate-buffered saline (PBS, pH 7.4, Invitrogen), 0.1% Triton X-100 and Complete protease inhibitor cocktail (Roche). Lysates were sonicated using a Sonic Dismembrator (Model FB-50, Thermo Scientific). Protein quantification was performed using the Micro BCA Protein Assay kit (Fisher Scientific). Five micrograms of NSC-34 cells and ten micrograms of fibroblasts were mixed with 0.2-volumes non-reducing 6 x loading dye (10.28% SDS, 36% glycerol and 0.012% bromophenol blue in 350 mM Tris-HCl, pH
6.8) and 0.1-volumes 1 M dithiothreitol (DTT) and were heated at 90°C-100°C for 10 minutes and briefly centrifuged. Prepared samples along with protein ladder (Kaleidoscope Precision Plus prestained standard, Bio-Rad) were resolved through 12% polyacrylamide gels, using the MiniProtean system (Bio-Rad). Samples were then transferred onto polyvinylidyne fluoride (PVDF) membranes (0.45 µm, GE Healthcare Life Sciences) using the Trans-Blot® semi dry transfer cell and blocked for 1 h at room temperature with PBS containing 5% milk and 0.1% Tween (PBST). Primary antibodies specific to SMN (a mouse anti-SMN mAb; MANSMA2, Young et al., 2000 was used at a titer of 1:100; and clone 8 SMN, BD Transduction Laboratories, was used at 1:1000) were incubated overnight at 4°C. The blots were extensively washed with PBST (3 X 10 min) and incubated for 1 h at room temperature with an HRP-linked anti-mouse IgG secondary antibody (Rockland, 1:5000) diluted in 0.2 X blocking buffer. After extensive washing, the bound antibody was detected by chemiluminescence using the Western Sure ECL Substrate kit and developed with the LI-COR C-DiGit Blot Scanner. To confirm equal loading of protein in each lane, the blots were stripped and re-probed with either a mouse anti-β-actin (Sigma-Aldrich, 1:5000) or a mouse anti-β-tubulin (Developmental Studies Hybridoma Bank, 1:100, Klymkowsky, 1989). Band intensities were measured using the Image Studio™ Lite Software version 5.2.
2.8 Cell Cycle Analysis

Cell cycle analysis was completed as described in Mohanan et al., 2013. GM03813 fibroblasts were plated at a density of 4 X 10^5 cells/dish and treated as previously described. Cells were trypsinized lightly in 0.05% Trypsin EDTA for several minutes; equal volume of serum-free medium was added to the cells and centrifuged at 800 rpm (IEC Central® CL3 Series, Thermo Scientific). The supernatant was aspirated and cell pellet was resuspended in 500µL PBS. Cells were fixed for 4 days in 70% ethanol and kept at -20°C. The fixed samples were rinsed once in PBS and resuspended in DNA staining solution (200 µg/mL propidium iodide, 200 µg/mL RNAse A and 0.1% Triton X-100 in PBS) for 1 hour at room temperature. The cells then were examined by a C6 flow cytometer (BD Biosciences) for DNA content.

2.9 Transfection and Lentiviral production

HEK293T cells were plated in 10 cm dishes at 2 X 10^6 cells/dish and lentiviruses were generated by co-transfecting the cells with a four plasmid combination as follow: 1 µg of each of the lentivirus packaging plasmid—pRSV-REV, pMDLg/pRRE and pMD2G—was combined with 3 µg of one of the following DcpS shRNAs—5570, 5571, or 5572 (Thermo Scientific)—using 30 µL lipofectamine 2000 (Invitrogen). pLV-EGFP was used as an empty vector control to ensure successful transfection of cells while pLK0.1-scramble shRNA was used as negative control. Approximately, 18 hours after transfection, 6 mL of fresh growth media was added to plates. Supernatant
containing lentiviral particles were harvested at 24 hours and 48 hours post-transfection. Supernatant was cleared by centrifugation and filtered using 0.45 µm filters (Millipore) before being used for subsequent infections of fibroblast cell lines.

2.10 Lentiviral Transduction of Fibroblasts

SMA type II fibroblasts—GM03813, GM22592, and AIDHC-SP22—were either plated at 3.2 X 10^4 cells/well in 6-well plates or 4 x 10^5 cells/dish in 10 cm dishes and incubated overnight. Supernatant containing the lentivirus was added to cells at 24 hours and 48 hours after initial overnight incubation. During each infection, hexadimethrine bromide (polybrene, Sigma) was added at a final concentration of 4 µg/mL. The effect of D156844, D157495, or DMSO was monitored by treating the cell lines with 1:1000 of each compound for 6 days starting on the first day of infection.

2.11 Data and Statistical Analysis

Data are expressed as mean ± standard error. The EC_{50} curve was generated by normalizing drug concentration to the log scale—log (x)—and EC_{50} value of each dose response curve for the SMN2 promoter assay was determined with the computer program Prism (GraphPad). Statistical significance was determined using a one-way analysis of variance (ANOVA). All statistical analyses were performed with Sigma
Plot version 12.0. Statistical significance between treated groups and control was set at 
$p < 0.05$. 
Chapter 3

RESULTS

3.1 Effect of 2,4-DAQs on SMN2 Promoter Activity

The NSC-34 clonal cell line 11 was used to determine the potency of the 2,4-DAQs at inducing SMN2 gene activity. This cell-based model is a β-lactamase driven reporter assay that monitors the activation of the SMN2 promoter. Trichostatin A (TSA), a histone deacetylase inhibitor (HDACi), previously shown to increase BLA expression twofold (Avila et al., 2007; Chang et al., 2001) was used in this study as a positive control (data not shown). When compared to the clone 11 cells treated with DMSO, those treated with 10 µM of each compound display ~3-fold increase in their 460/530 fluorescence emission ratios (Figure 3.1, A, n=4, p<0.05). Figures 3.1, B-E show the β-lactamase activity of the clone 11 cells post-treatment with differing concentrations of D156844, D158872, D157161, and D157495. All four compounds display sigmoidal dose response activity and increase the SMN2 promoter activity in a dose-dependent manner. D157495 has the lowest EC₅₀ value compared to the other three compounds and the EC₅₀ values for D156844 and D158872 are within similar
range ~50-60 nM (Table 3.1). These data suggest that D157495 is the most potent at activating the SMN2 promoter activity.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Average EC$_{50}$ (nM)</th>
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<tbody>
<tr>
<td>D156844</td>
<td>58.36 ± 0.0075</td>
</tr>
<tr>
<td>D158872</td>
<td>54.59 ± 0.0169</td>
</tr>
<tr>
<td>D157161</td>
<td>77.17 ± 0.0145</td>
</tr>
<tr>
<td>D157495</td>
<td>23.30 ± 0.0042</td>
</tr>
</tbody>
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Table 3.1. The EC$_{50}$ values of 2,4-DAQs using the SMN2 promoter assay. NSC-34 clone 11 cells were treated with increasing concentrations of compounds (1 nM-10 µM) for 19 hours and the ratio of fluorescence emission at 460 nm and 530 nm was fitted into a dose-response curve to obtain EC$_{50}$ values for each compound. Results are presented as means ± SEM of four replicates.
Figure 3.1. Dose response experiments of 2,4-DAQs on the SMN2 promoter assay. NSC-34 clone 11 cells were incubated with increasing concentration (1 nM-10 µM) of each compound for 19 hours prior to loading with BLA substrate, CCF2/AM (n=4/treatment group). β-lactamase activity of cells treated with 10 µM of each compound relative to treatment with DMSO for 19 hours is plotted to assess SMN2 promoter activation (A). Representative dose response curves for D156844 (B), D158872 (C), D157161 (D), and D157495 (E). Results are displayed as the ratio of fluorescence intensity of the blue channel (460 nM) to that of the green channel (530 nM). Higher 460/530 ratios indicate an increased in BLA reporter activity. All error bars in the figure indicate standard error of the mean. Asterisks indicate significant differences in comparison to the clone 11 cells treated with DMSO (*p <0.05, one-way ANOVA)
3.2 Effect of 2,4-DAQs on SMN2 mRNA Transcript Levels in SMA and Non-SMA Fibroblasts

To examine SMN2 expression in response to the quinazoline derivatives, a fibroblast cell line (GM03813) derived from a type II SMA patient (Scudiero et al., 1986) was treated with different doses of D156844, D158872, D157161 and D157495. The fibroblast cell line GM03814 was used for comparison as it is derived from the carrier mother of GM03813 and carries one copy of SMN1 (Zhong et al., 2011; Stably et al., 2015). Using qRT-PCR, the amounts of full-length SMN (FL-SMN) and SMN lacking exon 7 (SMNA7) mRNAs were examined in fibroblasts treated with the compounds or DMSO. Interestingly, these compounds did not increase FL-SMN (Figure 3.2, A) or SMNA7 (Figure 3.2, B) when compared to GM03813 cells treated with DMSO.

The effects of D156844, D158872, D157161 and D157495 were also tested in two additional type II SMA fibroblasts—GM22592, AIDHC-SP22— that have three copies of the SMN2 gene, similarly to GM03813 (Stabley et al., 2015). These experiments were conducted because the quinazolines had no significant effect on GM03813 fibroblasts and we wanted to ensure that such response to treatment with the compounds is not specific to GM03813. The compounds were also tested on non-SMA fibroblasts—AIDHC-NMC1, AIDHC-SC1, and AIDHC-SC2—that have two copies of each of each human SMN1 and SMN2 (Stabley et al., 2015). To monitor the effect of DMSO alone in the SMA and non-SMA cell lines, basal expression levels of
FL-SMN and SMNΔ7 expression were quantified by qRT-PCR. Treatment with DMSO alone demonstrated that the non-SMA fibroblasts have significantly higher basal expression of FL-SMN than the SMA type II cells (Figure 3.2, C). Although AIDHC-NMC1 showed a significant increase in SMNΔ7 expression when treated with DMSO, the other two non-SMA cell lines—AIDHC-SC1, and AIDHC-SC2—as well as GM03813 and GM22592 had similar basal levels of FL-SMN and SMNΔ7 (Figure 3.2, D). Treatment with 1 µM of each compound for 5 days resulted in no detectable increase FL-SMN (Fig. 3.2, E) or SMNΔ7 (Fig. 3.2, F) mRNAs relative to treatment with DMSO, in any of the fibroblast lines tested.

Figure 3.2. Effect of 2,4-DAQs on fold change of FL-SMN and SMNΔ7 mRNA levels in fibroblasts. (A) Changes in FL-SMN mRNA levels in SMA II fibroblasts GM03813 treated with the indicated concentrations of D156844, D158872, D157161, and D157495 or DMSO for five days (n=3/treatment group). (B) Changes in SMNΔ7 mRNA levels in GM03813 treated with indicated concentrations of the 2,4-DAQs or DMSO for 5 days (n=3/treatments group). Basal differences in FL-SMN (C) and SMNΔ7 (D) mRNA levels between fibroblast lines (*p<0.05, one-way ANOVA). qRT-PCR analyses of FL-SMN (E) and SMNΔ7 (F) mRNAs in SMA II fibroblasts—GM22592 and AIDHC-SP22 and non-SMA fibroblasts—AIDHC-NMC1, AIDHC-SC1, and AIDHC-SC2 treated with 1 µM of the 2,4-DAQs or DMSO for 5 days (n=3/treatment group). All error bars represent ± SEM. The carrier fibroblast cell line GM03814 represents the levels trying to be obtained. Data are shown as fold increase mRNA relative to three reference transcripts—ACTB, GAPDH, and RPLP0—mRNAs. Results are expressed as mean ± SEM. (Figure is on the next page).
3.3 Effect of 2,4-DAQs on SMN2 Splicing Activity

One important question was to investigate whether the 2,4-DAQs could also affect splicing of SMN2 in addition to their positive effect on SMN2 promoter activation. NSC-34 clonal cell line 5.3 was used to monitor the inclusion of exon 7 into the SMN2 transcripts (Andreassi et al., 2001) and treatment of these cells with 10 µM of D156844 and D158872 had no effect on the β-lactamase activity while 10 µM of D157161 and D157495 showed significant decrease in 460/530 fluorescence emission ratio (Figure 3.3, A). When the 460/530 ratios were fitted into a dose response curve for increasing concentrations (1 nM-10 µM) of each of the compound, no evident graded responses were detected to the varying doses of 2,4-DAQs (Figure 3.3, B-E).

Splicing patterns of FL-SMN and SMNΔ7 were also assayed in GM03813 SMA fibroblasts. The ratios of the PCR products following treatment with 1 µM of 2,4-DAQs was consistent across the range in comparison to DMSO treatment. Additionally, none of the compound altered the splicing of SMN2 to the level observed in the carrier fibroblast, GM03814 (figure 3.4, B). These data indicate that the 2,4-DAQs do not promote the inclusion of exon 7 into the spliced SMN2 transcripts.
Figure 3.3. Effect of 2,4-DAQs on the SMN2 splicing BLA-reporter gene assay. NSC34 clone 5.3 cells were treated with varying concentrations of 2,4-DAQs (1 nM to 10 µM) or DMSO for 19 hours prior to loading with BLA substrate, CCF2/AM (n=4/treatment group). β-lactamase activity of cells treated with 10 µM of each compound relative to DMSO is plotted to assess SMN7 inclusion (A). β-lactamase reporter activity was fitted into a dose response curve for each compound—D156844 (B), D158872 (C), D157161 (D), and D157495 (E). β-lactamase reporter activity is represented as the ratio of fluorescence intensity of the blue channel (460 nM) to that of the green channel (530 nM). Results are represented as mean ± SEM of n = 4 values. Asterisks indicate significant differences compared to DMSO-treated 5.3 cells (*p < 0.05, one-way ANOVA).
Figure 3.4. Effect of 2,4-DAQs on splicing of SMN2 in fibroblasts derived from patients. GM03813 cells were treated with 1 µM D156844, D158872, D157161, D157495 or DMSO for 5 days. RNA was extracted following treatment and amplified by RT-PCR using primers specific to SMNΔ7-8. PCR products were electrophoresed through a 2% agarose gel. A representative image is shown (A). The intensity of SMN-FL and SMNΔ7 in response to 2,4-DAQs treatment was quantified using ImageJ relative to DMSO and presented as SMN-FL/SMNΔ7 ratio for both GM03813 and SMA carrier fibroblasts, GM03814 (B). Results are presented as mean of three independent RT-PCR assays (*p<0.05, one-way ANOVA).
3.4 Effect of 2,4-DAQs on Stability of SMN mRNA

To assess whether the 2,4-DAQs alter the stability of FL-SMN and SMNΔ7 transcripts produced from the SMN2 gene, GM03813 and GM03814 were treated with 1 µM D156844 and D157495, or DMSO for five days. Following treatment with compounds, ActD was added to the cells at 5 µg/mL for its ability to inhibit transcription (Heier et al., 2007; Sobell, 1985; Perry and Kelley, 1970) and the cells were harvested at different time points (0, 1, 6, 12, 24 hours). The levels of FL-SMN and SMNΔ7 were assayed by RT-PCR using primers in SMN exons 6 and 8 and collagen IIIA (COL3A) was used as a control because previous studies have shown that it is highly expressed and stable in fibroblasts (Heier et al., 2007; Poulalhon et al., 2006). Representative agarose gels (2%) electrophoresis (Figure 3.5, A) show typical transcriptional and splicing patterns observed in the treated fibroblasts. In both GM03813 and GM03814, we observed no substantial difference in the rate of degradation of FL-SMN and SMNΔ7. Degradation of the SMN transcripts occurred at a steady rate in both cell lines. Treatment with D156844, D157495 or DMSO resulted in evident overlap between the SMN transcripts and this trend was consistent among the two fibroblast cell lines (Figure 3.5, B-E). Together, these results suggest that the 2,4-DAQs do not regulate the stability of FL-SMN and SMNΔ7.
Figure 3.5. Effect of 2,4-DAQs on stability of SMN2 transcripts. GM03813 and GM03814 fibroblasts were treated with 1µM of D156844 and D157495 or DMSO; they were further incubated with ActD (5 µg/mL) and harvested after 0, 1, 2, 6, 12, or 24 hours. The remaining mRNA was collected and assayed by RT-PCR on 2% agarose gel. SMN2 transcripts were amplified using primers in exons 6 and 8. (A) Representative 2% agarose gels show the remaining FL-SMN and SMNΔ7 at varying time points for the treated GM03813 (left panel) and GM03814 (right panel). The Points on the graphs represent densitometric values of FL-SMN and SMNΔ7 mRNA levels relative to 0-time point for GM03813 (B, C) and GM03814 (D, E). ImageJ was used to quantify band signals of FL-SMN and SMNΔ7.
3.5 **Effect of 2,4-DAQs on SMN Protein Levels in Fibroblasts**

Treatment of SMA and non-SMA fibroblasts with the 2,4-DAQs as previously described, resulted in no significant effect on *FL-SMN* and *SMNΔ7*. Moreover, the quinazoline compounds did not alter the basal levels of these transcripts. To validate these findings, SMA and non-SMA fibroblasts were treated with varying concentrations of the 2,4-DAQs for 5 days and SMN protein levels were determined through immunoblot analysis. Treatment of GM03813 SMA fibroblasts with increasing concentrations (1 nM-1 µM) of the drug compounds resulted in no significant change in SMN protein levels in these cells when compared to DMSO treatment (Figure 3.6, A-B). Additionally, SMN protein levels in 2,4-DAQs treated GM03813 fibroblasts did not reach the levels observed in the SMA carrier fibroblasts, GM03814. SMN expression in GM03814 is >3 fold higher than SMN protein levels in GM03813 (Figure 3.6, A-B). The basal levels of SMN protein in SMA and non-SMA fibroblasts were also established following DMSO treatment of each cell line for 5 days. Similar to what was seen at the *SMN2* mRNA levels, DMSO does not significantly alter the expression level of SMN protein in these fibroblasts (Figure 3.6, C-D). SMA and non-SMA fibroblasts were also treated with 1 µM of each quinazoline compound for 5 days and there was no significant difference in SMN protein levels in any of these cell lines (Figure 3.6, E-F). These data validate that the 2,4-DAQs have no significant effect on *SMN2* mRNA and SMN expression in patient-derived fibroblasts.
3.6 Effect of 2,4-DAQs on SMN Protein Levels in Fibroblasts

The NSC-34 motor neuron-like cell line was utilized to determine whether the effects of the 2,4-DAQs on SMN2 mRNA and SMN protein expression observed in fibroblasts were consistent among cell lines. SMA primarily affects motor neurons in patients and the NSC-34 cell line has been shown to have morphological and physiological characteristics that closely resemble primary motor neurons (Cashman et al., 1992; Durham et al., 1993). Hence, the NSC-34 cells are ideal for studying the effects of the 2,4-DAQs on SMA phenotype. NSC-34 cells were treated with 1 µM D156844, D158872, D157161, and D157495 for 5 days to assess Smn mRNA expression. qRT-PCR analysis showed that the compounds do not have any significant effect on the NSC-34 cells when compared to DMSO treatment (Figure 3.7, A).
Consistent with the RNA data, treatment of NSC-34 cells with 10 nM-1 μM of drug compounds resulted in no significant alteration in SMN protein levels (Figure 3.7, B-C).

Figure 3.7. Effect of 2,4-DAQs on the expression of Smn mRNA and SMN protein levels in the NSC-34 motor neuron-like cell line. Cells were treated for 5 days with 10 nM-1 μM of D156844, D158872, D157161, and D157495. DMSO was included as control. (n=3/treatment group). (A) Smn expression was analyzed by qRT-PCR and results are represented as fold change of Smn levels normalized to the expression levels of two reference transcripts—Pgtk1and Rplp13a. (B) SMN protein levels of NSC-34 cells after treatment with drug compounds as indicated. Protein levels were normalized to β-tubulin. (C) Representative images of immunoblot analysis of SMN levels. Band intensities were determined through LiCOR. Data are expressed as mean ± SEM.
3.7 Effect of 2,4-DAQs on DNA Content of Fibroblasts

Although 10 µM of each of the 2,4-DAQs was not cytotoxic to the NSC-34 motor neuron-like cells in the SMN2 reporter cell assays, we were unable to test this concentration on the expression levels of SMN mRNA, DcpS-inducible transcripts, and SMN protein. Treatment of SMA and non-SMA fibroblasts with 10 µM of 2,4-DAQs was cytotoxic to the cells. All the compounds triggered cell death within 24 hours following first day of treatment and approximately 90% of the cells underwent cell death by the last day of the treatment regimen. Representative images are included to show the effect of 10 µM D156844 on the SMA type II fibroblasts (GM03813) and non-SMA fibroblasts (AIDHC-NMC1) during the five-day treatment period (Figure 3.8, A-B). D158872, D157161 and D157495 had similar effect on both SMA and non-SMA fibroblasts.

Interestingly, 1 µM of the 2,4-DAQs resulted in a decrease in cell proliferation/viability (data not shown) when compared with 1 nM-100 nM of 2,4-DAQs treatment in the fibroblast cell lines. To assess these responses to the compounds, GM03813 fibroblasts were treated with 100 nM and 1 µM D156844 and D157495 or DMSO for five days and DNA content was assessed using propidium iodide (PI) staining and flow cytometry analysis. The compounds did not induce cell cycle arrest; however, the compounds reduced the number of cells in G1/G0, and S/G2M when compared to DMSO treatment (Figure 3.8, C).
Figure 3.8. Effect of 2,4-DAQs on cell cycle distribution in fibroblasts. Cells were treated with either 100 nM or 1 μM of D156844 and D157495 for five days. DMSO was included as control. (n=3/treatment group). (A) Representative image showing amount of GM03813 cells remaining following each treatment day. (B) Representative image showing amount of AIDHC-NMC1 remaining following each treatment day. (C) Representative cell cycle analysis histograms of DNA content of GM03813 fibroblasts treated with either 100 nM or 1 μM of D156844 and D157495 compared to DMSO. Cells were analyzed by flow cytometry for DNA content and the percentage of cells in different cell cycles was determined using BD Accuri C6 software. Histograms show DNA content (x-axis) versus cell number (y-axis). Labels show area of curves corresponding to G0/G1, S/G2M phases of cell cycle.
3.8 Effect of 2,4-DAQs Treatment on DcpS-inducible Transcripts

Recently, Zhou et al. (2015) explored additional functions of the human mRNA scavenger decapping enzyme, DcpS. They treated human-derived SH-SY5Y retinoblastoma cells with RG3039 (D157495) and conducted a microarray analysis of the RNA levels extracted from the cells, which revealed that the compound altered the levels of approximately 222 RNAs (Zhou et al., 2015). They further chose to validate the expression levels of a subset of 12 RNAs by qRT-PCR in the SH-SY5Y cells as well as HEK293T cells. In both cell lines, RG3039 treatment increased the levels of two long non-coding RNAs—DRNT1 and DRNT2—and ATOH7 and RAB26. However, RG3039 decreased the expression levels of PAQR8 and MAOB compared to untreated cells (Zhou et al., 2015).

The 2,4-DAQs were developed to act as potential treatment for SMA patients, hence the next step would be to study their effect on the transcript levels that were shown to be altered by DcpS, however, in patient samples. In this study, we investigated the impact of D157495 as well as D156844, D158872, D157161 or DMSO in GM03813 and GM03814. GM03813 SMA cells were treated with 1 µM of each compound for 5 days and qRT-PCR analysis resulted in similar changes, in which the compounds increased the expression levels of ATOH7, DRNT1 and DRNT2 more than two-fold (*p < 0.05) relative to DMSO treatment (Figure 3.9). The same patterns were also observed in GM03814. However, 2,4-DAQs treatment resulted in no statistically significant difference in the expression levels of MAOB and PAQR8 in
both cell lines (Figure 3.9). RAB26 was found not to be expressed in fibroblasts (data not shown) and was not used in subsequent experiments.

Figure 3.9. The effect of 2,4-DAQs on DcpS decapping activity in fibroblasts. GM03813 cells were treated with 1 µM of D156844, D158872, D157161, and D157495 or DMSO for 5 days (n=3/treatment group). Levels of five RNAs in both treated GM03813 and untreated GM03814 carrier fibroblasts were analyzed by qRT-PCR and results are presented as fold increase mRNA, normalized to the geometric mean of three reference transcripts—ACTB, GAPDH, RPLP0. All error bars indicate mean ± SEM (*p<0.05, one-way ANOVA).
3.9 Effect of SMN-deficiency on the Expression Levels of *ATOH7*, *DRNT1* and *DRNT2*

Given that the 2,4-DAQs significantly increased the levels of *ATOH7*, *DRNT1* and *DRNT2* in GM03813 SMA fibroblasts, we wanted to examine the effect of SMN deficiency itself on the expression of these three transcripts. Since the 2,4-DAQs were reconstituted in DMSO, the effect of DMSO alone was tested on the fibroblast cell lines by qRT-PCR to assess the basal expression levels of *ATOH7*, *DRNT1* and *DRNT2* in SMA and non-SMA fibroblasts. There was a significant increase in *ATOH7* level in the non-SMA fibroblasts compared to SMA fibroblasts (Figure 3.10, A). Although the basal levels of *DRNT1* (Figure 3.10, B) and *DRNT2* (Figure 3.10, C) in the non-SMA fibroblasts were slightly higher than those observed in the non-SMA cells, the fold change was not statistically significant for both transcripts. The low levels of *ATOH7* observed in the SMA fibroblasts suggest that defective expression of the SMN protein has a negative effect on *ATOH7* expression.
Figure 3.10. Basal levels of ATOH7, DRNT1, and DRNT2 expression in patient-derived fibroblasts. SMA fibroblasts, —GM03813, GM22592, AIDHC-SP22—non-SMA fibroblasts—AIDHC-NMC1, AIDHC-SC1, AIDHC-SC2—were treated with DMSO for five days (n=3/treatment group). RNA was harvested from treated cells and analyzed by qRT-PCR. Data are represented as mean ± SEM and normalized to the geometric mean of three reference genes—ACTB, GAPDH, RPLP0. The asterisks (*) indicate p < 0.05, one-way ANOVA.
3.10 Effect of 2,4-DAQs on the Expression Levels of ATOH7, DRNT1 and DRNT2

To investigate whether the changes in ATOH7 observed in quinazoline treated GM03813 is not specific to that cell line, we further tested the effects of the compounds in two additional SMA type II fibroblasts in comparison to non-SMA fibroblasts. We treated both cell lines with 1 µM of D156844, D158872, D157161, D157495 and quantified transcript fold change by qRT-PCR. The levels of ATOH7 was significantly increased (more than two-fold) in all SMA and non-SMA fibroblasts relative to DMSO treatment (Figure 3.11, A). Additionally, the expression of Atoh7 was tested in the motor neuron-like cell line, NSC-34 and treatment with 1 µM of each compound resulted in a significant increase in Atoh7 in comparison to DMSO treatment (Figure 3.11, B). ATOH7 encodes a basic helix-loop-helix (bHLH) transcription factor that regulates several aspects of retinal neuron formation (Cepko, 1999; Vetter and Brown, 2001) and in this study, the 2,4-DAQs significantly increased ATOH7 expression in fibroblasts derived from type II SMA patients and non-SMA fibroblasts. This was also true for the motor neuron-like, NSC-34 cells.

DRNT1 and DRNT2 levels have been shown to be altered upon quinazoline treatment and the stability of DRNT1 was also shown to be DcpS-dependent in HEK293T cells (Zhou et al., 2015). In this study, we also found that treatment of GM03813 SMA fibroblasts significantly increased the levels of DRNT1 and DRNT2.
Thus, we tested for the effects of the drug compounds on the expression levels of
*DRNT1* and *DRNT2* in SMA fibroblasts compared to non-SMA fibroblasts. The cells
were treated with drug compounds for 5 days and qRT-PCR analysis showed a
significant increase in *DRNT1* (Figure 3.11, A) and *DRNT2* in the fibroblast cell lines
(Figure 3.11, B). We were unable to confirm whether treatment of the motor neuron-
like, NSC-34 cell line would result in similar increase in the expression levels of
*DRNT1* and *DRNT2* because their murine orthologs have not yet been discovered.
Together, our results confirmed that the 2,4-DAQs compounds can alter the levels of
*ATOH7, DRNT1* and *DRNT2* and this is consistent among different patient cell lines.
Furthermore, these compounds increase the expression level of *Atoh7* in NSC-34 cells.
Figure 3.11. Effect of 2,4-DAQs on ATOH7 expression in patient-derived fibroblasts and NSC-34 cells. SMA fibroblasts, —GM03813, GM22592, AIDHC-SP22—non-SMA fibroblasts—AIDHC-NMC1, AIDHC-SC1, AIDHC-SC2—and the NSC-34 motor neuron-like cell line were treated with 1 μM D156844, D158872, D157161, D157495 or DMSO for five days (n=3/treatment group). RNA was harvested from treated cells and analyzed by qRT-PCR. Data are represented as mean ± SEM and normalized to the geometric mean of three reference genes—ACTB, GAPDH, RPLP0. The asterisks indicate (*) p < 0.05, one-way ANOVA.
Figure 3.12. The effect of 2,4-DAQs on the expression levels of DRNT1 and DRNT2. SMA type II fibroblasts—GM03813, GM22592, AIDHC-SP22—and non-SMA fibroblasts—AIDHC-NMC1, AIDHC-SC1, AIDHC-SC2—were treated with 1 μM D156844, D158872, D157161, D157495 or DMSO for 5 days (n=3/treatment group). The RNA expression levels of DRNT1 (A) and DRNT2 (B) were measured by qRT-PCR. Data are expressed as mean ± SEM and normalized to the expression levels of three reference transcripts—β-actin, GAPDH, RPLP0. The asterisks (*) indicate p<0.05, one-way ANOVA.
3.11 Effect of DcpS Knockdown on the Expression Level of SMN2 mRNA

The 2,4-DAQs have been shown to bind to and inhibit the activity of the mRNA scavenger decapping enzyme, DcpS (Singh et al., 2008). Although we saw no change in SMN expression in the fibroblast lines, we wanted to confirm whether reducing the levels of DcpS in these cell lines would result in similar effect by performing shRNA-mediated knockdown of DcpS. GM03813 fibroblasts were transduced using three different lentiviral shRNA vectors directed against DcpS—5570, 5571, and 5572. Enhanced green fluorescence protein (EGFP) shRNA was used as a positive control for positive transduction (Figure 3.13, A). The knockdown efficiency of each shRNA was first assessed by immunoblot analysis, revealing that DcpS shRNA 5571 was the most efficient at reducing the expression level of DcpS in GM03813 fibroblasts. DcpS shRNA 5571 reduced DcpS expression to approximately 90% when compared to the negative control—scrambled shRNA—and unmanipulated GM03813 (Figure 3.13, B). Both DcpS shRNAs 5570 and 5572 resulted in 70% and 80% DcpS reduction, respectively (Figure 3.13, B). Knockdown of DcpS, however, had no significant effect on SMN protein expression (figure 3.13, C-D). This finding suggests that reducing the activity of DcpS does not directly affect SMN expression in GM03813 SMA fibroblasts.
**Figure 3.13. DcpS knockdown and its effect on SMN expression in GM03813 fibroblasts.** Cells were infected with lentivirus vectors with shRNA directed against DcpS—5570, 5571, and 5572. Cells were grown for five days to mimic the standard treatment regimen with drug compounds. Enhanced green fluorescence protein (EGFP) labeled lentivirus were used as positive control and (A) a representative image of fibroblasts expressing EGFP 48 hours post transduction is shown. (B) Immunoblot analysis of SMN expression in lentivirus infected fibroblasts. SMN protein levels were normalized to the expression levels of β-actin. (C) Representative immunoblots for DcpS, SMN and β-actin are shown. (D) Immunoblot analysis of DcpS protein levels in lentivirus infected fibroblasts. Levels of DcpS were normalized to expression levels of β-actin. Data depicted are representative of 3 independent experiments. The asterisks (*) indicate p<0.05, one-way ANOVA.
3.12 Effect of DcpS knockdown on the Expression of SMN2 and DcpS-inducible Transcripts

DcpS shRNA 5571 was shown to decrease the level of DcpS protein by 90% in GM03813 SMA. Using this DcpS-directed shRNA, we investigated whether DcpS knockdown influences SMN2 mRNA and the levels of expression of ATOH7, DRNT1 and DRNT2 in GM03813 fibroblasts. qRT-PCR analysis indicated that DcpS knockdown alone has no detectable effect on SMN2 mRNA as the levels of FL-SMN or SMNΔ7 remained unchanged (Figure 3.14). In contrast, DcpS knockdown significantly increased the expression levels of ATOH7, DRNT1 and DRNT2 compared to the unmanipulated GM03813 cells (Figure 3.14). These data demonstrate that SMN2 expression does not depend on the activity of DcpS as the levels while the levels of ATOH7, DRNT1 and DRNT2 are responsive in a DcpS-dependent manner.
Figure 3.14. Effect of DcpS knockdown on SMN2 mRNA levels and DcpS-inducible transcripts in GM03813 fibroblasts. GM03813 SMA fibroblasts were infected with lentivirus vectors containing DcpS-directed shRNA (DcpS shRNA 5571) or scrambled shRNA as a negative control (n=3/treatment group). Cells were grown for 5 days and RNA levels for FL-SMN, SMNΔ7, ATOH7, DRNT1 and DRNT2 were analyzed by qRT-PCR and results are represented as geometric mean of three reference transcripts—ACTB, GAPDH, RPLPO. All error bars indicate mean ± SEM (*p<0.05, one-way ANOVA).
3.13 DcpS Knockdown and its Effect on the Expression Levels of SMN2, ATOH7, DRNT1 and DRNT2 in 2,4-DAQs Treated GM03813 Fibroblasts

To determine whether changes in the levels of expression of SMN2, ATOH7, DRNT1 and DRNT2 to 2,4-DAQs treatment are mediated through DcpS, we utilized DcpS shRNA 5571 to monitor changes in these transcripts in GM03813 SMA fibroblasts. The effect of the compounds was also tested on GM03813 fibroblasts transduced with scrambled shRNA control lentivirus. Cells were treated with D156844, D157495 or DMSO for 5 days and transcript fold change was quantified by qRT-PCR. Consistent with the outcome of 2,4-DAQs treated GM03813, D156844 and D157495 resulted in similar patterns in cells transduced with both, scrambled shRNA (Figure 3.15, A) and DcpS shRNA 5571 (Figure 3.15, B). The levels of FL-SMN and SMN17 remained unchanged while treatment with D156844 and D157495 significantly increased the expression levels of ATOH7, DRNT1 and DRNT2. It is evident from these data that the 2,4-DAQs elevate ATOH7, DRNT1 and DRNT2 RNA levels independent of DcpS.
Figure 3.15. Effect of 2,4-DAQs on SMN2 mRNA levels and DcpS-inducible in GM03813 fibroblasts knocked down for DcpS. GM03813 SMA fibroblasts were infected with lentivirus vectors containing a DcpS-directed shRNA (shRNA 5571) or scrambled shRNA and treated with D156844, D157495 or DMSO for 5 days (n=3/treatment group). RNA levels for FL-SMN, SMNΔ7, ATOH7, DRNT1 and DRNT2 were measured by qRT-PCR and results are represented as fold change of RNA levels normalized to the geometric mean of three reference transcripts—ACTB, GAPDH, RPLP0. All error bars indicate mean ± SEM (*p<0.05, one-way ANOVA).
A unique clinical feature of SMA is the retention of variable copy number of \textit{SMN2} gene, which inversely correlates with disease severity. Unlike the \textit{SMN1} gene, \textit{SMN2} primarily produces transcripts that lack exon 7 (\textit{SMN}Δ7) and only 10-20\% of \textit{SMN2} mRNA include exon 7 (\textit{FL-SMN}) and encodes for the fully functional SMN protein (Lorson et al., 1999; Monani et al., 1999). Hence, many drug discovery programs aim to develop compounds that increase the level of SMN protein produced from the \textit{SMN2} gene by exploring multiple mechanisms, including enhancing the inclusion of exon 7 in the \textit{SMN2} mRNA, increasing transcription from the \textit{SMN2} promoter and stabilizing SMN protein (Cherry et al., 2014). The \textit{SMN2} promoter and exon 7 splicing assays are two commonly used assays to predict changes that small molecules have on \textit{SMN2} expression levels. Both reporter assays were developed using the NSC-34 cells, which were generated by fusing neuroblastoma cells with mouse primary motor neurons. These cells retain many characteristics that are similar to those observed in motor neurons; they have the ability to generate action potentials and to synthesize, store, and release acetylcholine (Cashman et al., 1992; Durham et al., 1993). The NSC-34 cells were used in these assays because SMN-deficiency predominantly affects motor neurons.
In the SMN2 promoter assay, β-lactamase was cloned downstream of a 3.4 kb fragment of the SMN2 promoter and the reporter was transfected into the NSC-34 cells, generating the NSC-34 clone 11 cells. The 3.4 kb DNA fragment was chosen in this assay because it contains all the regulatory elements that facilitate SMN2 gene transcription (Echaniz-laguna et al., 1999; Monani et al., 1999). As a result, compounds that stimulate SMN2 promoter activation are expected to increase both the amount of FL-SMN mRNA and SMNΔ7 produced by the SMN2 gene without changing FL-SMN/SMNΔ7 ratios. This in turn would increase SMN protein levels.

The NSC-34 clone 5.3 cell line, in contrast is an ideal model for monitoring changes in SMN2 exon 7 inclusion. This clonal cell line contains a construct that was generated by adding a single nucleotide (+G) in exon 7 to remove the termination signal for translation (Andreassi et al., 2001). Hence, when exon 7 is incorporated in the transcript, β-lactamase is in frame and translated. When exon 7 is excluded, the β-lactamase translational reading frame is disrupted and the reporter is not expressed (Andreassi et al., 2001). Compounds that increase the inclusion of exon 7 into the SMN2 transcripts would result in an overall higher production of FL-SMN compared to SMNΔ7, which would change FL-SMN/SMNΔ7 ratios.

The 2,4-DAQs were identified using the SMN2 promoter assay (Jarecki et al., 2005) and were found to activate SMN2 promoter assay and also increases SMN protein levels in SMA patient fibroblasts (Thurmond et al., 2008). In this study, we looked at the effects of four 2,4-DAQs—D156844, D158872, D157161 and D157495.
Our results show that these compounds are effective at increasing SMN2 transcription in the motor neuron-like cell line, NSC-34. All four drug compounds resulted in >3-fold induction in the promoter assay. Dose response curves show that compound D157495 was the most potent at activating the SMN2 promoter with an EC50 of 23.30 ± 0.0042 (table 3.1). Compared to other 2,4-DAQs, D157495 was advanced to early phase clinical trials and is known as RG3039. D157495 has been shown to improve motor neuron function and extend survival of SMA mice models (Gogliotti et al., 2013; Van Meerbee et al., 2013).

Analysis of SMN2 splicing patterns by RT-PCR confirms that the 2,4-DAQs do not alter SMN-FL/SMNΔ7 ratios (Figure 3.4). This supports the conclusion that the 2,4-DAQs do not promote exon 7 inclusion in the SMN2 transcripts.

Considering the positive response of the 2,4-DAQs on the SMN2 promoter activity in the NSC-34 cells, we were expecting that treatment of SMA fibroblasts would increase both SMN-FL and SMNΔ7 as well as SMN protein levels. However, the 2,4-DAQs had no detectable effect on SMN2 mRNA or SMN expression in fibroblast from type II SMA and non-SMA patients. Several factors may contribute to these contradicting results. The promoter assay only uses a fragment (3.4 kb) of the SMN2 promoter whereas the entire SMN2 promoter is 5.2 kb. The 2,4-DAQs may have positive effects that are specific to the regulatory elements that are present within the 3.4 kb fragment. Studying their effects on the entire SMN2 promoter could be a more suitable approach at investigating the activity of these compounds. Another
possibility for the difference seeing between SMN2 promoter activity, SMN2 mRNA and SMN protein levels may be that the effects of the 2,4-DAQs are cell-line specific. The promoter assay is based on the NSC-34 cells, which are derived from mice. In contrast, SMN2 mRNA and SMN protein levels were studied in fibroblasts derived from human patients. These compounds may have different effects that are cell specific. Additionally, the lack of detectable changes in SMN2 mRNA or SMN protein in treated fibroblasts confound the mechanism by which the 2,4-DAQs promote an increase in SMN2 expression in the NSC-34 cells. These conflicting results suggest that the effect of the 2,4-DAQs on SMN2 transcriptional regulation is not conserved among the cell lines.

The 2,4-DAQs were found to bind to and inhibit the human mRNA scavenger decapping enzyme, DcpS. This enzyme has been shown to work at multiple levels in the regulation of gene expression. DcpS cleaves the cap structures resulting from exosome degradation of mRNAs. DcpS also influences the function of other cap-binding proteins including, the nuclear cap binding protein complex cbp20 and cbp80 as well as the cytoplasmic cap-binding protein eIF4E (Bail and Kiledjian, 2008). By inhibiting DcpS, the 2,4-DAQs disrupt its decapping activity which is expected to lead to accumulation of the m7GpppN cap structure. When it is not hydrolyzed, the m7GpppN cap structure can sequester the nuclear cap binding protein and decrease the efficiency of first intron splicing (Shen et al., 2008) and it can also potentially alter
cytoplasmic mRNA translation by sequestering the cytoplasmic cap binding protein (Bail and Kiledjian, 2008).

As previously shown by Zhou et al., (2015), DcpS has an additional function in regulating RNA levels. We addressed this by looking at the effect of the 2,4-DAQs on fibroblast lines derived from SMA and non-SMA patients. Treatment of these cell lines resulted in a robust increase in the expression levels of \textit{ATOH7}, \textit{DRNT1} and \textit{DRNT2}. DcpS knockdown alone altered the levels \textit{ATOH7}, \textit{DRNT1} and \textit{DRNT2} and 2,4 DAQs treatment of GM03813 fibroblasts also resulted in similar increase in these transcripts. It is evident that the increase in \textit{ATOH7}, \textit{DRNT1} and \textit{DRNT2} is not a result of a defect in DcpS’ decapping activity. Interestingly, neither compound treatment nor DcpS knockdown lead to statistically significant increase in \textit{SMN2} mRNA or SMN protein, suggesting that the compounds likely function through a pathway that is independent of SMN and possibly the effects of the 2,4-DAQs may be independent of DcpS.

SMN-deficient fibroblasts were also shown to have significant reduced levels of \textit{ATOH7} (Figure 3.10, A) and 2,4-DAQs treatment of these SMA fibroblasts cell lines restored \textit{ATOH7} expression to levels observed in non-SMA fibroblasts (Figure 3.11, A). \textit{ATOH7} (\textit{attonal homolog 7}) is a single exon gene that belongs to the basic helix-loop-helix (bHLH) transcription factors homologous to Drosophila achaete-scute and atonal (Khan et al., 2012; Tomita et al., 2000). Studies have shown that \textit{ATOH7} is required for optic nerve and ganglion cell development and plays key roles in ocular
embryogenesis (Brown et al., 2002; Jarman et al., 1994). Here, we show that SMN deficiency is associated with low levels of \textit{ATOH7} and 2,4-DAQs treatment restores \textit{ATOH7} expression in SMA fibroblasts to normal levels. \textit{ATOH7} could be a possible target for SMA therapeutics.

Although the 2,4-DAQs have positive effects on the expression levels of \textit{ATOH7}, \textit{DRNT1} and \textit{DRNT2}, they may have other cellular levels that should be addressed. Shalem et al., (2014) showed that DcpS deletion is lethal in cells, suggesting the requirement of DcpS for cellular viability. Hence, the efficacy of DcpS inhibitor compounds in SMA patients remains unclear.
To date, therapeutic strategies in SMA have been focused on drugs that target an increase in \( SMN2 \) function in order to compensate for the loss of the \( SMN1 \) gene. Several compounds, including the 2,4 DAQs were found to increase \( SMN2 \) promoter activation using the \( SMN2 \) promoter assay. However, 2,4-DAQs do not increase \( SMN2 \) mRNA or SMN expression in SMA fibroblasts. As evident from our results, the \( SMN2 \) promoter assay may not be a valid approach for finding drug compounds that can modulate \( SMN2 \) expression. This assay is based on a 3.4 kb fragment of the \( SMN2 \) promoter in NSC-34 cells and only accounts for the effects of compounds on that specific region of the \( SMN2 \) promoter. A better approach at studying the effects of drug compounds on \( SMN2 \) activity should include the entire 5.2 kb of the promoter.

This study also reveals that the 2,4-DAQs do not simply bind to and inhibit the human mRNA scavenger decapping enzyme, DcpS. Despite its initial described function in the last step of the mRNA 3’ end decay pathway, DcpS is a multifunctional protein involved in a greater network of regulated gene expression.

The 2,4-DAQs are most likely a group of molecules that modify the SMA phenotype independent of SMN. These compounds do not alter SMN expression in fibroblasts and the same effect was observed in DcpS knockdown GM03813 SMA fibroblasts. The 2,4-DAQs were found to positively modulate different RNA levels, in
particular *ATOH7, DRNT1* and *DRNT2*. This was also consistent in GM03813 cells knocked down for DcpS, suggesting that the modulation of these transcripts is likely to be mediated through DcpS. Similar effects of the 2,4-DAQs on DcpS knockdown should also be explored in other SMA fibroblasts. We also show that these compounds do not directly affect *SMN2* mRNA stability in GM03813 fibroblasts; however, it remains unknown whether these compounds have a direct implication in stabilizing *ATOH7, DRNT1* and *DRNT2* to increase their expression levels in fibroblasts.
REFERENCES


Vetter ML, Brown NL. The role of basic helix-loop-helix genes in vertebrate

Viollet L, Bertrand S, Bueno Brunialti AL, Burfevre S, Burlet P, Clermont O,
Cruaud C, Guenet JL, Munnich A, Melki J. cDNA isolation, expression, and
chromosomal localization of the mouse survival motor neuron gene (Smn).
PubMed PMID: 9070939.

Wang Z, Kiledjian M. Functional link between the mammalian exosome and mRNA
8674(01)00592-x. PubMed PMID: 11747811.

Wirth B, Brichta L, Schrank B, Lochmuller H, Blick S, Baasner A, Heller R. Mildly
affected patients with spinal muscular atrophy are partially protected by an

Young PJ, Le TT, thi Man N, Burghes AH, Morris GE. The relationship between
SMN, the spinal muscular atrophy protein, and nuclear coiled bodies in

Zhang HL, Pan F, Hong D, Shenoy SM, Singer RH, Bassell GJ. Active transport of
the survival motor neuron protein and the role of exon-7 in cytoplasmic
PMID: 12878704.

Zhong Q, Bhattacharya S, Kotsopoulos S, Olson J, Taly V, Griffiths AD, Link DR,
Larson JW. Multiplex digital PCR: breaking the one target per color barrier of
quantitative PCR. *Lab Chip*. 2011;11(13):2167-74. Epub 2011/05/18. doi:

Zhou M, Bail S, Plasterer HL, Rusche J, Kiledjian M. DcpS is a transcript-specific
PMCID: PMC4478349.