The First Orally Deliverable Small Molecule for the Treatment of Spinal Muscular Atrophy

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Abstract
Spinal muscular atrophy (SMA) is one of the leading causes of infant mortality. SMA is mostly caused by low levels of Survival Motor Neuron (SMN) protein due to deletion of or mutation in the SMN1 gene. Its nearly identical copy, SMN2, fails to compensate for the loss of SMN1 due to predominant skipping of exon 7. Correction of SMN2 exon 7 splicing by an antisense oligonucleotide (ASO), nusinersen (Spinraza™), that targets the intronic splicing silencer N1 (ISS-N1) became the first approved therapy for SMA. Restoration of SMN levels using gene therapy was the next. Very recently, an orally deliverable small molecule, risdiplam (Evrysdi™), became the third approved therapy for SMA. Here we discuss how these therapies are positioned to meet the needs of the broad phenotypic spectrum of SMA patients.

Keywords
SMA, splicing, SMN, ISS-N1, antisense oligonucleotide, risdiplam, Spinraza, nusinersen, Branaplam, Evrysdi, Zolgensma

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The First Orally Deliverable Small Molecule for the Treatment of Spinal Muscular Atrophy

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**ABSTRACT:** Spinal muscular atrophy (SMA) is 1 of the leading causes of infant mortality. SMA is mostly caused by low levels of Survival Motor Neuron (SMN) protein due to deletion of or mutation in the SMN1 gene. Its nearly identical copy, SMN2, fails to compensate for the loss of SMN1 due to predominant skipping of exon 7. Correction of SMN2 exon 7 splicing by an antisense oligonucleotide (ASO), nusinersen (Spinraza™), that targets the intronic splicing silencer N1 (ISS-N1) became the first approved therapy for SMA. Restoration of SMN levels using gene therapy was the next. Very recently, an orally deliverable small molecule, risdiplam (Evrysdi™), became the third approved therapy for SMA. Here we discuss how these therapies are positioned to the need of the broad phenotypic spectrum of SMA patients.

**KEYWORDS:** SMA, splicing, SMN, ISS-N1, antisense oligonucleotide, risdiplam, Spinraza, nusinersen, Branaplam, Evrysdì, Zolgensma

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Introduction

Spinal muscular atrophy (SMA) is the leading genetic cause of infant mortality affecting 1 in every ~10,000 live births.1,2 Low levels of the Survival Motor Neuron (SMN) protein due to deletion of or mutation in the SMN1 gene is the primary cause of SMA.3 A nearly identical copy of SMN1 universally present in humans, called SMN2, cannot compensate for the loss of SMN1 since SMN2 exon 7 is predominantly skipped. Skipping of exon 7 leads to production of a truncated unstable protein, SMN7.4 SMA has a broad disease spectrum that is categorized into 5 types: 0, 1, 2, 3, and 4.5 Type 0 is the most severe, in which patients die before their 2nd birthday.7 The onset of type 2 SMA (also called Dubowitz disease) occurs before 18 months of age; here patients cannot walk but can sit.7 Type 3 SMA (also called Kugelberg-Welander disease) is manifested after 18 months of age; and patients can walk but cannot sit or walk and succumb to death before their 2nd birthday.7 The severity of SMA correlates inversely with the higher the copy number, the lower the severity.53-56 Several factors, including Stat5 (PL53), Neuritin 1 (NRN1), Neurocalcin delta (NCALD), TIA1 cytotoxic granule associated RNA binding protein (TIA1), Ubiquitin specific peptide 9 X-linked (USP9X), and Senataxin (SETX), have been suggested to modify SMA severity.57-60 Due to broad differences in the age of the SMA onset and the diversity of SMA phenotypes, developing an ideal therapy for the disease remains a challenging task.

Considering SMN2 is universally present in SMA patients, correction of SMN2 exon 7 splicing remains one of the most promising avenues for the treatment of the disease.69 A critical C-to-T mutation at the 6th position (C6U substitution in RNA) of exon 7 is associated with the skipping of SMN2 exon 7.71,72 In general, skipping of exons is triggered by suboptimal splice sites defined by a combinatorial control of splicing cis-elements and transacting factors that recognize them.73 Being close to the 3’ splice site (3’ss), C6U substitution is proposed to weaken the 3’ss of SMN2 exon 7.74 Various other mechanisms including abrogation of an enhancer, creation of a silencer and strengthening of an extended inhibitory context (Exinct) have been put forward to explain...
the C6U substitution-induced skipping of exon 7. A breakthrough in our understanding of exon 7 splicing regulation came from a study performing in vivo selection of the entire exon that, among other important observations, confirmed that the 5′ss of exon 7 was suboptimal. Of note, in vivo selection of the entire exon also revealed the strong negative effect of an ‘A’ residue at the 54th position (54A) of exon 7 and a structural context of the 5′ss of SMN exon 7. Numbering is the same as described in panel A. Only a portion of exon 7 and intron 7 is shown. Cis-elements that promote exon 7 skipping are highlighted in colors. Abbreviations: 3′ss, 3′ splice site; 5′ss, 3′ splice site; Exinct, extended inhibitory context; GCRS, GC-rich sequence; ISS-N1, intronic splicing silencer; ISTL1, an internal stem (inhibitory RNA structure) formed by long-distance interaction; TSL2, terminal stem-loop structure 2; TSL3, terminal stem-loop structure 3.

Context of the Suboptimal 5′ss of SMN2 Exon 7

Most human introns, including all introns in the SMN genes, belong to the U2-type. The 5′ss of U2-type introns is defined by a total of eleven nucleotides, specifically the last 3 exonic residues and the first 8 intronic residues. An RNA:RNA duplex (U1:5′ss duplex) formed between these 11 nucleotides and the 5′-end of U1 snRNA, a component of U1 snRNP, sets the stage for exon definition and intron removal. With very few exceptions, a GU dinucleotide at the first 2 positions of U2-type introns is required for the exon definition. Additional splicing cis-elements come into play when the size of the U1:5′ss duplex is less than 6 base pairs and/or the 5′ss is sequenced in a RNA structure. The finding that the 5′ss of exon 7 is suboptimal as revealed by the in vivo selection paved the way for the discovery of a number of inhibitory cis-elements located in the 5′ss vicinity. These include the intronic splicing silencer N1 (ISS-N1), the terminal stem-loop structure 2 (TSL2), the GC-rich sequence (GCRS), the internal stem formed by long-distance interaction 1 (ISTL1) (Figure 1). The 5′ss of exon 7 is suboptimal as revealed by the in vivo selection paved the way for the discovery of a number of inhibitory cis-elements located in the 5′ss vicinity. These include the intronic splicing silencer N1 (ISS-N1), the terminal stem-loop structure 2 (TSL2), the GC-rich sequence (GCRS), the internal stem formed by long-distance interaction 1 (ISTL1) (Figure 1). The finding that the 5′ss of exon 7 is suboptimal as revealed by the in vivo selection paved the way for the discovery of a number of inhibitory cis-elements located in the 5′ss vicinity. These include the intronic splicing silencer N1 (ISS-N1), the terminal stem-loop structure 2 (TSL2), the GC-rich sequence (GCRS), the internal stem formed by long-distance interaction 1 (ISTL1) (Figure 1). The finding that the 5′ss of exon 7 is suboptimal as revealed by the in vivo selection paved the way for the discovery of a number of inhibitory cis-elements located in the 5′ss vicinity. These include the intronic splicing silencer N1 (ISS-N1), the terminal stem-loop structure 2 (TSL2), the GC-rich sequence (GCRS), the internal stem formed by long-distance interaction 1 (ISTL1) (Figure 1).
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splicing, turned out to be dispensable for inclusion of this exon in a Tra2-β1-deficient mouse model.97,98 These observations are not entirely surprising given the redundancy and cross-regulation of splicing factors as, for example, observed for PTB, CELF2, and hnRNP C.99-101

The discovery of the 15-nucleotide long ISS-N1 propelled the development of an antisense oligonucleotide (ASO)-directed therapy for SMA.89,102,103 Based on its strong inhibitory effect, ISS-N1 was dubbed as a master regulator of both splicing checkpoint and exon definition.104 Nusinersen (Spinraza™), the first FDA-approved drug for SMA, is an ISS-N1-targeting ASO that is intrathecally delivered for the treatment of the disease.105,106 Methods and mechanisms associated with the splicing correction by an ISS-N1-targeting ASO are reviewed elsewhere.107-109 Collaborative studies conducted in the Krainer lab at Cold Spring Harbor Laboratories, New York and by Ionis Pharmaceuticals, Carlsbad, California played a pivotal role in the therapeutic development of nusinersen.110 Several recent reports describe the efficacy of nusinersen in SMA patients.111-113 Of note, similar to ISS-N1, GCRS and ISS-N2 are additional targets that could be potentially exploited for correction of SMN2 exon 7 splicing by abrogating the inhibitory context at the 5′ss of exon 7.91,93,114,115 Indeed, in vivo studies employing ASOs targeting GCRS and ISS-N2 have shown therapeutic benefits in mouse models of SMA.116,117

Zolgensma®, an adeno-associated virus 9 (AAV9) based gene delivery, became the second FDA-approved therapy for SMA.118 The success of gene therapy was enabled by pre-clinical and clinical studies conducted by Kaspar and colleagues at Nationwide Children’s Hospital, Columbus, Ohio.119 Unlike nusinersen that relies on the endogenous SMN2 transcripts for the production of SMN, gene therapy produces SMN from exogenously delivered DNA coding for SMN1. Hence, risks of the generation of autoantibodies against SMN due to overexpression of this protein as a consequence of gene therapy could not be ruled out. Of note, a recent study has found a correlation between autoantibodies against SMN and systemic sclerosis.120 Both nusinersen and gene therapy have the limitations of an invasive administration process and having poor body-wide delivery/distribution.121 The recent approval of risdiplam (Evrysdi™), an orally deliverable small molecule, addresses these concerns.122-124 Here we review the mechanism of action of risdiplam, its target specificity, and potential off-target effects. We also discuss how available SMA drugs would potentially complement each other for a better treatment of the disease. Other SMA therapies currently in preclinical and clinical studies/trials have been described elsewhere.5,125,126

Discovery of Risdiplam as a Therapeutic Candidate

A joint endeavor by PTC-Roche (PTC Therapeutics, South Plainfield, New Jersey and Hoffmann-La Roche, Basel, Switzerland) to identify an orally available molecule for the treatment of SMA began about a decade ago. Investigators at these companies screened a library of small molecules and reported three orally deliverable compounds, namely SMN-C1 (isocoumarin), SMN-C2 (coumarin), and SMN-C3 (pyrido-pyrimidine derivative); each promoted exon 7 inclusion from SMN2 minigene expressed in HEK293H human embryonic kidney cell
Mechanism of Action of Risdiplam

Currently, there is no consensus on the mechanism by which risdiplam (molecular mass 401.46 Da) promotes $SMN2$ exon 7 inclusion with high specificity. A study led by investigators at California Institute for Biomedical Research (CIBR) showed that $SMN$-C, one of the analogs of risdiplam, interacts with an AG-rich motif, AGGAAG, located in the middle of exon 7 (Figure 3). Authors employed a series of in vitro and in vivo techniques to demonstrate a high specificity of direct interactions between $SMN$-C and this AG-rich motif. Further, binding of $SMN$-C to this AG-rich motif was proposed to recruit stimulatory splicing factors Far Upstream Element Binding Protein 1 (FUBP1) and its homolog KH-type Splicing Regulatory Protein (KHSRP) (Figure 3). Supporting this hypothesis, depletion of FUBP1/KHSRP diminished the effect of $SMN$-C on $SMN2$ exon 7 splicing, particularly at low nanomolar concentrations of $SMN$-C. A different study led by investigators at Hoffmann-La Roche suggested that the interaction of $SMN$-C class of compounds with the AG-rich motif displaces hnRNP G. Previous studies have implicated the role of hnRNP G in promoting of $SMN2$ exon 7 inclusion, although it has been also argued that the stimulatory effect of hnRNP G on exon 7 splicing is mediated through Tra2-$\beta$1, which in turn interacts with the purine-rich motif located in the middle of exon 7. It is likely that the displacement of hnRNP G is accompanied by the recruitment of stimulatory factors, including FUBP1/KHSRP as proposed by investigators at CIBR.

In addition to the interaction with the AG-rich motif, $SMN$-C class of compounds have been shown to interact with the 5’ss of exon 7, particularly with the adenosine residue at the last exonic position (54A) (Figure 3). Of note, the inhibitory effect of 54A was first uncovered by in vivo selection of the entire exon 7. Consistently, replacement of 54A with 54G (A54G substitution) fully restores exon 7 inclusion even when the Tra2-$\beta$1-binding site in exon 7 is destroyed. Importantly, 54A also strengthens a stem-loop structure (TSL2) that sequesters the 5’ss of exon 7 (Figure 1). When it comes to the 5’ss recognition, 54A creates a bulge (a mismatch base pair) in the duplex formed between the U1 snRNA and the 5’ss of exon 7. Strengthening of the U1:5’ss duplex by a compensatory mutation within U1 snRNA has been shown to have the similar stimulatory effect on $SMN2$ exon 7 inclusion as the one observed with the A54G substitution.

A recent study by Allain and colleagues employing NMR confirmed the interaction between $SMN$-C5 and 54A in the context of the U1:5’ss duplex (Figure 3). The authors proposed that $SMN$-C5 stabilizes the U1:5’ss duplex by “5’ss bulge repair,” restoring the accessibility of the U1-C zinc finger for the interaction with the minor groove of the duplex. While stabilization of the U1:5’ss duplex by $SMN$-C5 seems to be sufficient to promote $SMN2$ exon 7 inclusion, the authors did not rule out the role of additional factors recruited by $SMN$-C to the AG-rich motif. A caveat in the $SMN$-C5-induced U1:5’ss duplex model is its inability to explain why lower concentrations of $SMN$-C
series of compounds were ineffective in the promotion of SMN2 exon 7 inclusion upon depletion of splicing factors FUBP1/KHSRP.131
All mechanistic studies proposed thus far have been performed employing risdiplam analogs but not risdiplam itself. Some of the disparities in the proposed mechanisms of action could lie in the methods employed and risdiplam analogs used. Given the structural differences between risdiplam and its analogs (Figure 2), it is not a matter of fact that the mechanism proposed for a risdiplam analog will also hold true for risdiplam itself. Additional studies including analysis of exons associated with the off-target effects of risdiplam would be needed to fully understand its mechanism of action.

Off-Target Effects of Risdiplam
The first RNA-seq performed on transcripts isolated from SMN-C3-treated SMA type I fibroblasts provides insight into the nature of off-target effects of C-series of small molecules that are analogs of risdiplam.127 Analysis of this RNA-seq revealed that SMN-C3 treatment altered splicing of 42 exons, 6 of which underwent a change of greater than 40%. The effect on splicing of the top 10 candidate exons including SMN2 exon 7 is shown in Figure 4a. Analysis of the sequences surrounding the 3′ss of the affected exons revealed a slight, but not significant enrichment in the AG-rich motif (Figure 4b). However, similar motifs were not enriched in total exonic sequences or in the vicinity of the 5′ss (Figure 4b). SMN-C3-affected “off-target” exons had a strong enrichment for a GA dinucleotide at their two last positions followed by a consensus

\[\text{GUAGU} \ 5′\text{ss} \text{ sequence (Figure 4b and c). Interestingly, SMN-C3 also triggered the inclusion of previously unannotated exons and in at least one case promoted intron retention (Figure 4d). For instance, inclusion of an unannotated exon positioned between exons 3 and 4 of the SNAP23 gene was accompanied by a significant retention of the downstream intron (Figure 4d). As compared to its precursors, low concentrations of risdiplam showed similar off-target effects on pre-mRNA splicing in a cell culture model.123 However, it is predicted to exhibit superior target specificity and stability in vivo due to novel modifications that were introduced to prevent its conversion into potentially harmful active metabolite(s).123 It should be noted that at high concentrations, risdiplam did produce off-target effect on splicing of several genes, including STRN3, FOXM1, APLP2, MADD, and SLC25A17 (Figure 4).123 The off-target effect of risdiplam on splicing of exons of several genes could be attributed to the similarity of the 5′ss context and sequence motifs present within the affected “off-target” exons (Figure 4).

In Vivo Efficacy of Risdiplam in Mouse Models
Risdiplam showed enhancement in expression of SMN in brain and quadriceps muscle upon oral administration in a mild SMA mouse model (allele C model).123 Intraperitoneal (IP) mode of delivery was used to monitor the efficacy of risdiplam in severe (SMAΔ7) mouse model. IP administration of risdiplam at a concentration as low as 1 mg/kg of body weight produced a robust enhancement in SMN levels in brain and quadriceps muscle of these mice.123 Also, risdiplam-treated
SMAΔ7 mice showed a dose-dependent improvement of NMJ phenotype and an increase in the number of motor neurons and the size of the extensor digitorum longus (EDL) muscles. Higher IP doses of risdiplam (10 mg/kg of body weight) provided one of the best life expectancy gains reported in the literature. For instance, more than 70% of SMAΔ7 mice provided one of the best life expectancy gains reported in the literature.123 For instance, more than 70% of SMAΔ7 mice

Figure 4. Off-target effects of risdiplam or its analog SMN-C3. (a) Splicing pattern of SMN2 exon 7 and ten other splicing events affected by SMN-C3 treatment as reported by Naryshkin and coworkers.127 Y axis indicates the proportion of total spliced transcript that has the exon in question included. X axis labels indicate the host gene and exon number of the target exons. NE: novel (unannotated) exon, (b) top enriched sequence motifs near the 3′ and 5′ss of the exons, splicing of which was changed by SMN-C3. Letter height in each motif corresponds to nucleotide enrichment at that position, (c) the sequences of 5′off-target” exons, splicing of which was affected by risdiplam, as reported by Ratni and coworkers.123 Numbering is given relative to the first position of each exon. Uppercase letters represent exonic sequences, lowercase letters represent intronic sequences. The longest AG-rich motif in each exon is boxed. The last two exonic nucleotides and the first six intronic nucleotides of the 5′ss are shown in bold. Each shaded/clear area “cover” ten consecutive nucleotides. An additional 5′ss within exon 9 of FOXM1 is indicated with an asterisk, and (d) genomic overview of two examples of splicing events induced by SMN-C3 treatment. POMT2 (top panel) contains a novel, unannotated exon located in the region between exons 11 and 12. Inclusion of this unannotated exon is caused by SMN-C3 treatment, as shown by the increased read depth. SNAP23 (bottom panel) has a novel exon (between exons 3 and 4) that undergoes inclusion. This is coupled with intron retention, as indicated by increased read depth in the flanking introns.
survived beyond seven months upon treatment with risdiplam at 10 mg/kg of body weight. In further studies in several SMA mouse models as well as in rats and non-human primates, risdiplam displayed excellent pharmacokinetic and pharmacodynamic properties, such as body-wide distribution and stable plasma levels over extended dosing periods. These results were sufficient to launch clinical trials of risdiplam for its evaluation in SMA patients.

Clinical Trials and FDA Approval of Risdiplam

Several clinical trials of risdiplam have been performed to evaluate the safety, tolerability, and efficacy of the drug in both healthy and SMA patients. Two of these clinical trials, first in the infantile-onset population receiving risdiplam have been significant for the approval of risdiplam. The clinical trial for infantile-onset SMA was an open-label study in which 21 patients, whose average age was ~6.7 months, participated. About 41% patients showed ability to independently sit after 12-month treatment. Also, the patients showed more than 81% survival without permanent ventilation after 23 or more months of treatment. These results were considered as a significant improvement over the untreated patients in a similar category. The clinical trial with the later-onset SMA patients was randomized and placebo-controlled in which 180 SMA patients aged from 2 to 25 years participated. Risdiplam-treated patients performed significantly better in motor function tests than untreated patients. On August 7, 2020 FDA granted approval of risdiplam (Evrysdi™) under the fast-track designation and rare pediatric disease priority review process.

Side Effects of Risdiplam

The most common side effects in clinical trials of risdiplam were fever, rash, and/or immune response concerns when it comes to nusinersen and gene therapy. Storage and shipping at ambient temperatures as well as its comparatively low cost are added benefits of risdiplam for its worldwide availability/distribution. In comparison to its “parent analogs,” risdiplam is predicted to exhibit reduced off-target effect in vivo, particularly at lower concentrations. Similar to other approved drugs for SMA, side effects encountered during the clinical trials of risdiplam remain a cause of concern. Future studies will reveal if the side effects associated with the frequent administration of risdiplam would pose a hurdle for its acceptance for the long-term treatment. In addition, risdiplam may not be useful for SMA patients that carry pathogenic mutations at the 5’ss of SMN exon 7. Activation of a cryptic 5’ss downstream of exon 7 by an engineered U1 snRNP could be an alternative therapeutic approach in this case. In fact, in vivo efficacy of the engineered U1 snRNP has been validated in a mouse model of SMA. Future studies aimed at the activation of the cryptic 5’ss downstream of exon 7 of the SMN genes by a small molecule will cater to the needs of a broader patient population.

One of the exciting aspects of risdiplam’s approval is the validation of the utility of a small molecule for targeted splicing correction as a promising therapy. Another orally available small molecule, branaplam, that modulates SMN2 exon 7 splicing with high specificity is about to conclude the phase 2 clinical trial (NCT02268552) conducted by Novartis Pharmaceuticals (Figure 2). Branaplam (synonyms: NVS-SM1 and LMI070) was identified by high-throughput screening of the Novartis compound library, followed by chemistry optimization. It was shown to modulate splicing, elevate levels of the full-length SMN protein and increase the survival of a severe SMA mouse model. Despite structural differences between branaplam and risdiplam, the proposed mode of action of branaplam appears to be similar to that of risdiplam. Both drugs stabilize the U1:5’ss duplex at the 5’ss of SMN2 exon 7. Two more small molecules, PK4C9 and TEC-1, have been recently reported to enhance SMN2 exon 7 inclusion with high specificity (Figure 2). TEC-1 has been found to be permeable to the central nervous system and confer therapeutic efficacy in a mouse model of SMA. TEC-1 action has not yet been examined, PK4C9 has been shown to interact with a structural element, namely the tri-loop TEC-1 action has not yet been examined, PK4C9 has been shown to interact with a structural element, namely the tri-loop. Incidentally, sequences encompassing the tri-loop of TSL2 has been found to overlap the “3-cluster,” a negative element identified by in vivo selection of the entire exon 7 (Figure 1). These findings expand the number of potential targets that could be exploited for developing small molecules therapies for SMA. In addition, several orally available small molecules that work downstream of SMN or independent of SMN are currently undergoing pre-clinical and clinical studies.

Diverse treatment options currently being exploited for SMA are commensurate with the varied needs of the broad spectrum of SMA patients.

To harness the full potential of available treatment options, it is likely that the combined therapies would become the desired approach for the treatment of SMA. Recent studies of the combined therapies (in mouse models of SMA) in which one of the components was an ISS-N1 targeting ASO have shown promising results. Now that risdiplam is approved, future studies will reveal if it could be combined with other drugs for a better therapeutic outcome. For example, risdiplam could be
used together with an “SMN-independent” treatment(s) targeting muscle or neurological functional deficits observed in SMA to further alleviate symptoms of the disease. Using risdiplam together with other splicing-modulating drugs that work by complementary mechanisms, such as nusinersen, holds the promise to enhance the expression of full-length SMN, while maintaining minimum off-target effects on other splicing events due to lowering the treatment dose. With the prece-

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Author Contributions

RNS and NNS reviewed the literature and wrote and edited the manuscript. EWO reviewed the literature, performed the data analysis for Figure 4, and edited the manuscript. All authors reviewed and gave approval of the intellectual content of the final manuscript.

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