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 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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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, SMNΔ7.[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 birth.[6] Patients suffering from type 1 SMA (also called Werdnig-Hoffmann disease) need ventilatory support, cannot sit or walk and succumb to death 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 sit, walk, and survive into adulthood.[7,8] Type 4 SMA is characterized by mild symptoms, it manifests during early adulthood, and patients are expected to have a normal lifespan.[6,9] SMN is a multifunctional protein involved in RNA metabolism, DNA repair, cytoskeletal dynamics, and macromolecular trafficking.[10-13] Low levels of SMN were shown to affect multiple signaling cascades, including STAT5, RhoA/ROCK, AKT/CREB and JNK pathways.[14-18] SMN can bind RNA, preferentially interacting with GA-rich motifs.[19,20] A recently published study indicated that the protein might play a critical role in the translation of a specific subset of mRNAs linked to SMA pathogenesis by "priming" ribosomes in a tissue-specific manner.[21] A much broader role of the *SMN* genes could be envisioned based on a vast repertoire of transcripts generated from the *SMN* loci. These transcripts include alternatively spliced mRNA isoforms, non-coding antisense RNAs, and circular RNAs.[22-31] Loss of *SMN1* affects all tissues, including skeletal muscle, central, peripheral and autonomic nervous system, heart, liver, lung, kidney, pancreas, spleen, ovary, and testis.[32-52] Hence, an ideal therapy for SMA must "target/remedy" the body-wide defects caused by the loss of *SMN1*. The severity of SMA correlates inversely with the *SMN2* copy number: the higher the copy number, the lower the severity.[53-56] Several factors, including Plastin (PLS3), Neuritin 1 (NRN1), Neurocalcin delta (NCALD), TIA1 cytotoxic granule associated RNA binding protein (TIA1), Ubiquitin specific peptidase 9 X-linked (USP9X), Ubiquitin like modifier activating enzyme 1 (UBA1), Stathmin-1 (STMN1), Myostatin (MSTN), ZPR1 zinc finger protein (ZPR1), and Senataxin (SETX), have been suggested to modify SMA severity.[57-69] 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.[70] 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
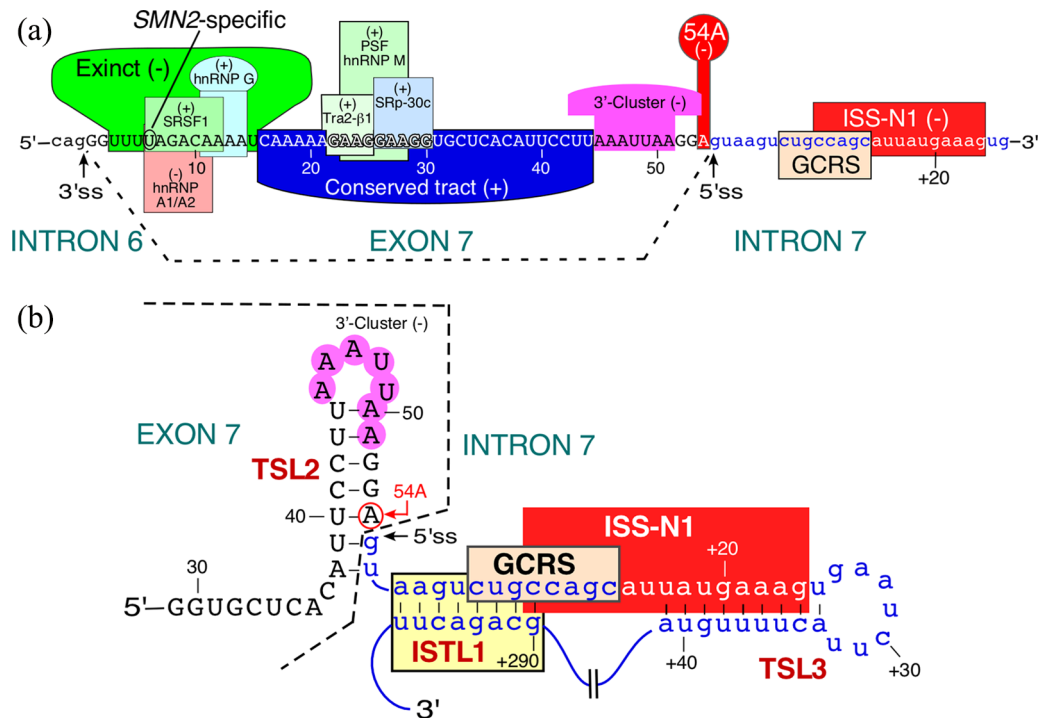
Figure 1. Diagrammatic representation of cis-elements and transacting factors that regulate *SMN2* exon 7 splicing. (a) Relative positioning of cis-elements within exon 7 and downstream intron 7 of *SMN*. Cis-elements and transacting factors that bind them are highlighted in different colors. Note, the diagram presented here is not inclusive of all reported exon 7 splicing regulators. Please refer to recent reviews for more comprehensive information.[95,96] Positive and negative regulators of exon 7 splicing are indicated by (+) and (–), respectively. Neutral numbering of nucleotides starts from the first position of exon 7. Positive numbering of nucleotides starts from the first position of intron 7. Exonic and intronic sequences are shown in upper- and lower-case letters, respectively. *SMN2*-specific C6U substitution is marked. Exinct, the Conserved tract and the 3′-Cluster were identified by in vivo selection of the entire exon 7. 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 (b) 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.

the C6U substitution-induced skipping of exon 7.[75-78] 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.[79] Of note, in vivo selection of the entire exon 7 was the first experiment of its kind: here the relative significance of every exonic nucleotide was functionally analyzed in a single experiment.[80] In addition, the unbiased method of in vivo selection revealed that not only the linear sequence(s) but also putative structural motifs were critical for inclusion of *SMN* exon 7.[81,82] Significantly, findings of in vivo selection of the entire exon 7 of *SMN1* turned out to be useful for validating the outcome of a machine learning program that analyzed the pathogenicity of all known point mutations within the human genome.[83] As discussed below, two splicing modulating compounds currently approved for SMA therapy as well as an additional small molecule are currently in clinical trials are linked to the strengthening of the 5′ss of *SMN2* exon 7.

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.[84] 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.[84] With very few exceptions, a GU dinucleotide at the first 2 positions of the U2-type introns is required for the exon definition.[85] 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 sequestered in a RNA structure.[86,87] 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.[88] These include the intronic splicing silencer N1 (ISS-N1), the terminal stem-loop 2 (TSL2), the GC-rich sequence (GCRS) overlapping ISS-N1, and the internal stem formed by a long-distance interaction 1 (ISTL1) (Figure 1).[89-94] Of note, as per recent estimates, more than 30 cis-elements and an even higher number of transacting factors have been implicated in the regulation of *SMN* exon 7 splicing.[95,96] However, subsequent studies showed that some of these splicing factors were dispensable. For instance, SF2/ASF was initially thought to be critical for exon 7 inclusion.[75] Yet, in cells lacking SF2/ASF no effect on exon 7 splicing was observed.[76] Similarly, Tra2-β1, a positive regulator of exon 7
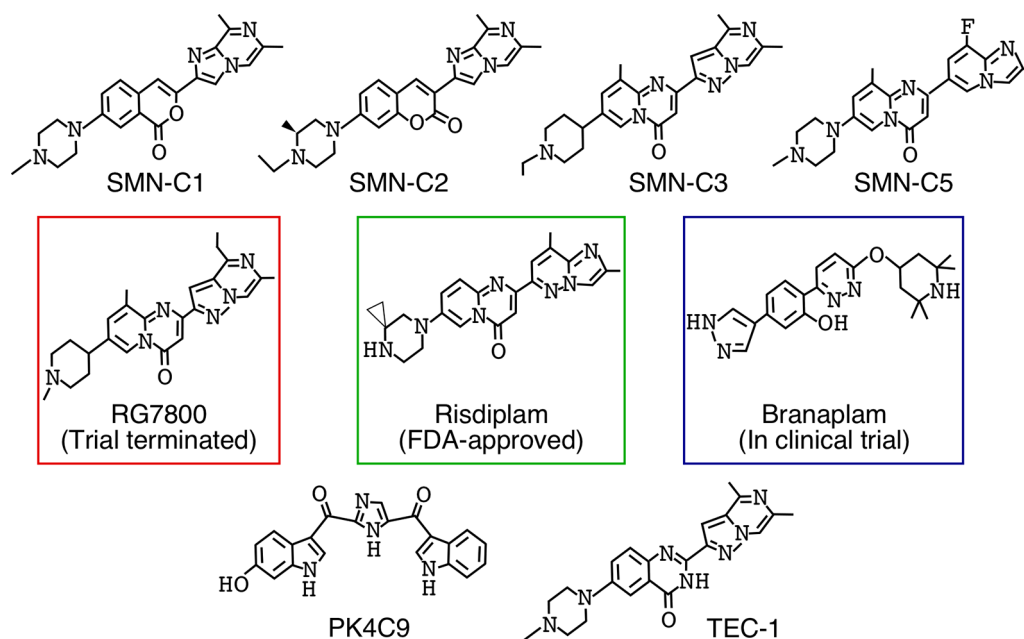
Figure 2. Structure of orally available small molecules used in pre-clinical and clinical studies for the treatment of SMA. SMN-C1, SMNC-2, and SMN-C3 were the first set of compounds reported by PTC-Roche (PTC Therapeutics, South Plainfield, New Jersey and Hoffmann-La Roche, Basel, Switzerland) to correct *SMN2* exon 7 splicing with high specificity. Most mechanistic studies have been done using SMN-C3 and SMN-C5. Clinical trial of RG-7800 by PTC-Roche was terminated due to its toxicity in cynomolgus monkeys. Risdiplam has gone through multiple clinical trials by PTC-Roche and has recently been approved by FDA. Branaplam is in clinical trial by Novartis Pharmaceuticals. PK4C9 and TEC-1 are the newly reported compounds to show specific splicing correction of *SMN2* exon 7.

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-pyrimidinone derivative); each promoted exon 7 inclusion from *SMN2* minigene expressed in HEK293H human embryonic kidney cell

line[127] (Figure 2). These compounds also promoted exon 7 inclusion in mRNAs generated from the endogenous *SMN2* and increased SMN levels in SMA patient fibroblasts and patient-derived induced pluripotent stem cells (iPSCs). The results of RNA-seq conducted using type 1 SMA fibroblasts treated with 500 nM of SMN-C3 showed high target specificity of the compound for *SMN2* exon 7 splicing correction, although limited off-target effects were also captured. For instance, expression of important genes including *DNA polymerase N* (*POLN*) and *PAP-associated domain containing 4 protein* (*PAPD4*) were significantly altered.[127] Also, SMN-C3 promoted inclusion of several exons in mRNAs generated from *Pyridoxal-dependent Decarboxylase Domain Containing 1* (*PDXDC1*), a gene associated with an increased risk for brain cancer.[127,128] In vivo studies employing a mild SMA mouse model (allele C model) as well as a severe SMA mouse model (SMA Δ7 model) confirmed splicing correction of *SMN2* exon 7 and upregulation of SMN upon oral or intraperitoneal (IP) administration of SMN-C3.[127] IP administration of SMN-C3 conferred substantial gain of lifespan and improvement of neuromuscular junction (NMJ) phenotype of Δ7 mice. However, based on their potential genotoxicity, phototoxicity, and/or chemical instability in plasma or aqueous buffers, none of the above-mentioned molecules advanced on to the human clinical trials.

With the realization that the compounds present in the existing library have a high potential for direct clinical applications, PTC-Roche began the process of designing improved versions of their active small molecules. As a result, three novel pyrido-pyrimidinone derivatives were "created," namely, compounds 3, 4, and 5; all displayed negative genotoxicity in the universally used Ames assay and showed very high therapeutic efficacy in the SMAΔ7 mouse model.[129] Compound 3, also known as RG7800, was selected for the subsequent human clinical trial that began in 2014. In parallel to the human clinical trial, RG7800 was also evaluated in cynomolgus monkeys for chronic toxicity. Due to nonreversible adverse effects on monkey retina, RG7800 clinical trial was put on hold.[129] Uncertain about the success of RG7800, PTC-Roche chose another small molecule, risdiplam (also known as RG7916), an improved version of RG7800, for clinical trials.[123] Risdiplam selection was based on its superior in vivo efficacy in the SMAΔ7 mouse model as well as its reduced off-target effects tested in SMA patient fibroblasts as compared to RG7800.[99] Risdiplam went through a relatively rapid clinical development, from the phase 1 clinical trial (January of 2016) to its FDA approval (August of 2020).[130] The fast approval of risdiplam was possible in part due to the well-defined parameters of therapeutic efficacy established during the preclinical and clinical studies of nusinersen and gene therapy.

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-C3, one of the analogs of risdiplam, interacts with an AG-rich motif, AGGAAG, located in the middle of exon 7 (Figure 3).[131] Authors employed a series of in vitro and in vivo techniques to demonstrate a high specificity of direct interactions between SMN-C3 and this AG-rich motif. Further, binding of SMN-C3 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).[131] Supporting this hypothesis, depletion of FUBP1/KHSRP diminished the effect of SMN-C3 on *SMN2* exon 7 splicing, particularly at low nanomolar concentrations of SMN-C3.[131] 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.[132] 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-β1, which in turn interacts with the purine-rich motif located in the middle of exon 7.[133,134] 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).[132] Of note, the inhibitory effect of 54A was first uncovered by in vivo selection of the entire exon 7.[79] Consistently, replacement of 54A with 54G (A54G substitution) fully restores *SMN2* exon 7 inclusion even when the Tra2-β1-binding site in exon 7 is destroyed.[79] 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.[90] 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.[90]

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).[135] 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.[135] 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-C5 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
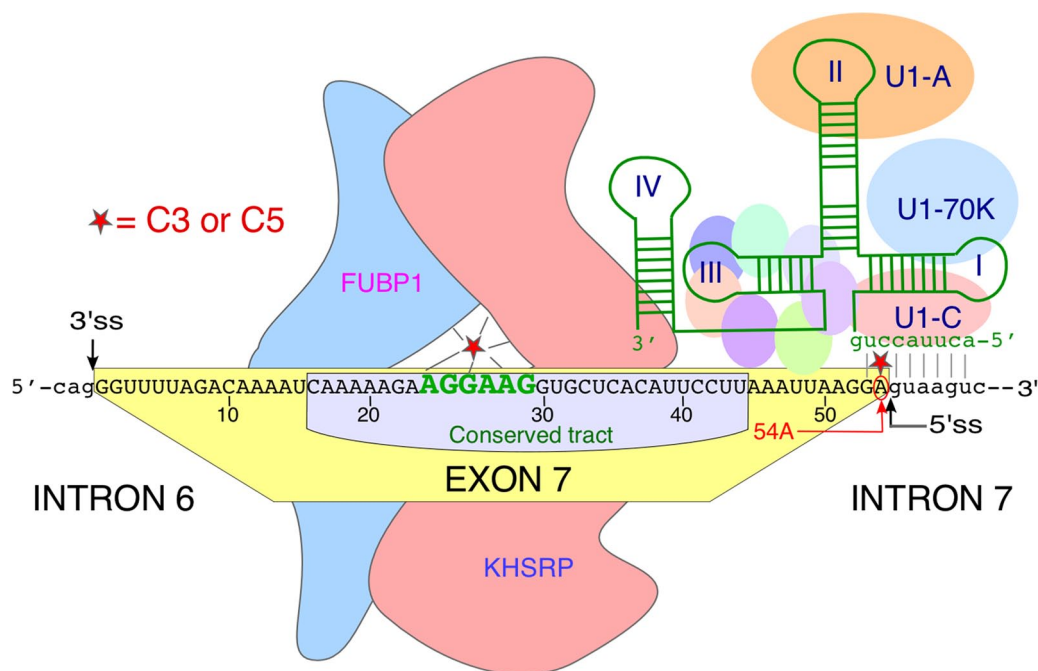
Figure 3. Potential mechanism of action of risdiplam. Risdiplam analogs SMN-C3 and -C5 are depicted as red stars. SMN-C3 has been shown to interact with an AG-rich motif (shown in green letters) located in the middle of exon 7.[131] SMN-C3 has been proposed to recruit splicing factors FUBP1 and KHSRP.[107] SMN-C5 has been proposed to promote recruitment of U1 snRNP by directly binding to 54A at the 5′ss of exon 7.[135] Interaction of U1 snRNP with the 5′ss of exon 7 has been depicted. Drawing is not to scale.

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

GUAAGU 5′ss 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
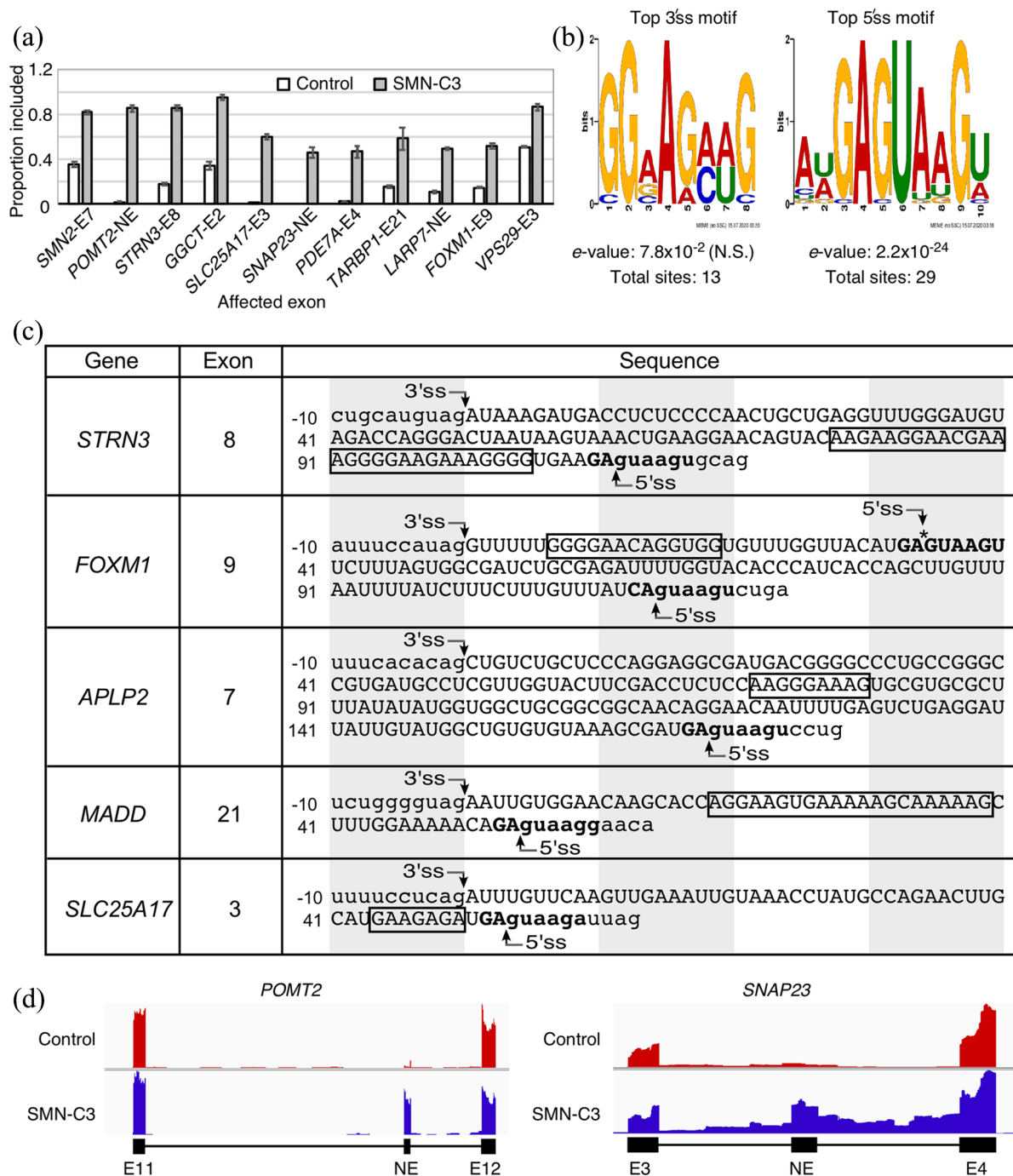
Figure 4. Off-target effects of risdiplam or its analog SMN-C3. (a) Splicing pattern of *SMN2* exon 7 and ten other splicing events affected by SMN-C3 treatment as reported by Naryshkin and coworkers.[127] Y axis indicates the proportion of total spliced transcript that has the exon in question included. 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.

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.[123] For instance, more than 70% of SMAΔ7 mice

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.[124] 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 (NCT02913482) and second in the later-onset SMA patients (NCT02908685) were significant for the approval of risdiplam.[130] The clinical trial for infantile-onset SMA was an open-label study in which 21 patients, whose average age was ~6.7 months, participated.[130] 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.[130] 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.[130]

Side Effects of Risdiplam

The most common side effects in clinical trials of risdiplam were fever, rash, ulcers of the mouth area, joint pain (arthralgia), diarrhea, and urinary tract infections.[130] The infantile-onset population receiving risdiplam had additional side effects including upper respiratory tract infection, pneumonia, vomiting, and constipation.[130] Currently, it is not known if the side effects are directly linked to the off-target effects of risdiplam.

Conclusion

Recent approval of risdiplam, an orally deliverable small molecule, is a major advancement for the treatment of SMA. The noninvasive mode of administration coupled with body-wide distribution provide risdiplam with clear advantages over other approved therapies. Risdiplam availability is particularly great news for a group of SMA patients that might have tolerability 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.[136] Activation of a cryptic 5′ss downstream of exon 7 by an engineered U1 snRNP could be an alternative therapeutic approach in this case.[136,137] In fact, in vivo efficacy of the engineered U1 snRNP has been validated in a mouse model of SMA.[138] 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).[139,140] Branaplam (synonyms: NVS-SM1 and LMI070) was identified by high-throughput screening of the Novartis compound library, followed by chemistry optimization.[139,141] It was shown to modulate splicing, elevate levels of the full-length SMN protein and increase the survival of a severe SMA mouse model.[141] Despite structural differences between branaplam and risdiplam, the proposed mode of action of branaplam appears to be similar to that of risdiplam.[141] Both drugs stabilize the U1:5′ss duplex at the 5′ss of *SMN2* exon 7.[135,141] Two more small molecules, PK4C9 and TEC-1, have been recently reported to enhance *SMN2* exon 7 inclusion with high specificity (Figure 2).[142,143] TEC-1 has been found to be permeable to the central nervous system and confer therapeutic efficacy in a mouse model of SMA.[142] While the mechanism of TEC-1 action has not yet been examined, PK4C9 has been shown to interact with a structural element, namely the tri-loop of TSL2.[142,144] Incidentally, sequences encompassing the triloop 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).[79,81] 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.[5,140,145] 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.[25,146-148] 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 precedence-setting success of risdiplam coupled with the discovery of additional small molecules capable of modulation of *SMN2* exon 7 splicing with high specificity, prospects of small molecule therapeutics for the treatment of SMA appears to be on the fast track. In addition, these advancements should serve as a catalyst for the development of novel therapeutics for other genetic diseases amenable by splicing modulation.

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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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