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Review

Spinal muscular atrophy: An update on therapeutic progress

Joonbae Seo [1], Matthew D. Howell [1], Natalia N. Singh, Ravindra N. Singh *

Department of Biomedical Sciences, Iowa State University, Ames, IA 50011, USA

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ABSTRACT

Humans have two nearly identical copies of survival motor neuron gene: *SMN1* and *SMN2*. Deletion or mutation of *SMN1* combined with the inability of *SMN2* to compensate for the loss of *SMN1* results in spinal muscular atrophy (SMA), a leading genetic cause of infant mortality. SMA affects 1 in ~6000 live births, a frequency much higher than in several genetic diseases. The major known defect of *SMN2* is the predominant exon 7 skipping that leads to production of a truncated protein (SMNΔ7), which is unstable. Therefore, SMA has emerged as a model genetic disorder in which almost the entire disease population could be linked to the aberrant splicing of a single exon (i.e. *SMN2* exon 7). Diverse treatment strategies aimed at improving the function of *SMN2* have been envisioned. These strategies include, but are not limited to, manipulation of transcription, correction of aberrant splicing and stabilization of mRNA, *SMN* and *SMNΔ7*. This review summarizes up to date progress and promise of various in vivo studies reported for the treatment of SMA.

1. Introduction

Spinal muscular atrophy (SMA) is a genetic disease caused by homozygous deletion, truncation, mutation or gene conversion of *survival motor neuron 1* (*SMN1*) [1–4]. *SMN2*, a nearly identical copy of *SMN1*, fails to compensate for the loss of *SMN1* owing to a cytosine to thymidine mutation at the 6th position (C6U in the transcript) of exon 7. C6U triggers predominant skipping of *SMN2* exon 7 due to disruption of an exonic splicing enhancer and/or creation of an exonic splicing silencer [5–7]. The resultant decrease in full-length transcript reduces functional SMN, since the translated product (SMNΔ7) of the truncated transcript is unstable and rapidly degraded [8–10]. The copy number of *SMN2* modulates the severity of SMA: the more *SMN2* copies the less severe the disease due to higher levels of the full-length transcript and functional SMN [11–13]. Thus, treatment strategies to halt the disease progression and ameliorate the symptoms have primarily focused on means to increase full-length *SMN2* transcript and functional SMN.

The multifunctional SMN has been implicated in snRNP biogenesis [14–17], transcription [18,19], splicing [20], translation [21], signal transduction [22], stress granule formation [23] and intra-cellular trafficking [24]. With respect to neuron-specific functions, SMN facilitates interaction of mRNA binding proteins and participates in mRNA transport across the axonal processes of motor neurons [25–27]. SMN modulates axon outgrowth and cytoskeletal dynamics through β actin localization [28–30]. Preventing SMN transport across axons causes growth cone collapse [31]. SMN also plays an important role in postnatal muscle nerve terminal maturation and reduction in SMN levels is predicted to negatively affect neurotransmission [32]. Defects in snRNP biogenesis correlate with the severity of SMA, although only a subset of snRNPs is preferentially affected [33]. Supporting these arguments, motor neurons of *Smn* deficient *Drosophila* show decreased expression of a subset of certain genes containing the U12 type introns [34].

Mice, unlike humans, possess only one *Smn* gene, and homozygous deletion of *Smn* is embryonically lethal [35]. Several transgenic mouse models that mimic the SMA pathology have been developed by introducing human *SMN2* into the mouse genome in the context of *Smn* knockout. Two recent excellent reviews describe these models in much detail [36,37]. Preclinical research to identify promising treatments for SMA has relied heavily upon these murine models [22,38–48]. Table 1 lists a few major mouse models utilized in preclinical trials as well as a few other models that may be exploited for these pursuits. Two severe mouse models account for the majority of preclinical studies: the Taiwanese model [38,48] and the Δ7 SMA model [40]. Generally, therapeutic strategies in SMA mice focused on increasing the amount

Abbreviations: ALS, amyotrophic lateral sclerosis; ASO, antisense oligonucleotide; BBB, blood brain barrier; CREB, cyclic AMP response element binding protein; FDA, Food and Drug Administration; GSK-3, glycogen synthase kinase 3; HDAC, histone deacetylase; hnRNP A1, heterogenous ribonucleoprotein A1; ICV, intracerebroventricular; IGF-1, insulin-like growth factor 1; IP, intraperitoneal; ISS-N1, intronic splicing silencer N1; ISS-N2, intronic splicing silencer N2; IV, intravascular; JAK, Janus kinase; NMDA, *N*-methyl D-aspartic acid; NSAID, non-steroidal anti-inflammatory drug; SAHA, suberoylanilide hydroxamic acid; SC, subcutaneous; scAAV, self-complimentary adeno-associated virus; SMA, spinal muscular atrophy; SMN, survival motor neuron; snRNP, small nuclear ribonucleoprotein; STAT5, signal transducer and activator of transcription 5; TIA1, T-cell restricted intracellular antigen 1; TSA, trichostatin A; VPA, valproic acid

* Corresponding author at: Department of Biomedical Sciences, Iowa State University, 2035 Veterinary Medicine, Ames, IA 50011, USA. Tel.: +1 515 294 8505; fax: +1 515 294 2315.
E-mail address: singhr@iastate.edu (R.N. Singh).
[1] These authors made equal contributions.

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Table 1
Mouse models useful for testing drug efficacy for potential SMA therapy.

Model	Genotype	Survival (days)	Outcome measures	References
Taiwanese	*Smn*−/−; *SMN2(2Hung)*+/± [mice carry 1 or 2 copies of transgene]	Type I: ~10 Type II: ~14 Type III: Normal	Survival; motor function; tail and limb necrosis; motor neuron, NMJ[a], muscle and heart morphology and/or function	[38,48]
Line89	*Smn*−/−; *SMN2(89Ahmb)*+/+	5	Survival; motor function; motor neuron, NMJ and muscle morphology and/or function	[39]
Δ7 SMA	*Smn*−/−; *SMN2(89Ahmb)*+/+; *SMNΔ7* +/+	~14	Survival; motor function; motor neuron, NMJ, muscle and cardiac morphology and/or function	[40]
3 copy *SMN2*	*Smn*−/−; *SMN2(N11)*; *SMN2*(N46)	14–16	Survival; motor function; motor neuron, NMJ and muscle morphology and/or function	[41]
F7 or exon 7 floxed	*SmnF7/Δ7; NSE-Cre* [exon 7 loss in neurons]	25	Survival; motor function; motor neuron, NMJ and muscle morphology and/or function	[43]
F7 or exon 7 floxed	*SmnF7/Δ7*; *HSA-Cre* [exon 7 loss in skeletal muscle]	33	Survival; motor function; motor neuron, NMJ and muscle morphology and/or function	[42]
2B	*Smn2B*/− [2B is defined by mutation in exon 7 splicing enhancer]	~30	Survival; motor function; neuromuscular junction and muscle morphology and/or function	[22]
SMN^{RT}	*Smn*−/−; *SMN2(89Ahmb)* +/+; $SMN\Delta7^{RT}$ +/+	34	Survival; motor function; motor neuron, NMJ and muscle morphology and/or function	[44]
Olig2-*Cre*	*Smn*F7/−; *SMN2(89Ahmb)*+/+; *Olig2-Cre* [exon 7 loss in motor neuron progenitor cells]	365[b]	Motor function; motor neuron, NMJ and muscle morphology and/or function	[45]
Smn C > T	*Smn*C > T [*SMN2* mutation inserted in mouse *Smn*]	Normal	Motor function; motor neuron, NMJ and muscle morphology and/or function	[46]
Allele C	*Smn*C/C [C is defined by a chimeric gene plus *SMN2*]	Normal	Ear, tail and limb necrosis; cardiac morphology and function	[47]

[a] NMJ, neuromuscular junction.
[b] 70% of these mice survived to 365 days.

of full-length *SMN2* transcript and SMN as a means to extend lifespan and correct tissue and motor function abnormalities. We summarize the major avenues of therapeutic interventions explored for SMA with particular emphasis on small molecules and antisense oligonucleotides (ASOs). This review complements a recent report that describes in detail the progress in the field of ASO-mediated therapy of SMA [49]. Due to lack of space and the staggering number of compounds tested for SMA therapy, we are unable to provide details on dose, duration and frequency of delivery for most of the compounds. For the purposes of comparison, we have put major emphasis on the life expectancy as the primary measure of the therapeutic efficacy in severe SMA mice. Until two years ago there was no report of a therapeutic compound that could extend the lifespan of a severe SMA mouse beyond 30 days. Recently, independent studies have shown an impressive increase in the life expectancy of severe SMA mice treated with ASOs that specifically target an intronic sequence within *SMN2* [refs. in 49]. The noticeable aspect of these studies is the cross validation of ASO efficacy among various mouse models and oligonucleotide chemistries against the same intronic target (described later). However, due to timing of blood brain barrier (BBB) formation and several other features distinct from humans, results in mouse models of SMA should be interpreted with caution. An overwhelming majority of small compounds confer a modest (<1.5-fold) increase in the life expectancy of severe SMA mice (Fig. 1). Consistently, these compounds display poor efficacy in clinical trials. However, possibilities remain that some of these compounds could be further improved to achieve a better therapeutic efficacy.

2. Treatment with small compounds

Small compounds offer several advantages, including an easy transport across biological barriers. Considering SMA is a neurodegenerative disease, compounds that are transported across BBB would be best suited for an effective therapy. A summary of the relative efficacy of small compounds and other treatments is given in Fig. 1 [based on refs. 50–82]. Available reports underscore the diversity of processes that may impact *SMN2* transcription, *SMN2* exon 7 splicing and/or SMN levels within a cell. Given below are the major classes of compounds that have been tested for their efficacy for SMA therapy (Table 2).

2.1. Histone deacetylase (HDAC) inhibitors

HDAC inhibitors prevent deacetylation of histones and increase gene expression through chromatin remodeling [83,84]. Various chemical classes of HDAC inhibitors have been shown to enhance the expression of *SMN2* in SMA patient cells and in mouse models of SMA (Fig. 1) [68,69,80,81,85–88]. Sodium butyrate modestly increased survival in Taiwanese type II mice and reduced tail necrosis [81]. Valproic acid (VPA), a FDA-approved compound with multiple functions including HDAC inhibition, increased motor neuron density in the lumbar spinal cord and ameliorated necrosis of the tail and ears of Taiwanese type III mice [89]. In a follow-up study, type III SMA mice treated with VPA exhibited decreased spinal cord motor neuron degeneration, decreased muscle atrophy and improved neuromuscular junction innervation [90]. However, the effects of VPA in severe SMA mice were less pronounced. Nevertheless, VPA has been extensively examined as a treatment for types I, II and III SMA patients in several clinical trials (Clinicaltrials.gov ID numbers NCT00661453, NCT00227266, NCT00374075, NCT00481013, and NCT01671384). The beneficial effects of VPA in SMA patients, however, have been nominal [91–95]. Another HDAC inhibitor, phenylbutyrate, has been trialed in SMA patients with modest results [96], although more extensive clinical trials have been terminated due to poor treatment compliance or slow enrollment (Clinicaltrials.gov ID numbers NCT00439569 and NCT00439218).

Trichostatin A (TSA), a second-generation HDAC inhibitor, improved motor function and modestly increased the survival of Δ7 SMA mice (Fig. 1) [69]. Addition of a nutritional supplement with TSA treatment augmented the beneficial effects, including a ~2.5-fold increase in lifespan of Δ7 SMA mice (Fig. 1) [68]. Suberoylanilide hydroxamic acid (SAHA), another second-generation HDAC inhibitor, rescued the embryonic lethality and modestly increased survival of Taiwanese type I SMA mice (Fig. 1) [80]. However, higher doses of SAHA resulted in toxicity even in heterozygous mice [80]. A recent study with SAHA showed weight gain and improved motor function in Taiwanese type I SMA

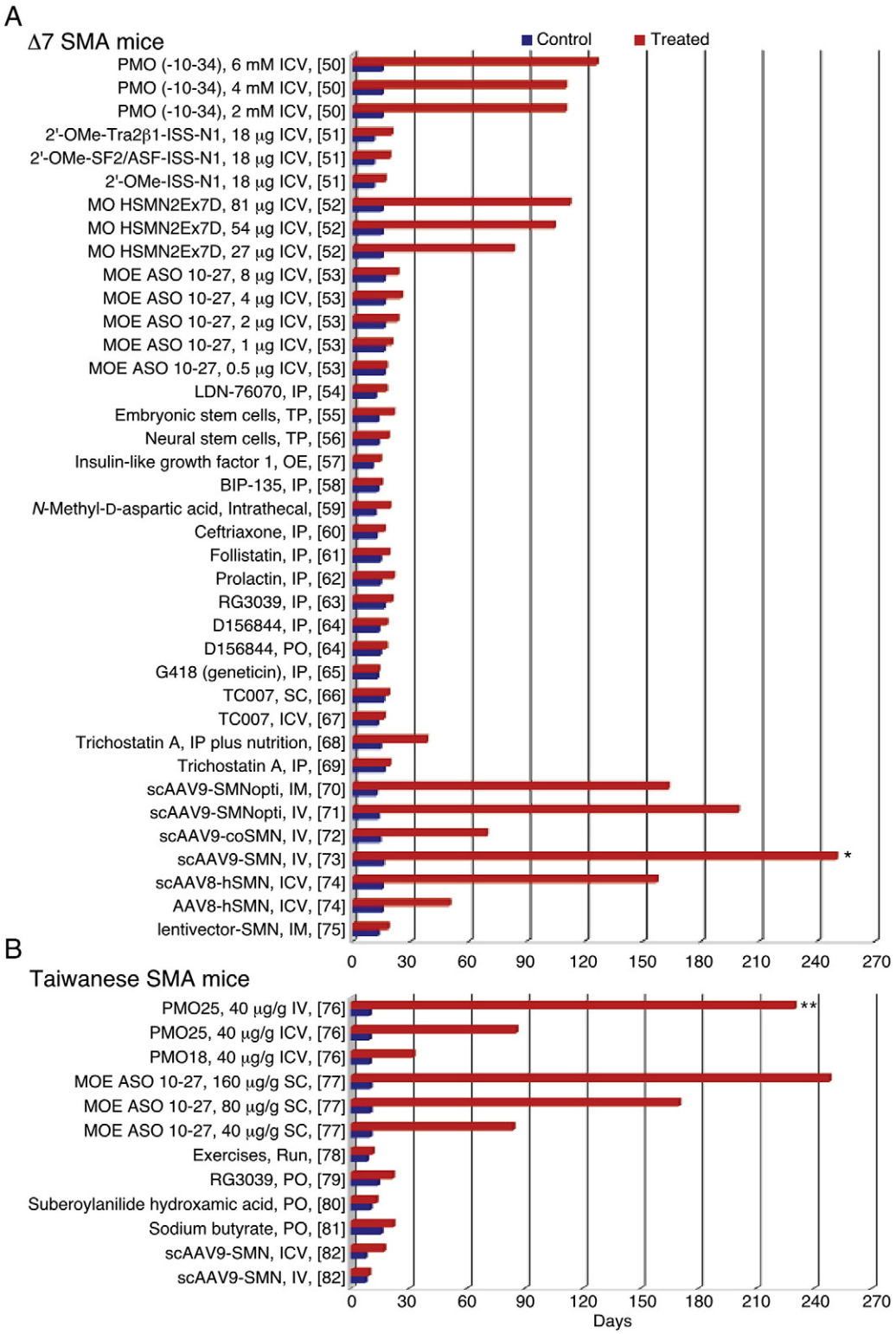


Fig. 1. Survival bar charts of treatments in Δ7 SMA mice and Taiwanese SMA mice. (A) Survival bar charts comparing control and treatment in Δ7 SMA mice. *, still alive at >250 days. (B) Survival bar charts comparing control and treatment in Taiwanese SMA mice. **, still alive at >230 days. References for the studies are denoted in the brackets. Abbreviations: AAV, adeno-associated virus; scAAV, self-complementary adeno-associated virus; coSMN, codon-optimized SMN; scAAV9-SMNopti, an optimized SMN-encoding scAAV9; IM, intramuscular injection; ICV, intracerebroventricular injection; IV, intravenous injection; IP, intraperitoneal injection; SC, subcutaneous injection; OE, overexpression; PO, per os; TP, transplantation; ASO, antisense oligonucleotide; MOE, an ASO with phophorothioate backbone and 2′-O-methoxyethyl modification; MO, morpholino; 2′-OMe, an ASO with phophorothioate backbone and 2′-O-methyl modification; ISS-N1, intronic splicing silencer N1; PMO, phosphorodiamidate morpholino oligonucleotides; Tra2β1, transformer-2 protein homolog β; SF2/ASF, splicing factor2/alternative splicing factor.

mice, although the effect on lifespan was not reported [97]. Treatment of Taiwanese type I SMA mice with JNJ-26481585, a novel second-generation HDAC inhibitor, did not provide any lifespan benefit [98]. Thus far, only modest benefits have been observed in SMA patients treated with HDAC inhibitors. HDAC inhibitors continue to be evaluated as potential SMA therapeutics.

Table 2
Pathways affected by compounds used for potential SMA therapy.

Treatment	Process	Models used	References
Histone deacetylase inhibitors			
Trichostatin A	Transcription	Δ7 SMA mice	[68,69]
Suberoylanilide hydroxamic acid	Transcription	Taiwanese mice	[80]
Sodium butyrate	Transcription	Taiwanese mice	[81]
Valproic acid	Transcription	Taiwanese mice	[90]
Translation read-through compounds			
G418 (geneticin)	Translation	Δ7 SMA mice	[65]
TC007	Translation	Δ7 SMA mice	[66,67]
Quinazolines			
RG3039	Transcription	Δ7 SMA mice	[63]
		Taiwanese mice	[79]
		2B	[79]
D156844	Transcription	Δ7 SMA mice	[64]
Other small molecules that increase SMN2 expression			
LDN-76070	Transcription	Δ7 SMA mice	[54]
Indoprofen	Splicing and/or translation	Line 89	[109]
Antibiotics			
Ceftriaxone	Cell signaling	Δ7 SMA mice	[60]
PTK-SMA1 (tetracycline-like)	Splicing	Taiwanese mice	[110]
Signal transducing molecules			
Sodium vanadate	Splicing	Taiwanese mice	[117]
Neuroprotective compounds			
BIP-135	Cell signaling	Δ7 SMA mice	[58]
N-Methyl-D-aspartic acid	Cell signaling	Δ7 SMA mice	[59]
Riluzole	Cell signaling	F7 or exon 7 floxed	[121]
Polypeptides and proteins			
Insulin-like growth factor 1	Cell signaling	Δ7 SMA mice	[57]
Follistatin	Cell signaling	Δ7 SMA mice	[61]
Prolactin	Transcription	Δ7 SMA mice	[62]
Gene therapy and trans-splicing			
SMN1 gene therapy	SMN-associated functions	Δ7 SMA mice	[70–75]
		Taiwanese mice	[82]
Trans-splicing	Splicing	Δ7 SMA mice	[133]
Stem cell based therapy of SMA			
Stem cell transplantation	SMN-associated functions	Δ7 SMA mice	[55,56]
SMN-independent treatment strategies			
Forced running	Multiple	Δ7 SMA mice	[148]
		Taiwanese mice	[78]
ASO-based therapy			
Antisense oligonucleotides	Splicing	Δ7 SMA mice	[50–53]
		Taiwanese mice	[76,77]

2.2. Translation read-through compounds

Skipping of *SMN2* exon 7 produces a truncated protein (SMNΔ7) with a novel extension of four amino acids (EMLA) coded by exon 8. EMLA serves as a degradation signal within SMNΔ7 [10]. Several aminoglycosides are known to interact with ribosomes and allow read-through at stop codons [99]. Read-through of the first stop codon in the exon 7-deleted transcript of *SMN2* is predicted to add another five amino acids to the C-terminus of SMNΔ7. Incidentally, SMA patient cells treated with aminoglycosides showed increased SMN bodies (gems), suggesting that addition of extra amino acids due to read-through has a stabilizing effect on SMNΔ7 [100,101]. A follow up study confirmed stabilization of SMNΔ7 upon addition of the five extra amino acids at the C-terminus of SMNΔ7 [102]. TC007, a novel aminoglycoside (an analog of neomycin not expected to cross the BBB), modestly extended the mean lifespan of Δ7 SMA mice when administered through intracerebroventricular (ICV) route (Fig. 1) [67]. However, chronic subcutaneous (SC) administration of this compound did not produce any life expectancy benefit (Fig. 1) [66]. Geneticin (G418), another aminoglycoside, improved motor function but did not provide survival benefit in Δ7 SMA mice (Fig. 1) [65]. Furthermore, chronic administration of G418 turned out to be toxic even for the wild type mice [65]. These findings underscore the limitations of the current generation of aminoglycosides as a potential drug candidate for SMA therapy.

2.3. Quinazolines

Quinazolines are a family of compounds that inhibit RNA decapping enzyme DcpS (involved in RNA turnover), and this inhibition can consequently increase *SMN2* expression [103]. A high-throughput screening identified a few quinazolines as lead compounds that increased full-length *SMN2* transcript and SMN [104]. These compounds had poor BBB penetration and required high doses to achieve sufficient upregulation of SMN. Based on a lead quinazoline, derivatives were developed to overcome the obstacles associated with the low BBB penetration [105]. One of these derivatives, D156844, crossed the BBB without any adverse effect in Δ7 SMA neonates. However, D156844 produced only a modest lifespan extension in Δ7 SMA mice (Fig. 1) [64]. Further optimization of this compound led to the identification of D157495, also called RG3039. In one study, daily intraperitoneal (IP) administration of RG3039 modestly extended the lifespan of Δ7 SMA mice and improved the morphology and function of the neuromuscular junction (NMJ) [63]. In another study, daily oral administration of RG3039 modestly extended the lifespan of Taiwanese type I mice (Fig. 1) [79]. Additionally, RG3039 markedly increased the lifespan in less severe Smn2B/− mouse model (median lifespan increased from 18 to 112 days) [79]. Currently, RG3039 is undergoing phase I clinical trial in which safety and efficacy of various doses will be evaluated.

2.4. Hydroxyurea

Hydroxyurea has been shown to increase *SMN2* expression in SMA patient fibroblasts, purportedly via an increase in nitric oxide [106]. Due to the positive in vitro effects and the BBB permeability of hydroxyurea, this compound was tested in SMA patients in several clinical trials (Clinicaltrials.gov ID numbers NCT00485511, NCT00568698, and NCT00568802). While one small study registered encouraging results in SMA patients treated with hydroxyurea [107], a larger, placebo-controlled study did not reveal any significant effects in SMA treated patients [108]. These findings limit the scope of hydroxyurea as an effective drug for SMA therapy.

2.5. Compounds from cell-based drug screenings

A luciferase reporter system coupled to *SMN2* minigene carrying CMV promoter was employed in a high-throughput screening to identify indoprofen, a non-steroidal anti-inflammatory drug (NSAID) that promoted *SMN2* exon 7 inclusion in C33a cervical carcinoma cells [109]. Other NSAID compounds failed to stimulate *SMN2* exon 7 inclusion, suggesting that the effect of indoprofen is mediated through a cyclooxygenase-independent pathway. Subsequent evaluation of indoprofen in SMA patient cells failed to show any stimulatory effect on *SMN2* exon 7 splicing from endogenous *SMN2*. Interestingly, through an unknown mechanism indoprofen produced a noticeable upregulation of SMN (protein) in SMA patient cells [109]. In vivo efficacy of indoprofen was tested employing a highly severe SMA model in which all SMA pups die by embryonic day 11 (E11). A single IP administration of indoprofen (5 mg/kg) was able to increase the mean litter size of embryos at E14 [109]. However, efficacy of indoprofen in less severe mouse models remains to be evaluated. Another reporter assay that used *SMN2* minigene coupled with *SMN2* promoter identified a set of compounds including LDN-75654 and LDN-76070. These compounds were found to increase SMN levels in SMA patient cells [54]. IP administration of LDN-76070 in Δ7 SMA mice modestly extended lifespan compared to untreated mice

(Fig. 1) [54]. However, in vivo efficacy of other LDN compounds could not be evaluated due to their extremely poor water solubility.

2.6. Antibiotics

Several antibiotics have been evaluated as potential therapeutic compounds for the treatment of SMA [60,110]. Screening of tetracycline-like compounds using a cell-free splicing assay identified PTK-SMA1 [110]. PTK-SMA1 increased SMN levels in SMA patient cells as well as in a type III SMA mouse model [110]. However, therapeutic efficacy of tetracycline-like compounds on phenotype and life expectancy of a mouse model of SMA is yet to be evaluated. Ceftriaxone is a third-generation cephalosporin antibiotic that has been tested for its therapeutic efficacy in a mouse model of amyotrophic lateral sclerosis (ALS), a devastating neurodegenerative disease [111]. Ceftriaxone prevented motor neuron loss and increased survival of ALS mice possibly through increased glutamate clearance and reduced excitotoxicity [111]. Treatment of Δ7 SMA mice with high doses of ceftriaxone (200 mg/kg) showed some benefits on muscle and NMJ morphology, however, the survival benefit was very modest (Fig. 1) [60].

2.7. Signal transducing molecules

Splicing factor Tra2β1 stimulates *SMN2* exon 7 inclusion by directly binding to exon 7 [112]. Dephosphorylation of Tra2β1 by protein phosphatase 1 (PP1) was implicated in the promotion of *SMN2* exon 7 skipping [113]. Recently, cantharidin analogs that modulate PP1 activity were shown to increase *SMN2* exon 7 inclusion and SMN levels in SMA patient cells [114]. Like several phosphatase inhibitors, cantharidins are known to affect transcription [115]. However, it remains to be seen if cantharidin analogs that promote *SMN2* exon 7 inclusion also stimulate *SMN2* transcription. Sodium vanadate is a tyrosine phosphatase inhibitor that markedly increases *SMN2* exon 7 inclusion in a cell-based splicing assay [116]. However, sodium vanadate was considered inappropriate for SMA therapy due to high toxicity. To overcome this toxicity, Taiwanese type III SMA mice were simultaneously treated with sodium vanadate and the detoxification agent L-ascorbic acid [117]. Indeed, the combination therapy prevented the morbidity and mortality induced by sodium vanadate. Also, the combination therapy delayed the onset of tail necrosis, increased lumbar spinal cord motor neurons and improved muscle histology. However, effects in a more severe mouse model of SMA remain to be determined.

2.8. Neuroprotective compounds

The neuroprotection conferred by small molecules can occur via several mechanisms and could potentially spare or protect motor neurons and thus ameliorate the disease. Glycogen synthase kinase-3 (GSK-3) has been linked to human pathologies, and inhibition of this signaling molecule is purported to activate cyclic AMP response element binding protein (CREB) and increase transcription of neurotrophic factors. Indeed, GSK-3 inhibition extended the lifespan of a mouse model of ALS [118]. When BIP-135 (a GSK-3 inhibitor) was administered to Δ7 SMA mice, median lifespan increased modestly compared to untreated mice (Fig. 1) [58]. Further studies would be needed to appropriately evaluate the effect of various treatment regimes. As a potential treatment for SMA, activation of the *N*-methyl-D-aspartic acid (NMDA) receptor has also been examined. Intrathecal NMDA administration in two SMA mouse models accelerated maturation of motor units, conferred neuroprotection to motor neurons and significantly extended the lifespan (Fig. 1) [59]. NMDA also led to CREB activation with a resultant increase in SMN levels [59]. However, the clinical utility of intrathecal NMDA administration is yet to be evaluated in SMA patients.

Olesoxime is a mitochondrial pore modulator that provides protective effects in motor neurons [119]. While the neuroprotective effect of olesoxime has not been tested in SMA mouse models, it is currently undergoing a phase 2 clinical trial for the treatment of SMA (Clinicaltrials.gov ID number NCT01302600). SMA type II and III patients are enrolled in this ongoing study; each patient will undergo two years of treatment to examine the beneficial effects of this compound.

Riluzole is the only FDA-approved compound for ALS treatment. Although the mechanism of riluzole action remains vague, it appears to exert its neuroprotective effect by modulating glutamate transmission [120]. Therapeutic efficacy of riluzole was evaluated in a SMA mouse model that was generated by neuron-specific knockout of *Smn* exon 7 [121]. Riluzole improved the architecture of motor neuron synaptic terminals and modestly increased the lifespan of SMA mice [121]. A phase I clinical trial in which infants with SMA type I were treated with riluzole produced intriguing results. Three of the seven patients lived well past the usual age of death with limited need for respiratory support, although conclusions were not definitive due to very small sample size [122]. A phase 2/3 clinical trial of riluzole with type II and type III patients has been conducted and findings of this study are still awaited (Clinicaltrials.gov ID number NCT00774423).

3. Polypeptide and protein-based therapies

Transcription of *SMN2* is modulated by transcription factor STAT5, a phosphorylation substrate of Janus kinase (JAK) [123]. Prolactin, a polypeptide hormone that activates JAK2/STAT5 signaling pathway has been shown to improve motor function and modestly increase the lifespan of Δ7 SMA mice (Fig. 1) [62]. Prolactin also increased SMN levels in the brain possibly through activation of transcription of *SMN2* [62]. However, it remains to be seen if other members of the JAK/STAT signaling pathway would have a better therapeutic efficacy.

Myostatin, a member of the transforming growth factor beta (TGFβ) family, is expressed almost exclusively in muscle and functions as a negative regulator of skeletal muscle growth [124]. Follistatin, a known myostatin inhibitor, improved motor function and modestly increased the lifespan of Δ7 SMA mice (Fig. 1) [61]. In another study, Δ7 SMA mice treated with activin IIB (ActIIB-Fc), a soluble receptor of myostatin, increased the mass of several muscles, but did not rescue motor function or extend survival [125]. Furthermore, knocking out of myostatin gene in Δ7 SMA model did not reduce the severity of disease [126]. These findings rule out the TGFβ signaling pathway modulating compounds as the potential therapeutic candidates of SMA.

Insulin-like growth factor 1 (IGF-1), a protein hormone, serves as an important regulator of skeletal muscle development and function [127]. Incidentally, IGF-1 serum levels are significantly reduced in both Taiwanese type I SMA [77] and Δ7 SMA mice [128]. To investigate the role of IGF-1 in SMA pathology, a novel mouse model that overexpressed a rat muscle-specific isoform of IGF-1 in skeletal muscle of Δ7 SMA model was developed [57]. IGF-1 overexpression modestly affected the phenotype: median lifespan was extended ~1.4-fold and several muscles increased in weight (Fig. 1) [57]. In a separate study, treatment of Δ7 SMA mice with IPLEX (recombinant human IGF-1 complexed with recombinant IGF binding protein 3) prevented muscle wasting and neuromuscular junction abnormalities [128]. However, this approach did not improve motor function and lifespan of Δ7 SMA mice [128].

4. Gene therapy and trans-splicing

Gene therapy promises a permanent solution for SMA through viral delivery and insertion of the entire *SMN1* gene or cDNA sequence into the genome of SMA patients. This process is generally irreversible and there are apparent risks if the exogenously delivered gene is inserted at a wrong location and/or overexpressed. The first gene therapy, conducted in Δ7 SMA mice, introduced the entire human *SMN1* gene and conferred some protection to brainstem motor neurons and increased lifespan from 13 to 18 days (Fig. 1) [75]. Despite the modest outcomes, findings provided the first direct evidence that the postnatal increase in SMN levels confers definite therapeutic benefits. In another

study, ICV administration of the *SMN1* gene substantially (~10-fold) increased the lifespan of Δ7 SMA mice (Fig. 1) [74]. This finding suggested that increasing SMN levels in the brain is critical for prolonging the life of SMA patients. However, numerous studies that delivered *SMN1* intravenously (IV) also demonstrated impressive results [71,72,129]. In particular, a codon optimized *SMN1* sequence injected into the temporal vein increased median survival ~15-fold (Fig. 1) [71]. Intramuscular delivery of the same sequence produced comparable results (Fig. 1) [70]. Another study conducted in Δ7 SMA mice demonstrated similar benefits from IV and ICV administrations of scAAV9-SMN [130]. On the other hand, experiments conducted in Taiwanese type I SMA mice yielded variable results and less promising survival benefits with ICV administration of *SMN1* sequence (Fig. 1) [82]. These findings underscore the caution that must be exercised when extrapolating the results of pre-clinical studies conducted in a specific mouse model to design clinical trials in humans. Overall, the results of gene therapy appear promising and may offer one of the best therapeutic alternatives to a specific group of SMA patients who are too weak to receive frequent invasive treatments.

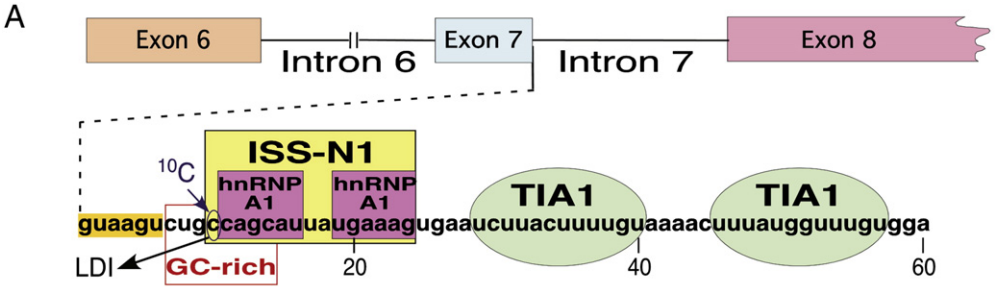
Trans-splicing is a gene-therapy-like approach with the potential to treat SMA patients. This approach involves exogenous delivery of a partial gene or mRNA that triggers production of a chimeric transcript that is equivalent to the full-length mRNA generated from the endogenous gene [131,132]. Considering that the trans-splicing process uses endogenous pre-mRNA as a substrate, it caps the level of mRNAs and protein produced. Therefore, trans-splicing has inbuilt potential to fully avoid complications of protein overexpression, which is an anticipated risk associated with the gene therapy. Of note, it must be brought forth that there is no evidence to suggest that gene therapy-based approaches would cause SMN overexpression that results into a recognizable harmful effect in pre-clinical studies. Experiments conducted in SMA mouse models have shown encouraging results of a trans-splicing-based approach [133]. However, the improvements in lifespan were less impressive [133]. In general, trans-splicing is a very inefficient process that remains an option only when other therapies fail to meet the high expectations.

5. Stem cell based therapy

Stem cell therapy is based on the assumption that intrathecal transplantation of healthy donor stem cells in the spinal cord of SMA patients would compensate for the eventual death of the endogenous motor neurons. Two major studies evaluated the effect of stem cells in mouse models of SMA [55,56]. In one study, spinal cord neural stem cells injected into cerebrospinal fluid increased the mean survival of Δ7 SMA mice ~39% (Fig. 1) [56]. In a separate study, intrathecal transplantation of embryonic stem cell-derived neural stem cells augmented the number and size of motor neurons, improved muscle innervation and increased mean survival of Δ7 SMA mice by 64% (Fig. 1) [55]. These modest benefits in pre-clinical studies somewhat undermine the promise of stem cell therapy for the treatment of SMA.

6. ASO-based therapy

ASO-based strategies employ specific *SMN2* sequences as targets for SMA therapy [49]. Earlier studies focused on bifunctional ASOs that blocked the C6U mutation site within *SMN2* exon 7 and provided a tailed sequence for the recruitment of the positive splicing factors at the weak 3′ splice site (3′ ss) of exon 7 [134,135]. These studies were driven by a well-founded belief that a weak 3′ ss was the sole cause of *SMN2* exon 7 skipping and forced recruitment of a positive regulator at the 3′ ss was the best mechanistic solution for the restoration of *SMN2* exon 7 inclusion. Subsequent studies shifted attention towards the 5′ ss as it became obvious that a series of inhibitory elements are positioned in the vicinity of the 5′ ss [136–139]. A major breakthrough came with the discovery of intronic splicing silencer N1 (ISS-N1), a fifteen-nucleotide sequence spanning from the 10th to the 24th positions of *SMN2* intron 7 (Fig. 2) [137]. Since an ASO annealing to an



B

ASO/Chemistry	Annealing positions	Outcomes	Ref.
	ISS-N1		
Target → 5'-	ccagcauuaugaaagugaaucuuac-3'		
2'-OMe ASO → 3'-	GGUCGUAAUACUUUCACUUA-5'	Original invention/high efficacy in GM	[137]
MOE ASO → 3'-	GGTCGTAATACTTTCACT-5'	~25-fold increase in lifespan of TW	[77]
MOE ASO → 3'-	GGTCGTAATACTTTCACT-5'	~2-fold increase in lifespan of Δ7	[53]
MO ASO → 3'-	GGTCGTAATACTTTCACTTA-5'	~8-fold increase in lifespan of Δ7	[52]
MO ASO → 3'-	GGTCGTAATACTTTCACTTAGAATG-5'	>20-fold increase in lifespan of TW	[76]

Fig. 2. ASO-mediated *SMN2* splicing correction as a therapy for SMA. (A) Diagrammatic representation of splicing cis-elements in *SMN2* intron 7. Numbering begins from the first position of intron 7. The negative cis-element ISS-N1 (yellow box) contains two hnRNP A1 binding sites (magenta boxes) and has emerged as a leading ASO target for splicing correction in SMA. Green ovals represent binding sites for TIA1, an enhancer of exon 7 splicing. (B) Representative ISS-N1-targeting ASOs tested in SMA mouse models. Intron 7 sequence is in lower caps and ASO sequences are in upper caps. The chemistry for each ASO is listed to the left of the sequence. The outcomes for studies in which the ASOs were tested are listed to the right of the sequence; references are denoted in brackets. Abbreviations: ASO, antisense oligonucleotide; ISS-N1, intronic splicing silencer N1; hnRNP A1, heterogenous ribonucleoprotein A1; TIA1, T-cell restricted intracellular antigen 1; LDI, long-distance interaction; 2′-OMe, an ASO with phosphorothioate backbone and 2′-O-methyl modification; MOE, an ASO with phosphorothioate backbone and 2′-O-methoxyethyl modification; MO, morpholino; GM, SMA patient fibroblast cells (cell line GM03813); TW, Taiwanese SMA mouse model; Δ7, Δ7 SMA mouse model.

intronic sequence does not interfere with the transport and translational machinery, ISS-N1 serves as an ideal target for an ASO-mediated splicing correction in SMA. Indeed, sequestration of ISS-N1 by an ASO fully restored *SMN2* exon 7 inclusion and substantially increased levels of SMN in SMA patient cells [137].

A vast number of independent reports have put ISS-N1 as one of the best-studied antisense targets for splicing correction in a human disease [49]. Therefore, lessons learned from these reports have wide implications for SMA therapy as well as for therapy of many other genetic diseases. Our understanding of the mechanism by which an ISS-N1-targeting ASO promotes *SMN2* exon 7 inclusion continues to evolve. ISS-N1 harbors two hnRNP A1/A2 binding sites that do not include a cytosine residue (^{10}C) at the 10th intronic position (the first nucleotide of ISS-N1) (Fig. 2). It was initially hypothesized that an ASO-mediated sequestration of hnRNP A1 motifs of ISS-N1 is necessary and sufficient to confer full stimulatory effect on *SMN2* exon 7 splicing [139]. However, a subsequent study suggested that the sequestration of ^{10}C is also critical for the robust stimulatory effect on *SMN2* exon 7 splicing [140]. Incidentally, ^{10}C falls within an 8-nucleotide GC-rich sequence that partially overlaps with the first hnRNP A1 motif of ISS-N1 (Fig. 2A) [141]. Sequestration of this GC-rich sequence alone by an ASO fully restored *SMN2* exon 7 inclusion [141,142]. A recent study puts ^{10}C and GC-rich sequence in a structural context that involves a unique long-distance interaction [143]. This study also brings a new perspective by demonstrating an ASO-mediated remodeling of RNA structure in the vicinity of the 5′ ss [143]. Intronic sequences downstream of ISS-N1 contain binding sites for T-cell restricted intracellular antigen 1 (TIA1), which is a positive regulator of *SMN2* exon 7 splicing [144]. A single amino acid substitution within TIA1 has been shown to negatively affect *SMN2* exon 7 splicing in patients of Welander distal myopathy [145]. Therefore, TIA1 emerges as the first and the only splicing factor whose mutation could be directly linked to *SMN2* exon 7 splicing in the context of a human disease. Based on a recent study, an ASO targeting ISS-N1 or GC-rich sequence brings a structural change that promotes TIA1 recruitment and consequently favors inclusion of *SMN2* exon 7 [143].

In order to validate the therapeutic efficacy of ISS-N1-targeting ASOs in vivo, oligonucleotides with three different modifications have been tested (Fig. 2) [49]. These modifications are: 2′-O-methyl with phosphorothioate backbone (2′-OMe), 2′-O-methoxyethyl with phosphorothioate backbone (MOE) and morpholino. A systematic study reported by Krainer and colleagues employed an ISS-N1-targeting 18-mer MOE ASO and demonstrated ~25-fold increase in the life expectancy of the severe Taiwanese model of SMA (Fig. 1) [77]. Surprisingly, subcutaneous injections produced the most beneficial effects, whereas ICV delivery resulted in only a moderate response. These findings supported the peripheral requirement of SMN for the avoidance of SMA. However, results of other studies conducted recently with ISS-N1-targeting morpholino ASOs were somewhat different. In particular, the Burghes group used a 20-mer morpholino ASO and showed ~8-fold increase in the life expectancy of Δ7 SMA mouse upon ICV administration (Fig. 1) [52]. The Muntoni group observed similar lifespan benefits in Taiwanese model mice upon ICV administration of a 25-mer morpholino ASO (PMO25) (Fig. 1) [76]. These findings are consistent with the early requirement of SMN in motor neurons. Interestingly, a single facial injection of PMO25 was found to be highly efficacious as it led to ~22-fold increase in the life expectancy of Taiwanese mouse model (Fig. 1) [76]. While these results support the peripheral requirement of SMN, they are confounded by the fact that BBB in mice remains permeable for a few days after birth. Therefore, it is likely that the beneficial effect at high doses of peripheral administration is due to leakage of ASOs into the brain. Overall, the results of these studies showed an unprecedented therapeutic benefit of ASOs targeting ISS-N1.

The observation that the efficacy of an ISS-N1-targeting ASO is on par with the gene replacement therapy underscores that ASO-mediated splicing correction is among the most promising options of SMA therapy (Fig. 1). Moving forward with clinical trials, concerns related to ASO delivery and toxicity would have to be addressed. ISIS Pharmaceuticals has recently concluded phase 1 and initiated a phase 2 clinical trial of ISIS-SMNRx, an ISS-N1 targeting MOE ASO (Clinicaltrials.gov ID NCT01839656). Encouraging outcomes of ISIS-SMNRx in a phase 1 clinical trial demand an expansion of ASO-based strategies to include additional targets and different oligonucleotide chemistries. Of note, a slight change in ASO sequence could confer entirely different pharmacokinetic and pharmacodynamic properties. Also, a change in the ASO backbone would generate an entirely new class of compound. Given the variable severity and age diversity among SMA patients, a variety of chemotherapy options would be needed for the treatment of SMA. In principle, multiple ASO-based drugs with different pharmacokinetic and pharmacodynamic properties could be developed against a single target, provided issues related to the proprietary rights on antisense target, oligonucleotide chemistry and delivery protocols are appropriately reconciled. Two recent clinical trials of a morpholino ASO have shown promising results for the treatment of Duchenne Muscular Dystrophy [146,147]. Incidentally, ISS-N1 targeting morpholino ASOs have demonstrated substantially better ICV efficacy than MOE ASOs (Fig. 1). Therefore, employment of morpholino chemistry for the development of the next ASO-based drug for SMA treatment remains an attractive proposition.

7. Other therapies

There are very-limited studies that utilize treatments that do not ostensibly affect SMN. Of these studies, the effects of physical exercise on the survival and phenotype of SMA mouse models have been most widely examined. Taiwanese type II SMA mice trained to run on an exercise wheel showed increased full-length *SMN2* transcript in the spinal cord, decreased neuronal loss and an extended lifespan (Fig. 1) [78]. In another study, forced running enhanced the development of several muscles compared to sedentary Δ7 SMA mice [148]. Interestingly, administration of MK-801, a NMDA receptor antagonist, abolished the lifespan extension conferred by exercise [148]. These results suggest the involvement of glutamate neurotransmission as an important mediator of exercise benefits. A recent study in Δ7 SMA mice showed tangible benefits of exercise on cardiac function such as improved conduction velocity, reduced fibrosis, improved bradycardia and decreased arrhythmias [149]. These findings support that exercise could be very useful as an adjunct therapy of SMA [150].

Proper supportive therapy is crucial to increase the survival and quality of life of SMA patients. Infants diagnosed with type I SMA will eventually require some sort of ventilation support to facilitate their breathing. Studies in type I SMA patients have shown respiratory interventions (invasive or noninvasive) increase survival compared to those patients who do not receive respiratory support [151,152]. Of course, patients with the more severe manifestations of the disease will likely require more invasive interventions. Respiratory support, as well as other supportive therapy such as nutritional supplementation or total parenteral nutrition, can certainly improve the quality of life for SMA patients.

8. Conclusions

SMA is a progressive neurodegenerative disease of infants and children. Although SMA has no known cure, the patient population possesses *SMN2*, which can be potentially targeted for therapy. The last ten years have witnessed a dramatic progress in the development of therapeutic strategies for SMA. Much of the success in the field of SMA therapy could be attributed to a better understanding of SMN function, *SMN2* exon 7 splicing regulation, SMA pathogenesis and the developments in the related fields. Small compounds continue to attract attention due to expected ease of their delivery across the BBB. However, all of small compounds tested thus far showed a very small lifespan extension benefit. SMN is an essential protein, which is involved in regulation of a number of vital processes within a cell. The low efficacy of small

compounds could be due to modulation of a limited number of downstream events regulated by SMN. It is also likely that some of these compounds have antagonistic effects on various critical pathways regulated by SMN. Therefore, there is a need for additional drug screening assays, which identify new classes of small compounds that affect upstream events such as transcription and splicing of *SMN2*. Exceptional preclinical results of ISS-N1-targeting ASOs and promising outcome of phase 1 clinical trial of ISIS-SMNRx provide one of the best hopes for SMA therapy. Recent discovery of ISS-N2, a novel antisense target [143], further expands the potential for the development of additional antisense-based drugs. Currently, invasive intrathecal administration remains the most effective mode of delivery of ASOs to SMA patients. Therefore, there is a need to work on modifications and non-invasive delivery protocols that allow an easy transport of ASOs across the BBB. A success in this direction would dramatically elevate the status of ASO-based therapy of SMA and possibly several other diseases requiring transport of a small nucleic acid molecule across BBB.

Despite admirable attempts by a number of investigators [153–155], one of the major challenges for the therapeutic development in SMA has been the availability of the robust biomarkers. The first therapy with substantial lifespan enhancements in SMA would likely offer additional biomarkers. Knowledge of such biomarkers would be extremely helpful for future drug screenings. Therapeutic progress in SMA thus far supports that compounds specifically aimed at correction of *SMN2* exon 7 splicing offer the most promising outcome. The challenge remains how to identify/design a small compound that fully corrects *SMN2* exon 7 splicing as well as is able to cross BBB. The answer could lie within intronic sequences that fold into secondary and high-order RNA structures. Indeed, small compounds can affect splicing through specific interactions with a unique RNA structure [156]. The complex process of pre-mRNA splicing of a specific exon comes at the expense of hundreds of structural rearrangements. As we move forward with uncovering of structural rearrangements during pre-mRNA splicing of *SMN2* exon 7, we hope to see new avenues open up for discovery of unique compounds for SMA therapy. Thanks to the partnership between academia and industry, SMA has an impressive track record of clinical trials of a variety of compounds covering different pathways. Many of these compounds are being tested for the first time to cure a genetic disease. Therefore, a potential success will likely translate into therapeutic development for a number of genetic diseases requiring splicing modulation.

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Disclosures and competing interests

ISS-N1 target (US patent # 7,838,657) was discovered in the Singh lab at UMASS Medical School (Worcester, MA, USA). Inventors, including RNS and UMASS Medical School, are currently benefiting from licensing of ISS-N1 target (US patent # 7,838,657) to ISIS Pharmaceuticals. A GC-rich target for a small ASO (Patent# US 20110269820) was discovered in the Singh lab at Iowa State University (Ames, IA, USA). Therefore, inventors including RNS and Iowa State University could potentially benefit from any future commercial exploitation of the abovementioned target.

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