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Drugging Pre-mRNA Splicing

Matthew G. Woll, Nikolai A. Naryshkin, and Gary M. Karp

Abstract The splicing of precursor messenger RNA (pre-mRNA) requires the precise cleavage and formation of multiple phosphodiester bonds in nascent pre-mRNA polymers in order to produce a protein coding message that can be properly translated by the ribosome. Despite the precision of this process, the spliceosome maintains considerable flexibility to include, or not include, defined segments in the final message, thus allowing for the production of diverse transcripts with distinct functions from a single gene sequence. The combination of control and flexibility displayed by the spliceosome, in conjunction with input from *cis*-acting sequences and *trans* factors, presents a unique opportunity for molecular intervention during gene expression. Various chemical agents have the capacity to alter the natural process of pre-mRNA splicing, thereby producing levels of splicing products different than those found under natural conditions. This approach has powerful therapeutic utility where mutation has caused certain splice variants to be under- or over-represented. The following chapter highlights the exceptional advances that have been achieved recently in splicing modulation with splice switching oligonucleotides (SSOs) and small molecules, the two leading therapeutic modalities in this field.

Keywords Alternative splicing, Duchenne muscular dystrophy, Exon skipping, Familial dysautonomia, Pre-mRNA splicing, Spinal muscular atrophy, Splice switching oligonucleotides, Spliceosome

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1 Introduction

Eukaryotic genes are discontinuous, with protein coding sequences (expressed regions or exons) being disrupted by non-coding sequences (intragenic regions or introns) [1]. Before the protein coding message can be properly translated by the ribosome, the introns must be removed from the precursor messenger RNA (pre-mRNA) and the remaining exons must be ligated together into a contiguous coding sequence in a process known as pre-mRNA splicing. When reduced to its simplest form, the splicing process is merely two sequential transphosphoesterification reactions (Fig. 1). And although the transformation seems unimposing from a purely chemical standpoint, the idea of facilitating a ring closing event (step 1) between two atoms that may be thousands of bonds apart in sequence space, amongst numerous functional groups with similar reactivity seems unfathomable. It is no wonder that nature employs a very complex cellular machine, known as the spliceosome, to attain high selectivity in this process. The spliceosome achieves great precision by recognizing conserved pre-mRNA sequence elements. Several small nuclear ribonucleoproteins (snRNPs) play an important role in defining the locations for exon junctions, organizing the pre-mRNA and forming the catalytically active spliceosome (Fig. 2). Additionally, dozens of proteins participate in the various stages and contribute to the high selectivity of this remarkable catalytic process [2].

Despite the high precision of the spliceosome, very often there are clearly marked exon junctions that are passed over and ignored. This apparent sloppiness is actually an essential element of a highly regulated system. The presence of nuclear *trans*-acting factors and pre-mRNA sequence elements (*cis*-acting elements) can either encourage or discourage the splicing machinery to choose a particular exon junction. These sequence elements are termed exonic and intronic splicing enhancers (ESEs and ISEs) and silencers (ESSs and ISSs). Nearly all

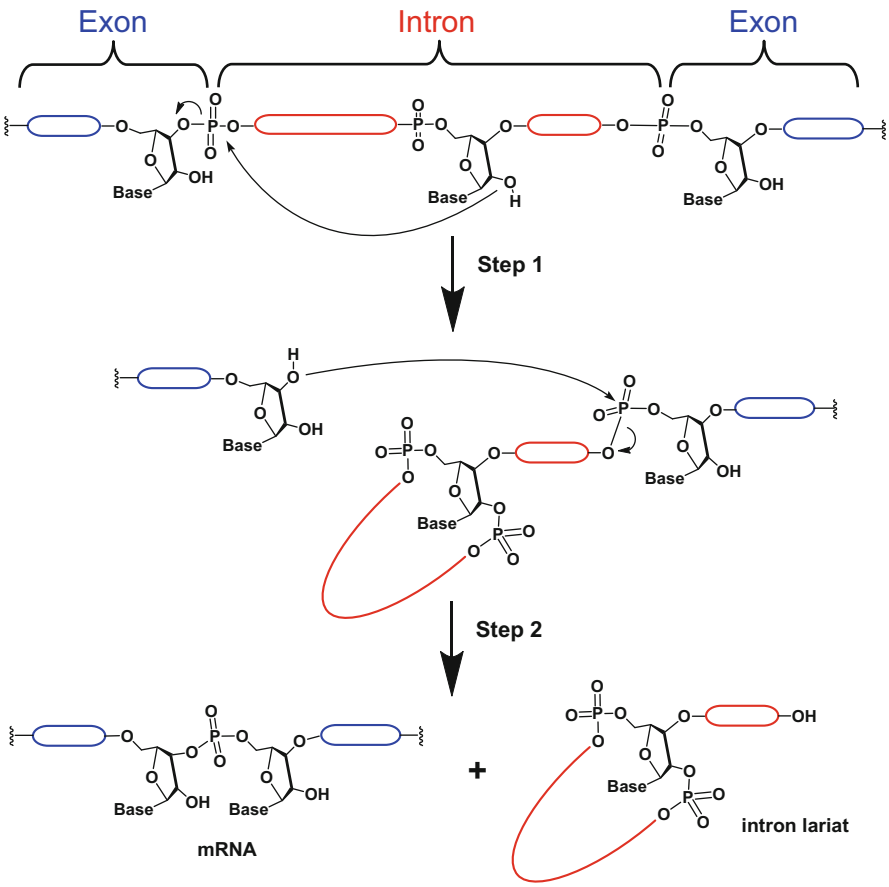

Fig. 1 Two sequential transphosphoesterification reactions are required for exon splicing

human genes that contain multiple exons can produce multiple distinct mRNA transcripts by alternative splicing [3]. Overall, the conceptual framework for mRNA production is relatively simple – the order of the exons is defined in the gene sequence. *Cis* and *trans* regulatory inputs determine the identity of exons (e.g., constitutive, cassette, or pseudo/cryptic) included in or excluded from the mature RNA transcript. Transcription start site selection and polyadenylation site selection can also be influenced by splicing control.

The flexibility of alternative splicing and the diversity of its outcomes are remarkable. It is thought to increase the diversity of the human proteome [4, 5] although typically a single major mRNA isoform is generated and alternative protein isoforms, expected to be expressed by minor alternative mRNA variants, are often undetectable. This coupled with the fact that the majority of alternative splicing lacks inter-species conservation have led to an on-going debate about the extent of splicing contribution to proteome complexity [6–8] and its role in gene evolution [9]. Nevertheless, many fundamental biological processes, such as

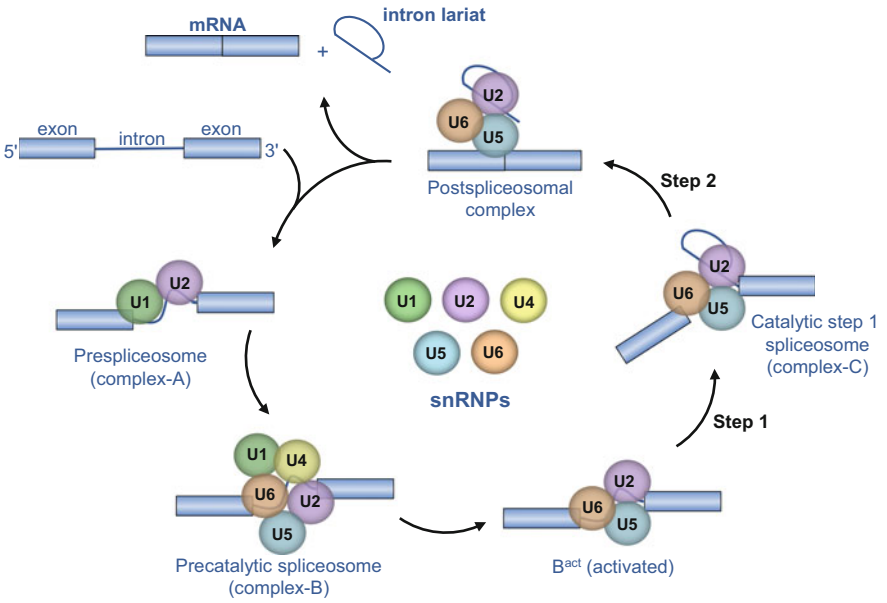

Fig. 2 The catalytic cycle for pre-mRNA splicing by the spliceosome. U1, U2, U4, U5, and U6 are snRNPs involved in the catalytic cycle and formation of the spliceosome. Many additional protein factors that are required for each of the complexes involved in the cycle are not shown for simplicity

organogenesis and development, apoptosis, signal transduction, cellular metabolism, and gene expression rely on alternative splicing to achieve a fast and sensitive switch in biological function (e.g., from a pro-apoptotic to an anti-apoptotic protein isoform) in response to developmental and environmental cues. For example, signal transduction via a single pass cell surface receptor can be attenuated by skipping the exon encoding the transmembrane domain (e.g., [10]); metabolic pathways can be modified by enzyme-inactivating exon skipping [11]; gene expression homeostasis can be maintained using a negative feedback loop that relies on an inclusion of a nonsense mediated decay-inducing "poison" exon [12].

The diversity of alternative outcomes also requires a precise control of pre-mRNA splicing to avoid potential deleterious effects. The dysregulation of splicing factors or gene mutations can lead to over- or under-representation of certain splice variants, which ultimately affects the level of proteins encoded by the corresponding sequences. Tissue-specific degeneration leading to disease can occur from the lack or overabundance of certain proteins required for normal tissue maintenance. The flexibility of pre-mRNA splicing also makes it a promising target for therapeutic intervention that may include switching between naturally occurring mRNA isoforms and the generation of novel isoforms with desired properties.

There are several potential scenarios where therapeutic modulation of splicing could be beneficial in disease therapy (also reviewed in [13, 14]). Abnormal

exclusion or inclusion of a cassette exon could lead to overexpression of an undesired mRNA isoform. This increased skipping of an essential exon can be the result of an intronic or translationally synonymous exonic mutation. An external stimulus that shifts splicing toward the inclusion of the cassette exon could be therapeutically beneficial (Fig. 3a). In another case, the overexpression of an isoform produced from a mutually exclusive splicing event may have detrimental effects on the cell. Treatment with a compound that shifts the splicing, thereby decreasing the expression of the overexpressed isoform would be desirable (Fig. 3b). In cases where an open reading frame is disrupted by an internal deletion or (in some cases) insertion, a stimulus that induces skipping of the exon flanking the mutation locus may restore the protein-encoding open reading frame and preserve protein function (Fig. 3c). An exon skipping approach could also be used in cases where a point mutation that disrupts protein function is present in an internal exon, provided that the resulting internally truncated mRNA maintains the protein-encoding open reading frame and that the expressed truncated protein maintains proper function (Fig. 3d). Finally, and in contrast to the previous examples, a splice altering stimulation may be used to create a faulty mRNA to reduce the expression level of a toxic or undesired mRNA or protein by generating a frameshifted message that undergoes subsequent degradation via nonsense-mediated decay or a related mRNA surveillance process and/or degradation of protein due to the presence of an unnatural C-terminus (Fig. 3e).

Several important questions arise when thinking about targeting pre-mRNA splicing as a means to treat human disease. What are the possible biomolecular targets for splicing modulation? How can selectivity be achieved? What are the practical limits for shifting an alternative splicing event? Therapeutics that target pre-mRNA splicing can be broadly classified into two groups: those that encourage the formation of a splice junction and those that discourage the formation of a splice junction. The practical output of these methods is to favor the expression of the splice variant that would be beneficial to address a particular disease.

Conceptually there are several conceivable modes of action for a molecule to modify pre-mRNA splicing. A drug may bind to a spliceosomal component or *trans* regulatory element to inhibit its interaction with pre-mRNA (*trans* blocker, Fig. 4a). The same biological outcome may result from a drug that directly engages the pre-mRNA, thereby blocking the binding of a spliceosomal component or *trans* regulatory element (*cis* blocker, Fig. 4b). Type B would also cover drug–RNA interactions that disrupt (or stabilize) a pre-mRNA structure that serves as a *cis* regulatory element. A type B scenario has the potential for greater specificity than type A if the drug has the ability to distinguish different pre-mRNAs by sequence. In another scenario, a drug may indirectly influence a spliceosomal or *trans* regulatory element by targeting upstream transcription factors, kinases, phosphatases, etc. (upstream regulator, Fig. 4c). In this case, no direct contacts are made with pre-mRNA, thus limiting the potential for high specificity. Lastly, there is the potential for a molecule to stabilize or activate a complex composed of a pre-mRNA transcript bound to a spliceosomal component or *trans* regulatory element (complex stabilizer, Fig. 4d). In this last scenario, since pre-mRNA is directly engaged there is potential for pre-mRNA sequence specificity. While molecules

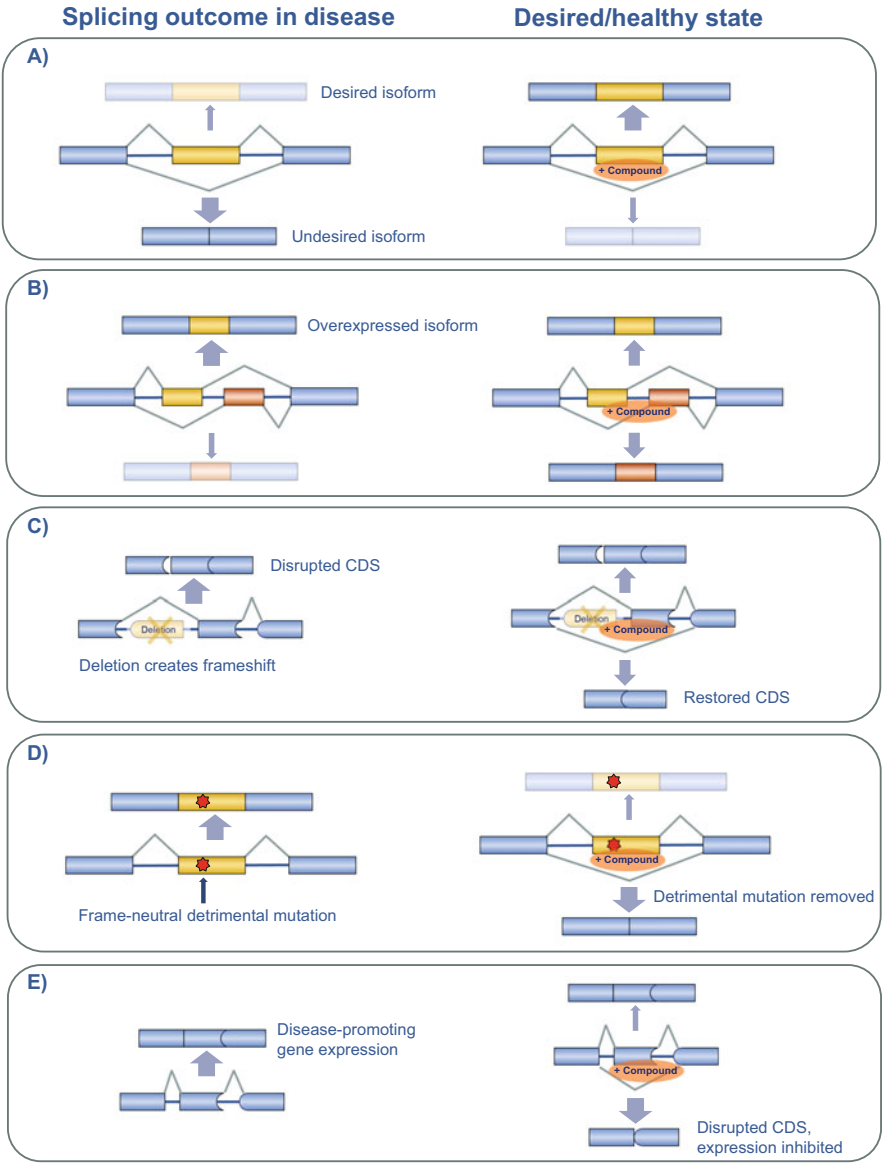

Fig. 3 Potentially druggable splicing events. (**a**) Inclusion (or exclusion) of a cassette exon. (**b**) Switch between mutually exclusive exons. (**c**) Exon skipping to restore reading frame. (**d**) Exon skipping to remove mutation. (**e**) Exon skipping to inhibit expression

that act by each of the described modes may elicit profound biological effects, the advancement of a molecule into the clinic will require a suitable therapeutic index, which is often directly linked to specificity. As is the case with any therapy that

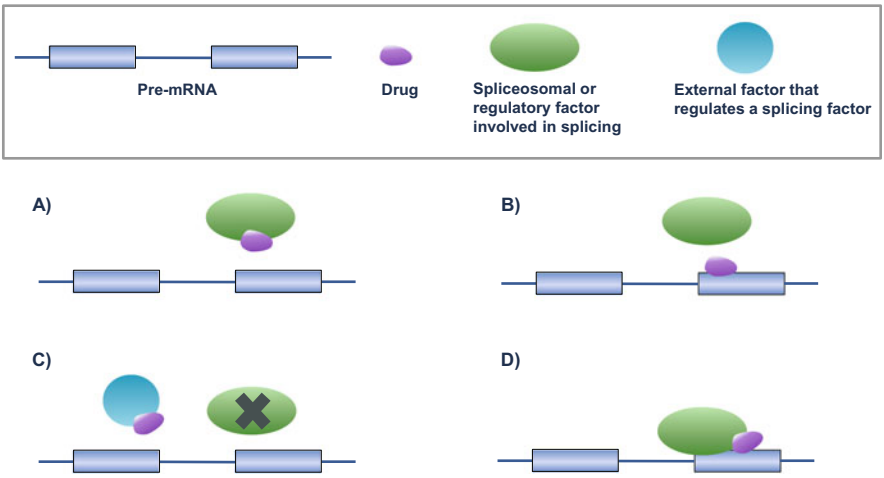

Fig. 4 Conceptual modes of action for drugs targeting pre-mRNA splicing. (**a**) *Trans* blocker. (**b**) *Cis* blocker. (**c**) Upstream regulator. (**d**) Stabilizer

modifies gene expression levels, the greater the number of off-target alterations, the more likely the chance of promoting undesired side effects.

In the last few years, novel therapeutic approaches utilizing different treatment modalities, including splice switching oligonucleotides (SSOs) and small molecule therapeutics, have targeted diseases caused by known splicing defects. New chemical entities (including SSOs and small molecules) targeting several distinct disease areas have been tested in human clinical studies. In 2016, two splice-switching oligonucleotides received regulatory approval. EXONDYS 51™ (eteplirsen), an SSO that induces the skipping of exon 51 of the *DMD* gene, was approved (under the accelerated approval mechanism) by the FDA for treatment of Duchenne muscular dystrophy in patients who have a confirmed mutation of the *DMD* gene that is amenable to exon 51 skipping (See [15]). Spinraza™ (nusinersen), an SSO that promotes the inclusion of exon 7 of the *SMN2* gene, was approved for the treatment of spinal muscular atrophy (SMA) in pediatric and adult patients (See [16]). Recent examples of methods used to promote exon inclusion induce exon skipping or inhibit some general aspect of splicing are described in the following sections.

2 Exon Inclusion

In some human diseases the underlying cause of disease can be directly linked to the reduced expression of a critical protein due to an impaired splicing event. Indeed, a single translationally synonymous exonic point mutation or intronic point mutation could lead to excessive skipping of an exon that is essential for the translation of fully functional protein product. Since some, albeit insufficient, desired isoform is

produced, a reasonable and rational approach to treat the disease would require the discovery of molecules that could take advantage of the mechanisms that impart spliceosomal flexibility to induce exon inclusion and hopefully ameliorate disease phenotype. Some exciting advances using this type of methodology are found in this section.

2.1 SSO Approach to Treat Spinal Muscular Atrophy

Patients with spinal muscular atrophy (SMA) have a deficit of the survival motor neuron (SMN) protein, which causes degeneration of α-motor neurons [17, 18]. This deficiency is almost always the result of a homozygous deletion of or mutation in the *SMN1* gene. Humans usually have two or more copies of the paralogous *SMN2* gene, but this gene produces much less functional SMN protein due to alternative splicing at exon 7. This alternative pathway is activated by a translationally synonymous C to T mutation at position 6 in exon 7. The seemingly innocuous mutation abrogates an exonic splicing enhancer sequence and creates an inhibitory motif. As a result, the majority of transcripts produced from *SMN2* lack exon 7 (*SMN2*-Δ7), and encode a truncated *SMN*-Δ7 protein that is rapidly degraded [19, 20]. Because *SMN2* contains all of the necessary coding information for wild type SMN protein, but just lacks the correct splicing impetus, any treatment that has the potential to shift the splicing of *SMN2* pre-mRNA to favor the desired full length transcript (*SMN2*-FL) should hold considerable promise for treating SMA.

One approach to modify a cellular splicing event is to treat cells with a splice switching oligonucleotide (SSO) that is complementary to a regulatory sequence within the pre-mRNA. The selective binding event that occurs between a specific pre-mRNA sequence and the SSO could have various consequences, depending on the location of the sequence within the pre-mRNA. To enhance exon inclusion, one might target an ESS or ISS proximal to the exon of interest. Another approach would be to slow a competing splicing event by binding to the competing exon junction. Recruiting splicing enhancers to a splicing region using a bifunctional SSO that contains a pre-mRNA binding motif and a splicing enhancer motif would be another means of influencing splicing events.

All of the above-mentioned techniques have been successfully employed to shift *SMN2* splicing in favor of the *SMN2*-FL transcript (Fig. 5). SSOs directed to repressor regions within intron 6 (element 1) [21], exon 7 (two ESSs) [22], and intron 7 (ISS-N1) [23, 24] have demonstrated *SMN2* splicing correction in patient-derived cells. Additionally, SSOs that bind to the intron 7–exon 8 junction have been shown to discourage splicing at the 3′ splice site of exon 8 and improve the ratio of *SMN2*-FL to *SMN2*-Δ7 [25]. In some cases a bifunctional oligomer, incorporating both a pre-mRNA binding motif and a splicing enhancer/repressor motif, has shown improved activity over the SSO alone [26, 27]. An even more elaborate example of a bifunctional activator of exon 7 inclusion is a U7 snRNA derivative delivered using a viral vector [28]. To date, SSOs targeting ISS-N1 have

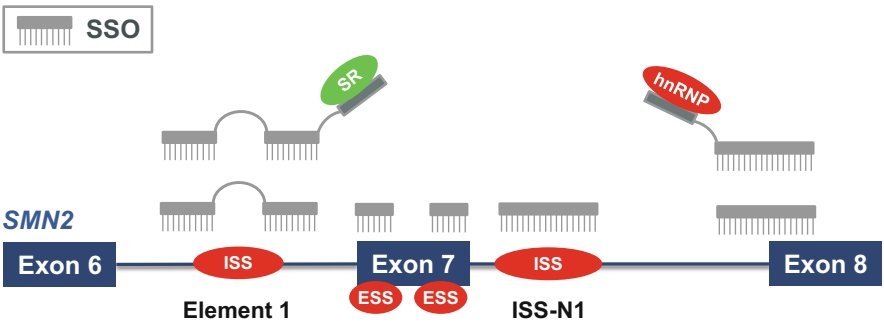

Fig. 5 Strategies for using SSOs to enhance exon 7 inclusion in *SMN2*

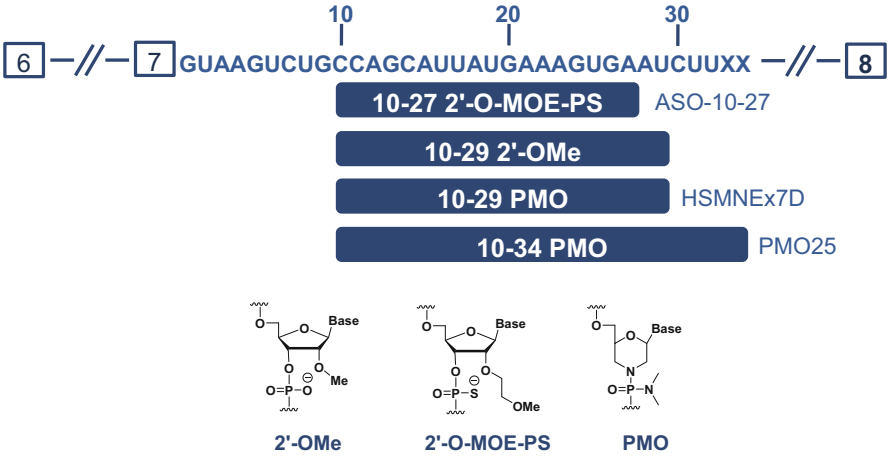

Fig. 6 SSOs targeting the ISS-N1 region of intron 7 of *SMN2*

had the most profound effect on splicing in vitro, and have shown the greatest therapeutic potential in animal models and SMA patients.

SSOs with varying length and backbone chemistry have demonstrated pharmacodynamic responses in SMA mouse models (Fig. 6) [23, 29–32]. The long-term benefit to motor function and survival depend on the delivery method and distribution of the SSO. An SSO complementary to positions 10–27 (targeting the ISS-N1 region) of intron 7 with 2′-*O*-methoxyethyl ribose phosphorothioate (2′-*O*-MOE-PS) modified backbone (ASO-10-27) was administered to *hSMN2* mice (mild SMA phenotype) intravenously twice a week at a dose of 25 mg/kg [23]. Tissues were harvested after 1, 2, 3, and 4 weeks. An increase in exon 7 inclusion was observed in the liver, kidney, and thigh muscle, but not in the spinal cord, suggesting that the SSO did not cross the blood–brain barrier (BBB).

Methodology for injecting SSOs intracerebroventricularly (ICV) to neonatal Δ7 SMA mice (severe SMA phenotype) was developed using a 2′-*O*-methyl ribose (2′-OMe) phosphodiester SSO spanning positions 10–29 of *SMN2* intron 7 (Fig. 6) [24, 29]. After injecting 1 μg SSO at post-natal days 1 (P1), 3, 5, 7, and 10, CNS tissue was harvested on P12. Western blot analysis indicated that SMN protein was increased greater than twofold in brain and spinal cord, with SMN levels in cervical spinal cord reaching as high as 3.6-fold. Analysis of the brain tissue using semi-quantitative reverse-transcription real-time polymerase chain reaction (RT-qPCR) indicated that *SMN2*-FL had significantly increased by ~1.4-fold in the brain. Modest improvement in body weight and righting reflex were observed, however the study was terminated at P12, thus no long-term benefits including survival were reported.

A subsequent study in a mouse model of severe SMA (median survival time 10 days) [33] demonstrated that ICV injection of 20 μg of ASO-10-27 on P1 produced significant increase of *SMN2*-FL and SMN protein in the brain (Fig. 7b, c), but a very modest increase in median survival time (MST) (16 vs. 10 d for untreated) (Fig. 7a) [30]. Strikingly, when the mice were administered 50 μg/g of ASO-10-27 by subcutaneous (SC) injections at P0 and P3 they had a median survival time of 108 days. Combining the ICV and SC injections further increased the median survival time to 173 days (Fig. 7d). Additional studies showed that increasing the SC dose at P0 and P3 to 160 μg/g increased the median survival time to 248 days, with some mice living longer than 500 days (Fig. 7e).

The SC injections increased the SMN levels not only in peripheral tissues (kidney, liver, muscle, and heart), but in the CNS (spinal cord and brain) as well. When the *SMN2* FL/Δ7 mRNA ratio was measured over time in the liver, a significant decrease in exon 7 inclusion was seen at P30 as compared to P10 after the 160 μg SC dose. Interestingly, exon 7 inclusion at P180 was still significantly higher than that observed for vehicle treated mice at P10. Since all of the vehicle treated mice die very early (<20 days), there is no direct vehicle comparison for P180. It is not entirely clear how much CNS penetration of the SSO is required for survival benefit. The SSO clearly achieves CNS penetration in mice after SC dosing at P0 and P3 when the blood–brain barrier is not completely developed. SC dosing at P5 and P7, when the BBB is more mature, produces a very modest survival benefit (Fig. 7d). The diminished benefit could also be rationalized by the need to treat these severe mice very early in their developmental cycle. Nonetheless, it seems clear that in this model system an increase of SMN in the CNS and periphery is preferable to SMN increase in the CNS alone.

Consistent with the dramatic increase in survival, histological examination of the mice treated SC with 160 μg/g of ASO-10-27 revealed α-motor neuron counts and neuromuscular junction integrity comparable to heterozygous littermates (Fig. 8a, b). Additionally, muscle fiber mean area cross sections and heart weight were nearly normalized for treated mice (Fig. 8c, d). Muscle strength, balance, and coordination were evaluated using the rotarod test in 3-month-old mice treated with 40, 80, or 160 μg/g ASO-10-27. The mice in the 80 and 160 μg/g groups performed better than the 40 μg/g group, but not as well as heterozygotes (Fig. 8e, f).

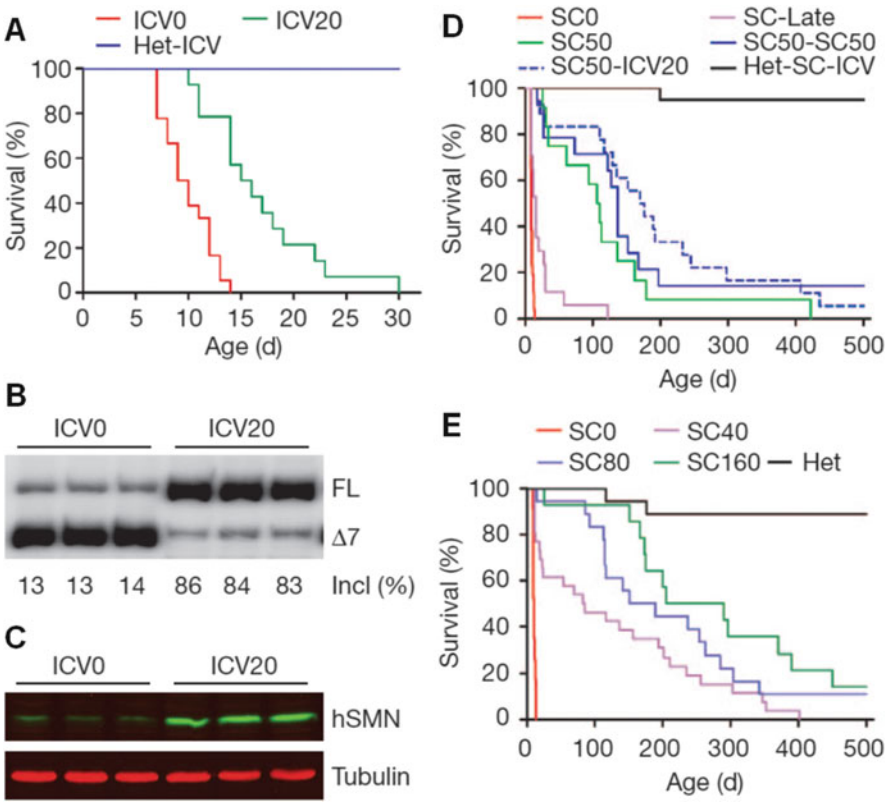

Fig. 7 (**a**) Survival curves for mice after ICV administration of 20 μg ASO-10-27 or vehicle on P1. (**b**) RT-PCR detecting *SMN*-FL and *SMN*-Δ7 transcripts in spinal cord tissue analyzed on P7 after ICV administration of 20 μg ASO-10-27 at P1. (**c**) Western blot detecting human SMN (hSMN) in spinal cord tissue analyzed on P7 after ICV administration of 20 μg ASO-10-27 at P1. (**d**) Survival curves after SC administration of saline (SC0) or 50 μg/g ASO-10-27 (SC50) twice between P0 and P3. SC50-SC50 mice received two additional SC injections on P5 and P7. Het-SC-ICV and SC50-ICV20 were heterozygous and SMA mice, respectively, that received combined P1 ICV and P0–P3 SC injections. SC-Late were SMA mice that received only two SC injections, on P5 and P7. (**e**) Dose-escalating SC injections at P0 and P3 with 40 (SC40), 80 (SC80), or 160 (SC160) μg/g of ASO-10-27. Saline-treated SMA (SC0) or heterozygous mice (Het) served as controls. Adapted from [30]. Copyright 2011 Macmillan Publishers Ltd

Superior in vivo efficacy can be achieved by switching from 2′-*O*-MOE-PS oligomers to phosphorodiamidate morpholino oligonucleotides (PMOs). A PMO targeting positions 10–29 of intron 7 (Fig. 6, HSMNEx7D), masking ISS-N1, showed long-term survival benefit in Δ7 mice after a single ICV injection (MST >100 days) [31, 32]. This is in contrast to the minimal survival benefit achieved with an ICV injection of 2′-*O*-MOE-PS oligomers of similar length [34]. When the

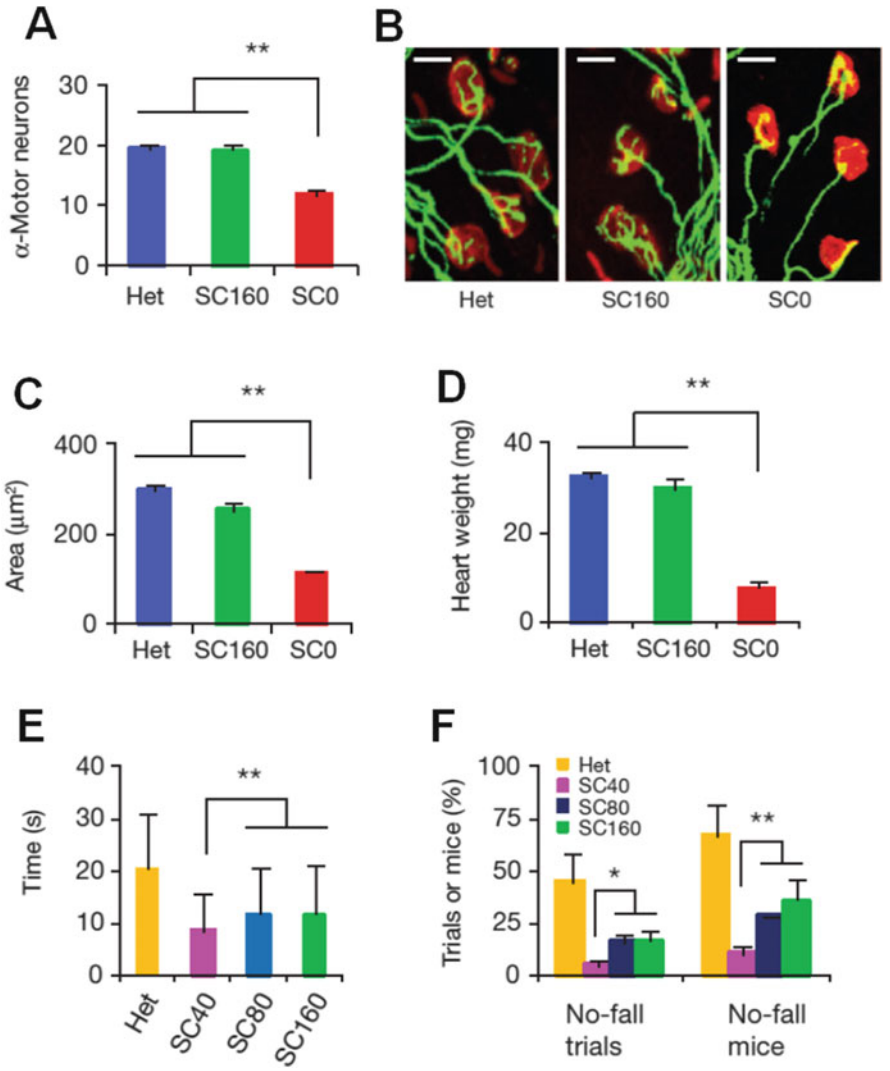

Fig. 8 SMA mice were treated with ASO-10-27 (SC160, two SC injections at 160 μg/g at P0 and P3), saline controls (SC0), and untreated heterozygotes (Het). (**a**) α-Motor neuron counts in each cross-section of the L1–L2 spinal cord. (**b**) Arborization complexity of neuromuscular junctions (*red* endplates; *green* neurofilament medium). (**c**) Mean fiber cross-sectional area of the rectus femoris muscle; (**d**) heart weight. (**e**, **f**) At P90, SC40, SC80, SC160 and untreated heterozygous (Het) mice were tested three to five times per day for 3 days on a rotarod, using an acceleration profile. The mean times for staying on the spinning rod (**e**) and the percentage of no-fall trials and of mice with ≥1 no-fall trial (**f**) are shown. Adapted from [30]. Copyright 2011 Macmillan Publishers Ltd

PMO was extended by five units (Fig. 6, PMO25), complementary to positions 10–34 of intron 7, additional activity improvement was observed both in vitro and in vivo [35]. No data were reported for SC dosing of PMOs targeting ISS-N1 as a comparison to results reported for ASO-10-27.

The very promising results achieved in pre-clinical animal studies with SSOs targeting *SMN2* splicing has led to multiple clinical trials in SMA patients with ASO-10-27 (nusinersen, Spinraza™, IONIS-SMN_{Rx}, ISIS 396443) being the most advanced. An open label phase 2 clinical trial in infants with Type I SMA receiving nusinersen via intrathecal injections showed increases in median event-free survival and muscle function scores (Available from: http://clinicaltrials.gov/show/NCT01494701). A 13-month phase 3 randomized, double-blind, sham-controlled study in 121 patients with infantile-onset SMA evaluated the efficacy and safety of a 12 mg dose of nusinersen (delivered via an intrathecal injection). A planned interim analysis of the trial revealed that a greater percentage of infants treated with nusinersen achieved a motor milestone response compared to those who did not receive treatment (40 versus 0%; $p < 0.0001$) as measured by the Hammersmith Infant Neurological Examination (HINE). At the end of the study, Spinraza also demonstrated a statistically significant 47% reduction in the risk of death or permanent ventilation ($p < 0.01$). A separate 15-month phase 3 study in 126 non-ambulatory patients with later-onset SMA also met the primary endpoint at the interim analysis, achieving a mean improvement of 4.0 points in the Hammersmith Functional Motor Scale Expanded (HFMSE). The results from these two phase 3 studies along with data from multiple other clinical trials were sufficient for the FDA to grant approval of Spinraza™ for the treatment of SMA in pediatric and adult patients (Available from: http://clinicaltrials.gov/show/NCT02193074; http://www.accessdata.fda.gov/drugsatfda_docs/nda/2016/209531Orig1s000MedR.pdf).

2.2 Small Molecule Approach to Treat SMA

Orally bioavailable small molecules are easier to administer and generally have much broader tissue distribution than SSOs. Despite these advantages, the advancement of small molecule drugs that target pre-mRNA splicing has been hampered by the difficulty of targeting specific sequences. Synthetic molecules have been elegantly designed to recognize specific DNA sequences through minor grove interactions [36], however, the same level of specificity has been more challenging to achieve when targeting RNA. There is no doubt that small molecules have the capacity to bind specific RNA structures with high affinity based on numerous examples of high affinity ligands designed to bind the bacterial ribosome and riboswitches [37]. Other synthetic molecules have shown promise for targeting specific mRNA sequences, such as trinucleotide repeats [38, 39]. However, as is typically the case with small molecule RNA interactions, high affinity comes at the cost of increased molecular weight, making the molecules less than ideal for oral delivery and broad distribution. The challenge therefore remains: Can a small

molecule with optimal pharmaceutical properties be designed to target specific mRNA sequences? One area that may be underexplored, perhaps due to the complexity of the target, is the design of small molecules that interact with RNA–protein complexes, where RNA sequence defined structural elements may be targeted (*vide infra*).

To identify small molecules that induce the inclusion of exon 7 in *SMN2* pre-mRNA, a HEK293 cell line was modified to contain an *SMN2* minigene (from exon 6 to the 5′ region of exon 8), followed by the firefly luciferase coding sequence (Fig. 9) [40]. The transcript would express the luciferase enzyme and increased chemoluminescence only when exon 7 was included. The exclusion of exon 7 results in a frameshift such that the upstream initiation codon and luciferase coding sequence reside in different reading frames. This minigene construct was used to conduct a high throughput screen of ~200,000 discrete compounds which led to the discovery of a weakly active coumarin compound, **2-1**, that increased the luminescence signal by >50% at 10 μM. The activity was confirmed using RT-qPCR, quantifying the change in *SMN2*-FL mRNA with increasing compound concentration. The concentration required to increase *SMN2*-FL mRNA by 50% ($EC_{1.5X\ RNA}$) was 9 μM. However, no measurable increase in SMN protein was observed upon compound treatment of SMA patient-derived fibroblasts.

Through an activity-based optimization process guided by structure–activity relationships (SAR), potency was steadily improved by more than 1,000-fold in the reporter assay. Although the initial lead (**2-1**) had no activity in patient-derived fibroblasts, the optimized compounds (e.g., **2-2**) demonstrated low nanomolar $EC_{1.5X}$ values for *SMN2*-FL mRNA ($EC_{1.5X\ RNA}$) in the minigene construct in HEK293 cells and SMN protein ($EC_{1.5X\ PRO}$) in patient fibroblasts. The dramatic increase in potency was linked to a few key structural modifications. An important pharmacophore was discovered by modifying the right-side heterocycle of **2-3**

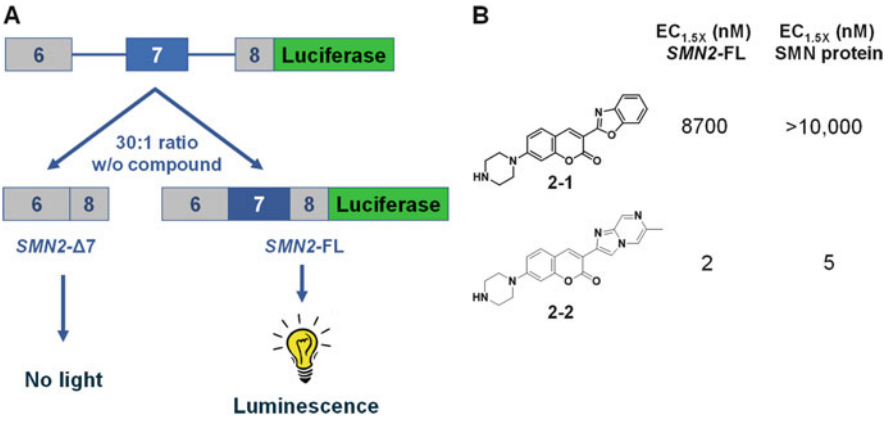

Fig. 9 (**a**) SMN2 minigene reporter construct in HEK293 cells was used to screen small molecules for exon 7 inclusion. (**b**) $EC_{1.5X}$ values for *SMN2*-FL increase in HEK293 cells and SMN protein in patient fibroblasts for a small molecule lead **2-1** and activity optimized compound **2-2**

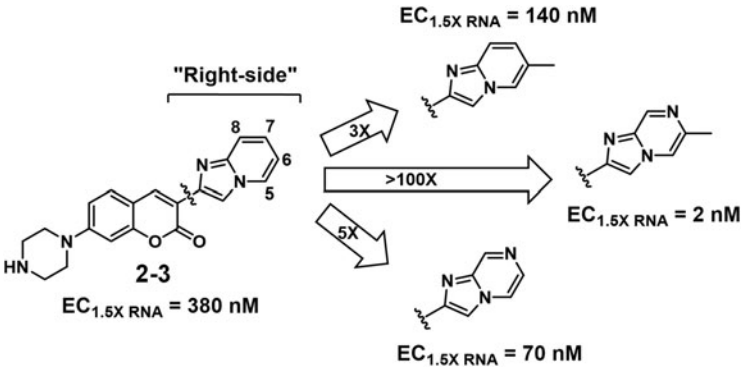

Fig. 10 Modification of the "right-side" heterocycle leads to >100-fold improvement in potency

(Fig. 10). The potency of **2-3** ($EC_{1.5X\ RNA}$ = 380 nM) was increased threefold by introducing a methyl group at the 6-position of the imidazopyridine. A fivefold potency improvement was achieved by replacing the ring carbon at the 7-position with nitrogen (7-aza). Interestingly, when both modifications were introduced (i.e., 6-methyl and 7-aza) a synergistic effect leading to >100-fold improvement in potency was observed. This "nitrogen-methyl pair" motif was incorporated into additional analogs during the optimization process.

Improved pharmaceutical properties could be achieved by replacing the 3,7-substitued coumarin core with a 4,8-substituted pyridopyrimidinone core (e.g., molecules **SMN-C3** and **RG7800**, Fig. 11) [41, 42]. The optimized molecules demonstrated a dose-dependent increase of SMN protein in the brain and spinal cord of Δ7 mice when administered by intraperitoneal (IP) injection. Sustained daily dosing of the compounds (IP from P3 to P23, then orally from P24 to P60) led to increased body weight, improved motor function, and longer survival (Fig. 12 for **SMN-C3**). In mice with a mild SMA phenotype (C/C-allele), both a shift in *SMN2* splicing and an increase in SMN protein were observed in the brain and whole blood. **RG7800**, a structural analog, was selected as the first small molecule *SMN2* splicing modifier to enter human clinical trials. A pharmacodynamic proof-of-concept was demonstrated by changes in the splicing ratio of *SMN2* mRNA in whole blood of healthy volunteers dosed with **RG7800**. In a subsequent study in SMA patients (Available from: http://clinicaltrials.gov/show/NCT02240355), RG7800, dosed once per day for 12 weeks, demonstrated an exposure-dependent increase in *SMN2*-FL mRNA with a corresponding decrease in *SMN*-Δ7 mRNA, as expected for a splicing modifier. SMN protein levels in whole blood increased up to 100% [43].

The specificity for **SMN-C3** was addressed in an RNA sequencing study, since one might surmise that a molecule exhibiting widespread changes in pre-mRNA splicing would be limited in its application due to potential toxicity related to the downstream effects. Comparing the differences in total transcript expression for 11,714 human genes in type 1 SMA patient-derived fibroblasts, treated with DMSO or **SMN-C3** (500 nM), demonstrated relatively few changes greater than twofold upon compound treatment (*SMN2* showed no significant change in total mRNA

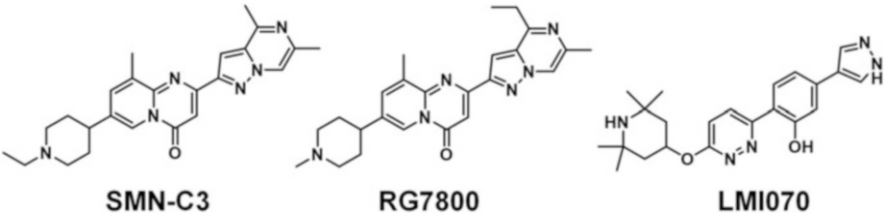

Fig. 11 Small molecule *SMN2* splicing modifiers

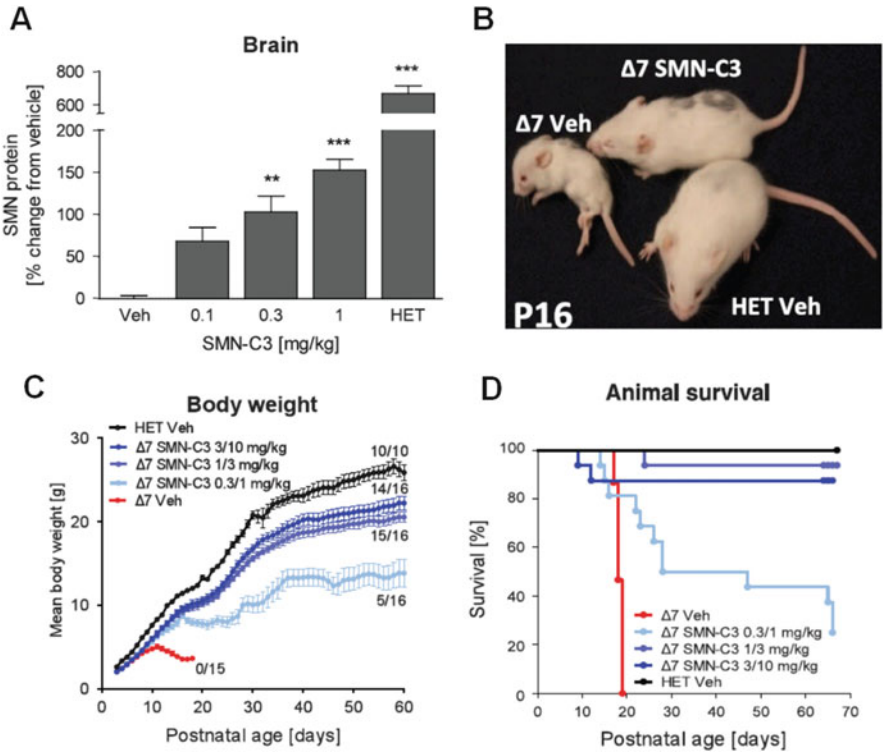

Fig. 12 (**a**) SMN protein levels in the brain of Δ7 mice after seven daily intraperitoneal doses (P3 through P9) of vehicle or SMN-C3 (0.1, 0.3, or 1 mg/kg). (**b**) Appearance of a vehicle-treated Δ7 mouse (Δ7 Veh), a SMN-C3–treated Δ7 mouse (Δ7 SMN-C3), and a vehicle-treated heterozygous mouse (HET Veh). (**c**) Body weight from P3 through P60. Numbers at right indicate survivors at P60 among 10 (HET) or 16 (Δ7) mice per group. (**d**) Kaplan–Meier survival curves from P3 to P65. Adapted from [41]

abundance), suggesting that compound treatment did not cause widespread changes in the transcription of genes or gene families (Fig. 13).

An additional analysis of changes to annotated splice junctions within the observed transcripts revealed that only a small group of splice junctions were

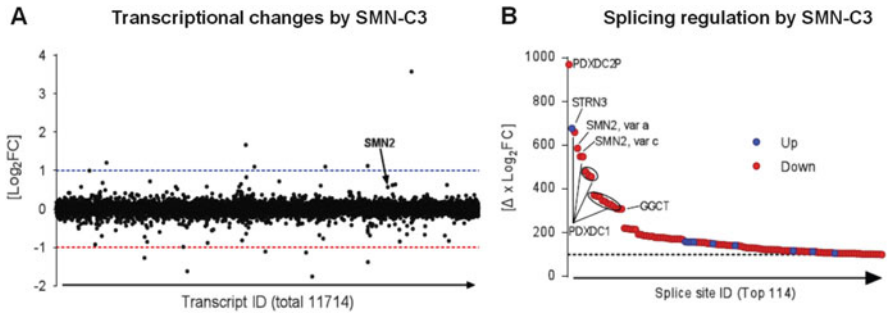

Fig. 13 (**a**) Difference in total transcript expression of **SMN-C3** (500 nM) versus DMSO-treated SMA type I patient fibroblasts for 11,714 human genes. (**b**) Differential effects of treatment on individual splice junctions in human transcripts. Affected splice junctions are characterized by either absolute difference in counts (Δ) or relative changes (Log2FC). The product $p = \Delta \times \text{Log2FC}$ was used to rank splice junctions (up-regulated in blue, downregulated in *red*). The top 114 splice junctions with $p > 100$ are shown (~300,000 splice junctions analyzed in total). Adapted from [41]

highly affected by compound treatment. Among these splice junctions were two mRNA variants of *SMN2* lacking exon 7 and several belonging to *PDXC1*. In effort to determine the molecular target, mutational analysis of sequences known to be involved with *SMN2* exon 7 splicing regulation was performed. **SMN-C3** appeared to work independently of the known splicing regulators.

Another class of small molecule *SMN2* splicing modifiers based on a pyridazine scaffold was independently discovered (e.g., **LMI070**, Fig. 11) [44]. SAR-based optimization aimed at maximizing cellular potency was driven by in vitro SMN ELISA activity in SMA patient-derived fibroblasts and Δ7 mouse-derived myoblasts. Oral dosing of **LMI070** resulted in changes in the splicing ratio of *SMN2* transcripts to favor *SMN2*-FL and increased SMN protein in C/+ mouse model of SMA as well as increased levels of SMN protein in the brain of Δ7 mice. A dose of 3 mg/kg/day of **LMI070** in Δ7 mouse model of SMA resulted in a MST >35 days compared to 14 days for mice treated with vehicle only. **LMI070** was the first small molecule splicing modifier to enter the clinic for studies in infants with type 1 SMA.

RNA sequencing analysis of **LMI070** at 100 nM identified 39 splice junction change events in 35 genes. Close inspection of the sequences of the affected splice junctions indicated a preferred 5′ splice site sequence, containing G and A at the −2 and −1 positions, respectively. This exon terminal "GA" sequence differs from the canonical "AG" sequence and forms a less stable pairing to the U1-snRNP. The hypothesis was proposed that the compounds bind to and stabilize U1-snRNP/mRNA complexes that contain the less common "GA" sequence. Additional binding experiments using size exclusion chromatography, surface plasmon resonance (SPR), and nuclear magnetic resonance (NMR) spectroscopy provided additional evidence of an interaction between **LMI070** and U1 snRNP in the presence of mRNA. No high resolution spectroscopic data have been published.

This hypothetical mode of action provides a rational explanation for compound-mediated splicing in the case of *SMN2* exon 7 (Fig. 14). As described previously,

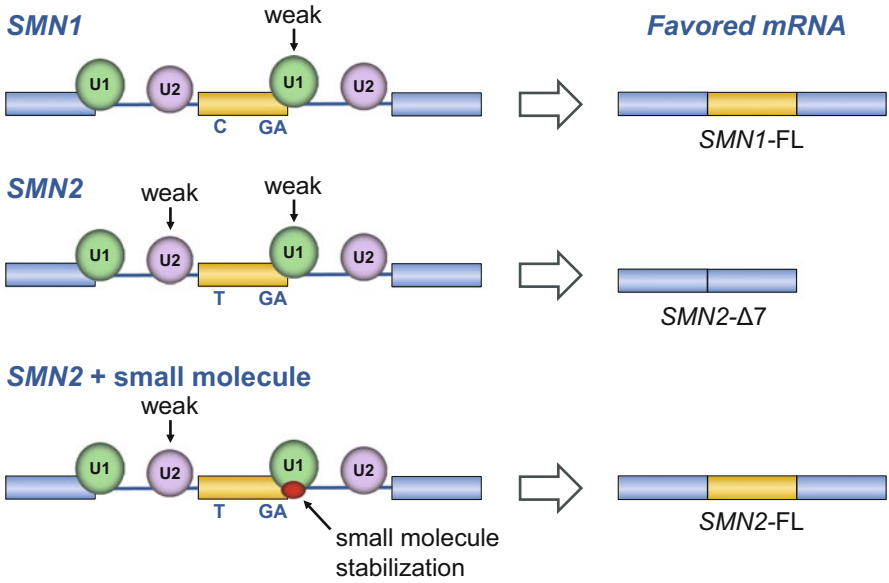

Fig. 14 Proposed mechanism of action for **LMI070**

the C to T transition in exon 7 of *SMN2* causes less favorable U2 recognition and, in conjunction with the less favorable "GA" sequence at the end of exon 7, impairs exon definition. This change is sufficient to cause exon 7 skipping 50–90% of the time. Compound treatment causes the stabilization of the U1/pre-mRNA complex, thus overcoming the weakness of the U2 recognition near the 3′ splice site. It is interesting to note that compound treatment also stabilizes the U1/exon 7–intron 7 complex in *SMN1* pre-mRNA and even though exon 7 is included in the final transcript in 95% of the *SMN1* mRNA, these small molecule splicing modifiers increase the fraction of full length *SMN1* mRNA to 100% (A. Dakka, N. A. Naryshkin, PTC Therapeutics, South Plainfield, NJ, unpublished work, 2012). This would suggest that on a global level additional sequence context around the 5′ terminal "GA" will largely determine the responsiveness to the treatment and could explain why the compounds show such remarkable selectivity.

2.3 Small Molecule Approach to Treat FD

Familial dysautonomia [FD, also known as Riley–Day syndrome or hereditary sensory and autonomic neuropathy (HSAN) III] is a recessive neurodegenerative disorder that is almost always caused by a noncoding point mutation at nucleotide 6 of the intron 20 donor splice site (IVS20+6T-C) of the *IKBKAP* gene (Fig. 15a) [46, 47]. In >99% of FD patients both alleles contain this same mutation, which

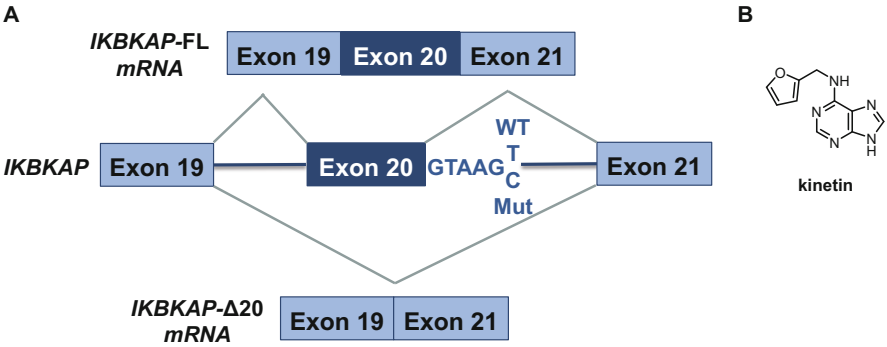

Fig. 15 (**a**) Alternative splicing of *IKBKAP* pre-mRNA. Mutation at nucleotide +6 of intron 20 causes a shift toward *IKBKAP*-Δ20. (**b**) Structure of the plant growth hormone kinetin

weakens the 5′ splice site. When mutant *IKBKAP* pre-mRNA is processed by the spliceosome, exon 20 is skipped much more frequently than would normally occur for the wild type *IKBKAP*, thus producing less full-length *IKBKAP* (*IKBKAP-FL*) and more exon-20-skipped IKBKAP (*IKBKAP-Δ20*) [48]. The frameshifted *IKBKAP-Δ20* transcript contains a premature termination codon in exon 21, which causes rapid degradation through the nonsense mediated decay (NMD) pathway. This is confirmed by the observation that *IKBKAP-Δ20* levels are increased in patient cells treated with cycloheximide, an NMD inhibitor [49]. Reduced levels of *IKBKAP*-FL lead to lower expression of functional IKAP, a protein that has roles in transcription elongation, histone acetylation, DNA methylation, and tRNA modification. The absolute amount of IKAP protein needed for normal cell function may vary by cell type. It is also known that the ratio of *IKBKAP-FL* to *IKBKAP-Δ20* varies between tissues, but tends to be lowest in the cells of the central and peripheral nervous system [50]. The level of IKAP protein produced in neuronal tissues of FD patients is not sufficient for normal function.

Because this devastating disease hinges on the balance of a single splicing event, several attempts have been made to identify a small molecule that could shift the splicing balance in favor of *IKBKAP*-FL [51–53]. The Slaugenhaupt lab developed an assay in FD patient lymphoblasts using RT-PCR to quantify the ratio of *IKBKAP*-FL/*IKBKAP*-Δ20 [49]. The assay was used to screen a small library of 1,040 bioactive compounds (mostly FDA approved drugs). A plant growth hormone, kinetin (Fig. 15b), demonstrated a shift in splicing at 10 μM. A full dose-response curve demonstrated that increasing the concentration of kinetin increases the ratio of *IKBKAP*-FL to *IKBKAP*-Δ20 from ~1:1 in the absence of compound to 12:1 in the presence of 400 μM kinetin. A corresponding dose dependent increase in IKAP protein was observed by Western blot.

To demonstrate the selectivity of kinetin on *IKBKAP* splicing, additional genes that either demonstrate alternative splicing in vivo or have been shown to have altered expression in the presence of kinetin were chosen for analysis [54]. Of the seven genes analyzed, the splicing ratios were unchanged in five of them upon

kinetin treatment. The two splicing responsive genes had a common feature with exon 20 of *IKBKAP*. Mutational analysis in and around exon 20 revealed the importance of the sequence CAA at the −3 through −1 positions of the 5′-splice site of exon 20. It is estimated that ~9% of internal exons contain this sequence at the 5′-splice site [54]. Three additional genes that contain the CAA sequence were shown to be kinetin sensitive; *ABI2* exon 2, *BMP2K* exon 14, and *NF1* exon 36 (*NF1* exon 36 ends in CAAG).

To test the efficacy of kinetin in vivo, a humanized transgenic mouse line carrying the complete human *IKBKAP* locus with the FD mutation was generated [55]. The tgFD mice appropriately express the human *IKBKAP* from the transgene with tissue-specific splicing patterns that match those in FD patients. In pharmacokinetic studies in mice, kinetin was observed in all tissues including those within the central nervous system [56]. Mice dosed orally with kinetin at 400 mg/kg/day achieved serum concentrations >30 μM, well above the 10 μM concentration required for measurable activity in cultured cells. After 30 days of dosing, harvested tissues were analyzed. RT-PCR analysis showed a shift in splicing toward *IKBKAP-FL* mRNA in all tissues analyzed. This shift translated to higher levels of *IKBKAP-FL* mRNA transcript and increased IKAP protein.

To determine whether orally administered kinetin could alter mRNA splicing in FD patients, eight patients that were homozygous for the splice mutation were administered 23.5 mg/kg/day of kinetin for 28 days [57]. Plasma concentrations consistent with the levels required for activity in vitro were achieved in most of the patients at this dose. The levels of *IKBKAP-FL* in patient peripheral leukocytes after 8 and 28 days of dosing were compared to baseline levels measured prior to dosing. At baseline the mean percent inclusion of exon 20 was 54 ± 10%. After 8 days of dosing, six of eight individuals showed increased levels of *IKBKAP-FL*, with a mean percent inclusion of 57 ± 10%. After receiving kinetin for 28 days, patients achieved an even greater percent inclusion of 71 ± 9% ($p = 0.002$, Fig. 16). No serious adverse effects related to treatment were noted for the study. Based on proof of concept results both in preclinical and clinical studies, additional kinetin analogs have been designed by the Slaugenhaupt lab with improved potency and pharmaceutical properties [58].

Roughly a decade after the initial discovery of kinetin as an *IKBKAP* pre-mRNA modifier, a screening effort in the Hagiwara lab led to the discovery of a more potent molecule mediating *IKBKAP* exon 20 inclusion [59]. A dual color reporter assay was designed and used to quantify the ratio of *IKBKAP-FL* to *IKBKAP-Δ20*. Exon 21 of the *IKBKAP* minigene construct contained sequences for enhanced green fluorescent protein (EGFP) and monomeric red fluorescent protein (mRFP). EGFP is placed in the proper translational reading frame only when exon 20 is included in the mRNA transcript. Conversely, when exon 20 is excluded, the resulting transcript places mRFP in the proper translational reading frame (Fig. 17a). The relative amounts of the two reporters can be quantified using fluorescence microscopy. The construct was used to screen 638 molecules from a chemical library and some additional approved pharmaceuticals.

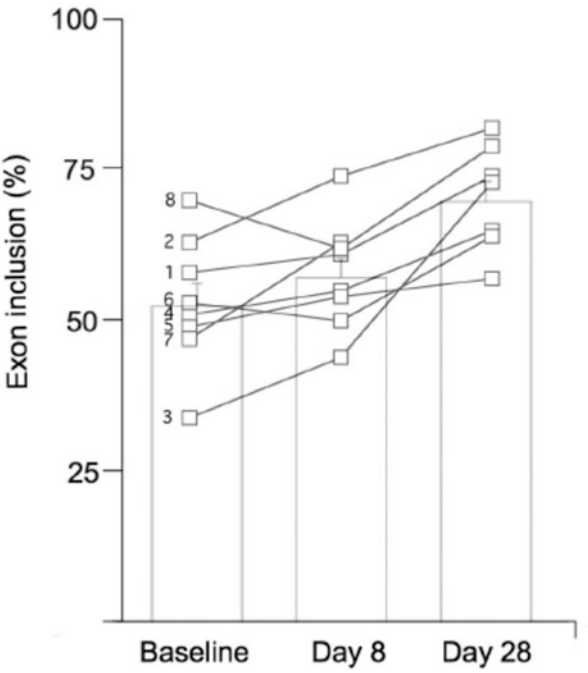

Fig. 16 The percent of the IKBKAP transcript that includes exon 20 in FD patients treated with 23.5 mg/kg/day kinetin at baseline, after 8 days of dosing, and after 28 days of dosing. Individual data points shown with open squares. Adapted from [57]. Copyright 2011 Macmillan Publishers Ltd

Interestingly, the most active molecule identified from the screen was a close structural analog of kinetin, differing only by the addition of chlorine at the 2-position of the purine ring (Fig. 17b, RECTAS). RECTAS (*rect*ifier of *a*berrant *s*plicing) demonstrated roughly a 25-fold improvement in activity over kinetin in the fluorescence reporter assay (Fig. 17c). In addition, RECTAS increased the inclusion of exon 20 in patient fibroblasts over kinetin when cells were treated at similar concentrations. The increased levels of *IKBKAP-FL* led to a corresponding increase in IKAP protein as demonstrated by western blot.

3 Exon Skipping

Skipping of an exon during pre-mRNA splicing can be exploited in several different genetic circumstances. The most common applications include (1) generation of a shorter isoform with altered function, (2) skipping of a downstream exon in order to restore an open reading frame with at least partial functional recovery, (3) skipping of a deleterious mutation-containing exon (provided that the shorter isoform retains at least some biological function), and (4) skipping to disrupt an open reading frame in order to suppress the expression of the target gene. The utility of exon skipping has now been documented for many therapeutically relevant genes, some of which are reviewed in detail below.

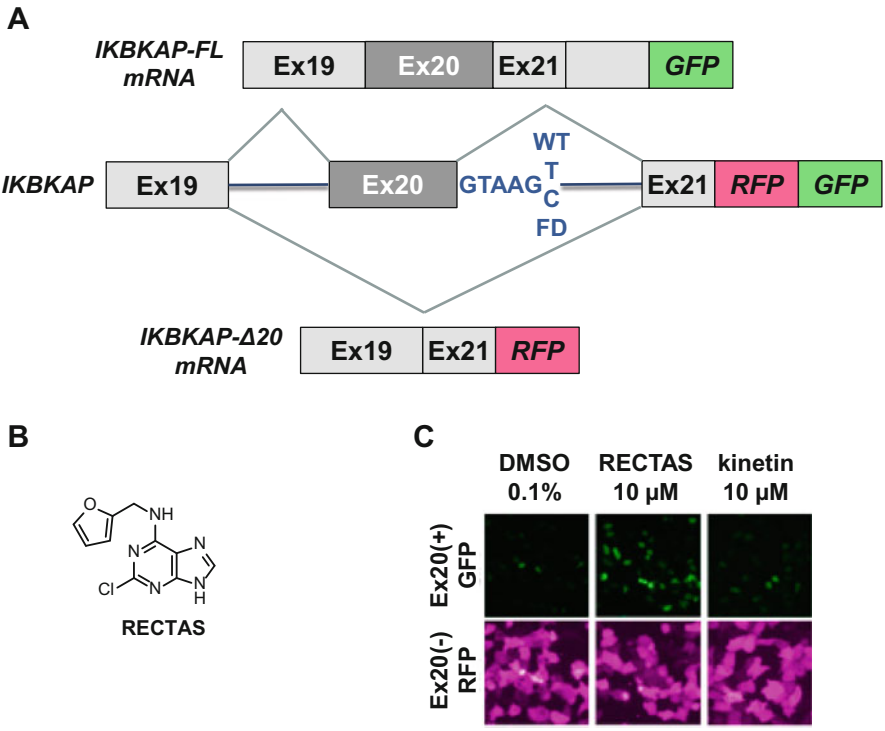

Fig. 17 (**a**) *IKBKAP-FD* reporter construct used to screen small molecules for exon 20 inclusion. (**b**) Small molecule identified in a screen that shifts *IKBKAP* pre-mRNA splicing toward *IKBKAP-FL*. (**c**) Microscopic analysis of HeLa cells expressing the *IKBKAP-FD* reporter treated with the indicated small molecules. Adapted with permission from [59]

3.1 Treatment of Duchenne Muscular Dystrophy

The application and clinical advancement of exon skipping for disease therapy has received the most attention in the field of Duchenne muscular dystrophy (DMD; MIM #310200). DMD is an X chromosome-linked progressive degenerative myopathy caused by mutations in the dystrophin gene, whose main function in muscle tissue is to connect cytoskeleton with the sarcolemma to maintain the structural integrity of the muscle fiber. The N-terminal domain of dystrophin binds actin filaments and, via 24 spectrin-like (SR) domains and four hinge regions, is connected to the C-terminal domain which interacts with the inner side of the sarcolemma where it joins a large multiprotein assembly called the dystrophin-associated protein complex (DAP). The complete list of biological functions for dystrophin is still being elucidated. In the muscle, the main role of dystrophin is to act as both a linker and a mechanical stress absorber during the cycles of contraction and relaxation. This function requires the presence of both attachment domains and at least some of the internal domains (much like a car suspension's strut

system). Therefore, mutations that maintain this general three-part structure would be predicted to retain some function, whereas mutations that result in the absence of either terminal domains or the internal "stress absorber" would be expected to lose all muscle-supporting properties. Indeed, following the discovery of the *DMD* gene in 1986 [60] and subsequent genotyping of a sufficient number of DMD patients, it was realized that a sizable fraction of *DMD* gene mutations do result in a considerably milder Becker muscular dystrophy (BMD; MIM #300376) in which affected individuals have reduced dystrophin function due to an internal truncation or an amino acid substitution. Thus, "the ORF rule" was put forward stating that genetic alterations retaining the open reading frame (ORF) of dystrophin are much more likely to result in the milder Becker dystrophy (Fig. 18) [61].

The ORF rule suggests a practical approach to the treatment of Duchenne muscular dystrophy in which the detrimental effect of a frame-disrupting deletion could be partly reversed during pre-mRNA splicing by skipping an exon flanking the deletion if such skipping restores the ORF (see Fig. 19 for a graphical overview highlighting exons that would maintain the ORF when skipped). A deleterious point

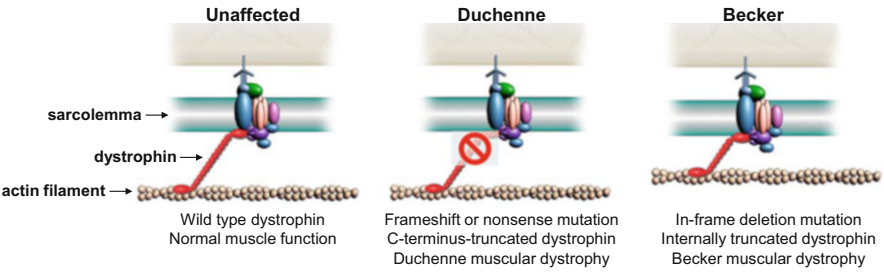

Fig. 18 Cartoon depiction of the role of dystrophin in wild type muscle fiber (*left*), muscle fiber with nonfunctional terminally truncated dystrophin leading to Duchenne muscular dystrophy (*middle*) and muscle fiber with an internally truncated dystrophin protein leading to Becker muscular dystrophy (*right*)

Fig. 19 Overview of the exons coding for the *DMD* gene. In-frame exons are shown in light blue and out-of-frame exons are shown in dark blue. Exon deletions lead to in-frame messages when the exons flanking the deletion have matching shapes. When the flanking exons do not match, the deletions disrupt the reading frame. Adapted from [62]

mutation in *DMD* could also be partly ameliorated by skipping the mutation-carrying exon provided the ORF is maintained in the shortened transcript (Fig. 3c, d). This phenomenon may be responsible for the production of dystrophin-positive ("revertant") fibers in human DMD and BMD patients and in animal models of Duchenne, although the origin of revertant fibers is still being elucidated [63–66]. The unexpectedly mild phenotype of the commonly used mdx mouse model of DMD [67] carrying an inactivating nonsense mutation in exon 23 is driven by massive sporadic exon skipping that generates functional internally deleted dystrophin isoforms [68, 69].

Additional appeal for utilizing an exon skipping strategy to treat DMD arises from the fact that a high prevalence of *DMD* mutations are amenable to this approach. Dystrophin is the third largest human gene comprising at least 7 characterized promoters, 79 exons, and introns whose size reaches over 248,000 nucleotides. Dystrophin's pre-mRNA takes about 20 h to be synthesized and undergoes complex splicing. According to the recent analysis of the TREAT-NMD DMD Global database, 80% of deletions and 55% of all *DMD* mutations can be potentially corrected using exon skipping therapy [70]. The top ten target exons for skipping, nine of which are located in the mutational "hot spot" area of exons 45–55, are shown in Table 1.

A naturally occurring mutation provided some of the initial insight leading to the development of methods for pharmacologically induced exon skipping. The so-called dystrophin Kobe, in which 52 nucleotides spanning an exonic splicing enhancer (ESE) region in the middle of *DMD* exon 19 are deleted, leads to native skipping of exon 19 [71]. Using a splice switching oligonucleotide (2′-OMe backbone) complementary to the first 31 nucleotides of the sequence deleted in the Kobe mutation, the Matsuo group first demonstrated, in 1995, the inhibition of *DMD* intron 18 removal from a *DMD* minigene pre-mRNA in HeLa cell nuclear extract [72]. Then, in 1996, the Matsuo group demonstrated that a DNA-PS oligomer (**AO19**, Fig. 20) complementary to the same region caused the skipping of DMD exon 19 during the splicing of wild type *DMD* pre-mRNA in cultured human lymphoblastoid cells [73]. Cellular delivery of **AO19** required the use of a

Table 1 Prevalence of mutations in *DMD* exons in Duchenne muscular dystrophy patients (top ten shown)

DMD exon	Mutation (%)	Deletion (%)
51	14.0	20.5
53	9.0	13.1
45	8.1	11.8
44	7.6	11.1
43	3.8	5.6
46	3.1	4.5
50	2.0	2.9
52	1.7	2.5
55	0.9	1.3
8	0.9	1.3

Column 2 contains the percentage of all mutations, while column 3 contains only the percentage of deletions [70]

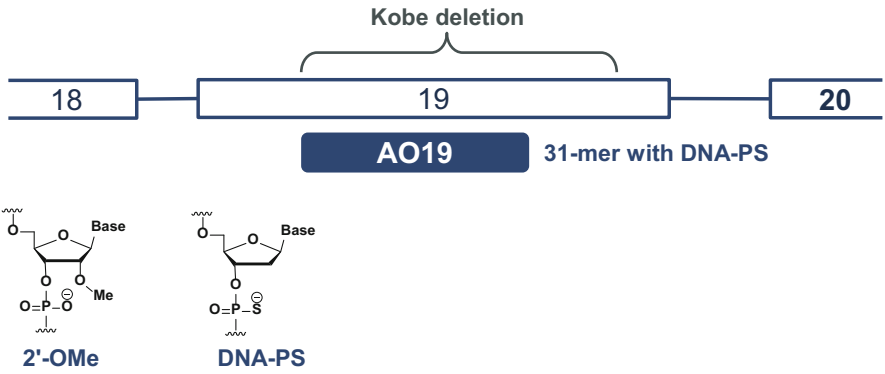

Fig. 20 SSO developed to mimic the Kobe deletion in *DMD*

cationic lipid vehicle. When cells were treated with 200 nM of **AO19**, only *DMD*-Δex19 mRNA was observed, demonstrating for the first time the feasibility of completely skipping a constitutive human exon through SSO treatment. Importantly, other *DMD* exons were not affected and the negative control, a DNA-PS sense-strand, did not induce exon skipping. Utilizing **AO19**, *DMD* exon 19 was skipped in myotubes generated from myogenic cells isolated from a DMD patient harboring a deletion of the entire exon 20 region of the dystrophin gene [74]. On this genetic background, skipping of exon 19 restores the open reading frame and is predicted to restore, at least partially, the expression of dystrophin. The authors observed up to 20% of dystrophin-positive fibers in cultures that were treated with the SSO.

When a single dose of **AO19** was delivered intraperitoneally as an aqueous solution to mdx mice, measurable skipping of exon 19 was detected, with an effect lasting up to 14 days post injection [75]. When a fluorescent label was linked to **AO19**, the authors were able to detect the compound in interstitial tissue, cytoplasm, and myocyte nuclei, including centered nuclei representing the regenerating myocytes. Thus, in vivo, **AO19** delivered as a naked molecule in solution was reaching the intended sites of action in skeletal muscle cells. These results gave the Matsuo group confidence to test **AO19** in a DMD patient with an exon 20 deletion [74]. Four doses of the **AO19**, given once a week, delivered as an intravenous infusion resulted in skipping of exon 19 in both lymphocytes and biceps muscle, achieving ~6% skipping in the muscle. Immunostaining of the biopsied muscles with antibodies detecting the N- and C-termini and the rod region of dystrophin showed a modest increase in dystrophin production in post-treatment samples [76].

In the late 1990s–early 2000s, several other groups initiated research efforts in exon skipping for DMD. The Wilton and Dunckley groups were inspired by another natural phenomenon observed in mdx mice. This mouse model carries a premature translation termination codon in exon 23 of the dystrophin gene, which would be expected to result in a severe dystrophic phenotype. However, mdx mice have a nearly normal lifespan and only mild dystrophy, primarily attributable to the

presence of a small but measurable number (typically less than 1%) of dystrophin-positive muscle fibers, thought to be caused by intrinsic exon skipping during pre-mRNA splicing or due to somatic mutation [69, 77, 78]. SSOs were designed to bind to the 3′ and 5′ splice sites at the ends of exon 23 and the branch point in intron 22. Effective exon skipping was achieved upon transfection of both mouse myoblast cell line C2C12 and primary mdx mouse myoblasts [79, 80]. Interestingly, while Wilton et al. found blocking of the 5′ splice site to be most effective in eliciting exon 23 skipping, Dunckley et al. achieved maximal skipping with an SSO blocking the 3′ splice site (the nucleotide sequences of the SSOs were different in the two studies). Dunckley et al. also observed increased levels of dystrophin protein at the sarcolemma in about 1% of cultured myotubes. Around the same time, van Deutekom et al. demonstrated the utility of 2′-OMe-PS oligomers in skipping murine and human *DMD* exon 46. A series of oligonucleotides that bind to predicted exonic splicing enhancers in exon 46 were used to transfect murine C2C12 cells and human primary muscle cells isolated from a healthy individual and two unrelated DMD patients. Efficient skipping of exon 46 was achieved and, in DMD patients-derived myotubes, skipping resulted in the appearance of dystrophin staining in the sarcolemma region [81]. Human *DMD*-specific skipping of exons 44, 46, and 49 was demonstrated in the hDMD mouse model carrying the full 2.4 Mb human dystrophin gene [82].

This seminal work clearly established the feasibility of exon skipping during *DMD* pre-mRNA splicing and confirmed the hypothesis that a translation-compatible open reading frame can be generated by skipping an exon carrying a point mutation or flanking a frame-disrupting deletion. To determine the general applicability of exon skipping as a potential therapy for DMD, several groups performed comprehensive systematic surveys of "skippability" of all internal exons in dystrophin using splice switching oligonucleotides [83, 84]. Every exon was found to be skippable; however, the ease of eliciting the skipping varied considerably between exons. For some exons treatment with two or more oligonucleotides was required. Simultaneous skipping of multiple exons, necessary for certain classes of *DMD* mutations, was also shown to be possible [85, 86].

Initial exon skipping studies in DMD relied mostly on oligoribonucleotides with a 2′-methoxy substituted phosphorothioate backbone (2′-OMe-PS). These molecules satisfy two key requirements for an SSO – they are nuclease-resistant and do not support RNase H-mediated cleavage. However, due to their negative charge, crossing biological membranes (which are also negatively charged) presents a challenge which is particularly severe in cardiac muscle and the CNS. Indeed, the extent of exon skipping observed in the in vivo studies presented to this point was typically a few percent at best.

Several additional oligonucleotide chemistries (Fig. 21), including those based on locked nucleic acids (LNA), peptide nucleic acids (PNA), phosphorodiamidate morpholino oligonucleotides (PMO) and, recently, tricycle-DNA (tcDNA) have been tested for DMD skipping (reviewed in [87, 88]). The presence of LNA units results in much stronger hybridization energy, with each monomer adding at least 5 °C to the duplex melting temperature [89]. The stronger binding has effectively

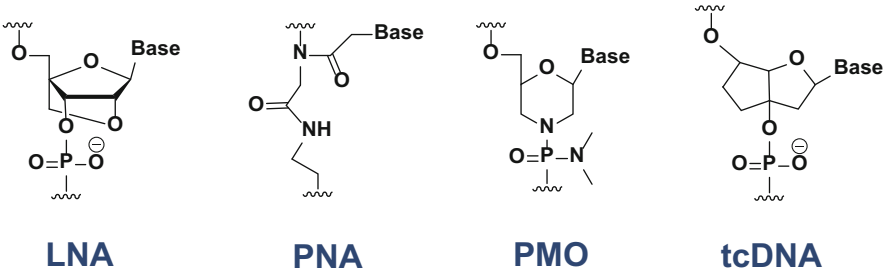

Fig. 21 Alternative SSO backbone chemistries

precluded the use of pure LNA oligomers as splice-switching reagents, because oligomers with mismatches maintain high binding affinity, raising concerns with regard to specificity. Shorter LNA oligomers (14-mers are typical) suffer from the same consequence of reduced specificity. Copolymers comprised of LNA and 2′-OMe-PS sequences may hold promise for maintaining the high nuclease stability afforded by the LNA segment while achieving higher specificity from the increased length permitted by the addition of the lower affinity 2′-OMe-PS segment [90]. However, the application of such chimeras has not produced the expected improvement in exon skipping activity in vivo [91]. The application of PNA oligomers showed early promise in modulating the splicing of pre-mRNAs for several membrane receptors, but the activity in *DMD* exon skipping proved to be much less consistent [89, 92, 93]. This inconsistency combined with higher reagent costs has caused PNAs to receive less attention than other classes.

The tcDNA backbone may be emerging as the next promising scaffold for SSO design due to its ability to induce exon skipping not only in skeletal muscle but also in the heart and even, to a small but measurable extent, in the brain of the mdx mouse [94] without signs of overt toxicity. In this study the tcDNA was designed to induce *Dmd* exon 23 skipping, which is one of the most easily skipped exons. Future work will need to address both the general utility of tcDNA for exon skipping and the tolerability of this backbone in vivo.

PMOs have emerged as the leading type of SSO owing to a unique combination of efficient hybridization, stability to nucleases, lack of immune response, and aqueous solubility (reviewed in [95]). This class of SSO has shown robust *DMD* exon skipping in cultured cells [96, 97]; in the mdx and hDMD mouse models [98–100], and in the CXMD dog model [101]. Aoki and coworkers innovatively proposed using a cocktail of PMOs to cause the skipping of the entire region covering DMD exons 45–55 [102]. This approach would be theoretically applicable to ~60% of DMD patients carrying a frame-changing deletion. The corresponding internally truncated protein is known to be associated with mild myodystrophy. The approach showed early promise in the mdx52 mouse model [102, 103]. Several review articles provide excellent surveys of the efforts directed toward multiple exon skipping in DMD [85, 86, 104].

There are divergent estimates, likely driven by the differences in sequence and biological models used, of unassisted cellular uptake of backbone-neutral PMOs vs. negatively charged 2-OMe-PS oligonucleotides, with some groups demonstrating better uptake of PMOs and others giving the edge to 2-OMe-PS oligonucleotides [91, 98]. A considerable advantage of the PMO backbone is its compatibility with covalent attachment of cellular uptake-promoting moieties.

Cell-penetrating peptide-conjugated oligonucleotides have emerged as another very exciting class of splicing modifiers due to their enhanced systemic bioavailability (reviewed in [91, 105, 106]). This approach turned a challenge – the presence of negative charges on the cell membrane and their interference with the uptake of negatively charged oligonucleotides – into an opportunity. By the mid-1990s several short peptides containing a high proportion of positively charged amino acids (arginines and lysines) were shown to facilitate the delivery of various kinds of molecular payload to the cytoplasm and nucleus of the cell. Research from several laboratories has now expanded the arsenal to several dozens of cell-penetrating peptides (CPPs) and work in this area continues. The mechanism of CPP-mediated internalization is not completely understood and, depending on the structure and cell type, can involve either the direct membrane translocation or an endocytic pathway. The positive charge of CPPs constrains the type of molecular cargo that can be covalently conjugated; only charge-neutral backbones such as PNA and PMO are compatible. Oligomers carrying negative charge (e.g., siRNA duplexes) can only be delivered using non-cationic peptides or as noncovalent complexes with amphipathic peptides. Non-charged homing peptides are being developed for oligonucleotides with charged backbones [107]. CPP-modified SSOs offer enhanced potency and tissue penetration upon systemic delivery. A very recent example is the work by Hammond and colleagues in which considerable efficacy was achieved upon treating severe SMA mice with systemically delivered PMO conjugated with Pip6a delivery peptide [108]. Of relevance to DMD, the intravenously delivered Pip6a-PMO conjugate induced efficient *SMN2* splicing correction in several tissues including brain and spinal cord. For treatment of DMD, the challenges of tissue distribution for SSO therapies are particularly acute with respect to penetration into the cardiac muscle. Arginine-rich CPPs have been the first SSO class to show DMD exon skipping in the heart. Additionally, they confer benefit at doses lower than those required for unconjugated PMOs, and prevent exercise-induced myopathy in mdx mice [106, 109–112]. Pharmacokinetics, biodistribution, stability, and toxicity of an early version of a cell-penetrating peptide attached to a PMO were studied in rats by Amantana and coworkers [113]. Intravenous administration of the conjugate in rats produced an LD_{50} of 210–250 mg/kg. Doses of 15 and 30 mg/kg were well tolerated, with no evidence of clinical abnormalities. The target organ for toxicity appeared to be the kidney, adding a cautionary note to this class of SSO. However, it should also be noted that CPP-PMO conjugates are biologically active at much lower doses (typically in the 1–10 mg/kg range) than PMOs by themselves. Active research focused on CPP optimization, including the reduction of kidney damage, is ongoing in several laboratories opening the way to commercial and therapeutic applications.

Two additional splice-switching modalities that rely on nucleic acid hybridization have been developed to achieve exon skipping. They are based on U1 [114–117] and U7 [118–120] small nuclear RNAs engineered to interact with various *cis* elements essential for splicing and interfere with the formation of a splicing-competent spliceosome. These RNAs are delivered using lentivirus- and AAV-based vectors and therefore achieve more consistent expression, proper cellular localization, and readily associate with spliceosome components. The downsides of this approach as a therapy are inherent to virus-mediated therapies and include the need for production of large number of viral particles and existing and induced immune response.

A comprehensive treatment for DMD would require not only a dystrophin-restoring compound but also pharmacological agents addressing other aspects such as chronic inflammation, fibrosis, the depletion of satellite cells and reduced muscle mass. Myostatin (*MSTN*) is a well-characterized muscle growth suppressor [121]. Animals with two null *MSTN* alleles develop overtly hypertrophic muscles. The *MSTN* gene consists of three exons. Skipping of the second exon disrupts the open reading frame and inhibits myostatin protein synthesis. Several groups achieved successful skipping of *MSTN* exon 2 using both 2-OMe-PS oligonucleotides and PMOs, typically in conjunction with frame-restoring exon skipping in DMD pre-mRNA [122–124]. It is important to note that in many myopathies the inhibition of myostatin alone, while increasing muscle mass, may not contribute substantially to muscle strength. To be effective, MSTN inhibition needs to be coupled with agents that restore muscle structure [125].

3.2 Clinical Development of Exon Skipping SSOs

Three classes of splice switching oligonucleotides, DNA-PS oligomers, 2′-OMe-PS oligomers and PMOs, have been undergoing extensive clinical development, although the bulk of the work has involved the 2-OMe-PS and PMO classes (reviewed in [126–128]). As described earlier, the first human trial of a *DMD* exon skipping oligonucleotide was performed by the Matsuo group using AO19 in a patient with a *DMD* exon 20 deletion [74]. This study served as a proof of concept, but left several open questions. What role does RNase H-driven degradation of *DMD* mRNA play? Will exon skipping studies in the mdx mouse translate to humans? And what cells and tissues are relevant for the analysis of drug efficacy (summarized in [87]). Since the skipping of *DMD* exon 51 can restore dystrophin's open reading frame in the largest cohort (13–15%) of DMD patients [62, 70], the largest clinical effort has been devoted to SSOs targeting this exon.

Drisapersen (PRO051, Kyndrisa™), a 20-mer 2′-OMe-PS oligonucleotide from Prosensa/GSK/BioMarin, was identified by van Deutekom and co-workers as a candidate for testing in DMD patients with mutations amenable to ORF restoration by *DMD* exon 51 skipping. The first clinical trial, at a single-center, was an open label study of a single intramuscular dose of drisapersen in four DMD patients. No adverse events were observed. A variable degree of exon 51 skipping was detected

in extracted muscle samples as measured by RT-PCR. Samples displayed an increase in dystrophin-positive fibers with a sarcolemma-localized signal and an upregulation of dystrophin production shown by western blotting; however, the increase in protein did not appear to correlate with the relative fraction of Δ51 mRNA [129]. Encouraged by the initial results, several phase 2 and 3 studies on drisapersen were initiated to investigate dose response, dosing regimens, and patient cohorts. In total more than 300 DMD patients were dosed over the course of the trials. The primary outcome measure was the distance traveled during a 6 min walk (6 min walk test or 6MWT). Drisapersen, while showing positive trends in subgroups of patients, did not achieve a statistically significant difference in the primary endpoint, which in combination with drug-related side effects made regulatory approval challenging. Further development of drisapersen and other DMD exon skipping candidates in BioMarin's pipeline was halted.

With similar timing to the development of drisapersen, eteplirsen (AVI-4658, EXONDYS 51™), a 30-mer PMO, was identified by researchers at Sarepta Therapeutics (AVI BioPharma at the time). Eteplirsen also targets patients with mutations amenable to ORF restoration by *DMD* exon 51 skipping. The first human clinical trial for eteplirsen was a single-blind placebo-controlled dose-escalation study of a single intramuscular dose in seven DMD patients. No-treatment-related adverse events were reported. Extensor digitorum brevis muscle biopsies were analyzed for *DMD* gene expression. The high dose (0.9 mg) resulted in measurable increases in Δ51 *DMD* mRNA, dystrophin-positive fibers and dystrophin protein. There appeared to be a correlation between the level of Δ51 *DMD* mRNA and dystrophin protein [130]. Several phase 2 studies of eteplirsen were conducted to select a dosing regimen and a therapeutic dose. Based on the results from one of these trials, a phase 2 study (including the study extension) in 12 patients, the company sought a regulatory approval in the USA. The primary outcome measure was an increase in the number of dystrophin positive fibers, while the 6 min walk test was a secondary outcome. Following an extended regulatory review that included additional analyses of dystrophin protein levels in an ongoing phase 3 study using western blotting, in September 2016 EXONDYS 51™ (eteplirsen) received accelerated approval for the treatment of Duchenne muscular dystrophy in patients who have a confirmed mutation of the *DMD* gene that is amenable to exon 51 skipping (Available from: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2016/206488Orig1s000Approv.pdf). Using a similar technology, SSOs that induce the skipping of exons 45, 51, and 53 are currently in clinical development for the corresponding DMD patients (Available from: http://www.sarepta.com/our-pipeline). Additional SSOs targeting DMD exons 8, 44, 50, 52, and 55, as well as a CPP-modified PMO to skip exon 51, are in preclinical development.

3.3 Small Molecules that Enhance Exon Skipping

Antisense oligonucleotide and virally delivered small nucleic acids dominate the *DMD* exon skipping field; nonetheless, small molecule splicing modifiers have been pursued as a potential therapeutic modality for this cause. Nishida and co-workers screened a library of over 100,000 compounds for inhibitors of kinases responsible for phosphorylation of SR proteins which regulate splicing. One of the hits, TG003 (Fig. 22), was shown to be an inhibitor of Cdc2-like kinases and to inhibit SF2/ASF-dependent splicing [131]. Upon treatment with TG003, a *DMD* exon 31 minigene was responsive to increased exon skipping in HeLa nuclear extracts. Additionally, when myotubes generated from DMD patient cells carrying a nonsense mutation in *DMD* exon 31 were treated with TG003, the inherent sporadic exon 31 skipping was increased [132].

Dantrolene, a postsynaptic muscle relaxant and ryanodine receptor antagonist, was identified in a chemical screen of 300 compounds as a potentiator of SSO-driven exon skipping [133]. In addition to the screening assay, SSO-enhancing activity was demonstrated in vitro in mdx mouse myoblasts (exon 23 skipping) and human DMD patient myoblasts (exon 51 skipping) and in vivo in mdx mice. Both 2′-OMe-PS oligonucleotides and PMOs were responsive to dantrolene enhancement.

Hu and coworkers performed a screen of 2,000 compounds using a construct that contained an interrupted green fluorescent protein coding sequence that carried either murine *Dmd* exon 23 or human *DMD* exon 50, both flanked by their corresponding intronic sequences [134]. The small molecule 6-thioguanine (6TG) was found to be a weak inducer of exon skipping when administered alone. When used in combination with a PMO, 6TG enhances the exon skipping activity of the PMO in C2C12 mouse myoblasts. The combination therapy was also reported to enhance exon skipping in mdx mice, after an intramuscular injection. Verhaart and Aartsma-Rus further examined the effect of 6TG on *DMD* exon skipping in cultured human muscle cells derived from a healthy donor, in C2C12 murine myoblasts, and in mdx mice [135]. They concluded that indeed 6TG enhances the effect of both 2′-OMe-PS oligomers and PMOs. Additionally, it was found that the activity of 6TG in the absence of SSO was non-selective, causing exon skipping of various exons throughout the transcript, suggesting a general splicing disruption and not an activity directly coupled to the binding of the SSO.

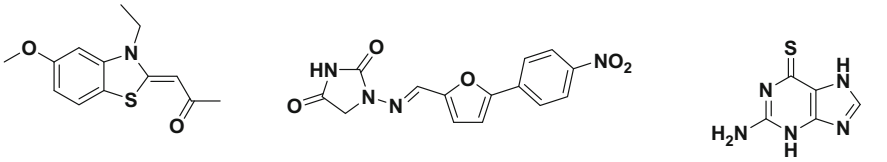

Fig. 22 Small molecules found to increase exon skipping

4 Splicing Inhibition

Various small molecules that inhibit some general aspect of the splicing process have been discovered in screening efforts with assays that were either designed specifically to identify splicing inhibitors or designed to identify cytotoxic molecules [136]. In the latter case, detailed mechanistic work was required to link the cytotoxic behavior of the molecule to the inhibition of a splicing process. Because these molecules often stall the assembly of a catalytically competent spliceosome or the catalytic splicing cycle at specific stages, they have served as useful tools in understanding certain aspects of pre-mRNA splicing. The therapeutic application of such molecules has largely been linked to cancer, since most display strong antiproliferative activity [137]. However, other applications may be possible depending on the splicing target, since some splicing events may be more strongly inhibited than others.

4.1 Targeting SF3b to Inhibit Splicing

The natural product FR901464 (Fig. 23) was isolated from the fermentation broth of *Pseudomonas* sp. due to its potent cytotoxicity. It was determined that FR901464 caused cell cycle arrest at the G1 and G2/M phases [138]. A close analysis of the

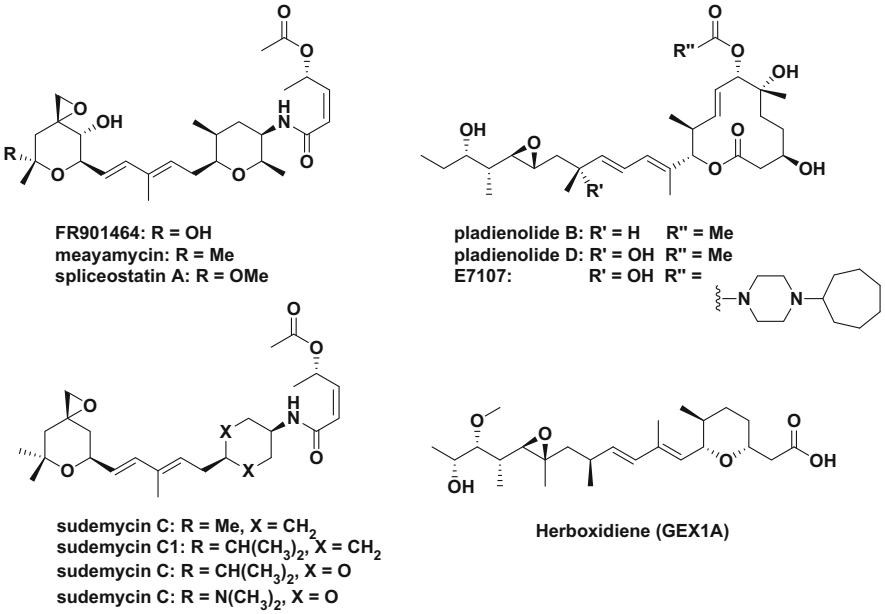

Fig. 23 Small molecules that inhibit pre-mRNA splicing by binding the SF3b complex

proteins involved in cell cycle regulation, after treatment with FR901464 or a more stable methyl ketal analog spliceostatin A, indicated that the protein p27 was upregulated [139]. Further analysis indicated that the upregulated protein was a C-terminally truncated variant of p27, and that the truncated variant did not arise from proteolytic processing. A biotinylated derivative of SSA was used in conjunction with streptavidin beads to pull out possible binding partners. Three proteins were enriched in the 130–160 kDa range. Using LC-MS these proteins were shown to be SAP155, SAP145, and SAP130, all of which are components of the SF3b sub-complex of the U2 snRNP. Although FR901464 and SSA both have reactive epoxides, they do not appear to form covalent attachments to the binding partners as evidenced by wash out experiments.

A class of macrolides, with similar structural features as spliceostatin A, revealed a similar mode of action. Pladienolides B and D (Fig. 23) were isolated from culture of *Streptomyces platensis* [140]. Treatment of cells with ^{3}H and fluorescently labeled analogs of pladienolide B showed that the compound was localized to the nucleus and present in nuclear speckles [141]. The nuclear fraction of cells treated with the ^{3}H labeled pladienolide B was subjected to immunoprecipitation using antibodies that bind nuclear speckle proteins. The results suggested compound binding to components of the U2 snRNP. Of the various antibodies raised against the components of the U2 snRNP, anti-SAP155 most efficiently coprecipitated the ^{3}H labeled pladienolide B. Using a similar coprecipitation strategy with anti-SAP155 antibody and the nuclear fraction, a pladienolide B analog containing a diazirine photo-reactive group was crosslinked to its binding partner after UV irradiation. The pladienolide B analog was also linked to biotin, allowing detection using streptavidin horseradish peroxidase (streptavidin-HRP). A single prominent band of approximately 140 kDa was seen only after treatment with both probe and UV irradiation. SAP130 was determined to be the target of the crosslinking agent.

Spliceostatin A, pladienolide B, and other molecules with a similar chemical architecture (Fig. 23) [142–144] influence splicing by binding to proteins in the SF3b complex, particularly SF3b1 (SAP155). It has been suggested that the binding event alone is not responsible for the activity, since inactive analogs effectively outcompete active compounds at the binding site to restore splicing activity in treated cells [145], leaving room for speculation that active molecules induce a conformation change upon binding the complex. Mechanistic studies have shown that SF3b interacting splicing inhibitors may impede the transition between complex A and B [146], compromise the fidelity of branch point recognition by U2 snRNP [147], and/or impede exon ligation [145]. Regardless of the exact mechanism, it is clear that although a core component of the spliceosome is being compromised, not all splicing events are equally affected [147]. The underlying principle may be rooted in the fact that not all pre-mRNA substrates have the same affinity for spliceosomal components (i.e., there is competition between introns for U2 snRNP association). RNA sequence and abundance, and associated splicing factors all play a role in determining the favorability of the pre-mRNA/U2 snRNP interaction. Ultimately, in an inhibitory environment pre-mRNA sequences that

lead to more weakly associated complexes may experience greater changes in splicing than their more stable counterparts [148].

So far, the therapeutic application of SF3b binders has been focused on cancer, since many analogs arrest cancer cell growth at nanomolar concentration. An analog of pladienolide D with improved pharmaceutical properties, E7107 (Fig. 23), showed promising results in oncology-related preclinical studies with several xenograft models [149], leading to entry into a phase 1 clinical trial [150]. The trial was halted due to vision loss experienced by two patients. The exact underlying mechanism for the visual toxicity remains unclear. Additional therapeutic applications of these molecules remain to be seen.

4.2 *Spliceosome Stalling with Isoginkgetin*

Whereas the SF3b binding compounds were identified from screening methods designed to identify cytotoxic or cytostatic compounds, followed by detailed mechanistic work to identify the target, a more direct approach of screening molecules in assays designed to detect splicing inhibition has led to the discovery of various other splicing inhibitors. In one such assay developed in HEK293 cells, the open reading frame of firefly luciferase was placed downstream of a minigene cassette containing exon 6-intron 6-exon 7 of human *TPI* (Fig. 24a) [151]. All the in-frame stop codons were removed from intron 6 plus an additional base was added to exon 7 such that luciferase only remains in the proper translational reading frame

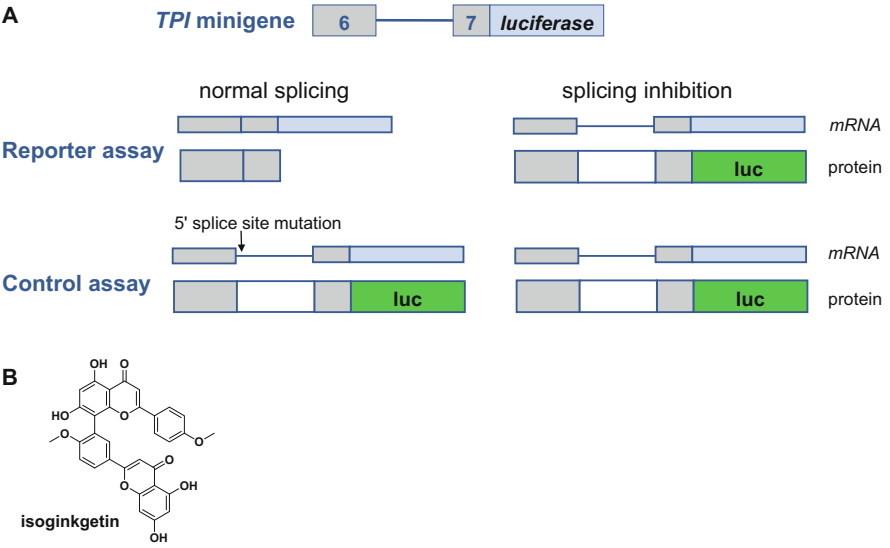

Fig. 24 (**a**) Construct designed to identify pre-mRNA splicing inhibitors. (**b**) Small molecule isoginkgetin identified to inhibit pre-mRNA splicing

in the intron-containing mRNA. Although transcripts that retain introns are not typically exported from the nucleus for translation, a small amount of exported transcript is sufficient to generate a luminescence signal that distinguishes changes in splicing. An additional control construct was created with a mutation at the 5′ splice site of exon 6 that effectively abolishes splicing. Comparing the effect of compound treatment in the reporter assay vs. the control assay distinguishes splicing changes from other effects (e.g., transport). Approximately 8,000 compounds from natural product and synthetic libraries were screened in the assay. A bisflavanoid natural product, isoginkgetin, stood far above the rest of the compounds in its ability to inhibit splicing (Fig. 24b). Isoginkgetin had been previously described as a potential anti-cancer agent due to its unique activity [152]. To test whether the activity was unique to the reporter assay, several endogenous transcripts were evaluated, including those encoding β-tubulin, actin, DNAJB1, TPI, glyceraldehyde-3-phosphate dehydrogenase, and RIOK3. All of these transcripts exhibited pre-mRNA accumulation (2–20-fold) upon treatment with isoginkgetin. It appears that isoginkgetin blocks spliceosome transition from complex A to complex B. The binding target for isoginkgetin has not been determined. One possible candidate is the SRPK2 kinase that phosphorylates the DEAD-box ATPase PRP28 responsible for complex B formation.

4.3 Spliceosome Stalling with Madrasin

A splicing inhibition assay was developed in HeLa cell nuclear extract, utilizing a FLAG-tagged spliceosomal protein and anti-FLAG peroxidase conjugated antibody [153]. The pre-mRNA substrate in the assay lacks the 3′ exon thus allowing spliceosome assembly and step 1, but not step 2 in the catalytic cycle (Fig. 2). The quantification of complex C formation is possible due to the stalled splicing process (Fig. 25). Any inhibition of early spliceosome formation or of step 1 in the catalytic cycle would be detected by a lower abundance of complex C. Screening a library of 30,000 compounds at 50 μM concentration revealed two cyclic esters, psoromic acid and norstictic acid, which inhibited splicing by more than 50%. In a separate study, a very similar assay was used to screen a library of ~72,000 compounds [154]. Madrasin (i.e., 2-((7-methoxy-4-methylquinazolin-2-yl)*a*mino)-5,6-*di*methylpyrimidin-4(3H)-one *RNA splicing inhibitor*) was identified as the most active compound. Madrasin completely inhibits splicing of the Ad1 pre-mRNA at 62.5 μM, whereas splicing of the MINX pre-mRNA was completely inhibited at 150 μM. Analysis using native agarose gel electrophoresis indicates that madrasin interferes with one or more of the early steps in spliceosome assembly, allowing the formation of the complex A but blocking the formation of subsequent larger spliceosome complexes. When HeLa and HEK293 cells were treated with up to 30 μM madrasin, pre-mRNA splicing inhibition was observed for several transcripts, ultimately causing cell cycle arrest. As is the case with the other general

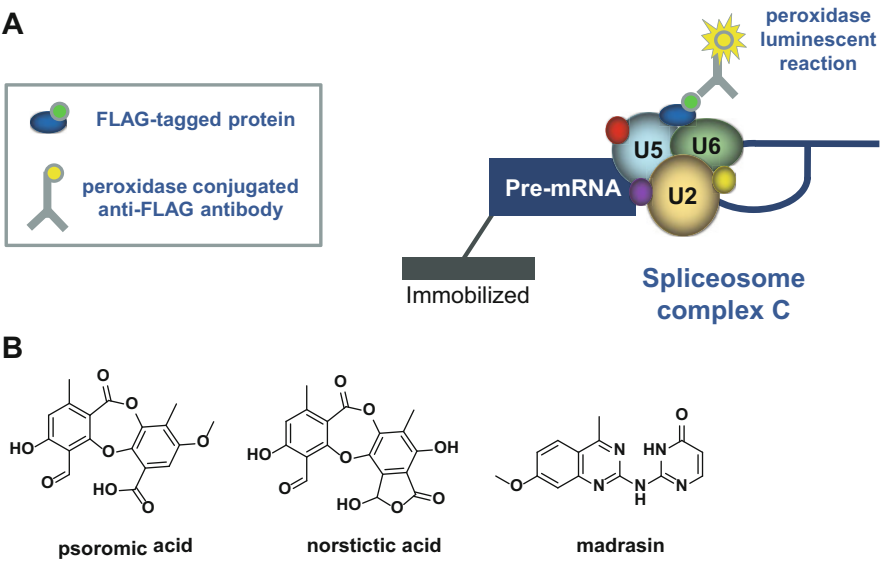

Fig. 25 (**a**) Assay designed to measure the amount of spliceosome complex-C formed nuclear extracts. (**b**) Small molecules that inhibit complex-C formation

splicing inhibitors, madrasin may have limited clinical benefit due to a lack of selectivity.

5 Conclusion

In humans the assembly of most mRNA transcripts requires the precise cleavage and formation of multiple phosphodiester bonds in nascent pre-mRNA polymers. This process of splicing is highly controlled, yet maintains sufficient flexibility to proceed down alternative paths. The combination of control and flexibility presents a unique opportunity for molecular intervention during gene expression. We have described in this chapter various chemical agents that have the capacity to alter the natural process of pre-mRNA splicing, thereby producing different levels of splicing products than found under natural conditions. This technique has powerful therapeutic application where mutation has caused certain splice variants to be under- or over-represented. Additionally, splicing manipulation may be used to bypass a deleterious section of mRNA and to reduce the expression of an undesired gene product.

Over the past several years exceptional advances have been made with SSOs and small molecules, the two leading therapeutic modalities for splicing modulation. SSOs have long been recognized for sequence selectivity, but often knocked for difficulty in administration and tissue penetration. Recent advances in delivery and

penetration-enhancing agents have greatly improved the in vivo efficacy of SSOs. Small molecules, on the other hand, rely on the strength of oral delivery and broad distribution, but traditionally have suffered from the lack of selectivity in splicing biology. Recent discoveries of selective small molecule splicing modifiers in the areas of SMA and FD have begun to challenge this selectivity paradigm. We expect that additional advances in drugging pre-mRNA splicing will be achieved as our understanding of the potential targets and mechanisms increases. A combination of creative design and applied knowledge should lead to promising new drugs in this field in the future.

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