Modulating splicing with small molecular inhibitors of the spliceosome

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Abstract

Small molecule inhibitors that target components of the spliceosome have great potential as tools to probe splicing mechanism and to dissect the splicing regulatory networks in cells. These compounds also hold promise as drug leads for diseases in which splicing regulation plays a critical role, including many cancers. Because the spliceosome is a complicated and dynamic macromolecular machine comprised of many RNA and protein components, a variety of compounds that interfere with different aspects of spliceosome assembly is needed to probe its function. By screening chemical libraries with high-throughput splicing assays, several labs have added to the collection of splicing inhibitors, although the mechanistic insight into splicing yielded from the initial compound hits is somewhat limited so far. In contrast, SF3B1 inhibitors stand out as a great example of what can be accomplished with small molecule tools. This group of compounds were first discovered as natural products that are cytotoxic to cancer cells, and then later shown to target the core spliceosome protein SF3B1. The inhibitors have since been used to uncover details of SF3B1 mechanism in the spliceosome and its impact on gene expression in cells. Continuing structure activity relationship analysis of the compounds is also making progress in identifying chemical features key to their function, which is critical to understanding the mechanism of SF3B1 inhibition. The knowledge is also key for the design of analogs with new and useful features for both splicing researchers and clinicians hoping to exploit splicing as pressure point to target in cancer therapy.

Graphical abstract
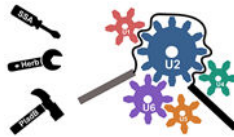
Keywords

Splicing; pre-mRNA; spliceosome; Inhibitor; Alternative splicing

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Introduction

Pre-mRNA splicing is a key regulatory step in gene expression

During eukaryotic gene expression the genetic information in DNA that is transcribed into RNA is discontinuous due to the presence of intron sequences. Pre-messenger RNA (pre-mRNA) splicing is required to remove the introns and join the flanking exon sequences, which creates the open reading frames in mRNA that will be translated into protein. Chemically, splicing is a straightforward sequence of two phosphodiester transfer reactions (Figure 1). The 1st chemical step results in cleavage of the bond at the beginning of the intron (5' splice site) and transfer to 2' OH of an adenosine near the end of the intron (branch point). The 2nd chemical step results in cleavage of the bond at the end of the intron (3' splice site) and transfer to the 3' OH created in the first reaction, which join the flanking exons. These reactions are catalyzed by the spliceosome, a complicated assembly of five small nuclear RNAs (snRNAs) and dozens of proteins.

In addition to the challenge of orienting the phosphodiester bonds at the 5' and 3' splice sites for catalysis and coordinating the two reactions, the spliceosome is responsible for the equally important task of selecting the right pair of splice sites, which are situated 100's to 10,000's of nucleotides apart. A choice of the wrong phosphodiester bond, even by a single nucleotide, will disrupt the final transcript message by creating a frameshift in the open reading frame. Notably, many human genetic diseases are linked to aberrant splicing [1].

In many organisms, the output of splicing is often further elaborated through alternative splicing, which tweaks the protein coding capacity and the stability of the resulting mRNAs by the selective inclusion or skipping of exons, retention of introns, and choice of alternate 5' or 3' splice sites. Alternative splicing adds an important layer of gene regulation that is exploited in many biological pathways including developmental programs, neuron identity and metabolic potential, to name just a few. Regulation of alternative splicing is often described in terms of splicing factors that guide or block spliceosome assembly at particular splice sites. Additionally, competition between splice sites that is influenced by the strength of splice site sequences, the order of their emergence from RNA polymerase II, and the availability of the spliceosome, among other factors, are other themes of regulation.

Spliceosome assembly / function is complicated

Likely reflecting the complexity involved in recognizing a wide variety of intron substrates and alternative splicing regulation, the spliceosome is a complicated macromolecule. It assembles on each intron to be spliced through the addition and rearrangement of the U1, U2, U4, U5 and U6 small nuclear ribonucleoproteins (snRNPs), which along with their snRNAs associate with specific and shared proteins. Dozens of additional proteins also participate in the assembly. The spliceosome is not a static entity and can be modeled as evolving through series of stereotypic intermediate splicing complexes (Figure 1). In a very simplistic view, spliceosome assembly starts when the U1 snRNP recognizes the 5' splice site to form E complex, followed by U2 snRNP addition at the branch point sequence to form A complex. The U4/U6.U5 tri-snRNP is then recruited to form B complex. Next,

structural rearrangements that include ejection of U1 and U4 snRNPs create the catalytic <u>C complex</u>, in which splicing chemistry takes place. For the purpose of this review, we will refer to this simplified model of the spliceosome, but note that characterization of more RNA/RNA and RNA/protein rearrangements have led to additional assembly intermediates to the splicing process (*e.g.* B[act], B*, P complex) [2-4]. These new intermediates point to our increasing understanding of the intricate interactions that take place during splicing, but they likely still represent only a subset of the conformations that the snRNAs and myriad of spliceosome proteins take on in the process.

The dynamics of the spliceosome pose a challenge to detailed mechanistic studies. Furthermore, while the list of spliceosome components is long, the list of known functions for these components is small, in large part because the means to manipulate them is lacking. Small molecules that target individual spliceosome components provide a flexible way to probe their function and uncover new details of spliceosome assembly. In this review, we summarize the identification and characterization of compounds that have been shown to interfere with spliceosome function, which we term splicing inhibitors. We also consider the potential for inhibitors to study the numerous cellular pathways that are impacted by splicing, and as drug leads for diseases in which splicing regulation plays a critical role. Table 1 highlights some of the most pertinent information gathered from a wide literature for representative groups of splicing inhibitors. With a few exceptions, we will not discuss molecules that have been identified to change alternative splicing choices of single genes but do not appear to generally inhibit splicing at the level of the spliceosome. Such compounds presumably target factors that regulate select splicing events upstream of the spliceosome.

Searching for splicing inhibitors

High throughput screens with *in vitro* splicing assays

One approach to finding splicing inhibitors has been through cell-free *in vitro* splicing assays, which can be adapted for high-throughput screening of small molecule libraries. By bypassing other steps of gene expression, use of *in vitro* systems increase the chances of finding direct spliceosome inhibitors. They are also not limited to identifying molecules that can penetrate cell membranes. On the down side, compounds that affect splicing regulation and coordination with other processes, such as transcription, are likely to be missed.

Several labs have identified splicing inhibitors from range of compound libraries by assaying *in vitro* splicing in HeLa nuclear extract, although different strategies were used to measure splicing. Our group used RT-qPCR to detect production of spliced mRNA in the presence of known bioactive compounds and natural products from marine bacteria and characterized four new splicing inhibitors [5, 6]. Three other groups employed enzyme-linked-antibodies to detect different proteins associated with the formation of catalytic spliceosomes on an immobilized pre-mRNA splicing substrate and together screened small molecule libraries ranging from ~2000-70,000 compounds [7-9]. So far, all the compounds identified from the different screens display relatively low potency, with IC_{50} for *in vitro* splicing in the micromolar range. It is possible that the activity of some of these compounds could be improved by structure activity relationship (SAR) approaches, but those efforts have been limited [5]. There is still impetus to continue high throughput screening for splicing inhibitors.

With over 100 components participating in the dynamic assembly pathway, many activities in the spliceosome remain available as small molecules targets, such as the enzymatic function of distinct RNA-dependent ATPases that facilitate structural rearrangements. Furthermore, a huge swath of chemical space that likely includes new spliceosome-targeting compounds still waits to be explored.

High throughput screens with cell-based assays

Cell-based assays have also been used to identify splicing inhibitors in a more physiological setting. Compounds with activity will necessarily have an ability to penetrate cell membranes and make their way to the nucleus to affect splicing. These characteristics are especially important if the compounds will be used *in vivo* or as drug leads. Two groups have developed mini-gene splicing reporters expressed in cells to screen for inhibitors. The Moore lab engineered a HEK293 cell line that expresses luciferase when the intron in the reporter is not removed, with which they identified the natural product isoginkgetin in a library of about 8000 compounds [10]. They showed that isoginkgetin interferes with spliceosome assembly *in vitro*, suggesting a direct effect on splicing. Taking an opposite tact, the Dreyfuss lab created a HeLa cell line that expressed luciferase only when intron in the reporter is removed and found three inhibitors in a screen of more than 23,000 small molecules [11]. However, the compounds did not inhibit splicing of a model pre-mRNA *in vitro*, suggesting that they target a splicing regulator that is specific to the reporter intron. In line with that idea, one compound, chlorhexidine, was shown to inhibit Clks, i.e. the kinases that regulate SR proteins. SR proteins are a family of RNA binding proteins that are controlled by phosphorylation of a domain consisting of extended repeats of Arg/Ser. Several SR proteins are characterized as enhancers of spliceosome assembly at exons and often modulate alternative splicing.

In some cases, searching for molecules that alter splicing of a specific gene leads to compounds that nonetheless target spliceosome components, and in some cases enhance, rather than inhibit splicing. Two groups used luciferase mini-gene reporter based on the SMN2 gene to screen for compounds that selectively promote inclusion of exon 7 [12, 13]. This splicing event has been shown as a potential avenue for treating the disease spinal muscular atrophy (SMA). Both groups identified separate sets of related compounds that enhanced exon 7 inclusion and, notably, also relieved SMA disease symptoms in a mouse model. At the concentrations tested, the compounds are each very selective for a small of set splicing substrates, which suggests that their targets are gene specific splicing factors or regulatory sequences. Remarkably, however, Palacino et al showed that their compounds stabilize U1 snRNP interactions with the 5' splice site of SMN2 exon 7 to affect splicing [13].

Inhibitor Effects on the Spliceosome Are Varied

Interfering with spliceosome assembly

Splicing inhibitors, by definition, block splicing. Their effect can be quantified by measuring the amounts of 1st step and 2nd chemical step splicing products from *in vitro* splicing relative to inhibitor concentration. However, a compound can interfere with any number of spliceosome assembly steps prior to catalysis to confer a loss of splicing chemistry. For

example, they could inhibit enzymatic activity of RNA-dependent ATPases that promote specific rearrangements, block interactions between spliceosome components, hyperstabilize interactions between spliceosome components and or freeze components in non-productive conformations, any of which could stall assembly. Spliceosome assembly can be visualized in native gels by the discrete shifts in mobility of a model pre-mRNA as intermediate complexes form *in vitro* in nuclear extract (E -> A -> B -> C complex) [14, 15]. When an intermediate splicing complex accumulate in the presence of a splicing inhibitor, the list of potential components/steps that the compound may be targeting is limited to some extent (Figure 1). The following discussion of compounds that interfere with early to late assembly stages provides several examples.

A number of splicing inhibitors, including NSC635326 and several related pyrido-carbazoles, benzo-pyrido-indoles, and pyrido-pyrrolo-isoquinolines (termed "indole derivatives") interrupt the earliest stage of spliceosome assembly such that no splicing complex formation is observed [6, 16]. With such an early block, it is not easy to rule out the possibility that the compounds are affecting the HeLa nuclear extract nonspecifically, instead of targeting a specific spliceosome component to elicit splicing inhibition. In the case of some indole derivatives, however, the compounds interact with the SR protein SRSF1 (ASF/SF2). SRSF1 is known to regulate initiation of spliceosome assembly by recognizing enhancer elements in many different exons, which is consistent with the effect of the inhibitor [16].

Many splicing inhibitors identified to date cause spliceosome assembly to stall at A complex, the stage where U2 snRNP joins the branch point sequence. With the exception of the compound madrasin, the inhibitors are natural products with distinct structures. They include isoginkgetin, tetrocarcin A and members of the FR901464, pladienolide B and herboxidiene families [6, 7, 10, 17-21]. Notably, compounds from these three different families have been shown to target the same spliceosome protein, SF3B1, which is a component of the U2 snRNP [17, 22, 23]. We will cover details of SF3B1 inhibitor mechanism in a following section. Whether the function of the natural products in the organisms that produce them has anything to do with splicing is not known, but if so, it suggests that the A complex assembly stage and/or SF3B1 may be a particularly responsive target.

Several splicing inhibitors interfere with late assembly stages. However, the effect is often concentration dependent because increasing drug begins to elicit earlier assembly defects [5, 6, 8, 9]. For example, naphthazarine compounds and psoromic acid at 100 μM both cause assembly to stall at a B/C-like complex, but at 250 μM produce an A-like complex accumulation [6, 8]. Correspondingly, at lower concentrations, the compounds predominantly inhibit 2nd step chemistry, but as concentrations increase, 1st step chemistry is also affected. It is important to note that many of the late spliceosome assembly inhibitors are quinones that are capable of generating reactive oxygen species (ROS), and in some cases their effect can be rescued by adding the reducing agent dithiothreitol (DTT) [6]. Therefore, the inhibitors may not act by simply binding spliceosome components, but instead trigger oxidation of a labile cysteine required for the activity of a spliceosome protein.

Targeting known enzymatic activities

Some compounds that inhibit splicing are also known to target certain classes of enzymes, which points to a potential mechanism of action. For example, in a candidate approach to identifying splicing inhibitors, three histone deacetylase (HDAC) inhibitors, suberoylanili dehydroxamic acid (SAHA), splitomicin, and dihydrocoumarin (DHC), were shown to inhibit splicing *in vitro* and stall assembly at a B-like complex [24]. Three histone acetyltransferase (HAT) inhibitors, garcinol, AA, and BA3, also inhibit *in vitro* splicing, but do not block complex formation at the same stages [24]. Garcinol and AA cause accumulation of an A-like complex, while BA3 stalls at a B-like complex. It is possible that these compounds disrupt the acetylation state of a spliceosome protein that is important for splicing, but a specific target has yet to be identified. In the same vein, ubistatin A, which interferes with ubiquitin protein/protein interactions, inhibits *in vitro* splicing in yeast extracts [25]. A specific ubiquitinated target in the spliceosome is not known, but ubistatin A's effect was linked to the destabilization of the U5.U4/U6 tri-snRNP. With all these compounds, however, relatively high IC_{50} values for splicing (μM to mM range) raises concerns of specificity, and limits their use to *in vitro* model systems.

Some splicing inhibitors are known to target kinases and phosphatases, and may be related to phosphorylation events already linked to multiple stages of spliceosome assembly. For example, changes in phosphorylation of the core spliceosome factors SF3B1 and DDX23 (hPrp28) and the activity of the kinase PRP4K correlate with spliceosome rearrangements [26-30]. As previously noted, SR protein activity is also regulated by phosphorylation [31]. Inhibitors of the kinase activities of topoisomerase I, MNB-506 [32, 33] and the Cdc2-like kinases (Clks) [11] interfere with splicing *in vitro* of particular substrates known to be regulated by SR protein phosphorylation. The compounds, however, do not interfere with splicing of all substrates, which may reflect the function of SR proteins as alternative splicing factors and not necessarily as core spliceosome components.

The phosphatases okadaic acid, tautomycin and microcystin-LR, which are known to inhibit PP1 and/or PP2A, inhibit splicing *in vitro* but do not disrupt spliceosome assembly up to B/C complex formation [34, 35]. Interestingly, their effects on splicing chemistry vary with concentration [34]. At lower concentrations, 1st step chemistry takes place while 2nd step chemistry is lost. As the amounts of inhibitors increase, 1st step chemistry is also lost. These results are consistent with at least two dephosphorylation events regulating both steps of splicing catalysis. Again, if/how *in vitro* splicing inhibition is linked to loss of phosphatase action on specific spliceosome proteins is not known.

Antibiotics as splicing inhibitors

Three compounds described as splicing inhibitors, Cl-tetracycline, streptomycin and erythromycin, are also antibiotics [36]. These drugs are known to bind specific RNA structure in the ribosome and inhibit translation [37]. The spliceosome is also an RNA-dependent enzyme, which could imply a similar mechanism of inhibition. However, the IC_{50} values of the drugs for *in vitro* splicing inhibition are in the high micromolar range, supporting a less specific mode of action. Indeed, tetracycline and streptomycin form complexes with pre-mRNA at high concentration, which interferes with the earliest stages of spliceosome

assembly likely through steric effects [36]. On the other hand, erythromycin does not impede spliceosome assembly and effects 2nd step chemistry most strongly [36]. In the ribosome, erythromycin blocks the exit tunnel of the growing peptide [38, 39], so there may be a similar structure in the spliceosome. Perhaps there is a relationship with the tunnel-like structure that the Moore lab suggested plays a role in 3' splice site selection during 2nd step chemistry [40].

SF3B1 Inhibitors: Powerful Tools to Study Splicing and Beyond

Inhibitors Suggest Multiple Roles for SF3B1 in the Spliceosome

As noted previously, the spliceosome protein SF3B1 was identified as the target of three compounds, pladienolide B, spliceostatin A and herboxidiene, which all strongly inhibit splicing [17, 22, 23]. SF3B1 and six other proteins comprise the SF3B complex, which is necessary for U2 snRNP function in the initiation of spliceosome assembly [41]. Genetic interactions between SF3B and U2 snRNA in yeast suggest that the complex has a role in U2 snRNA structural dynamics involved in early intron recognition at the branch point region [42]. Individually, SF3B1 has been linked to the recruitment and/or stabilization of U2 snRNP at the branch point sequence during A complex formation [43-46]. SF3B1 is a large protein predicted to form an extended set of alpha helical bundles and to have an intrinsically disordered N-terminal domain [47]. SF3B1 interacts with the early spliceosome assembly factor U2AF65 [43, 44] and with another SF3B component called p14, which directly contacts the branch point adenosine [48]. SF3B1 also directly cross-links to the intron upstream and downstream of the branch point sequence coincident with the ATP-dependent stabilization of A complex [45, 46]

As research tools, SF3B1 inhibitors exemplify how small molecule can be used to explore spliceosome mechanism. For example, the Reed lab reported that a pladienolide B derivative (E7107) effects U2 snRNP interactions with a branch point sequence, and concluded that SF3B1 plays a role in mediating a conformational change in U2 snRNP [20]. The Valcarcel lab showed that the fidelity of U2 snRNA interactions with the branch point decreases in the presence of spliceostatin A, suggesting that SF3B1 participates in branch point discrimination [18]. Our lab recently showed that after bypassing the first effects of the drugs, the SF3B1 inhibitors pladienolide B, spliceostatin A and herboxidiene interfere with spliceosome progression after A complex stabilization [49]. We also probed the catalytic stage of splicing by taking the spliceosome through the 1st step chemistry before adding drug, and found that SF3B1 inhibitors block the 2nd step chemistry, *i.e.* exon ligation [49]. These results suggest that there are additional roles for SF3B1 throughout the splicing process. That function may be linked to changes in the affinity of the SF3B complex for U2 snRNP during spliceosome activation and with exon ligation [2, 50, 51].

SF3B1 Inhibitor Links to Cancer

Discoveries from two different lines of cancer research have raised the potential of the splicing machinery as a target for chemotherapeutics. First, common mutations in a number of splicing factors have been identified by whole-exome sequencing of cancer cells [52]. Notably, specific SF3B1 mutations are frequent in malignant cells from patients with

myelodysplastic syndrome (MDS), chronic lymphocytic leukemia (CLL), breast cancer, and pancreatic cancer [53-56]. The presence of the mutations implies that modulation of SF3B1 function perturbs gene expression programs that contribute to cancer, and recent data links SF3B1 mutations to alterations in branch point selection that leads to aberrant 3' splice site choice [12, 57, 58]. Second, as note previously, several of the splicing inhibitors that target SF3B1 were first identified in screens for cell growth inhibitors. With most cancer cells in culture, SF3B1 inhibitors arrest growth at low nanomolar concentrations [17, 22, 23, 59-79]. Not all cell lines are equally sensitive, and members of the sudemycin family, in particular, have been shown to inhibit growth of only a subset of cancer cell lines [65, 70, 72, 80]. Cells derived from normal tissues show more resistance to SF3B1 inhibitors relative to cancer cells [70, 77, 81, 82], and tumor cells in mouse xenografts die at concentrations of these compounds that appear to have no effect on the host animal [70, 76, 81, 83, 84]. Unfortunately, an early clinical trial with the pladienolide B derivative E7107 in human patients was cut short because of side effects related to toxicity [85]. Still, there is therapeutic promise that the compounds can be either directly targeted to tumors or more selectively used to treat specific cancers that show higher sensitivity to the drugs, such a CLL [81-83, 86, 87].

Cellular splicing changes with inhibitors

Because of the essential role that splicing plays in gene regulation, it is not surprising that splicing inhibitors significantly impact cellular function. With many inhibitors, however, a direct link between the inhibitor effects and cellular splicing has not been demonstrated. Without that determination, the off-target effects of low potency compounds are a substantial concern. Again, the SF3B1 inhibitors stand out as highly potent and selective compounds. Where examined, the impact of SF3B1 inhibitors on cells closely parallel those of SF3B1 knockdown, which is consistent with inhibitor effects being indeed mediated by SF3B1 [18, 23, 88, 89]. Furthermore, identification of a point mutation in the protein (R1074H) that conferred resistance to the cytotoxicity of pladienolide B strongly links SF3B1 as the target [90]. We also observed that the activity of SF3B1 inhibitor derivatives for splicing *in vitro* strictly correlates with the phenotypic changes that they produce in cells [19].

Looking specifically at cellular splicing, the influence of SF3B1 inhibitors was first examined on a gene-by-gene basis. Instances of intron retention, aberrant splicing, and alternative splicing changes were all noted in a small collection of gene transcripts [22, 23]. Later, Corrionero et al. used splicing specific microarrays to examine 1800 alternative splicing events in HeLa cells treated with spliceostatin A and found that less than 10% of the monitored splicing events were affected [18]. The authors noted that there were splicing changes in genes associated with cell-cycle regulation and apoptosis, which are pathways both regulated and connected at the level of alternative splicing [91]. That result is consistent with SF3B1 inhibitors ability to inhibit growth of cancer cells in culture [76, 77, 79, 92]. More recently, a number of groups have shown that SF3B1 inhibitor effects can be linked to splicing changes in the apoptosis genes MCL1 [81, 82, 86, 88, 93] and BCL-X [86]. Both of these genes are alternatively spliced such that one isoform encodes a pro-apoptotic protein, while the other encodes an anti-apoptotic protein. [94, 95]. Kashyap et al. reported that pladienolide B and its analog FD-985 promote pro-apoptotic splicing of MCL1 and BCL-X, but that the cellular context is important: CLL cells showed the splicing isoform shifts with treatment,

but normal blood cells did not [86]. Larrayoz et al. also showed MCL1 splicing changes in CLL cells with SSA treatment, and further found that overexpression of the anti-apoptotic MCL1-L isoform in a human Burkitt's lymphoma cell line rescued drug-induced apoptosis [82]. Consistent with these findings, SF3B1 inhibitors show synergism with pro-apoptotic compounds in culture [81, 82, 88].

One question raised by the cellular effects of SF3B1 inhibitors is "How does targeting a core splicing factor only affect splicing of select genes and result in cell-type specific phenotypes?" On its face, it seems that blocking splicing in cells should be equally catastrophic for the expression of every intron-containing gene. Splicing inhibitors would thereby appear to be very blunt tools with limited selectivity for cancer cells *versus* healthy cells. Hints to a resolution of this paradox come from a recent study of the meiotic splicing program in yeast, which pointed to competition between introns as a means to regulate gene expression programs [96]. With that framework in mind, two related circumstances are important to consider for splicing inhibitor effects in cells. 1) The spliceosome does not have a unique substrate, but a large number of competing substrate variants, and 2) the cellular effects of splicing inhibitors are typically evaluated on the basis of cell growth.

Splice sites vary in a number of ways including splicing sequence strength, the existence of alternative splicing choices and the influence of factors that regulate those choices. All of these parameters influence the rate of spliceosome recognition and assembly at different splice sites. Furthermore, the levels a pre-mRNA transcript will influence the likelihood that the splicing machinery will engage particular splice sites. In this context, splice sites that are overall weaker competitors for the spliceosome will be affected first as even low concentrations of a splicing inhibitor begins to limit the amount of active enzyme (Figure 2). With alternative splice sites, which typically have weak splicing consensus sequences, a change in competition would most likely manifest as a switch of splice isoforms, like those seen with MCL1 and BCL-X. In low abundance transcripts, even constitutive splice sites with strong splice sites may no longer compete well, resulting in intron retention. Importantly, a decrease in efficiency of only one splicing event in a transcript could have a big impact on the cells, depending on the function of the resulting mRNA. In the context of cell growth, splicing of genes controlling proliferation or apoptosis will therefore act as the meter by which splicing inhibitors activity is evaluated.

The sensitivity of specific splicing events likely explains the difference in concentration of SF3B1 inhibitors typically required to affect cell growth (low nanomlar) vs. inhibit splicing *in vitro* (high nanomolar). It also can point to differences between cancer vs. normal cells in the relative abundance of different transcripts and their competitiveness for the splicing machinery as a potential foothold for therapeutics. A recent study looking at cancers driven by the Myc oncogene illustrates this point [83]. A decrease in the levels of core spliceosome proteins, including SF3B1, resulted in death of cancer cells expressing activated Myc oncogene relative to cells not driven by Myc. Notably, the presence of activated Myc in several cancer cell lines also correlated with higher sensitivity to sudemycin D6, a SF3B1 inhibitor structurally related to spliceostatin A [83]. Myc activity increases the overall transcription load in cells[97]. More transcripts means more splice sites competing for the splicing machinery, which in combination with decreased spliceosome function, may result

in a lethal splicing change for one or more important genes. Further systematic analysis of the splicing changes induced by SF3B1 inhibitors will be needed to fully determine which introns drive the cellular response to the drugs. In addition to informing therapeutic strategies for splicing inhibitors, the data may also help untangle the intricate web of splicing regulation.

Structure Activity Relationships of SF3B1 Inhibitors

Relationships between SF3B1 inhibitor families

The SF3B1 inhibitors can be classified into three general structural families. The FR901464 [79] family includes spliceostatin A [23], meayamycin[71] and thailanstatins [64]. The architecture of these compounds is based on two highly functionalized tetrahydropyran rings connected by diene chain and differs the most in structure relative to the other two compounds. Members of the pladienolide B family, which includes E7107 and FD-895 [22, 67], are polyketides with a large macrolide ring. The GEX1 family of compounds [77], including herboxidiene, has a similar structure, but with a smaller tetrahydropyran ring. Despite these structural differences, the compounds exhibit nearly identical effects on spliceosome assembly and in cells, presumably by interfering with SF3B1 function. Still, specifically how and where the compounds interact with SF3B1 to interfere with splicing is not currently understood. We recently reported that inactive analogs of pladienolide B, spliceostatin A and herboxidiene, in terms of their ability to inhibit splicing, nevertheless are able to somehow out-compete their active counterparts [49]. Our data are consistent with both active and inactive versions being able to interact with SF3B1, although only the active version impacts SF3B1 activity. Remarkably, we also found that the inactive version of each compound also competes with the active versions of the other two compounds (*i.e.* inactive pladienolide competes with both active spliceostatin A and herboxidiene). This result suggests that all three compounds have the same interaction with SF3B1, despite their different structures, and lends credence to the idea of a common pharmacophore. Still, we likely will need structures of the different inhibitor scaffolds interacting with SF3B1 to know whether a shared pharmacophore exists.

SF3B1 Inhibitor analogs

Structure activity relationship (SAR) studies identify important chemical features by altering individual moieties of the inhibitor structure and testing the resulting derivative for biological activity. If activity is lost, the feature is required, but if the derivative has the same activity as the original compound, the feature is dispensable. Multiple rounds of SAR analysis will hopefully lead to improvements in inhibitor potency, stability, membrane permeability, and simplicity of chemical synthesis. Furthermore, the information can be used to guide addition of functional groups to the inhibitor and generate biological probes. For example, SAR studies guided attachment of biotin and cross-linker moieties that were critical to first identifying SF3B1 as the target of SSA, pladienolide B and herboxidiene [17, 22, 23]. Biotinylated SSA was also used to show that the drug is present in stalled spliceosomes [21]. Addition of a fluorescent label showed that pladienolide B molecule localizes to the nucleus of cells in a SF3B dependent manner [22].

One critical constraint for SAR studies is the availability of a wide range of related compounds to test. In some cases, nature provides related molecules. For example, several naturally occurring analogs of SF3B1 inhibitors show differential activity in cell growth assays [64, 74, 76, 79, 84, 92] and, in some case, *in vitro* splicing [20, 64]. Unfortunately, the access to the compounds is limited, which impedes medicinal chemistry efforts with natural products. In some cases, semi-synthetic approaches in which natural products are chemically modified have also been informative [22, 60]. Still, detailed pharmacological studies of these molecules have not been reported.

Complete chemical synthesis provides the most flexibility in systematically creating derivatives to test specific features of a molecule. However, the complex chemical structures of SF3B1 inhibitors, in particular, make total synthesis very challenging. Nonetheless, several groups successfully generated SF3B1 inhibitors and derivatives for the three main structural families: FR901464 [23, 68, 71, 73, 75, 78, 98-100], pladienolides (including FD-985) [19, 62, 67, 69, 101, 102], and herboxidiene [59, 103, 104]. Taking SAR analysis another step further, the Webb lab compared the structures of FR901464 and pladienolide B to predict common pharmacophore consisting of epoxy and carbonyloxy groups joined and relatively positioned by a diene linker [72]. Based on this scaffold, they synthesized a series of compounds termed "sudemycins" [65, 70, 80, 105]. An optimized compound, sudemycin D6, is in the preclinical development stage and has fewer stereocenters and increased stability relative to the natural products [65].

Assaying SF3B1 inhibitor activity

To build a picture of key and potentially shared features that confer splicing inhibition by SF3B1 inhibitor, we ideally want to directly compare the activity of all the derivatives. The situation is complicated, however, by the different assays used to test compound activity reported in the literature. The majority of studies use growth inhibition or cytotoxicity of cells in culture, which are straightforward and biologically relevant assays [17, 22, 23, 59-79]. However, results for the same compounds vary between different cell lines and assay conditions (*e.g.* [60, 68-70, 79]) and cytotoxicity is, at best, an indirect readout for splicing inhibition. As discussed above, effects of SF3B1 inhibitor in cells hinges on the dependency on specific splicing events, which also vary between cells types. Other cell-based assays used to test SF3B1 inhibitors measured splicing of select endogenous gene transcripts, the morphology of nuclear speckles, which is linked to splicing machinery activity, or a constellation of cellular features to create a cytological profile [18, 19, 22, 23, 59, 65, 66, 69, 71, 80, 106]. With cell-based assays, it is not easy to determine whether differences in activity are due to the effect of the analog on splicing or to properties like solubility, stability and membrane permeability. For the few cases where these characteristics have been reported, there is generally a correlation between activity and stability [64, 68, 75] or permeability [60]. *In vitro* assays bypass some of these issues by more directly measuring splicing activity. Several FR901464, pladienolide B, and herboxidiene derivatives have been tested for effects on *in vitro* splicing in HeLa cell extract [18-21, 23, 49, 62, 71, 99, 104, 107]. Generally, *in vitro* data correlates well with cell-based assay results. One exception is the FR901464 analog spliceostatin E, which is cytotoxic [60] but does not inhibit *in vitro* splicing or cause nuclear speckles to aggregate, as seen with

other SF3B1 inhibitors [99]. The discrepancy may be base on residual activity that cannot be detected with a model splicing substrate, but nonetheless is sufficient to alter splicing of an important gene transcript in cells.

With these issues in mind, in Figure 3 we graphically summarized SAR analysis for FR901464 and pladienolide B inhibitor families based on the relative activity of natural analogs and synthetic derivatives that have been tested. Consistent with a critical function, most modifications of FR901464 removing the C3 epoxy group results in a loss of measurable activity [68, 78]. However, substitution at that position with chlorine reduces cell growth inhibition by only ~2-4 fold, depending on the cell line, which is still in the low nanomolar range [68]. The same substitution also drops *in vitro* splicing inhibition by 10-fold [68]. Changes in the C11-15 tetrahydropyran ring can also reduce potency by several orders of magnitude [68]. An acetyl group at the C4' position (the presumable carbonyloxy of the Sudemycin scaffold) contributes to potency, but it loss still produces low nanomolar cell growth inhibition [68, 78]. The relative conformations of other FR901464 chemical features throughout the molecule are also important, as changes in stereochemistry negatively affects potency by over 100-fold [78, 107]. In contrast, changes in side groups at C1 and C4 in the other tetrahydropyran ring have been shown to improved potency, evidently by improving compound stability in aqueous solution [60, 68, 73]. Remarkably, Koide and co-workers achieved low picomolar potency for cell growth inhibition with the derivative meaymycin B [68].

The epoxide group in pladienolide B at C18-19 also contributes to, but is not required for, activity, because its absence causes up to a five-fold decrease in potency [19, 63]. The presence of the carbonyloxyl group off the C7 position is critical, but several functional groups can be appended to it with out a significant loss of activity [22]. Derivatives of pladienolide B-related compounds indicate that constituents at the C6 position in the macrolide ring also contribute to [63], as well as at the C16/C20 positions in the extended "arm" [19]. Several stereocenters of pladienolide B, such as C11-C12, are not important [19]. Modifying the stereochemistry C16/C17 is the only change identified thus far that improve activity [67].

Conclusion

Small molecules that target different components of the spliceosome can be powerful tools that allow researchers to study such a complex and dynamic macromolecular machine. They also can serve as promising drug leads for treating human disease. SF3B1 inhibitors, in particular, demonstrate that potential through the progress they have fuelled in the combined arenas of medicinal chemistry, cancer biology and splicing molecular biology. The inhibitors are revealing the role of SF3B1 in the spliceosome and showing that affecting splicing in cells is complicated. Rather than targeting specific splicing events, the compounds likely affect the competition between introns for the spliceosome. Fortunately, the introns that lose out when SF3B1 is inhibited are involved in apoptosis and cell cycle control, leading to the death of selective cancer types. Continued SAR analysis will hopefully further improve the SF3B1 inhibitors as research tools and chemotherapeutics. Finally, several other splicing inhibitors have been found by screening and candidate approaches, but they have yet to be fully characterized in terms of their targets and mechanisms of inhibition. Additional SAR

and mechanistic studies of these molecules and expanded screens for new inhibitor compounds hold many opportunities for splicing researchers in the future.

Acknowledgements

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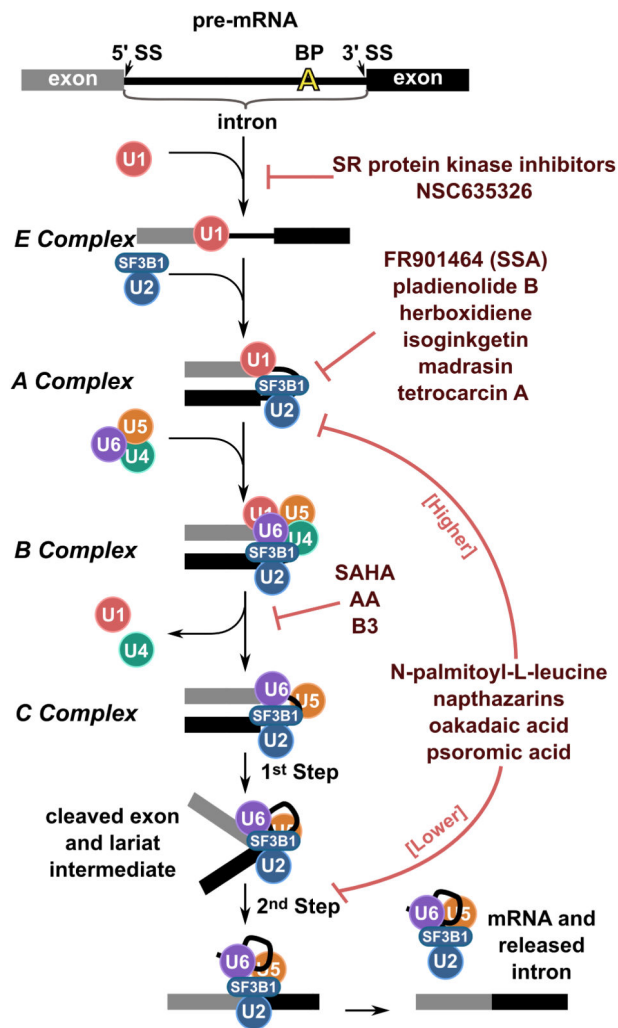
Figure 1. Spliceosome assembly pathway is blocked at different stages by splicing inhibitors
Simplified schematic of the step-wise assembly of a spliceosome on a model pre-mRNA
substrate consisting of two exons flanking an intron. Key splicing sequences are highlighted
for the topmost substrate: 5' splice site (5' SS), branch point sequence (BP) and 3' splice site
(3'SS). In actuality, the U snRNPs (U1, U2, U4, U5 and U6) are dynamic complexes that
each contains a structured RNA and several proteins. The SF3B1 subunit of U2 snRNP,
which is a target of many inhibitors, is indicated. The U snRNPs form several RNA-RNA
interactions with each other and the pre-mRNA to first recognize the splice sites in E and A
complex, before rearranging in B complex to create the catalytically active C complex. After
the 1st and 2nd steps of splicing chemistry take place, the spliceosome disassociates into
released mRNA product and intron lariat complex. Not illustrated are the dozens of
additional protein components of the spliceosome. The assembly transitions affected by
select splicing inhibitors are also highlighted.
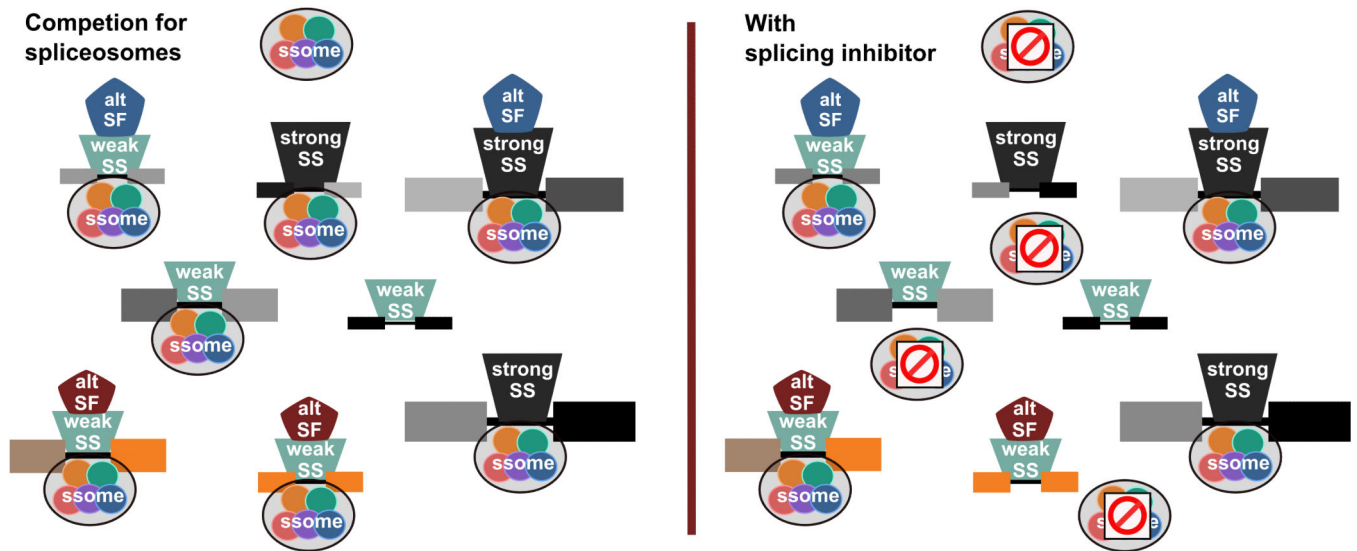
Figure 2. Splicing inhibitors may impact competition between splicing substrates in cells
Different splice sites in pre-mRNA substrates (indicated by differently shaded exons flanking introns) compete for the spliceosome (ssome). Competitiveness is determined by a combination of splice site (SS) sequence strength, the presence of positive alternative splicing factors (alt SF) and total amount of pre-mRNA transcript (indicated by pre-mRNA size). Under normal cellular conditions (left panel) the weakest competitors "lose" for splicing (not engaged by ssome), typically resulting in alternative splicing changes. With splicing inhibitors (red and white "no" sign) additional splice sites will lose for splicing (right panel), resulting in alternative splicing changes and intron retention. Splicing events required for a cells growth (golden pre-mRNAs) will drive the response to the splicing inhibitor. Importantly, the relative competitiveness of an intron will differ in different cell types and under different conditions because the presence of alternative splicing factors differ along with pre-mRNA transcript levels.
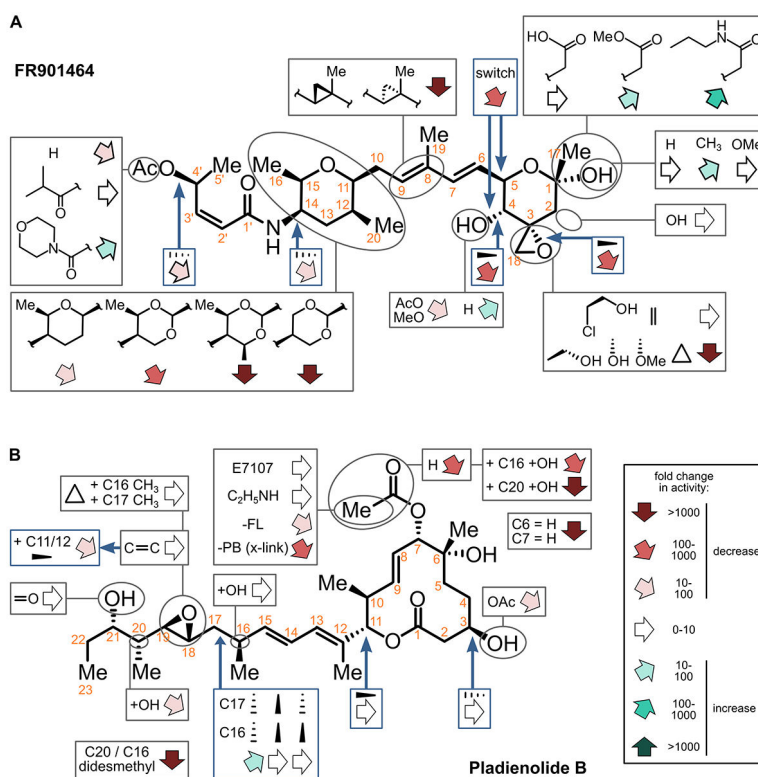
Figure 3. Graphical summary of structure activity relationship studies for two SF3B1 inhibitors
A. FR901464. Functional group changes (in black outlined boxes) are linked by lines to the corresponding positions in the FR901464 structure (circled). Blue arrows point to the position of bond orientations changes (in blue outlined boxes). The effect and relative magnitude of specific changes on the compound's activity is indicated by the open arrow direction and color as indicated in the inset box legend. B. Pladienolide B. Same as panel A.

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Table 1

Splicing inhibitors

Inhibitor class: Prototype and select members	Discovery as splicing inhibitor	~ IC$_{50}$ for *in vitro* splicing / first ssome block	Features of prototype inhibitor	Other activities
FR901464 FR901463/5 spliceostatins A-G thailanstatins A-C meamycins	Spliceosome protein SF3B1 identified as anti-cancer compound target[23]	50 nM – 200 µM; A complex	Targets SF3B1; natural product isolated from FERM BP-3421 (*Burkholderia* sp.); affects cellular splicing in low nM range; SAR data summarized in Fig. 2A; synthetic pathway available	Causes mega-speckles phenotype; cytotoxic at low nM range in multiple cell lines; arrests cell cycle at G1 and G2/M; anti-tumor activity in mouse xenograft model[23],[84]
Pladienolide B pladienolides A-G E7107 FD-895	Spliceosome protein SF3B identified as anti-cancer compound target[22]	0.9 – >200 µM; A complex	Targets SF3B1; natural product isolated from *Streptomyces platensis* Mer-11107; 12-memberedmacrolide; affects cellular splicing in low nM range; SAR data summarized in Fig. 2B; synthetic pathway available	Same as listed for FR901464; derivative E7107 was tested in preliminary clinical trials[85]
Herboxidiene (GEX1A) GEX1Q1-5 RQN-18690A (18-deoxyherboxidiene)	Spliceosome protein SF3B1 identified as anti-cancer compound target[17]	230 – 800 nM; A complex	Targets SF3B1; natural product isolated from *Streptomyces* sp.; polyketide; affects cellular splicing in 1 µM range; SAR data available; synthetic pathway available	Same as listed for FR901464; herbicidal activity[108],[109,110], inhibits angiogenesis
Sudemycins C1, D1, F1, E, D6	Design based on pharmacophore model between FR901464 and pladienolide B[65]	N.D.; N.D.	Targets SF3B1; affects cellular splicing in low nM range of some cell lines; multiple rounds of SAR simplified structure and synthesis for potential drug use/clinical trials; synthetic pathway available.	Cytotoxic at low nM range in multiple cell lines; anti-tumor activity in mouse xenograft model; synergistic effect with some known anticancer drugs[65,81]
Isoginkgetin	HTS with cell-based splicing reporter assay[10]	30 µM; A complex	Natural product found in a variety of plants including *Ginkgo biloba*; biflavonoid; affects cellular splicing in low nM range; inhibits multiple pre-mRNA substrates *in vitro* and major and minor spliceosome in cells	Inhibits tumor cell invasion[111], cyto-protective effects against Ab42-induced toxicity in Alzheimer's disease[112]
Madrasin (DDD00107587)	HTS with *in vitro* splicing assay with enzyme-linked antibody to DDX41[7]	<62.5-150 µM; A complex	Derived by SAR analysis of hit compound from screen; affects cellular splicing at 30 µM; inhibits multiple substrates *in vitro* and in cells (HeLa and HEK293), affects alternative splicing	Cytotoxic; arrests cell cycle; reduces DNA synthesis, disrupts Cajal bodies[7]
Tetrocarcin A (NSC333856)	HTS using *in vitro* splicing assay with RT-qPCR readout[6]	25 µM; A complex	Natural product isolated from the culture broth of *Micromonospora chalcea* KY11091; also inhibits splicing in yeast extracts	Anti-tumor activity[113,114], Cytotoxic in several cell lines; antibiotic activity[115]; promotes apoptosis by blocking BCL2, activating caspase-9 or inhibiting PI3K kinase[116-118]
N-palmitoyl-L-leucine	HTS using *in vitro* splicing assay with RT-qPCR readout[5]	35 µM; B/C complex	Natural product isolated from *Streptomyces* sp. RL10-300-HVF-B (marine sediment sample, Dolan Canyon, CA); inhibits multiple pre-mRNA substrates *in vitro*; tail length most important for activity with head group and absolute orientation also contributing; synthesizable	Impacts HeLa cytological features in 35 µM range[5]
Psoromic acid	HTS using *in vitro* splicing assay with enzyme-linked antibody to Abstrakt[8]	56 µM; B/C complex	Cell-membrane permeable depsidone produced as secondary metabolite by lichens; SAR suggest the functional core is a depsidone containing 3-aldehyde and 4-hydroxy groups	Induces apoptosis in rat hepatocytes, activates caspase 3[119], inhibits Rab geranylgeranyl-transferase[120]

Inhibitor class: Prototype and select members	Discovery as splicing inhibitor	~ IC_{50} for *in vitro* splicing / first ssome block	Features of prototype inhibitor	Other activities
Clotrimazole	HTS using cell-based splicing reporter assay[11]	N.D.	Imidazole derivative; inhibits the major and minor spliceosome in cells but is not a general splicing inhibitor, changes 874 alternative splicing events	IC_{50} 45 μM for cell-based splicing-reporter assay; cytotoxic for several cell lines; antifungal medication; inhibits cytochrome P450; interferes with cellular calcium homeostasis [11]
		N.D.		
NSC635326	HTS using *in vitro* splicing assay with RT-qPCR readout[6]	50 μM	Indole derivative from an NCI library; SAR shows that presence and orientation of nitrophenyl ring is important	
		H/E complex		
Napthazarin: NSC659999 BN82865 NSC95397	HTS using *in vitro* splicing assay with RT-qPCR readout[6]	10 – 20 μM	Naphthazarin derivative from an NCI library; inhibition of 2nd step is more sensitive than inhibition of 1st step; inhibits yeast splicing; mechanism involves modification of redox-sensitive cysteine(s) by reactive oxygen species; SAR suggests nahthazarin scaffold is important; similar compounds include naphthoquinone derivatives	Suppresses tumor growth; NSC95397 inhibits CDC25 dual-specificity phosphatase [121, 122]
		B/C complex		
Translation Inhibitors: Erythromycin Cl-tetracycline streptomycin	Candidate-approach screen using *in vitro* splicing assay [36, 123]	160 – 230 μM	Natural product produced by *Saccharopolyspora erythraea*, macrolide; likely inhibits indirectly by non-specific binding to pre-mRNA; commercially available	Antibiotic; translation inhibitor; binds ribosome peptide exit tunnel [38, 39]
		B/C complex		
HDAC inhibitors: SAHA Splitomicin DHC	Candidate-approach screen using *in vitro* splicing assay [24]	1.5 – 2 mM	Synthetic hydroxamic acid suberoylanilide derivative; inhibits major and minor spliceosome *in vitro*, stalled complexes are functional intermediates; commercially available	Inhibitor of Zn^{2+}–dependent classes I and II of HDACs; antineoplastic activity; used for treatment of cutaneous T cell lymphoma [124, 125]
		B complex		
HAT inhibitors: Garcinol AA BA3	Candidate-approach screen using *in vitro* splicing assay [24]	25 – 500 μM	Polyisoprenylated benzophenone derivative isolated from *Garcinia indica*; inhibits major and minor spliceosome *in vitro*, stalled complexes are functional intermediates; commercially available	Inhibitor for the p300/CBP and Gcn5/PCAF families of HATs[126, 127]
		A complex B complex B complex		
PP1/PP2A inhibitors: Okadaic acid Tautomycin microcystin–LR	Testing importance of Ser/Thr protein phosphatases in splicing [34]	<1 μM	Ser/Thr phosphatase inhibitor; polyketal fatty acids produced by several dinoflagellates; addition of purified catalytic PP1 or PP2A subunits restores splicing; commercially available	
		B/C complex		
SR Protein Kinase inhibitors: NB-506 diospyrin Chlorhexidine TG003	Testing importance of DNA topoisomerase I phosphorylation of SR proteins in splicing[32]	50 – <100 μM	DNA topoisomerase I kinase function inhibitor; 13-/V-glucopyranosyl and 6-IV-formylamino derivative of BE-13793C; isolated from *Streptoverticillium* sp.; affects cellular splicing at 1 μM; inhibits SF2/ASF phosphorylation; synthesizable	Anti-tumor activity in mouse xenograft model [128]; Chlorhexidine and TG003 are Clk family of SR protein kinase inhibitors [11, 129]
		H/E complex		
Ubistatin A	Testing importance of ubiquitination in splicing[25]	<15 mM	Small molecule from a NCI library; decreases U4/U6 unwinding time ~10-fold	Inhibits proteasome-dependent degradation of ubiquitinated proteins[130]
		N.D.		
G5	Candidate-approach using *in vitro* splicing	0.8 mM (in yeast extract)	Oxaspiro compound; SAR shows requirement of spirolactone ring and contribution of spirolactone	Oxaspiro compounds have antibacterial, insecticidal, tumoricidal, and anti-inflammatory activities[123, 131]

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Inhibitor class: Prototype and select members	Discovery as splicing inhibitor	~ IC_{50} for *in vitro* splicing / first ssome block	Features of prototype inhibitor	Other activities
	assay in yeast extract[123]	B/C complex	structure with larger R1 groups improve splicing inhibition	