Title
Dissecting spliceosome function with small-molecule inhibitors

Permalink
https://escholarship.org/uc/item/4ht1998w

Author
Effenberger, Kerstin

Publication Date
2015

License
CC BY-NC-ND 4.0

Peer reviewed|Thesis/dissertation
DISSECTING SPLICEOSOME FUNCTION WITH SMALL-MOLECULE INHIBITORS

A dissertation submitted in partial satisfaction of the requirement for the degree of

DOCTOR OF PHILOSOPHY

in

MOLECULAR, CELL, AND DEVELOPMENTAL BIOLOGY

by

Kerstin A. Effenberger

June 2015

The Dissertation of Kerstin A. Effenberger is approved:

Professor Melissa S. Jurica, Chair

Professor Manuel Ares, Jr.

Professor R. Scott Lokey

Tyrus Miller
Vice Provost and Dean of Graduate Studies
# Table of contents

## Table of contents

1. List of figures ............................................................................................................................. viii
2. List of supplementary figures ........................................................................................................ viii
3. List of tables ................................................................................................................................. ix

## Abstract ................................................................................................................................. x

## Acknowledgements ..................................................................................................................... xii

## Chapter I: Introduction ................................................................................................................... 1

1. Goal of this dissertation ............................................................................................................... 1
2. Current state of the field ............................................................................................................... 1
   2.1. Introns are removed by two consecutive transesterification reactions .................. 1
   2.2. The spliceosome assembles stepwise in an ordered process ................................. 4
   2.3. The dynamic nature of the spliceosome makes it adjustable ................................. 5
   2.4. Only few tools are available to study the spliceosome ............................................. 6
   2.5. Strategies to identify new small-molecule inhibitors of the spliceosome .......... 9
   2.6. Pre-mRNA splicing is linked to cancer ....................................................................... 11
   2.7. Splicing inhibitors hold the potential to expand the currently limited
       mechanistic understanding of the spliceosome ................................................................. 16
3. Contributions of this dissertation ............................................................................................... 17
Chapter II: A high throughput chemical screen identifies new classes of inhibitors of human and yeast spliceosomes.................................19

1. Abstract................................................................................................................. 19
2. Introduction ........................................................................................................... 20
3. Results .................................................................................................................. 22
   3.1. RT-qPCR assay to screen for inhibitors of in vitro splicing ...................... 22
   3.2. High-throughput screen for splicing inhibitors ........................................... 25
   3.3. Candidate compounds inhibit human and yeast pre-mRNA splicing ........... 25
   3.4. C1 stalls spliceosome assembly early in human and yeast extracts .......... 28
   3.5. C3 causes accumulation of a B-like complex .............................................. 31
   3.6. C3 activity is related to redox potential in HeLa extracts ......................... 33
   3.7. C2 inhibits spliceosome assembly early in human extracts ....................... 36
4. Discussion ............................................................................................................. 38
5. Supplemental Material .......................................................................................... 41
   5.1. Supplementary Figure 1 .................................................................................. 41
   5.2. Supplementary Figure 2 .................................................................................. 42

Chapter III: Coherence between cellular response and in vitro splicing inhibition for the anti-tumor drug pladienolide B and its analogs.........43

1. Abstract.................................................................................................................. 43
2. Introduction ............................................................................................................. 44
3. Results .................................................................................................................... 46
   3.1. Synthesis of PB structural analogs ................................................................. 46
   3.2. Structural requirements for PB splicing inhibition in vitro ......................... 48
3.3. Cellular changes produced by PB and analogs correlate with
splicing inhibition ................................................................. 52
3.4. Nuclear speckle morphology changes with PB treatment .............. 55
3.5. Impact of PB modifications on splicing in cells .................................. 58
4. Discussion ........................................................................................................... 61
5. Supplemental material .................................................................................... 66
   5.1. Structure of PB analogs as determined by NMR ......................... 66
   5.2. Structure-activity studies for FR901464, SSA, and key derivatives .... 66
   5.3. Evaluation of in vitro and cellular effects of the putative splicing inhibitor
        spliceostatin E ..................................................................... 70
   5.4. Determining the importance of the C5 hydroxyl group stereo-chemistry for
        the putative splicing inhibitor GEX1Q1 ........................................ 72

Chapter IV: Structurally distinct SF3B1 inhibitors bind the same site and
extend SF3B1 function in the spliceosome ............................................ 75
1. Abstract................................................................................................................. 75
2. Introduction ......................................................................................................... 75
3. Results .................................................................................................................. 79
   3.1. Inactive analogs compete with SF3B1 inhibitors ......................... 79
   3.2. Three distinct SF3B1 inhibitors bind to the same site ................. 81
   3.3. SF3B1 inhibition is independent of branch point sequence in vitro .... 83
   3.4. SF3B1 activity is required after stable A complex formation ............. 87
   3.5. SF3B1 inhibitors block exon ligation ............................................. 90
4. Discussion ......................................................................................................... 92
5. Supplemental Material ........................................................................................................ 97
  5.1. Supplementary Figure 1 ............................................................................................... 97
  5.2. Supplementary Figure 2 ............................................................................................... 98

Chapter V: Summary, impact, and future directions .............................................................. 99

Chapter VI: Methods and materials ...................................................................................... 103
  1. *In Vitro* splicing reactions ............................................................................................. 103
  2. Bi-molecular exon ligation ............................................................................................... 103
  3. Denaturing gel analysis .................................................................................................... 104
  4. Native gel analysis ........................................................................................................... 104
  5. snRNA-depletion of HeLa nuclear extracts ..................................................................... 105
  6. High-throughput screening ............................................................................................. 105
     6.1. RT-qPCR reagents ..................................................................................................... 105
     6.2. High-throughput splicing assay ................................................................................ 106
     6.3. RT-qPCR analysis ..................................................................................................... 106
     6.4. Z’ calculation ............................................................................................................ 107
  7. Synthesis of SSA, BP, and HB structural analogs ............................................................. 107
  8. Cytological profiling ....................................................................................................... 108
  9. Immunostaining for nuclear speckle analysis .................................................................. 108
 10. Monitoring endogenous splicing changes by semiquantitative RT-PCR ..................... 109
Appendix ..........................................................................................................................110

1. Detailed NMR data for different PB analogs ...................................................................... 110

2. Notes Chapter II: A high-throughput splicing assay identifies new classes of inhibitors of human and yeast spliceosomes ............................................................................. 112

3. Notes Chapter III: Coherence between cellular responses and \textit{in vitro} splicing inhibition for the anti-tumor drug pladienolide B and its analogs ....................................................... 113

References ..........................................................................................................................116
1. List of figures

Figure I-1. Pre-mRNA splicing is an essential part of human gene expression .......... 3
Figure I-2. Pre-mRNA splicing is closely related to cancer ................................. 15
Figure II-1. High-throughput approach to identify splicing inhibitors .................. 23
Figure II-2. Three compounds inhibit splicing chemistry dose-dependent ............ 26
Figure II-3. C1 stalls human and yeast spliceosomes at an A-like complex .......... 29
Figure II-4. A B-like complex accumulates in the presence of the C3 compound .. 32
Figure II-5: Inhibition by naphthazarins is partially rescued by excess DTT ............ 35
Figure II-6. A nitrophenyl ring is important for splicing inhibition by C2 ............ 37
Figure III-1. Impact of PB analogs on in vitro splicing ..................................... 49
Figure III-2. Native gel analysis of spliceosome assembly .................................. 51
Figure III-3. Cellular effects of PB and analogs ............................................... 54
Figure III-4. Changes in nuclear speckle morphology ....................................... 57
Figure III-5. Splicing changes in cells treated with PB analogs ........................... 60
Figure IV-1. Chemical structures of compounds used in this study ..................... 77
Figure IV-2. SSA, PB, and HB bind to the same site on SF3B1 ............................ 82
Figure IV-3. Branch point region and PYT do not correlate with drug-sensitivity .. 86
Figure IV-4. Inhibitors interfere with assembly after stable A complex formation. ... 89
Figure IV-5. Bimolecular assay shows that PB inhibits exon ligation ................... 91
Figure IV-6. Model of SF3B1 function in the spliceosome cycle .......................... 92
2. List of supplementary figures

Suppl. Figure II-1. The RT-qPCR assay has a broad dynamic range and specifically detects mRNA produced by \textit{in vitro} splicing in HeLa nuclear extract ..... 41
Suppl. Figure II-2. Quantification of first and second step splicing efficiency \textit{vs.} inhibitor concentration for the splicing reactions shown in Figure II-2A ................. 42
Suppl. Figure III-1. Structures of the PB analogs as determined by NMR .............. 66
Suppl. Figure III-2. Impact of FR901464/ SSA analogues on \textit{in vitro} splicing .......... 68
Suppl. Figure III-3. Impact of SSE analogues on \textit{in vitro} splicing ....................... 71
Suppl. Figure III-4. Impact of GEX1Q1 analogues on \textit{in vitro} splicing............... 74
Suppl. Figure IV-1. Splicing efficiency decreases with weak branch point region or shorter polypyrimidine tract................................................................. 97
Suppl. Figure IV-2. Splicing and rescue in snRNA-depleted nuclear extracts .......... 98

3. List of tables

Table I-1. Overview of known splicing inhibitors, sorted by \textit{in vitro} potency .......... 8
Table I-2. SF3B1 mutations in cancer........................................................................ 12
Table III-1. Chemical structure of compounds tested in this study .................... 47
Abstract

Kerstin A. Effenberger

Dissecting spliceosome function with small-molecule inhibitors

In eukaryotes, a crucial step in gene expression is pre-mRNA splicing by the spliceosome. The spliceosome is a macromolecular machine that removes intervening intron sequences from over 95% of human transcripts to create a functional template for protein synthesis. Precise splicing is essential for correct gene expression, and mutations in the spliceosome are associated with diseases including cancer. Mass spectrometry has identified over 100 spliceosome proteins, but deciphering the function for the vast majority of them has been challenging due to the highly dynamic and complex nature of the spliceosome. My dissertation focuses on small-molecule inhibitors as tools to expand the currently limited mechanistic understanding of the spliceosome:

First, I developed a high-throughput assay to rapidly and efficiently screen large compound libraries for small molecules that inhibit splicing. I found three new splicing inhibitors and determined their effect on spliceosome assembly and splicing chemistry. Second, I used synthetic inhibitor analogs for structure-activity studies to identify chemical groups that are responsible for compound activity. I found that the same structural features are required for in vitro and in vivo splicing inhibition, and that the cellular response was mainly due to inhibition of the spliceosome. Third, I used biochemical assays to show that three structurally distinct inhibitors bind to the same site of the spliceosome core protein SF3B1, and that SF3B1 has a functional
role throughout the multi-step splicing process. SF3B1 is of particular interest because it is often mutated in cancer, which makes it a promising target for the development of novel chemotherapeutics.

My work provides a fast, reliable assay to identify small-molecule splicing inhibitors, a series of in vitro and in vivo assays to characterize their effect on complex assembly, splicing chemistry, and cellular phenotype, and an excellent example of how inhibitors can be utilized to decipher the function of spliceosome proteins. In addition to expanding the mechanistic model of one of the most complex macromolecular machines in the cell, identifying the function of individual spliceosome parts in healthy situations is the necessary first step to determine how changes of these functions in aberrant situations can lead to cancer.
Acknowledgements

I am grateful for the support and encouragement from the following people. Professor Melissa Jurica has been all I could possibly ask for in an advisor and mentor. I thank her for supporting my research and allowing me to grow as a scientist, for encouraging and training me, and for her guidance, advice, and excitement. Thanks also goes to present and past members of the Jurica lab, especially Dr. Janine Ilagan, Beth Prichard, Veronica Urabe, Andrew MacRae, Dr. Patricia Coltri, and Dr. Gabriel Roybal, for helping me master the art of splicing, providing insightful (scientific) discussions and reassurance, and making the Jurica lab a welcoming, friendly workplace. I also appreciate the help and advice from the members of my dissertation committee, Professor Manny Ares and Professor Scott Lokey, who have given me new perspectives and different points of view on my research. Finally, I want to thank Nicolas Weber for all his unlimited support, reassurance, and patience, and my family for encouragement and chocolate care packages throughout my graduate years.

This dissertation contains previously published material as specified in the Appendix and was supported by the Paul and Anne Irwin Graduate Fellowship in Cancer Research.
Chapter I: Introduction

1. Goal of this dissertation

Pre-mRNA splicing by the spliceosome is an essential step in human gene expression. Despite the fact that over 95% of all human genes have to be spliced to produce a functional protein, we do not have a detailed mechanistic understanding of the responsible macromolecular machine. This is in part because of the high complexity and dynamic nature of the spliceosome with over 100 proteins and five RNAs being involved.

My dissertation focuses on reaching two goals: First, I wanted to stall the constantly moving and rearranging spliceosome as a first step for further in depth functional and structural analyses. Second, I wanted to determine the function of individual spliceosome components to expand the mechanistic understanding of the machine. I used a combination of high-throughput screening, structure-function relationship analysis, biochemical methods, and in vitro and cell-based assays to reach these goals.

2. Current state of the field

2.1. Introns are removed by two consecutive transesterification reactions

Pre-mRNA splicing is an essential step of eukaryotic gene expression, and thus a crucial part of the central dogma (Figure I-1A). During gene expression, DNA is first transcribed into a precursor mRNA (pre-mRNA), which is 5’ capped and 3’
poly-adenylated. Then, during splicing, intervening intron sequences are removed and the remaining exons are ligated. The mature mRNA is transported into the cytoplasm and translated into a protein. Since over 95% of human transcripts require splicing in order to function as a template for protein synthesis, intron removal and exon ligation need to be precise on a base-by-base level, because even a single nucleotide mistake would lead to an alteration of the open reading frame and thus to a non-functional protein.

Chemically, splicing involves two well-understood $S_N2$ transesterification reactions (Figure 1-1B). To remove an intron, first the 2' hydroxyl group of a highly conserved adenosine, the so-called branch point A, functions as a nucleophile and attacks the phosphodiester bond at the 5' splice site at the 5' end of the intron. This reaction results in a covalent lariat-3’ exon intermediate and a free 5’ exon. Second, the 3’ hydroxyl group of the free 5’ exon attacks the phosphodiester bond at the 3’ splice site at the 3’ end of the intron, which results in ligation of the two exons and a free lariat-intron. Since two identical chemical bonds are broken and reformed, the splicing reaction itself is energy neutral, but the machine that catalyzed the process needs ATP for assembly and extensive rearrangements (Wahl et al. 2009; Will and Lührmann 2011).
Figure I-1. **Pre-mRNA splicing is an essential part of human gene expression.**

(A) Overview of eukaryotic gene expression. In the nucleus, DNA gets transcribed into pre-mRNA, modified by addition of a 5’ cap and a 3’ poly(A) tail, and spliced. The resulting mature mRNA gets transported into the cytoplasm and translated into protein. For RNA, exons are represented as boxes and intron are represented as lines. (B) The two steps of splicing chemistry. First step intermediates are free 5’ exon and lariat-3’exon, and second step products are mRNA and free lariat-intron. GU, 3’ splice site; A, branch point adenosine; AG, 5’ splice site; OH, nucleophilic hydroxyl group. (C) Alternative splicing expands the proteome. Depending on cell type and developmental status, mature mRNAs contain different exons and thus encode for different proteins. (D) Stepwise spliceosome assembly. The circles represent snRNPs and are color coded as U1 snRNP, dark blue; U2 snRNP, red; U4 snRNP, light blue; U5 snRNP, yellow; U6 snRNP, orange.
2.2. The spliceosome assembles stepwise in an ordered process

Splicing is catalyzed by the spliceosome, a large macromolecular machine that consists of over 100 proteins and five uridine-rich small nuclear RNAs (snRNAs) (see Cvitkovic and Jurica 2013 for an overview). The main components are five so-called small nuclear ribonucleoproteins (snRNPs), which are pre-assembled and consist of an snRNA and associated proteins: U1, U2, U4, U5, and U6 snRNP. The spliceosome also contains many additional proteins, often with regulatory function (Wahl et al. 2009).

On each intron that will be removed, the spliceosome dynamically assembles de novo through a series of intermediate complexes, which are mainly characterized by their snRNP composition and their ability to be separated on native gels (Figure I-1D, reviewed in Wahl et al. 2009). First, U1 snRNA base pairs with the 5′ splice site to form early or E complex. Next, U2 snRNP gets recruited to the branch point region and the interaction gets stabilized by at least one ATP-dependent step, forming A complex. After that, the so-called U5:U4/U6 tri-snRNP joins the spliceosome to form the fully assembled B complex. A series of ATP-dependent rearrangement steps catalytically activate the spliceosome, which results in the dissociation of U1 and U4 snRNP from the spliceosome. This reorganization allows for the first step of splicing chemistry and results in the catalytic or C complex that contains the first step intermediates lariat-3′ exon and free 5′ exon. Post-catalytic P complex forms after exon ligation and before the mature mRNA are released (Ilagan et al. 2013). The whole process is also called the spliceosome cycle, because the individual building blocks are reused to remove the next intron.
In addition to the complexes described above (E \rightarrow A \rightarrow B \rightarrow C \rightarrow P), there are variations like B^\text{act} (Bessonov et al. 2010) or B^* (Ohrt et al. 2012) that contain the same snRNP composition and are indistinguishable on native gels. These complexes vary in protein composition and/or are characterized by key rearrangement steps like changes in RNA-RNA interactions. Furthermore, there are likely many more intermediates states that are currently unknown and cannot yet be captured or analyzed (Jurica 2008). Although more and more information emerge about the dynamic spliceosome architecture, many open questions remain. These questions include: How exactly do spliceosome components rearrange? How is the pre-mRNA positioned? How are the intermediates held in place? Is RNA the only catalytic component? How does the active site change after the first chemical step?

2.3. The dynamic nature of the spliceosome makes it adjustable

The dynamic and complex nature of the spliceosome allows for high flexibility, which makes the machine adaptable and allows regulation. These abilities are especially important in the cellular context, where often one pre-mRNA substrate can be alternatively spliced to generate multiple different mRNAs consisting of different exons (Figure I-1C). The specific pattern can vary from cell type to cell type, and can be regulated throughout development, which results in the translation of multiple proteins from the same pre-mRNA and greatly expands the proteome (Nilsen and Graveley 2010).

On the other hand, the highly dynamic nature of the spliceosome stymies detailed structural and functional analyses and makes the spliceosome a challenging research target. Most methods in molecular biology are based on averaging
populations, which in case of the spliceosome leads to mixing different intermediates states. To prevent assaying spliceosomes at different assembly stages, the complexes need to be stalled at a particular conformation and purified. But even with this extra step, the high dynamic nature of the spliceosome along with potential “contamination” with different assembly stages only led to relatively low-resolution models, which is in contrast to other molecular machines of the central dogma like RNA polymerase or the ribosome. Also, while the identity of the proteins in different spliceosome complexes was determined by mass spectrometry, functional information for the vast majority of these proteins is not available, mainly due to the lack of adequate tools.

2.4. Only few tools are available to study the spliceosome

There are several ways to stall, accumulate, and purify different spliceosome intermediates. For example, the pre-mRNA substrate can be mutated to prevent the spliceosome from moving to the next intermediate complex, which generates a more homogenous population for structural analysis (Jurica et al. 2002). Using this method, spliceosomes were accumulated after the first step of splicing chemistry (C complex) by mutating the 3’ splice site, which lead to a ~30 Å resolution 3D model (Jurica et al. 2004). Similarly, shortening of the 3’ exon leads to accumulation of spliceosomes after the second step of chemistry (P complex) (Ilagan et al. 2013), and using substrates with a short polypyrimidine tracks and no 3’ splice site stall assembly before the first step of chemistry (B act) (Bessonov et al. 2010). Protein composition and overall structure of both purified B act and P complexes was determined by mass spectrometry and electron microscopy, respectively. While these methods add much
needed structural details to the spliceosome, they alone cannot identify the functions of individual proteins.

Another way to lock the spliceosome in a certain conformation is the use of small-molecule inhibitors, similar to the use of antibiotics to accumulate ribosomes in various stages of protein synthesis. This approach is promising for the spliceosome as well, especially because the great diversity and dynamic nature of the complex likely allows for multiple ways to lock a conformation (Jurica 2008). For example, small molecules could inhibit an enzyme, block protein-protein, protein-RNA, and RNA-RNA interactions, or prevent a protein modification. Inhibitors that target specific proteins are likely also tools to decipher protein function and increase our mechanistic understanding of the spliceosome. Currently, there are only few spliceosome inhibitors known (see Table I-1 for an overview), most of which are not commercially available to researchers, so there is great need to identify additional inhibitors.
Table I-1. Overview of known splicing inhibitors, sorted by in vitro potency. In vitro IC$_{50}$, compound concentration that inhibits 50% splicing in an in vitro assay system; ND, not determined. For explanation of the stalled complexes, see Figure I-1D.

<table>
<thead>
<tr>
<th>Compound</th>
<th>In vitro IC$_{50}$ (µM)</th>
<th>Stalled complex</th>
<th>Commercial?</th>
<th>Synthesis?</th>
<th>Notes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spliceostatin A</td>
<td>~0.07</td>
<td>A-like</td>
<td>No</td>
<td>Yes</td>
<td>Anti-tumor activity</td>
<td>Kaida et al. 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Targets SF3B1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Related compounds:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FR901464, meayamycin</td>
<td></td>
</tr>
<tr>
<td>Pladienolide B</td>
<td>~0.09</td>
<td>A-like</td>
<td>Yes</td>
<td>No</td>
<td>Anti-tumor activity</td>
<td>Kotake et al. 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Targets SF3B1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Related compounds:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pladienolides A + C-G, E7107, FD-895</td>
<td></td>
</tr>
<tr>
<td>Herboxidiene</td>
<td>~0.23</td>
<td>A-like</td>
<td>No</td>
<td>Yes</td>
<td>Anti-tumor activity</td>
<td>Hasegawa et al. 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Targets SF3B1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Related compounds:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GEX1Q1-5</td>
<td></td>
</tr>
<tr>
<td>Sudemycin C1</td>
<td>~0.5</td>
<td>ND</td>
<td>No</td>
<td>Yes</td>
<td>Anti-tumor activity, modulates alternative splicing</td>
<td>Fan et al. 2011</td>
</tr>
<tr>
<td>Thailanstatin A</td>
<td>~0.65</td>
<td>ND</td>
<td>No</td>
<td>No</td>
<td>Anti-cancer activity</td>
<td>Liu et al. 2013a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Targets Thailanstatin B, C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Related compounds:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Thailanstatin B, C</td>
<td></td>
</tr>
<tr>
<td>BN82685</td>
<td>~7</td>
<td>C-like</td>
<td>Yes</td>
<td>No</td>
<td>Blocks 2nd step chemistry</td>
<td>Berg et al. 2012</td>
</tr>
<tr>
<td>Isoginkgetin</td>
<td>~30</td>
<td>A-like</td>
<td>No</td>
<td>No</td>
<td>Anti-tumor activity</td>
<td>O’Brien et al. 2008</td>
</tr>
<tr>
<td>Psoromic acid</td>
<td>~56</td>
<td>B-like</td>
<td>No</td>
<td>No</td>
<td>Might stall after spliceosome activation</td>
<td>Samatov et al. 2012</td>
</tr>
<tr>
<td>SAHA (Vorinostat)</td>
<td>~1,500</td>
<td>B-like</td>
<td>Yes</td>
<td>Yes</td>
<td>HDAC-inhibitor</td>
<td>Kuhn et al. 2009</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>~1,800</td>
<td>ND</td>
<td>Yes</td>
<td>Yes</td>
<td>Broad-range protein kinase inhibitor</td>
<td>Aukema et al. 2009</td>
</tr>
</tbody>
</table>
2.5. Strategies to identify new small-molecule inhibitors of the spliceosome

There are two approaches to identify new small-molecule inhibitors of pre-mRNA splicing. The first possibility is to use high-throughput screening to test hundreds or even thousands of compounds in a fast and efficient way for their ability to interfere with splicing. Screening can take place both in cells or in an in vitro system.

To identify spliceosome inhibitors in cells, a common approach is the use of a reporter that produces an easily quantifiable readout like the luciferase protein. The Moore lab developed such a genome-integrated construct where the start codon of the luciferase open reading frame is separated from the rest of the coding sequence by an intron (O'Brien et al. 2008). Normal splicing removes the intron, leading to a frame shift and non-functional luciferase, whereas splicing inhibition results in an mRNA that can be translated into luciferase. The assay was used successfully to screen ~8,000 compounds and identified the natural product isoginkgetin as splicing inhibitor.

Common problems with cell-based assays include labor-intensive engineering of cell lines and often multi-day periods for cell growth, which reduced the high-throughput capability. Also, small molecules might not penetrate the cell membrane, and inhibitory effects might take hours to be detectable. Additionally, there is always the possibility of off-target effects on other processes during gene expression.
Alternatives to cell-based screens include *in vitro* assay systems that use splicing extracts and allow for a more direct measurement of spliceosome activity. One such assay was developed in the Dreyfuss lab and quantifies splicing efficiency by using an antibody specific to a protein complex that gets deposited onto mRNA after successful exon-exon ligation (Berg et al. 2012). Compared to uninhibited reactions, the fluorescent signal from the antibody is reduced when splicing is stalled. Screening of ~2,100 compounds revealed multiple related quinones to be splicing inhibitors.

The second approach to identify splicing inhibitors is to take advantage of specific functional information about individual spliceosome proteins. For example, if a protein has an (predicted) enzymatic function, a limited set of known inhibitors for that function could be tested for the ability to also inhibit splicing. There are successful examples using kinase inhibitors (Aukema et al. 2009) or protein acetylation/deacetylation inhibitors (Kuhn et al. 2009) to interfere with splicing. However, in both cases the inhibition was early in the spliceosome cycle and required un-physiological high concentrations of the inhibitors, raising the concern of an indirect effect. Also, this approach limits the search to known enzymatic activities within the spliceosome and does not take advantage of rearrangements, blocking interactions between components, or novel enzymatic activities.

Finally, for any small-molecule inhibitors that can be chemically synthesized, there is the possibility of structure-activity analysis. In these studies, the initial hit is the so-called lead compound that serves as the starting point for synthetic tweaking and modification with the goal to improve activity, chemical properties like stability,
and physiological activities like cell membrane permeability. Structure-function studies might also simplify the synthesis pathway and point out regions of the molecule that can be modified as biological probes, for example by attaching affinity tags or fluorescent probes to the molecule. One example for that process is a class of compounds called sudemycins. These compounds are based on natural products, but their structures have been simplified by removing stereocenters and non-required functional groups while still retaining biological activity (Fan et al. 2011).

2.6. Pre-mRNA splicing is linked to cancer

The most potent splicing inhibitors spliceostatin A (SSA), pladienolide B (PB), and herboxidiene (HB) (Table I-2, Figure I-1A) revealed a strong link between splicing and cancer. All three compounds were initially identified as natural products that are cytotoxic in cancer cells, and were later discovered to inhibit splicing. Early on, PB was shown to shrink tumor size in mice (Figure I-2B) (Mizui et al. 2004), and together with the discovery that derivatives of SSA are about 10-fold more cytotoxic for cancer cell lines than healthy cell lines (Lagisetti et al. 2009) this raised the hope to develop splicing inhibitors into novel chemotherapeutics. One compound, the PB derivative E7107, was even used in a phase I clinical trial to determine its safety and pharmacokinetics (Eskens et al. 2013). Unfortunately, E7107 caused bilateral optic neuritis (inflammation of the optical nerve), an unexpected toxicity that led to discontinuation of the clinical trial.
Table I-2. **SF3B1 mutations in cancer.** Data are based on April 2015 entries from the COSMIC database for somatically acquired mutations found in human cancers (Forbes et al. 2015).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mutations</th>
<th>Samples</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematopoietic / lymphoid</td>
<td>946</td>
<td>10,221</td>
<td>9.26 %</td>
</tr>
<tr>
<td>Uveal melanoma (eye)</td>
<td>60</td>
<td>371</td>
<td>16.17 %</td>
</tr>
<tr>
<td>Nervous system</td>
<td>30</td>
<td>998</td>
<td>3.01 %</td>
</tr>
<tr>
<td>Breast</td>
<td>17</td>
<td>1,393</td>
<td>1.22 %</td>
</tr>
<tr>
<td>Pancreas</td>
<td>15</td>
<td>1,187</td>
<td>1.26 %</td>
</tr>
<tr>
<td>Skin</td>
<td>13</td>
<td>940</td>
<td>1.38 %</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>11</td>
<td>1,959</td>
<td>0.56 %</td>
</tr>
<tr>
<td>Liver</td>
<td>10</td>
<td>1,331</td>
<td>0.75 %</td>
</tr>
<tr>
<td>Lung</td>
<td>8</td>
<td>1,728</td>
<td>0.46 %</td>
</tr>
<tr>
<td>Large intestines</td>
<td>7</td>
<td>1,478</td>
<td>0.47 %</td>
</tr>
<tr>
<td>Prostate</td>
<td>5</td>
<td>1,025</td>
<td>0.49 %</td>
</tr>
<tr>
<td>Endometrium</td>
<td>5</td>
<td>603</td>
<td>0.83 %</td>
</tr>
<tr>
<td>Kidney</td>
<td>4</td>
<td>1,216</td>
<td>0.33 %</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>3</td>
<td>88</td>
<td>3.41 %</td>
</tr>
<tr>
<td>Esophagus</td>
<td>2</td>
<td>664</td>
<td>0.30 %</td>
</tr>
<tr>
<td>Urinary tract</td>
<td>2</td>
<td>500</td>
<td>0.40 %</td>
</tr>
<tr>
<td>Stomach</td>
<td>1</td>
<td>729</td>
<td>0.14 %</td>
</tr>
<tr>
<td>Thyroid</td>
<td>1</td>
<td>550</td>
<td>0.18 %</td>
</tr>
<tr>
<td>Biliary tract</td>
<td>1</td>
<td>152</td>
<td>0.66 %</td>
</tr>
<tr>
<td>Testis</td>
<td>1</td>
<td>4</td>
<td>25.00 %</td>
</tr>
</tbody>
</table>
SSA, PB, and HB all target the same core spliceosome protein, SF3B1, which is part of U2 snRNP and has a role early in spliceosome assembly during branch point recognition. Given its central role in gene expression, it is not surprising that interfering with the spliceosome is related to diseases including cancer. In fact, multiple recent whole-exome sequencing analyses of cancer patients have revealed that spliceosome core proteins including SF3B1 are among the most commonly mutated proteins, although it is not clear if the mutations drive tumor development or are a consequence of tumor progression. SF3B1 mutations were first identified in hematopoietic cancers like chronic lymphocytic leukemia (CLL) (Wang et al. 2011; Quesada et al. 2012) and myelodysplastic syndrome (MDS, Figure I-2C) (Yoshida et al. 2011), but since then were also found in solid tumors of multiple tissues (Ellis et al. 2012; Biankin et al. 2012; Harbour et al. 2013). Table I-2 summarizes the currently reported cases, and most likely mutations in more cancers will be reported. Most mutations (84%) are amino acid missense substitutions clustering in the carboxy-terminal region of SF3B1 that is organized in 22 so-called HEAT repeats (huntingtin, elongation factor 3, protein phosphatase 2A, target of rapamycin 1). Notably, there are several hot-spot positions located in exons 14-18 (nucleotides 620-750 / 1,305) that are mutated in many patients across multiple types of cancer, with K700E being the most common cancer mutation (Figure I-2D).

Even with sequencing data accumulating, the functional consequences of SF3B1 mutations in cancer remain elusive. There is no clear correlation between SF3B1 mutation and disease outcome. For MDS and uveal melanomas, SF3B1 mutations are associated with good prognosis (Papaemmanuil et al. 2011; Harbour et al. 2013), which is in contrast to the link with poor prognosis for CLL (Wang et al. 2011; Quesada et al. 2012).
2011). Also, and maybe more surprisingly, there is only very little data available about the effect of SF3B1 mutations on splicing in cancer cells. Given that SF3B1 is a core splicing protein, it seems obvious that the splice pattern of many different pre-mRNA transcripts would change. But so far, only few individual splicing events were reported to be altered, like increased intron retention in BRD2 and RIOK3 in CLL (Wang et al. 2011) and changes in five alternative splicing events in tumor samples from patients with CLL, MDS, and uveal melanoma (Gentien et al. 2014). As a consequence, the importance of SF3B1 mutations in cancer is currently not clear, and it remains to be tested to which extent pre-mRNA splicing changes on a genome-wide level and which of the altered splicing events have the biggest effect on the cells.

Despite the growing evidence that SF3B1 mutations play an important role in cancer development and/or progression, very little is known about how splicing inhibitors change splicing on a cell-wide level. It seems clear that some events are more sensitive than others, and that inhibitors cause a change in alternative splicing and/or intron retention rather than affecting all splicing events at the same level (Corrionero et al. 2011; Kashyap et al. 2015). Currently, it is still an open question whether splicing inhibitors function differently in cells with SF3B1 mutations, and which role the inhibitors might play in new approaches for drug development.
Figure I-2. **Pre-mRNA splicing is closely related to cancer.** (A) Chemical structures of three potent anti-tumor compounds that also inhibit splicing. SSA, spliceostatin A; PB, pladienolide B; HB, herboxidiene. IC$_{50}$ refers to the concentration required to reduce in vitro splicing by half compared to DMSO-treated control reactions (Effenberger et al. 2014). (B) Antitumor effect of PB on mouse xenograft models. Mice were intravenously treated with 5 mg/kg/day PB for 5 days. Modified from Mizui et al. 2004. (C) SF3B1 is the most commonly mutated gene in specific myeloid neoplasms (refractory anaemia with ring sideroblasts (RARS) / refractory cytopenia with multilineage dysplasia with >15% ring sideroblasts (RCMD-RS)). Modified from Yoshida et al. 2011. (D) Homology-based predicted model of the splicing core protein SF3B1. Cancer mutations cluster in the indicated HEAT-repeats. The most common amino acid substitutions are listed (data based on the COSMIC database, April 2015).
2.7. Splicing inhibitors hold the potential to expand the currently limited mechanistic understanding of the spliceosome

The crucial first step in connecting changes in splicing or mutations in splicing factors to diseases like cancer is to understand how the spliceosome functions under normal, healthy conditions. Splicing inhibitors are tools that can help answering multiple questions in order to build a better mechanistic understanding of the spliceosome.

First, inhibitors can add detail to known assembly steps of the spliceosome and reveal novel intermediate conformations. One example is the use of E7107, a PB derivative, to determine U2 snRNA conformation requirements to associate with the spliceosome (Folco et al. 2011). The study found that U2 snRNA likely undergoes an ATP-dependent conformational change before it can base pair with the branch point region to form A complex. Another study using SSA shows that during the transition from A complex to B complex, tri-snRNP is first loosely associated with the spliceosome before it gets stably integrated (Roybal and Jurica 2010). If inhibitor-stalled complexes are stable, they can also be enriched and affinity-purified to determine their protein composition by mass spectrometry and their overall shape by electron microscopy.

Second, inhibitors can identify or further specify the function of individual proteins. Using SSA as a tool, the Valcárcel group showed that SF3B1 interacts with the pre-mRNA to ensure correct U2 snRNA base pairing with the branch point region (Corrionero et al. 2011). In the presence of SSA, this interaction is blocked and splice site choice becomes less precise, leading to changes in alternative splicing.
3. Contributions of this dissertation

While the general composition and broad assembly steps of the spliceosome are well understood, we are lacking a detailed mechanistic understanding of most components of this dynamic macromolecular machine. Given its essential role during gene expression and its close link to cancer, it is important to determine in detail how introns are removed and exons ligated. My dissertation work expands the current knowledge by identifying new tools to study the spliceosome and using these tools to add to our incomplete picture by deciphering the function of a core protein.

In Chapter II, I describe the development of a fast and reliable high-throughput assay to identify new splicing inhibitors. In an initial screen, I found and characterized three new compounds that stall spliceosomes at different assembly stages. In Chapter III, I use structure-activity relationship analysis to determine important functional groups in the splicing inhibitor pladienolide B. I used a series of assays to determine how the compounds affect splicing in vitro and in cells, and how cellular phenotypes changed upon compound treatment. I also show detailed in vitro structure-activity relationship analyses for the two splicing inhibitors spliceostatin A and herboxidiene. In Chapter IV, I focus on the functional role of the core spliceosome proteins SF3B1 and show that three structurally distinct inhibitors all bind to the same site on the protein. Furthermore, I determined that in addition to its known early role in spliceosome assembly, SF3B1 also functions after the first chemical step before exon-exon ligation. In Chapter V, I summarize my results, discuss how they expand the current knowledge and advance the field, and point out future directions.
In summary, my work combines high-throughput screening, biochemistry, and medicinal chemistry to identify new tools for mechanistic analyses and use them to add puzzle pieces to our incomplete picture of the spliceosome. My methods and data increase the mechanistic understanding of one of the most complex macromolecular machines in the cell.
Chapter II: A high throughput chemical screen identifies new classes of inhibitors of human and yeast spliceosomes

1. Abstract

Spliceosomes are the macromolecular machinery responsible for pre-mRNA splicing, an essential step in eukaryotic gene expression. Given the myriad of subunits that join and leave while the spliceosome assembles on each intron to be spliced, along with constantly rearranging interactions that regulate the splicing process, it is surprising that very few inhibitors of the spliceosome are known. Splicing inhibitors could be used to capture transient spliceosome conformations and probe important functional components. They may also have chemotherapeutic potential, as links between splicing and cancer are increasingly uncovered. To identify new splicing inhibitors, we have developed a high throughput assay for \textit{in vitro} splicing in HeLa extract using an RT-qPCR readout. In a pilot screen of 3,080 compounds we identified three small molecules that inhibit splicing in HeLa extract by interfering with different stages of human spliceosome assembly. Two of the compounds similarly affect spliceosomes in yeast extracts, suggesting selective targeting of conserved components. By examining related molecules, we identified chemical features required for the activity of two of the splicing inhibitors. In addition to verifying our assay procedure and paving the way to larger screens, these studies establish new compounds as chemical probes for investigating the splicing machinery.
2. Introduction

Pre-mRNA splicing is a critical process in eukaryotic gene expression. While the chemistry behind removing introns and ligating exons is well understood, the mechanisms by which the spliceosome catalyzes and regulates splicing remain unclear. Spliceosome assemble de novo at intron in a stepwise process from five small nuclear RNAs with associated proteins (snRNPs, U1, U2, U4, U5, and U6) and a large number of additional protein components (Wahl et al. 2009). *In vitro* studies using native gels have defined an ordered series of intermediate splicing complexes (Konarska and Sharp 1986). In the first complex (E) U1 snRNP joins the pre-mRNA, followed by addition of U2 snRNP to create the pre-spliceosome complex (A). The U4, U5, and U6 tri-snRNP then join to create B complex, which is activated by release of U1 and U4 for splicing catalysis in C complex. Complex rearrangements of protein-protein, protein-RNA and RNA-RNA interactions drive spliceosome assembly and, given its complexity, many additional spliceosome complexes surely remain to be captured and characterized.

To make these new intermediate complexes available for biochemical and structural analysis, small molecule inhibitors that selectively target different spliceosome components are needed to arrest spliceosome progression at discrete steps. With the large number of enzymatic activities and regulated rearrangements in spliceosomes, it is clear that a diverse set of compounds will be required. Some splicing inhibitors may also be useful as biological probes of spliceosome function in cells. With the recent finding of spliceosome mutations associated with progression of chronic lymphocytic leukemia and myelodysplastic syndrome (Landau et al. 2013;

High-throughput screening (HTS) with a sensitive and robust assay is an important strategy for identifying small molecules inhibitor candidates. An established human in vitro splicing system allows spliceosome function to be assessed in isolation from other cellular processes and provides a means to probe all of its ~100 components simultaneously. Here we describe HTS of ~3,000 compounds for splicing inhibitors using a new RT-qPCR assay. We identified three structurally distinct small molecules that inhibit human in vitro splicing reactions in a dose-dependant manner. We characterized the effects of these compounds on splicing chemistry and spliceosome assembly using extracts and substrates in human and yeast to examine their selectivity. One compound, Tetrocarcin A (C1), an antibiotic with anti-tumor activity (Tomita et al. 1980), inhibits first step chemistry and an early stage of spliceosome assembly in extracts from both organisms. A family of naphthazarin compounds (C3) affects later stages of spliceosome assembly in human and yeast extracts, while a third indole derivative (C2) blocks the earliest stages of assembly in the human system only. With these results it is clear that we have an assay system that is robust in identifying new modulators of splicing. Furthermore, we can attribute effects of candidate inhibitors to discrete steps of splicing chemistry and spliceosome assembly. These results are key to eventually establishing these compounds as drug leads that target splicing in cells.
3. Results

3.1. RT-qPCR assay to screen for inhibitors of \textit{in vitro} splicing

To search for inhibitors of the human spliceosome, we used a synthetic pre-mRNA substrate consisting of two exons separated by an intron and HeLa nuclear "splicing" extract (Padgett et al. 1983). Spliceosomes assemble on the pre-mRNAs, which are then spliced in two chemical steps forming mRNA (Figure II-1A, (Ruskin et al. 1984; Padgett et al. 1984)). Typically, the reaction is analyzed by denaturing gel electrophoresis to quantify the amount of splicing products, which is not conducive to scale up or automation. For HTS, we developed a reverse transcription followed by quantitative PCR (RT-qPCR) assay that employs a TaqMan® probe complementary to the unique splice junction sequence created by exon ligation (Figure II-1B). This assay reports a threshold cycle (C$_T$), which directly correlates to the amount of mRNA produced in the reaction by the spliceosome. A lower C$_T$ value indicates more mRNA and more splicing, while an increased C$_T$ value corresponds to reduced mRNA and less splicing. Using this system, we detect mRNA produced by \textit{in vitro} splicing (Supplementary Figure II-1A). We also verified that 2% DMSO, the compound in which most library molecules are dissolved, does not affect the assay (Supplemental Figure 1b). Finally, we do not detect mRNA in splicing reactions supplemented with the known splicing inhibitor SSA (Supplementary Figure II-1B, (Kaida et al. 2007)).
Figure II-1. **High-throughput approach to identify splicing inhibitors.** (A) Two-step splicing reaction showing pre-mRNA, first-step intermediates (5' exon and lariat intron intermediates), and second-step products (mRNA and intron lariat). (B) Schematic of TaqMan®-based RT-qPCR assay. mRNA is reverse transcribed (RT) and quantified via a dual-labeled oligo probe to the splice junction by release of a fluorophore reporter (R) from a quencher (Q). (C) Schematic of automated splicing assay. (D) Data used to calculate the Z’ value for the assay using a no-splicing control (black diamonds) and normal splicing (gray diamonds) reactions. For each reaction, C_T is plotted versus well number. (E) Screening results of the National Cancer Institute library collection. The histogram plots the number of compounds screened versus C_T value. The chemical structures of three verified hits (C1, C2, and C3) are shown.
To screen large chemical libraries for splicing inhibitors, we used liquid handling robots to set up the assay in 384-well plates. The steps of the protocol are schematized in Figure II-1C: (1) 5 µl nuclear extract is dispensed to wells, (2) a pin robot transfers 0.2 µl of test compounds from a library plate into the nuclear extract, (3) 5 µl of pre-mRNA substrate in splicing buffer is dispensed into the nuclear extract, (4) splicing proceeds for sixty minutes, (5) the reactions are diluted with 10 µl of water, (6) 5 µl of the RT-qPCR reaction components are dispensed into a second 384-well plate, (7) a pin robot transfers 15 nl of the diluted splicing reactions as template to the RT-qPCR plate, and (8) RT-qPCR is carried out. To analyze the data, we compare the \( C_T \) value for each well with control splicing wells into which either DMSO alone or SSA was added.

Before screening, we characterized the assay system by setting up a plate with alternating rows of splicing reactions containing DMSO alone or SSA and measured mRNA amounts by RT-qPCR for each condition. DMSO-treated splicing reactions cluster with an average \( C_T \) value of 19.0±0.8, while SSA-treated (inhibited) splicing reactions yield an average CT value of 38.8±2.2 (Figure II-1D). From these \( C_T \) distribution we calculate a \( Z' \) value (Zhang et al. 1999) for the assay of 0.55 with good separation (10 \( C_T \)) between fully inhibited and uninhibited splicing reactions. We conclude that the automated splicing assay can measure splicing inhibition allowing us to screen large chemical libraries for splicing inhibitors.
3.2. High-throughput screen for splicing inhibitors

Using the RT-qPCR splicing assay, we screened of 3,080 compounds library combining the Structural Diversity Set, Challenge Set, Natural Products Set, and Mechanistic Diversity Set from the National Cancer Institute (NCI). The vast majority of compounds in the library did not have an effect on in vitro splicing and showed low C_T values within a baseline range centered at a C_T value of 21 (Figure II-1E). About 3% or 100 compounds resulted in C_T values in the fully inhibited splicing range (Figure II-1E, C_T >40). For these, we returned to the original splicing plate and manually repeated the RT-qPCR analysis of the candidate inhibitor wells in triplicate, which reconfirmed 40 (1.3%) as yielding high C_T values. We ordered the compounds added to those wells and retested their effects on in vitro splicing by the same RT-qPCR assay. Six of these consistently interfered with in vitro splicing, but only three showed clear dose-dependent effects expected for a specific splicing inhibitor.

3.3. Candidate compounds inhibit human and yeast pre-mRNA splicing

The three candidate splicing inhibitors that we identified from the pilot screen have very distinct structures as shown in the inset in Figure II-1E. Compound C1 is a large, complex natural compound known as Tetrocarcin A (NSC333856). It has been described as an antibiotic (Tomita et al. 1980) and as an antitumor compound that inhibits the anti-apoptotic gene Bcl2 (Tomita et al. 1980; Nakashima et al. 2000; Shoemaker 2006). The compound C2 is an indole derivative (NSC635326) with no known biological activity. Compound C3 is a naphthazarin derivative (NSC659999) and like several other naphthazarin compounds, has shown activity in a variety of biological contexts, including suppression of tumor growth (Shoemaker 2006).
Figure II-2. **Three compounds inhibit splicing chemistry in a dose-dependent manner.** Denaturing gel analysis of RNA from splicing reactions with an indicated concentration of C1, C2, and C3. Quantification of splicing efficiency vs. inhibitor concentration is plotted below each gel along with estimated IC\textsubscript{50} values. (A) Inhibition in HeLa nuclear extract. Identities of bands are schematized to the left as (from top to bottom) lariat intermediate, pre-mRNA, mRNA, 5’ exon intermediate. (B) Inhibition in yeast extract with an RP51A splicing substrate. Identities of bands are schematized to the left as (from top to bottom) lariat intermediate, intron lariat, pre-mRNA, mRNA, 5’ exon intermediate.
The RT-qPCR splicing assay detects inhibition of mRNA accumulation, but does not identify the molecular basis of this inhibition. To determine how the three compounds affect splicing chemistry, we performed *in vitro* splicing assays in HeLa nuclear extract using radiolabeled pre-mRNA substrate, followed by gel electrophoresis to visualize splicing intermediates and products. We found that all three compounds do not impair RNA stability in the extract (which could result in mRNA loss after a successful splicing reaction). Instead, their addition to splicing reactions results in a loss of splicing chemistry in a concentration-dependent manner (Figure II-2). The presence of C1 primarily affected the first step of splicing chemistry, which was completely lost at 100 µM, and we determined an IC$\text{_{50}}$ for the compound of ~25 µM. Second step chemistry appeared more sensitive to the C2 and C3 compounds, although first step chemistry was also lost at higher concentrations (Supplementary Figure II-2). For C2, second step chemistry was completely lost at 200 µM, with IC$\text{_{50}}$ of ~50 µM. C3 was the most potent splicing inhibitor. At 60 µM, its presence resulted in full loss of second step chemistry with an IC$\text{_{50}}$ of ~20 µM. First step was lost at 200 µM, with an IC$\text{_{50}}$ of ~50 µM (Supplementary Figure II-2). The distinct sensitivities of 1st and 2nd splicing chemistry could mean that these compounds affects a spliceosome component differentially required for both steps. Alternatively, they may be selective for more than one target. Notably, splicing inhibition in the presence of all three compounds is reproducible with different preparations of nuclear extract and does not depend upon pre-incubation of the extract or pre-mRNA with the inhibitor.
We also examined the effects of these compounds on \textit{in vitro} splicing in \textit{S. cerevisiae} extracts (Figure II-2B, Pikielny and Rosbash 1986). Again, addition of C1 resulted in loss of first step chemistry, although higher concentrations were required to see an effect (IC$_{50}$ \textasciitilde 250 \(\mu\)M). Similar to its effect in human extracts, addition of C3 primarily resulted in loss of second step, although much higher concentrations are required and splicing is never completely blocked. In contrast, C2 had no effect on yeast splicing. These results suggest that C1 and C3 interfere with a conserved mechanism of the splicing process, whereas C2 is selective for a human factor and does not simply inactivate all extracts.

\textbf{3.4. C1 stalls spliceosome assembly early in human and yeast extracts}

The spliceosome assembles through a series of complex intermediates, and we expect that a splicing inhibitor selective for a component involved in complex formation will interfere with a specific assembly stage. We used native agarose gels to investigate the effect of inhibitor compounds on human spliceosome assembly (Das and Reed 1999). In the absence of inhibitor (2\% DMSO), these gels show the progression of assembly through complex intermediates of E/H, A, B, and C complexes (Figure II-3B, lanes 1–5). Both steps of splicing chemistry take place in C complex, which disassembles immediately after catalysis. Direct inhibition of splicing chemistry typically causes C complex accumulation, whereas interference with a specific assembly step results in accumulation of the preceding complex.
Figure II-3. C1 stalls human and yeast spliceosomes at an A-like complex. Native gel analysis of spliceosome assembly. (A) Thirty minute time points of splicing reactions in HeLa nuclear extract supplemented with 2% DMSO or indicated concentration of C1. The identity of complexes is denoted with assembly occurring in the following order: H/E → A → B → C. The arrow indicates the A-like complex. (B) Time course analysis of splicing reactions in HeLa nuclear extract in 2% DMSO or 1 mM C1. Time points are indicated in minutes. (C) Time course analysis of splicing reactions in HeLa nuclear extract in the presence or absence of ATP and C1 as indicated using a pre-mRNA with wild type or mutant branch point. (D) Twenty minute time points of splicing reactions in yeast extract supplemented with 1% DMSO or indicated concentration of C1. The identity of complexes is denoted with assembly occurring in the following order: CC1 → CC2 → PS/SP. (E) Time course analysis of splicing reactions in yeast extract in 1% DMSO or 500 µM C1.
In the presence of increasing amounts of C1, splicing assembly is affected in parallel to the loss that we observed in splicing chemistry (Figure II-3A and Figure II-2A). At a concentration that completely blocks splicing a complex that migrates near the position of A complex ("A-like" complex) accumulates. Furthermore, B and C complexes do not appear over time, but instead an apparent conversion of the A-like complex to a stable lower migrating species becomes evident (Figure II-3B, lanes 6–10).

Formation of A complex requires recognition of the branch point sequence and ATP hydrolysis (Konarska and Sharp 1986; Reed and Maniatis 1988). If the A-like complex is related to normal spliceosome assembly, it should have the same substrate and ATP requirements. Splicing reactions assembled in the presence of C1 with a pre-mRNA mutated at the branch point sequence no longer produce the A-like complex (Figure II-3C, lanes 8–11). Like a branchpoint in the substrate, ATP is also required to form the A-like complex (Figure II-3C, lanes 12–15). This result shows that compound does not generally disturb all components of the extract, because specific dependencies of spliceosome assembly are maintained.

We also looked at spliceosome assembly in yeast extracts treated with C1, and again see a dose dependent loss of spliceosome assembly compared to DMSO alone (Figure II-3D). At lower concentrations the effect of C1 is most evident in the appearance of prespliceosomes/spliceosomes (PS/SP) complex bands, which are equivalent to A/B/C complexes in human extracts; Figure II-3D, lanes 1–4), while at higher concentrations all PS/SP accumulation is lost (lanes 5–6). At 500 µM concentration C1 also dramatically reduces stable accumulation of commitment
complexes CC1 and 2, which are formally equivalent to E/H complex in human extracts (Figure II-3E, lanes 7–12). We conclude that C1 does not directly inhibit 1st splicing chemistry, but instead interferes with early stages of spliceosome assembly in both systems potentially by destabilizing complexes that form. These results also suggest that it targets a core component conserved in both human and yeast spliceosomes.

3.5. C3 causes accumulation of a B-like complex

C3 had a different effect on spliceosome assembly in human extracts. As the amount of C3 is increased in splicing reactions, C complex decreases with the same dose dependence as loss of second step chemistry (Figure II-4A, lanes 2–7 and 2a). The compound does not block or change the timing of A and B complex formation, even at concentrations that completely block both steps of splicing chemistry (Figure II-4A, lanes 13–17). This result shows that C3 does not generally disrupt complex assembly. Instead, considering the different sensitivities of 1st and 2nd step chemistry to C3, there must be a factor(s) involved in late assembly that is selectively targeted by the presence of the compound.

In yeast extracts there is a similar a dose dependent loss of spliceosome assembly (Figure II-4B, lanes 2–7) with C3. In comparison to no inhibitor (1% DMSO- lanes 8–14), progression from commitment complexes (CC1/CC2) to prespliceosomes/spliceosomes (PS/SP) is decreased (Figure II-4B, lanes15–21), which is formally equivalent to a decrease in A/B/C complex formation in human extracts.
Figure II-4. A B-like complex accumulates in the presence of the C3 compound. (A) Native gel analysis of spliceosome assembly. The left panel shows 30 min time points of splicing reaction in HeLa nuclear extract supplemented with 2% DMSO or indicated concentration of C3. The right panel shows time course analysis of splicing reactions in HeLa nuclear extract in 2% DMSO or 1 mM C3. Complexes are labeled as in Figure II-3. (B) The left panel shows 20 min time points of splicing reactions in yeast extract supplemented with 1% DMSO or indicated concentration of C3. The right panel shows time course analysis of splicing reactions in yeast extract in 1% DMSO or 1 mM C3. Complexes are labeled as in Figure 3. (C) Chemical structure of C3 and related compounds. (D) Denaturing gel analysis of in vitro splicing reactions with increasing concentrations of C3 or indicated compounds in HeLa nuclear extract. Bands are schematized as in Figure II-2. (E) Denaturing gel analysis of in vitro splicing reactions with increasing concentrations of C3 or NSC224124 in yeast extract. (F) Quantification of the splicing efficiency relative to compound concentration of the splicing reactions shown in (D) (top panel) and (E) (bottom panel). Estimated IC\textsubscript{50} values are indicated.
3.6. C3 activity is related to redox potential in HeLa extracts

To examine the structure activity relationships of the C3 inhibitor, which contains a naphthazarin backbone, we tested the effects of related compounds on in vitro splicing. In human extracts, another naphthazarin derivative (NSC659997) inhibits splicing primarily at second step, although with a slightly higher IC<sub>50</sub> of ~50 µM (Figure II-4C, D, F). Interestingly, NSC659997 also shows growth inhibition in a panel of tumor cell lines that is similar to the original C3 compound (Shoemaker 2006). A second naphthazarin derivative (NSC224124) had a more limited effect on splicing (Figure II-4C, D, F), and no effect on same panel of tumor cells. Finally, we tested the naphthazarin backbone alone (NZ), and find that it also inhibits splicing with an IC<sub>50</sub> similar to C3 (Figure II-4C, D, F). Together, these results suggest that the naphthazarin backbone alone mediates splicing inhibition, but that substitutions within the ring structure can affect the activity.

These results are in some contrast to what we observe with naphthazarin derivatives in yeast splicing. In this case, the naphthazarin backbone alone was not sufficient to inhibit splicing chemistry (data not shown). In contrast, NCS224124 is more potent in yeast than the C3 compound, but still has a relatively high IC<sub>50</sub> of ~150µM (Figure II-4E, F). This difference in activity points to the importance of substitutions on the naphthazarin ring in yeast splicing as well, but is it is not clear why the sensitivity to the substitutions is different for human splicing.

Naphthazarin and many of its derivatives are reactive compounds with well-known redox properties proposed to interfere with proteins by two mechanisms (You et al. 1998). First, they can serve as electrophiles to covalently modify proteins, most
commonly by thioether linkage with labile cysteine residues, which cannot by reverse by DTT. Alternatively, they also generate reactive oxygen species (ROS) that, among other effects, can oxidize thiol groups of cysteines, a process which can be blocked by addition of DTT. We tested the effect of DTT on HeLa splicing and found that excess DTT recovers about 70% of splicing in reactions inhibited by the naphthazarin compounds (Figure II-5A, B). DTT alone does not affect splicing efficiency up to 20 mM concentration. Relief of splicing inhibition by DTT suggests that the compounds inhibit splicing through ROS generation that results in modification of a redox-sensitive cysteine(s). Because we still see assembly to B complex in the presence of the naphthazarin compounds at all concentrations, the redox-sensitive cysteine is specific and appears to be required for formation of a catalytically active spliceosome. Surprisingly, in yeast extracts, DTT did not reverse splicing inhibition by the most potent naphthazarin derivative (NCS224124) (Figure II-5C, D), which suggests that the compound functions by a different mechanism in yeast splicing. Again, we cannot specify why different ring substitutions in the naphthazarin backbone affect the inhibitory properties of the different compounds. With human splicing, they may modulate the redox potential of the compounds, while splicing in yeast appears sensitive to their structure.
Figure II-5: Inhibition by naphthazarins is partially rescued by excess DTT. (A) Denaturing gel analysis of *in vitro* splicing reactions in HeLa nuclear extract inhibited by C3 or related compounds supplemented with increasing concentrations of DTT. Bands are schematized as in Figure II-2. (B) Quantification of the splicing efficiency relative to compound concentration of the splicing reactions shown in (A). (C) Same analysis as in (A) but with yeast splicing. (D) Quantification of the splicing efficiency relative to compound concentration of the splicing reactions shown in (C). Estimated IC₅₀ values are indicated.
3.7. C2 inhibits spliceosome assembly early in human extracts

The C2 compound only inhibits in the human splicing system and primarily affects very early spliceosome assembly. With increasing concentrations of C2, there is a loss of higher complexes that correlates with the loss of splicing chemistry (Figure II-6A, Figure II-2A, and Supplementary Figure II-2B). At lower concentrations, where 2nd step is more sensitive to the compound, the spliceosome fully assembles to some extent indicating selectivity for a late-acting splicing factor (Figure II-6A). In the presence of 1 mM C2, where all splicing chemistry is completely blocked no complexes form beyond E/H (Figure II-6B, lanes 6–10), which could indicate that the extract is generally inactivated. However, 1 mM C2 does not affect splicing in yeast extracts at all (Figure II-2B, C), suggesting instead that C2 also has selectivity for a splicing factor involved in the earliest assembly stages.

We also examined structure activity relationships for C2. Two related compounds with different substitutions in place of the nitrophenyl group had very little effect on splicing chemistry (Figure II-6C, D, E). A third compound, which differs only in the linkage position of the nitrophenyl group, inhibits splicing but with less potency than the original C2 compound (Figure II-6C, D, E). We conclude that presence and orientation of a nitrophenyl ring from the oxoethyl group at the 3' position of the indole ring structure are important for the mechanism of C2 inhibition of splicing.
Figure II-6. Presence and orientation of a nitrophenyl ring are important for splicing inhibition by C2. (A) Thirty minute time points of splicing reactions in HeLa nuclear extract supplemented with 2% DMSO or indicated concentration of C2. (B) Time course analysis of splicing reactions in HeLa nuclear extract in 2% DMSO or 1 mM C2. (C) Denaturing gel analysis of in vitro splicing reactions with increasing concentrations of C2 or indicated compounds in HeLa nuclear extract. (D) Chemical structure of C2 and related compounds. (E) Quantification of the splicing efficiency relative to compound concentration of the splicing reactions shown in (C).
4. Discussion

Using a highly sensitive RT-qPCR splicing assay we identified three structurally diverse molecules (C1, C2 and C3) that inhibit pre-mRNA splicing at specific stages in spliceosome assembly in both HeLa and S. cerevisiae extracts. In HeLa extracts this inhibition has an IC\textsubscript{50} in the 20–50 µM range. These three compounds are structurally distinct from previously identified splicing inhibitors (O’Brien et al. 2008; Berg et al. 2012; Samatov et al. 2012) and offer a unique opportunity to dissect important spliceosomal complexes not previously captured in either human or yeast splicing systems.

C1, also known as Tetrocarcin A, has demonstrated antibiotic activity, as well as cytotoxic effects in several cancer cell lines (Tomita et al. 1980). It has been shown to promote apoptosis by blocking BCL2 activity (Nakashima et al. 2000), activating caspase-9 (Anether et al. 2003) or inhibiting PI3K kinase activity (Nakajima et al. 2007), depending on cell type, although its molecular mechanisms are not clear. One possibility for this wide range of effects is that Tetrocarcin A inhibits the splicing of one or more key gene products in the apoptosis pathway.

C2 impedes all stages of spliceosome assembly, eventually resulting in loss of all complexes beyond E/H. C2 has no effect on yeast in vitro splicing, therefore its target(s) are likely specific to higher eukaryotes. By comparing the activity of C2 to several structurally related compounds, we find that a nitrophenyl substituent is key to its inhibition of splicing. The lack of previous data demonstrating an effect of C2 on cell growth suggests cells may not readily take up the compound. But its in vitro
utility remains in allowing concentration dependent access to the earliest stages of spliceosome assembly.

The influence of C3 (NSC659999) on splicing is somewhat more complex. At lower concentrations this compound primarily affects second step chemistry, which is mirrored by a clear loss of C complex accumulation. In yeast, C3 shows similar effects on splicing chemistry and complex assembly, although only at significantly higher concentration. In the human system, the effect of the naphthazarin inhibitors can be reversed by the addition of excess DTT, which suggests that ROS generation by these compounds plays a role in their inhibition of splicing. It also indicates that a specific redox-sensitive cysteine in a splicing component functions in the transition from first to second step chemistry. Recently, the Dreyfuss lab identified a CDC25 phosphatase inhibitor in their screen for compounds that block *in vitro* splicing (Berg et al. 2012). This compound is a naphthoquinone (NSC95397) with similar redox properties and affects second step chemistry and C complex formation like the naphthazarins. It is notable that the phosphatase PP2A is also required for second step chemistry in human extracts and that the PP2A inhibitor Okadaic acid is also a splicing inhibitor (Shi et al. 2006; Mermoud et al. 1992). PP2A is sensitive to oxidation by H$_2$O$_2$, which can also be rescued by excess DTT (Foley et al. 2004). We propose that at least some of the splicing inhibition observed with naphthoquinones, which includes the naphthazarins, is conferred by indirect inactivation of PP2A by oxidation. As of yet, there is no known role in splicing for the yeast PP2A ortholog. This may explain why most of the naphthazarin derivatives had no effect on yeast splicing and why the modest effect of NSC2241124 in yeast extracts cannot be rescued by DTT.
There are several directions of study that may be pursued with these new splicing inhibitors. Our results underscore their potential as tools *in vitro* to study splicing mechanisms. For those that can be synthesized and derivatized, their structure activity relationships can be further explored and their targets potentially identified addition of affinity tags. These studies also open the door to their use as probes in studying the role of splicing in cells. Many splicing inhibitors have shown bioactivity in the growth of different tumor cell lines, including Tetrocarcin A and naphthazarins. An important next step will be to determine whether this activity is due to inhibition of spliceosomes and, if so, which splicing pathways are particularly affected.

Finally, with the extreme complexity of the spliceosome, the need is still great for a larger arsenal of compounds that modulate enzymatic functions and rearranging interactions involved in splicing. Fortunately, a great deal of chemical space remains unexplored. As we screen more compound libraries, particularly those containing structurally diverse natural products, we will certainly increase the number of small molecule tools that will be useful for studying splicing mechanisms and cellular functions.
5. Supplemental Material

5.1. Supplementary Figure 1

Supplementary Figure II-1. The RT-qPCR assay has a broad dynamic range and specifically detects mRNA produced by in vitro splicing in HeLa nuclear extract. (A) The assay reliably detects mRNA in the low pM range. Plotted is threshold cycle (C\text{\text{\textsubscript{T}}}) vs. concentration of synthetic mRNA in RT-qPCR. Regression analysis determined a linear range of detection over six orders of magnitude with a correlation coefficient of 0.994. The PCR efficiency is 90%, as determined with the following equation: qPCR efficiency = 10^{(-1/slope)} – 10. (B) Assay specificity represented another concern because in vitro splicing is not 100% efficient. A significant amount of pre-mRNA will remain in the reaction, especially if splicing is inhibited. We tested whether pre-mRNA will be also be detected with the exon-junction RT-qPCR probe. Whereas 5 nM mRNA was readily detected with a low C\text{\text{\textsubscript{T}}} value, we see no signal for pre-mRNA at twice the amount (left part, naked RNA). Next, we tested how the assay performs with mRNA produced by in vitro splicing. With normal splicing reactions we consistently observed a C\text{\text{\textsubscript{T}}} value of ~20 cycles, which is consistent with the amount of mRNA expected from uninhibited splicing. Because most small molecule libraries that we will screen for inhibitors are solubilized in DMSO, we tested whether DMSO alone affects splicing chemistry. We found that, compared to uninhibited splicing reactions, 2% DMSO did not change the C\text{\text{\textsubscript{T}}} value significantly (right part, splicing reactions). Finally, to ensure that we can detect splicing inhibition, we examined splicing reactions supplemented with the known splicing inhibitor SSA or depleted of ATP, which is required for splicing. In both cases, no reporter fluorescence was observed after 40 cycles of qPCR, indicating that no mRNA was produced in the reaction (right part, splicing reactions).
5.2. Supplementary Figure 2

Supplementary Figure II-2. Quantification of first and second step splicing efficiency vs. inhibitor concentration for the splicing reactions shown in Figure II-2A. (A) C1. (B) C2. (C) C3. Splicing efficiency for first step is the amount of intermediates relative to total RNA and normalized to a DMSO control reaction. Splicing efficiency for second step is the amount of mRNA relative to total RNA and normalized to a DMSO control reaction.
Chapter III: Coherence between cellular response and *in vitro* splicing inhibition for the anti-tumor drug pladienolide B and its analogs

1. Abstract

Pladienolide B (PB) is a potent cancer cell growth inhibitor that targets the SF3B1 subunit of the spliceosome. There is considerable interest in the compound as a potential chemotherapeutic, as well as a tool to study SF3B1 function in splicing and cancer development. The molecular structure of PB, a bacterial natural product, contains a 12-member macrolide ring with an extended epoxide-containing side chain. Using a novel concise enantioselective synthesis, we created a series of PB structural analogs and the structurally related compound herboxidiene. We show that two methyl groups in the PB side chain, as well as a feature of the macrolide ring shared with herboxidiene, are required for splicing inhibition *in vitro*. Unexpectedly, we find that the epoxy group contributes only modestly to PB potency and is not absolutely necessary for activity. The orientations of at least two chiral centers off the macrolide ring have no effect on PB activity. Importantly, the ability of analogs to inhibit splicing *in vitro* directly correlated with their effects in a series of cellular assays. Those effects likely arise from inhibition of some, but not all, endogenous splicing events in cells, as previously reported for the structurally distinct SF3B1 inhibitor spliceostatin A. Together, our data support the idea that the impact of PB on cells is derived from its ability to impair the function of SF3B1 in splicing and also demonstrate that simplification of the PB scaffold is feasible.
2. Introduction

An essential step in expression of human genes is pre-mRNA splicing, the process by which intron sequences are removed from gene transcripts to create functional mRNA for protein translation. Splicing is facilitated and regulated by the spliceosome, a highly dynamic macromolecular complex that assembles de novo at each intron from five small nuclear ribonucleoproteins (snRNPs) and dozens of additional proteins. Increasing evidence connects mutations in components of the spliceosome to various types of cancer and points to the splicing machinery as a target for new anticancer drugs. A recent example is the core spliceosome component SF3B1 and myelodysplastic syndrome, a heterogeneous group of diseases caused by abnormal proliferation of hematopoietic stem cells. Whole exome sequencing showed that 75% of a myelodysplastic syndrome subtype had mutations in SF3B1, which cluster in a particular region of the protein (Papaemmanuil et al. 2011; Quesada et al. 2012; Yoshida et al. 2011). SF3B1 mutations are also present in cancers of several other tissues (Harbour and Chao 2014; Ellis et al. 2012; Biankin et al. 2012).

Interestingly, SF3B1 is also the target of pladienolide B (PB), a natural product with potent cytotoxicity and antitumor activity both in cancer cell lines and mouse xenograft models (Mizui et al. 2004; Kotake et al. 2007; Yokoi et al. 2011). In cell culture, mutation of a single amino acid in SF3B1 confers resistance to PB, making it likely that its cytotoxicity is directly related to SF3B1 function (Yokoi et al. 2011), although how these activities are connected to splicing inhibition is not known. Two other natural products, herboxidiene (GEX1A) and FR901464 (and the related
molecules spliceostatin A (SSA) and meayamycin), also interact with SF3B1 and have similar cytotoxic effects (Kaida et al. 2007; Hasegawa et al. 2011; Gao et al. 2013). *In vitro* studies indicate that both PB and FR901464 analogs SSA and meayamycin interfere with the role of SF3B1 in stabilizing the addition of U2 snRNP to the spliceosome and identifying the intron branch point sequence (Corrionero et al. 2011; Roybal and Jurica 2010; Folco et al. 2011; Albert et al. 2009). However, it has been difficult to use structure activity studies to define the precise mechanism by which these compounds interact with SF3B1 because of their complex structures.

Novel concise enantioselective syntheses (Ghosh and Anderson 2012; Ghosh and Li 2011) have now opened the door to systematic structure activity relationship studies for PB and herboxidiene. In this study, we identify structural features of PB that are responsible for inhibition of human *in vitro* splicing. We find that the same features are responsible for a wide ranging set of phenotypic effects in cells, eliminating the hypothesis that splicing inhibition and cellular phenotypes arise from different parts of the same molecule and further underscoring the link between inhibition of the spliceosome and the cellular response to PB. There are also several positions in the molecule that can be modified with no change in activity. Our data point toward more straightforward synthetic pathways and modifications in PB, which will be key to dissecting the function of its target SF3B1 in the spliceosome and studying the relationship between splicing pathways and cancer cell growth. They may also lead to structurally less complex new PB analogs that are better tuned to selectively target cancer cell growth and increase the therapeutic potential of the drug.
3. Results

3.1. Synthesis of PB structural analogs

Although it is established that PB targets the spliceosomal core protein SF3B1 and inhibits pre-mRNA splicing, the structural elements of PB responsible for this biological activity are unknown. PB is structurally complex with multiple stereocenters, and the design of less complex PB structural variants is of interest for several reasons, including enabling the creation of probes for studying spliceosome function. To probe the structural elements of PB that are dispensable for splicing inhibition, we utilized our recent total synthesis of PB (1) (Ghosh and Anderson 2012) and herboxidiene (10) (Ghosh and Li 2011) to synthesize a number of key analogs as depicted in Table III-1 and in the supplemental materials. The details of the synthesis will be described elsewhere. We first synthesized C3-hydroxyl epimer (2) and C10-C11 anti-diastereomer (3). Furthermore, we synthesized PB analogs where specific functional groups were deleted from the side chain of PB. These analogs include preparation of C18-C19 desoxy PB (4), C18-C19 desoxy PB containing C10-C11 anti-diastereomer (5), C16, C20-didesmethyl PB (6), C16, C20-didesmethyl with C10-C11 anti-diastereomer PB (8), and their combination with C21-hydroxyl epimer (7, 9). All test compounds were purified by HPLC to 95% purity. Notably, the synthetic pathway for the desoxy compounds, which have a distinct retention time, would not allow for formation of epoxide.
Table III-1. **Chemical structure of compounds tested in this study.** IC\textsubscript{50} refers to the concentration required to reduce *in vitro* splicing by half compared to DMSO control reactions and is based on the averaged values of 3-6 individual experiments. *, the IC\textsubscript{50} for SSA was obtained from Roybal and Jurica 2010.

<table>
<thead>
<tr>
<th>Compound class</th>
<th>#</th>
<th>Changes relative to PB (1)</th>
<th>IC\textsubscript{50} for <em>in vitro</em> splicing (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>epimer</td>
<td>(2)</td>
<td>C3-hydroxy epimer</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td>epimer</td>
<td>(3)</td>
<td>C10-C11 <em>anti</em>-diastereomer</td>
<td>0.31 ± 0.23</td>
</tr>
<tr>
<td>desoxy</td>
<td>(4)</td>
<td>C18-C19 desoxy</td>
<td>0.48 ± 0.27</td>
</tr>
<tr>
<td>desoxy</td>
<td>(5)</td>
<td>C18-C19 desoxy, C10-C11 <em>anti</em>-diastereomer</td>
<td>1.16 ± 0.88</td>
</tr>
<tr>
<td>didesmethyl</td>
<td>(6)</td>
<td>C16, C20-didesmethyl</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>didesmethyl</td>
<td>(7)</td>
<td>C16, C20-didesmethyl, C21-hydroxyl epimer</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>didesmethyl</td>
<td>(8)</td>
<td>C16, C20-didesmethyl, C10-C11 <em>anti</em>-diastereomer</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>didesmethyl</td>
<td>(9)</td>
<td>C16, C20-didesmethyl, C10-C11 <em>anti</em>-diastereomer, C21-hydroxyl epimer</td>
<td>&gt; 200</td>
</tr>
</tbody>
</table>
3.2. Structural requirements for PB splicing inhibition in vitro

To test the effects of the PB analogs on splicing inhibition, we added the compounds to in vitro splicing reactions containing a synthetic pre-mRNA substrate, ATP, and nuclear extract from HeLa cells. The reaction is monitored by denaturing PAGE to separate the substrate and product mRNA, and splicing efficiency is quantified as the percentage of pre-mRNA converted to mRNA. In this system, DMSO alone has no effect on splicing (Figure III-1A, lane 1), whereas PB (1) inhibits splicing with an IC$_{50}$ of 0.1 µM (Figure III-1A, lanes 2–7, and B). Herboxidiene (10) also inhibits splicing in this system with the same IC$_{50}$ (Figure III-1B and C, lanes 29–34). The PB analogs showed different potencies, allowing us to classify several PB molecular features with respect to their contribution to in vitro splicing inhibition.

With all the C16-C20 didesmethyl compounds (6–9), absence of a pair of methyl groups flanking an epoxy moiety resulted in a loss of splicing inhibition, indicating that one or both methyl groups are key to splicing inhibition by PB (Figure III-1C, lanes 1–28, and B). Removal of the epoxy group (desoxy 4, 5) reduces splicing inhibition by more than 5-fold, indicating that the epoxy group contributes to splicing inhibition by PB but is not absolutely required (Figure III-1A, lanes 20–31, and B). Epimerization of the hydroxyl group at C3 in the ring (2), as well as of the C10-C11 linkage between the “arm” and macrolide ring (3), has no significant effect on splicing inhibition (Figure III-1A, lanes 8–19, and B), which means that the conformation of these stereocenters does not contribute to splicing inhibition by PB.
Figure III-1. **Impact of PB analogs on in vitro splicing.** (A) and (C) show denaturing gel analysis of RNA isolated from splicing reactions incubated with the indicated concentrations of dimethyl sulfoxide (DMSO), PB, PB analogs (epimer, desoxy, and didesmethyl), and herboxidiene (herb). Identities of bands are schematized to the left as (from top to bottom) lariat intermediate, pre-mRNA, mRNA, and 5' exon intermediate. The free lariat is indicated by an asterisk. (B) Quantification of normalized splicing efficiency vs. inhibitor concentration for the splicing reactions shown in (A) and (C).
In addition to assaying splicing chemistry, we also tested the effect of the compounds on spliceosome assembly. Spliceosome assembly on intron substrates via an ordered series of intermediate complexes. A subset of these complexes (H/E, A, B, and C) can be visualized by native gel analysis of the same *in vitro* splicing reactions described above. H/E and A complexes form as early intermediates that convert to B and then to C complex, at which point the splicing reaction is catalyzed. As before, DMSO alone has no effect, and spliceosomes assemble over time in the normal progression (Figure III-2A, lanes 2–5). With increasing concentrations of PB (1) (Figure III-2A, lanes 6–11) or herboxidiene (10) (Figure III-2B, lanes 29–34), spliceosome assembly appears to halt before B and C complex formation at a pre-mRNA-containing complex that migrates near the position of the early intermediate known as A complex. This A-like complex is indistinguishable from the one that forms in the presence of SSA under the same experimental conditions (Roybal and Jurica 2010). Because the amount of the complex does not increase commensurate with the loss of higher order complexes, the results are consistent with a model in which drug treatment results with a less stable A complex that is not able to proceed to the next assembly step (Corrionero et al. 2011; Roybal and Jurica 2010; Folco et al. 2011).
Figure III-2. **Native gel analysis of spliceosome assembly.** (A) and (B) show the results from time course analysis of splicing reactions in HeLa nuclear extract (NE) in 1% DMSO or 30 min time points of splicing reactions with the indicated concentrations of PB, PB analogs (epimer, desoxy, and didesmethyl), and herboxidiene (herb). The identities of complexes are denoted with assembly occurring in the following order: H/E → A → B → C. DMSO, dimethyl sulfoxide.
With the PB analogs, the effects on spliceosome assembly directly correspond to the effects on mRNA production. The didesmethyl compounds (6 – 9), which do not inhibit splicing, also do not interfere with spliceosome assembly (Figure III-2B, lanes 1–28). C3 and C10-C11 epimers (2, 3) give a block at an A-like complex at the same concentrations as PB (Figure III-2A, lanes 12–23). With the compounds lacking the epoxide (4, 5), the block at the A-like complex is not complete, and a small amount of higher order assembly still occurs (Figure III-2A, lanes 24–37), which correlates with the decrease, but not complete loss, of splicing chemistry in their presence. The similar effects of active analogs and PB indicate that they are likely inhibiting splicing assembly by the same mechanism: interference with U2 snRNA branch site recognition/progression of the A complex intermediate (Folco et al. 2011).

3.3. Cellular changes produced by PB and analogs correlate with splicing inhibition

We hypothesized that the effect of PB in cells is a function of its ability to inhibit pre-mRNA splicing. To address this subject, we used a cytological profiling assay to monitor the effect of PB and analogs on HeLa cells (Schulze et al. 2013). In the assay, HeLa cells cultured in 384-well plates were treated with test compounds in a range from nanomolar to micromolar concentration or with DMSO alone for 20 h and then stained with fluorescent probes targeting actin (phalloidin), tubulin (α-tub), total DNA (Hoechst), newly synthesized DNA (EdU), and phosphor-histone H3 (α-pHH3). The cells were imaged through automated microscopy, and a phenotypic profile for each compound was created using an algorithm that measured 244
cellular features based on the staining patterns, which provides information pertaining to cell number, cytoskeletal structure, nuclear size and morphology, DNA replication, and mitosis (Schulze et al. 2013). The profiles were compared by cluster analysis and plotted as differences in feature value relative to DMSO treatment versus compound concentration.

Treatment of HeLa cells with PB (1) results in a distinct profile relative to DMSO even at low nanomolar concentrations (Figure III-3A). The profile is primarily a result of decreases in signal for features associated with DNA synthesis and mitosis and an increase signal for features corresponding to nuclear size and shape. The C3 and C10–11 epimer compounds (2, 3) give the same strong profile as PB, which is consistent with its nearly identical inhibition of splicing chemistry and spliceosome assembly in vitro (Figure III-3A). At higher concentrations (>~100 nM), the desoxy compounds (4, 5) also give a very similar profile, but they show little change relative to DMSO at lower concentrations (Figure III-3A). This result is consistent with the desoxy compounds behaving in a manner similar to PB but with lower potency. The cytological profile of herboxidiene (10) strongly resembles that of PB (Figure III-3A). The subtle differences in the herboxidiene profile are likely due to assay variability rather than to a significantly different response. Notably, treatment with the didesmethyl compounds (6 – 9) even at the highest concentration of 67 µM yields few minimal phenotypic differences in HeLa cells in the assay (Figure III-3A), consistent with the inability of these analogs to inhibit in vitro splicing and spliceosome assembly. This result suggests that the PB scaffold does not have strong off target effects, and is consistent with PB selectivity for SF3B1.
Figure III-3. Cellular effects of PB and analogs. (A) Cytological profiles of HeLa cells treated with increasing concentrations of PB, PB analogs (epimer, desoxy, and desemethyl), and herboxidiene (herb) (2 nM to 67 µM) and SSA (0.02 nM to 0.67 µM). Each row represents a different drug condition, whereas columns indicate the change in different cell parameters relative to DMSO treatment. Blue represents an increase in the parameter, yellow represents a decrease, and black indicates no difference. Color intensity indicates the magnitude of the difference. Features associated with DNA synthesis (EdU), mitosis (pHH3), and nuclear size/shape are highlighted. (B) Cluster analysis of cytological profiles of SF3B1 inhibitors with those produced by compounds with known molecular targets/cellular effects.
We also compared the PB (1) profile with that of SSA (11), and they are nearly indistinguishable (Figure III-3A). Because SSA is a splicing inhibitor with the same molecular target in the spliceosome, this result further strengthens the hypothesis that the changes in HeLa cells are due to the ability of PB to inhibit splicing through SF3B1. Remarkably, PB/SSA profiles do not cluster with those produced by compounds that cause cell death or apoptosis, which shows distinctly different changes in the measured parameters (Figure III-3B) (Schulze et al. 2013). Instead, they most closely cluster with profiles of compounds that cause a G₁ cell cycle arrest (Figure III-3B), consistent with previous studies showing that PB and SSA arrest cell cycle progression during the G₁ phase and the G₂/M transition (Mizui et al. 2004; Kaida et al. 2007; Nakajima et al. 1996). The result may reflect a higher sensitivity of intron(s) in gene(s) required for cell cycle progression with inhibition of SF3B1 by PB, which has been reported for SSA (Corrionero et al. 2011).

### 3.4. Nuclear speckle morphology changes with PB treatment

In addition to the automated cytological profiling, we also examined the morphology of nuclear speckles in HeLa cells treated 4 h with PB or analogs (Figure III-4A). Nuclear speckles are cellular bodies that contain transcription and pre-mRNA processing components (Spector and Lamond 2011). Knockdown of PB target SF3B1 causes a “mega-speckles” phenotype in which the speckles coalesce into large bodies (Allende-Vega et al. 2013). PB treatment of cells also induces formation of mega-speckles (Kotake et al. 2007). Using immunofluorescence of SFRS2 (also called SC35), a common speckle marker, we observed mega-speckles in cells treated with PB (1) from 0.01 to 1 µM, which were absent in DMSO-treated control
cells (Figure III-4A and B). In line with the cytological profiling data, treatment with a
didesmethyl compound (6) does not cause mega-speckles to form, and treatment
with a desoxy (4) compound results in an intermediate phenotype, with fewer mega-
speckles present relative to the same concentration of PB (Figure III-4A and B). This
result underscores the correlation between in vitro splicing inhibition and the cellular
response to the drugs, i.e., the molecular features that are responsible for loss of
splicing and block in spliceosome assembly are required in the same degree for
changes in HeLa cell appearance. Mega-speckles also appear in cells treated with
herboxidiene (10), but higher concentration of the drug is required (Figure III-4A).

Finally, because PB (1) gave cytological profiles similar to cell cycle inhibitors
(Figure III-3B), we also looked at speckle morphology in cells treated with the cell
cycle inhibitors etoposide (topo-isomerase II inhibitor) and genistein (tyrosine kinase
inhibitor). Although the overall appearance of treated cells changed with respect to
DMSO, the compounds did not cause formation of mega-speckles (Figure III-4C).
This result indicates that mega-speckles do not form simply in response to cell cycle
inhibition, but to another effect of PB treatment.
Figure III-4. Changes in nuclear speckle morphology. (A) SFRS2 (SC35) immune-fluorescence (magenta) over DAPI stain (blue) of HeLa cells treated with DMSO or 0.1–1 µM of the indicated compounds for 4 h. herb, herboxidiene. (B) Box plots of relative speckle size from 20–40 nuclei images for cells treated with DMSO or 1 µM of the indicated compounds. Nonparametric Mann-Whitney U value tests show that differences between treatment and DMSO treatment are highly significant for PB (**, p<0.001) and moderately significant (*, p<0.05) for the desoxy compound. (C) Immunofluorescence images as described in (A) of cells treated with DMSO or 0.6–67 µM of the indicated cell cycle inhibitor.
3.5. Impact of PB modifications on splicing in cells

To further corroborate the relationship between the cellular phenotype produced by PB and its impact on splicing, we used RT-PCR to examine splicing of three genes in HeLa cells treated with increasing concentrations of the drug. As a control for RNA isolation and RT-PCR activity, we also examined the intron-less U6 mRNA, which was not affected by treatment with any of the compounds, even at the highest concentration of 1 µM (Figure III-5D). For the genes RBM5 and CCNA2, we designed primers to assay for skipping of selected exons that exhibited alternative splicing changes in cells treated with SSA (Corrionero et al. 2011) (Figure III-5A and B). We see similar changes in exon skipping with PB (1) treatment: increased skipping of exon 16 in RMB5 and increased skipping of exon 5 in CCNA2. However, the effect does not directly correlate with the amount of the drug, in that the highest concentration of PB resulted in a smaller change in exon skipping relative to lower concentrations. A desoxy compound (4) also increased exon skipping in both genes, but higher amounts of the drug were required to obtain the same magnitude of change. A didesmethyl compound (6), which was inactive in all previous assays, did not affect CCNA2 splicing. Surprisingly, it did alter RBM5 splicing, although the increase in exon 16 skipping was not as large as with PB or the desoxy compound. This result suggests that the didesmethyl compound still affects SF3B1 at some level and that the effect can impact certain splicing events. We did not detect splicing changes for either gene in cells treated with herboxidiene (10) (data not shown).

Notably, not all splicing events appear affected by PB treatment. For example, we did not detect a decrease in splicing of the intron between exons 6 and 7 of
SF3A1 in cells treated with any of the compounds (Figure III-5C), which had previously been observed upon knockdown of the spliceosome proteins in the SF3A complex of the U2 snRNP (Tanackovic and Krämer 2005). Together these results show that in cells PB and analogs do not affect every intron at the same level and that changes in splicing of endogenous transcripts with drug treatment are likely modulated by more than SF3B1 activity alone. Splicing consensus sequences, particularly the branch point signal, probably play a role, as has been shown for SSA (Corrionero et al. 2011). Additionally, indirect effects through splicing of other factors and differential compound stability or cellular uptake may also come into play. Still, there is a general trend that cellular splicing events appear to be most sensitive to the strongest in vitro splicing inhibitors.
Figure III-5. **Splicing changes in cells treated with PB analogs.** Semi-quantitative RT-PCR analysis of RNA isolated from cells treated with increasing drug concentrations for RBM5 exon 16 inclusion (A), CCNA2 exon 5 inclusion (B), SF3A1 intron 6 removal (C), and U6 snRNA (D). The positions of different cDNA products and molecular weight markers are diagrammed at the right and left of each gel image. The percentage of exon skipping for RBM5 and CCNA2 is indicated below each lane.
4. Discussion

PB exhibits a remarkable combination of both antitumor activity and splicing inhibition, which gives it potential as lead compound for a new class of anticancer drug. PB is a member of a family of several pladienolide natural products from *Streptomyces platensis*, all of which share a complex macrolide ring structure (Mizui et al. 2004; Sakai et al. 2002b). The other family members and a handful of synthetic analogs have varying effects on cell growth and splicing, which provides some clues as to which molecular features of PB may be important for activity (Mizui et al. 2004; Kotake et al. 2007; Müller et al. 2011; Gundluru et al. 2011; Villa et al. 2013). In this study, we used an expanded series of PB analogs to directly test the requirement of PB features for *in vitro* splicing inhibition and effects in cells and identified both dispensable and indispensable functional groups.

Our results clearly show that one or both of the methyl groups at C16 and C20 are key to inhibition of splicing and most effects of PB on cells. This finding is in line with previous reports of compounds created on a simplified PB scaffold that lacked these methyl groups and that had >10,000-fold less activity in splicing and cell growth (Gundluru et al. 2011). Although we have not yet evaluated each position independently, previous studies suggest that both of them contribute to PB activity. The presence of an additional hydroxyl group at C20 in pladienolide F results in a ∼10-fold drop in cytotoxicity (Mizui et al. 2004). Similarly, addition of a hydroxyl group at the position equivalent to C16 in the herboxidiene analog GEX1Q2 lowers activity in cell growth assays considerably (Sakai et al. 2002a). In the context of loss of the acetyl group at C7, the presence of an additional hydroxyl group at C16 in
pladienolide E or at C20 in pladienolide G has a drastic effect on activity. Absence of the acetyl group at C7 alone in pladienolide A reduces cell growth inhibition by ~250-fold relative to PB, but this reduction is further exacerbated ~1,000-fold with pladienolide E and >50,000-fold with pladienolide G (Mizui et al. 2004). However, it is notable that a hydroxyl group is also present at C16 in two other PB analogs (pladienolide D and E7107), both of which mimic the effects of PB on splicing and cells with similar potencies (Kotake et al. 2007; Folco et al. 2011). Also, isomerization of the C16-C17 bond in the context of FD-895, another related natural product with an additional hydroxyl group at C17 (Seki-Asano et al. 1994), has limited effect on the cytotoxicity of the compound (Villa et al. 2013; 2012). More studies focusing on each position independently will be required to clear up the contribution of functional groups to PB activity at these sites.

Some feature(s) of the macrolide ring distal to the arm linkage appears to contribute to the effects of PB on spliceosome assembly and thus splicing inhibition. As noted above, absence of the acetyl group at C7 in pladienolide A reduces activity (Mizui et al. 2004). Combined loss of side groups at C3, C7 and C10 abrogates bioactivity completely (Villa et al. 2013). Interestingly, herboxidiene, which has a less complex ring structure but maintains an acetyl group, inhibits splicing in vitro to the same extent as PB. Its cytological profile is very similar to PB, and the slight differences can be attributed to assay variability and clustering parameters. Notably, herboxidiene is less potent in cellular splicing assays and in impacting nuclear speckles. More work will be needed to determine whether differences between PB and herboxidiene change activity toward SF3B1 or instead alter its uptake or stability in cells. Notably, our results with the C3 epimers show that at least the
stereochemistry at this position in the macrolide ring is not important for splicing inhibition.

Absence of the epoxide at C18-C19 resulted in only a 10-fold loss of splicing inhibition or cell activity. Because PB, herboxidiene, and FR901464 share an epoxy group, it was proposed to be a required feature of a pharmacophore common to the compounds (Lagisetti et al. 2009). Based on that pharmacophore model, a set of compounds termed sudomycins was synthesized on an FR901464-like scaffold with an epoxide, and these compounds exhibit only limited cytotoxicity and effects on splicing (Lagisetti et al. 2009; Fan et al. 2011). Our data indicate that the epoxide contributes to PB activity but is not absolutely required. Corroborating this observation, a recent report provides evidence that changing the epoxide of FD-985 to a cyclopropane does not drastically alter its cytotoxicity but instead appears to stabilize the compound (Villa et al. 2013). Furthermore, recent papers identified an analog of FR901464, termed spliceostatin B, and two members of the thailanstatin family of natural products that resemble FR901464 and that do not contain the epoxide (Liu et al. 2013a; 2013b). All three compounds are considerably less cytotoxic than their epoxide-containing counterparts. Thailanstatins B and C were also shown to inhibit splicing of a reporter gene but required ~10-fold higher concentrations. These findings parallel our results with the PB desoxy compounds.

PB exhibits a remarkable combination of both antitumor activity and splicing inhibition, which gives it potential as lead compound for a new class of anticancer drug (Hong et al. 2014; Eskens et al. 2013). Our current structure-activity relationship studies demonstrate that creating less complex PB analogs that retain bioactivity is
quite feasible. As it turns out, the C3-hydroxyl stereochemistry is not critical to spliceosome inhibition, which indicates that the hydroxyl group can be replaced with isosteric functionalities, and even gem-dimethyl groups may be accommodated. Similarly, C10-C11 stereochemistry is not critical to activity, and it can be eliminated with the incorporation of gem-dimethyl groups. Furthermore, C18-C19 desoxy PB (4, 5) retains good potency, which indicates that stable PB analogs without labile epoxide functionality can be developed (Villa et al. 2013). Finally, because the activity of herboxidiene resembles PB in many aspects, the macrolide ring of PB may also be simplified. Thus, the design of structurally less complex and more stable PB analogs promises to greatly facilitate medicinal chemistry efforts toward PB analog development for further in-depth studies.

The cytostatic effect of PB along with the discovery of SF3B1 as its molecular target suggests a relationship between splicing and cancer cell growth (Kotake et al. 2007). The relationship is strengthened by the observation that PB inhibits splicing of cell cycle genes (Kotake et al. 2007) and by the identification of an SF3B1 mutation that abrogate the ability of PB to halt cell growth (Yokoi et al. 2011). Our data further underscore the link by demonstrating that the ability of PB to inhibit splicing \textit{in vitro} is tied to its ability to impact a diverse set of cellular phenotypes. However, it is important to note that changes in cell growth do not correlate with a general inhibition of splicing of all introns but are more likely to result from more subtle changes in alternative splicing of a key set of transcripts. This is evident from the differential effects that we observe in cellular splicing for different genes and from the difference in drug potency for \textit{in vitro versus in vivo} effects, which also been noted for SSA, another SF3B1 targeting drug (Corrionero et al. 2011; Furumai et al. 2010).
Presumably, our *in vitro* splicing substrate is not as dependent on robust SF3B1 activity, and more drug is required to impact its splicing, whereas certain splicing events in cells are very sensitive. These sensitive events are likely to correlate with alternative splicing choices, and the cellular effect of PB analogs will depend both on how many splicing events they modulate and on how changes in isoform ratios of the affected genes impact phenotype. So, even though the didesmethyl PB analogs affect some splicing events in cells, the absence of an effect on cytological profiles and nuclear structure suggests off target effects that would hamper development of PB as a potential cancer drug will be limited. Ultimately, how the important features of PB that we identified contribute to its activity is still an open question. It may be that they affect its affinity for SF3B1, its ability to interfere with SF3B1 function, or its stability in extracts and cells. Future studies addressing these possibilities will be key for understanding the mechanisms of PB splicing inhibition and its chemotherapeutic promise.
5. Supplemental material

5.1. Structure of PB analogs as determined by NMR

Supplementary Figure III-1. Structures of the different PB analogs as determined by NMR. For detailed NMR data, see Appendix.

5.2. Structure-activity studies for FR901464, SSA, and key derivatives

The natural product FR901464 (1) and spliceostatin A (SSA, 2), its C1 methoxy derivative, have similar splicing-inhibitory and anti-cancer activities as PB. Furthermore, they also interact with the same spliceosome protein, SF3B1 (Kaida et al. 2007; Gao et al. 2013). The exceptional combination of these biological activities led to several approaches for total synthesis of these compounds (Thompson et al. 2001; Albert et al. 2006; Ghosh and Chen 2013). We recently published a novel enantioselective total synthesis of FR901464 (1), SSA (2), and six key diastereomers.
(Ghosh et al. 2014a). The longest linear sequence was 9 or 10 steps, and the synthesis approach allowed to probe the importance of specific stereochemical features for *in vitro* activity.

We determined the biological properties of FR901464 (1), SSA (2), along with their six diasteromers (3-6) in an *in vitro* splicing system (Supplementary Figure III-2) (Roybal and Jurica 2010). We added the compounds to splicing reactions containing a synthetic pre-mRNA substrate, ATP, and nuclear extract from HeLa cells. Splicing chemistry was examined by denaturing PAGE to separate the substrate and product mRNA, while splicing efficiency was quantified as the percent of pre-mRNA converted to mRNA. In this system, DMSO alone has no effect on splicing efficiency, while SSA (2) and FR901464 (1) both inhibit splicing with an IC$_{50}$ of 0.01 and 0.05 µM, respectively (Supplementary Figure III-2A, C, D). Surprisingly, compounds 1, 5, and 6 showed an approximately 100-fold reduction in potency relative to spliceostatin A, with IC$_{50}$ values between 1 and 1.5 µM (Supplementary Figure III-2, A–D). Additionally, compounds 4, 7, and 8 were the least potent splicing inhibitors with IC$_{50}$ between 10 and 35 µM.

We also examined the effect of the compounds on spliceosome assembly. Spliceosome assembles on pre-mRNA substrates via an ordered series of intermediate complexes. A subset of these complexes (H/E, A, B, and C) can be visualized by native gel analysis of the same *in vitro* splicing reactions described above. H/E and A complexes form as early intermediates that convert to B and subsequently to C complexes, at which point the splicing reaction is catalyzed. As with splicing chemistry, DMSO alone has no effect, and spliceosomes assemble over
time in the normal progression from H/E \(\rightarrow\) A \(\rightarrow\) B \(\rightarrow\) C complex (Supplementary Figure III-2A). With increasing concentrations of SSA (2) and FR901464 (1), spliceosome assembly halts at a previously observed A-like complex (Roybal and Jurica 2010). The six diastereomers have the same effect on spliceosome assembly, but with decreased potencies that coincide directly with inhibition of splicing chemistry (Supplementary Figure III-2A, B).

In summary, we utilized six diastereomers of FR901464 (1) and SSA (2) to probe the importance of certain stereochemical features in terms of biological activity. Strikingly, all diastereomers showed over 100-fold reduction in potency relative to SSA (2), which indicates that each modification strongly impacts their activity at some level. In particular, the stereochemistry at C4 had the largest influence on splicing inhibitory activity, although there appears to be some synergistic effects with the modifications at C1 and C14.

Supplementary Figure III-2. **Impact of FR901464/spliceostatin A (SSA) analogues on in vitro splicing.** (A) and (B): (1) Top panels: Denaturing gel analysis of radiolabeled RNA isolated from splicing reactions incubated for 30 min. The first lane is 1% DMSO followed by splicing reactions incubated with indicated concentration of compound. Identities of bands are schematized to the left as (from top to bottom) lariat intermediate, pre-mRNA, mRNA, 5’ exon intermediate. (2) Bottom panels: Native gel analysis of spliceosome assembly. Aliquots of the splicing reactions described above were separated under native conditions. The identity of splicing complexes is denoted with assembly occurring in the following order: H/E \(\rightarrow\) A \(\rightarrow\) B \(\rightarrow\) C. (3) Structures of the compounds are indicated to the right of the gels. (C) Quantification of normalized splicing efficiency vs. inhibitor concentration for the splicing reactions shown in (A) and (B), respectively. (D) Summary of splicing inhibition data. IC\(_{50}\) refers to the concentration required to reduce *in vitro* splicing efficiency by half compared to DMSO control.
5.3. Evaluation of *in vitro* and cellular effects of the putative splicing inhibitor spliceostatin E

Recently a number of additional spliceostatin class natural products from the same fermentation broth that FR901464 was isolated (FERM BP3421, previously thought to be *Pseudomonas* sp., later renamed as *Burkholderia* sp. based on 16S rRNA analysis), (He et al. 2014). One of these new compounds, spliceostatin E (SSE), was of particular interest because it is structurally less complex while exhibiting good potency against multiple human cancer cell lines with IC$_{50}$ values ranging from 1.5 to 4.1 nM. This combination holds the promise of a simpler, more efficient chemical synthesis without compromising anti-tumor activity.

We reported the first enantioselective synthesis of SSE (Ghosh et al. 2014c) and tested the biological properties in an *in vitro* splicing system as previously described (Effenberger et al. 2014). Strikingly, the compound showed no inhibition of splicing in this system, even at 200 µM concentration (Supplementary Figure III-3B, C). In contrast, spliceostatin A (SSA) strongly inhibits. This result was counter to our expectations because of the recent demonstration of comparable cytotoxicity for both compounds (He et al. 2014). Therefore, we examined the effect of SSE on the structure of nuclear speckles in HeLa cells (Supplementary Figure III-3D). Potent splicing inhibitors including pladienolide B and SSA (Kaida et al. 2007) cause speckles to increase in size. We see no strong difference between cells treated with DMSO and SSE, which correlates with the *in vitro* splicing results.

In summary, we have evaluated spliceosome inhibitory activity of SSE and compared its activity with SSA. Surprisingly, SSE does not inhibit splicing *in vitro* and
does not impact speckle morphology in cells. Further analogs are required to tease apart which of the structural features altered between SSA and SSE cause the loss in activity.

Supplementary Figure III-3. **Impact of spliceostatin E (SSE) analogues on *in vitro* splicing.** (A) Structure of SSE. (B) Denaturing gel analysis of radiolabeled RNA isolated from splicing reactions incubated for 30 min. The first lane is 1% DMSO followed by splicing reactions incubated with the indicated concentration of compound. Identities of bands are schematized to the left as (from top to bottom) lariat intermediate, pre-mRNA, mRNA, 5’ exon intermediate, free intron. SSA, spliceostatin A. (C) Average splicing efficiency vs. inhibitor concentration normalized to DMSO control. Average is from three independent experiments. (D) Changes in nuclear speckle morphology. SFRS2 (SC35) immunofluorescence in HeLa cells nuclei treated with 1% DMSO, 1 µM pladienolide B (PB), and 0.1, 1, and 10 µM SSE, left to right.
5.4. Determining the importance of the C5 hydroxyl group stereochemistry for the putative splicing inhibitor GEX1Q1

Herboxidiene (HB, GEX1A) is another splicing inhibitor that also has anti-tumor activity and targets the spliceosome protein SF3B1. HB was isolated from a culture broth of *Streptomyces* sp., together with five other natural products (Sakai et al. 2002a). Another compound from that broth, GEX1Q1 (1, Supplementary Figure III-4) has shown cytotoxicity with an IC$_{50}$ value of 0.93 µM against human tumor cell lines *in vitro* (Sakai et al. 2002a), making it another promising candidate to use as a tool for structural spliceosome analysis and as the lead for novel chemotherapeutics.

A recent enantioselective total synthesis (Ghosh et al. 2014b) allowed us to test the biological properties of GEX1Q1 (1) and compound 2, (5-epi-GEX1Q1) in an *in vitro* splicing system as previously described (Effenberger et al. 2014). We added the compounds to splicing reactions containing a synthetic pre-mRNA substrate, ATP, and nuclear extract from HeLa cells. As previously characterized for this extract system, spliceosomes assemble on only a portion of the pre-mRNA substrate and catalyze intron removal (Effenberger et al. 2014). We examined splicing activity by denaturing PAGE to separate the substrate and product mRNA, and splicing efficiency was quantified as the percent of pre-mRNA converted to mRNA. In the system, DMSO alone, which has no effect on splicing efficiency, is used as a control (Effenberger et al. 2014). GEX1Q1 (1) inhibits splicing relative to DMSO with an IC$_{50}$ of 0.3 µM (Supplementary Figure III-4A, C). Compound 2 showed a slight reduction in potency with an IC$_{50}$ of 0.8 µM. However, the difference is within the variation of splicing efficiency measured by the assay. These values are also comparable to what we have previously observed for herboxidiene (Effenberger et al. 2014).
We also examined the effect of these compounds on spliceosome assembly. Spliceosomes assemble on pre-mRNA substrates via an ordered series of intermediate complexes. A subset of these complexes can be visualized by native gel analysis of the same in vitro splicing reactions described above. H/E and A complexes form as early intermediates that convert to B and then to C complex, at which point the splicing reaction is catalyzed and the complexes immediately disassemble. As with splicing chemistry, DMSO alone has no effect and spliceosomes assemble normally over time (Supplementary Figure III-4B). With increasing concentrations of GEX1Q1 (1) and compound 2, spliceosome assembly halts at an A-like complex. The block in spliceosome assembly appears to be identical to that produced by herboxidiene and two other splicing inhibitors, pladienolide B and FR901464 (Effenberger et al. 2014).

In summary, we showed that GEX1Q1 (1) is a potent splicing inhibitor, with an IC₅₀ value that is similar to other SF3B1 inhibitors like pladienolide B or spliceostatin A. We probed the importance of the C-5 hydroxyl group stereochemistry of GEX1Q1 (1) and its epimer in terms of their effect on spliceosome activity. Interestingly, both GEX1Q1 and its C-5 epimer (2) showed nearly identical potency relative to herboxidiene (Effenberger et al. 2014). Therefore, the C-5 hydroxyl group stereochemistry does not significantly influence spliceosome inhibitory activity. The design and synthesis of novel herboxidiene and GEX1Q1 derivatives are in progress.
Supplementary Figure III-4. Impact of GEX1Q1 analogues on *in vitro* splicing. (A) Denaturing gel analysis of radiolabeled RNA isolated from splicing reactions. The first five lanes include a time course of splicing reactions in 1% DMSO followed by 30 min time points of splicing reactions incubated with indicated concentration of compound. Identities of bands are schematized to the left as (from top to bottom) lariat intermediate, pre-mRNA, mRNA, 5′ exon intermediate, free intron. Structures of the compounds are indicated to the right of the gel. (B) Native gel analysis of spliceosome assembly. Aliquots of the splicing reactions described above were separated under native conditions. The identity of splicing complexes is denoted with assembly occurring in the following order: H/E → A → B → C. (C) Quantification of normalized splicing efficiency vs. inhibitor concentration for the splicing reactions shown in A. IC$_{50}$ refers to the concentration required to reduce *in vitro* splicing efficiency by half compared to DMSO control. HB, herboxidiene.
Chapter IV: Structurally distinct SF3B1 inhibitors bind the same site and extend SF3B1 function in the spliceosome

1. Abstract

The spliceosome protein SF3B1 is the target of the three structurally distinct inhibitors spliceostatin A, pladienolide B, and herboxidiene, which all have been shown to block early spliceosome assembly. By bypassing this early block, we demonstrate that in vitro the inhibitors affect later stages of the spliceosome, including exon ligation. This is the first evidence that SF3B1 activity is required throughout the splicing process. Additionally, we show that inactive analogs of each compound interchangeably compete with the active inhibitors to restore splicing. This remarkable result shows that all three compounds bind the same site on SF3B1 and likely interfere with its function by the same mechanism, supporting a common pharmacophore model. It also indicates that SF3B1 inhibition does not result from binding alone, and suggests that active compounds affect a conformational change in in the protein. Together, our data offer new mechanistic insight into SF3B1 as core player in the spliceosome.

2. Introduction

In the search for compounds that kill tumor cells, it is notable three potent and structurally distinct cytotoxic compounds, spliceostatin A (SSA, 1), pladienolide B (PB, 2) and herboxidiene (HB, 3), all target the same core protein of the spliceosome called SF3B1 (Figure IV-1) (Hasegawa et al. 2011; Kaida et al. 2007; Kotake et al. 2007). The spliceosome is the macromolecular complex responsible for pre-mRNA
splicing, which is the removal of introns present in nearly all of human gene transcripts. The SF3B1 inhibitors presumably elicit tumor cell death by interfering with a critical role of SF3B1 in splicing. SF3B1 with six other proteins forms the SF3B complex, which is a part of the U2 small nuclear ribonucleoprotein (snRNP). When SF3B1 is depleted or targeted by inhibitors in in vitro splicing assays, spliceosome assembly halts early at the point in which U2 snRNP recognizes a sequence in introns called the branch point (Folco et al. 2011; Roybal and Jurica 2010; Effenberger et al. 2014; Corrionero et al. 2011; Brosi et al. 1993). In cells, however, the three SF3B1 inhibitors do not generally block intron removal but, instead, trigger changes in alternative splicing of genes involved in apoptosis and the cell cycle, among others. Microarray analysis of 2000 splicing events from almost 500 genes suggested that SSA affects alternative splicing of introns with weak branch points (Corrionero et al. 2011). The selective effect of SF3B1 inhibitors on a limited set of gene transcripts presumably results in tumor cells showing higher sensitivity than healthy cells, which has raised interest in the compounds as drug candidates. Still, there are many open questions relating to how SF3B1 functions in the spliceosome and, in turn, how the inhibitors affect that function. Answers to these questions are important to understanding the link between spliceosome function and cancer, as well as to the potential use of SF3B1 inhibitors as chemotherapeutics.
Figure IV-1. **Chemical structures of compounds used in this study.** SSA (1), spliceostatin A; PB (2), pladienolide B; HB (3), herboxidiene; iSSA (4), inactive spliceostatin A (*aka* SSE, spliceostatin E); iPB (5), inactive pladienolide B; iHB (6), inactive herboxidiene. IC$_{50}$ refers to the concentration required to reduce *in vitro* splicing by half compared to DMSO-treated control reactions and was previously determined (Effenberger et al. 2014; Ghosh et al. 2014c), manuscript in preparation for iHB. Inactive compounds do not interfere with *in vitro* splicing up to a concentration of 200 µM.

One question is how do three compounds with very different chemical structures produce nearly identical effects in cells and on spliceosome assembly? SAR data for the three compounds and related molecules has been steadily accumulating. SSA (1), and the related compounds FR901464, meamycin, thailanstatins, and sudemycins, have the most distinct structure. PB (2), which is related to E7107 and FD-895, and HB (3) a member of GEX1 family have a similar side chain, but different ring structures (macrolide vs. tetrahydropyran). A common pharmacophore for the three classes has been suggested (Lagisetti et al. 2008; 2014), but no shared feature has been shown to be critical for activity. Knowing how
the compounds each interact with SF3B1 and how those interactions interfere with its activity is important for determining the best candidate for drug development.

Another question is why are splicing events differentially affected by SF3B1 inhibition? At some level, pre-mRNA sequence must play a role, but how sequence information is communicated to the spliceosome is poorly understood. By directly examining the relationship between intron sequence and SF3B1 activity, we may be able to understand how alternative splicing is modulated by the drugs, and why tumor cells exhibit higher sensitivity.

A third question is at which points of spliceosome function is SF3B1 activity required? There are hints that it may function at multiple stages because the stability of its association with the spliceosome appears to be regulated (Lardelli et al. 2010; Coltri et al. 2011; Ilagan et al. 2013). The spliceosome assembles through a dynamic series of intermediates called the "spliceosome cycle" in which different components associate, rearrange and leave to create a catalytically competent complex. As described above, SF3B1 joins early with U2 snRNP during A complex formation. However, unlike most other spliceosome components that play an early role in intron recognition and leave, SF3B1 remains associated with the spliceosome after full assembly to B complex and through activation and catalysis in C complex (Agafonov et al. 2011).

In this paper, we present data that address these questions by demonstrating that the three SF3B1 inhibitors share a common binding site and likely affect a conformational change in the protein to inhibit its activity. We also show that the requirement for SF3B1 is not directly affected by intron sequence, indicating that in
cells other factors are impacting which genes the drugs target. Finally, by bypassing the earlier stages of inhibition, we show that SF3B1 activity is required at additional stages of spliceosome assembly and catalysis. Together, our results expand the role of SF3B1 function in the spliceosome and show that SF3B1 inhibitors share the same mechanism, which is fundamental to understanding what happens when splicing is altered in cells.

3. Results

3.1. Inactive analogs compete with SF3B1 inhibitors

It is well established that SSA (1), PB (2), and HB (3) bind to the core splicing protein SF3B1 and inhibit splicing in the in vitro system. We previously identified inactive analogs of the three compounds (Figure IV-1; iSSA, 4; iP, 5; iHB, 6) that revealed different structural features crucial for splicing inhibition (Roybal and Jurica 2010; Effenberger et al. 2014; 2013; Ghosh et al. 2014b; 2014c). However, we do not know whether the compounds are inactive because they cannot bind to SF3B1 any longer, or because they bind but do not interfere with the function of SF3B1. To distinguish between the two scenarios, we set up competition assays in which we incubated a pre-mRNA substrate under splicing conditions with different ratios of active to inactive compounds (Figure IV-2A). If the inactive compounds still bind to SF3B1, then it will outcompete the active version and, when in excess, restore splicing. On the other hand, if inactive compounds do not bind, then even large excess will not be able to outcompete the active version and splicing will not be restored. As previously shown, SSA (1), PB (2), and HB (3) each completely inhibit in
vitro splicing at 1 µM, whereas the corresponding inactive derivatives have no effect (Figure IV-2A, lanes 3-5 vs. 6-7). Surprisingly, addition of the corresponding inactive compound at increasing concentrations restores splicing completely for PB (2) and HB (3), and partially for SSA (1) (Figure IV-2A, lanes 9-17). For example, 2 µM iHB (6) restores 50% of splicing in the presence of 1 µM HB (3), and 100 µM iHB (6) restores essentially all splicing. iSSA (4) also restores splicing in the presence of SSA (1), but only partially, even at the highest concentration tested. We conclude that all the inactive compounds still bind SF3B1 and compete with their active counterparts, although with different affinities.

Additionally, because the inactive analogs do not interfere with splicing, we also conclude that SF3B1 binding is not sufficient for splicing inhibition. Instead, the compounds could impair SF3B1 by modulating a conformational change in the protein or a binding partner. Alternatively, the active compounds could block an interaction between SF3B1 and another spliceosome protein. This later scenario seems less likely because it is hard to imagine how three different small changes (i.e. the features altered in the inactive compounds) would each prevent an inhibitor that is occupying a binding site from blocking a protein/protein interaction. Whereas, if the bound inhibitors alter a conformational change in SF3B1, small differences in many chemical features could affect how the compounds modulate a structural shift.
### 3.2. Three distinct SF3B1 inhibitors bind to the same site

Given the similar effects that the three SF3B1 inhibitors have on cell growth and morphology, and the potential of a common pharmacophore, we hypothesized that SSA (1), PB (2), and HB (3) interact with the same binding site on SF3B1. We tested this hypothesis using the same competition assay with an active inhibitor and increasing concentrations of inactive analogs of the two structurally distinct inhibitors (e.g. SSA (1) with increasing concentrations of iPB (5) and iHB (6)). Strikingly, all three inactive analogs outcompete any active compound (Figure IV-2B). This result provides the first evidence that all three inhibitors bind to the same site in SF3B1.

Together our data also show that the three compounds differ in their binding affinities for SF3B1 (Figure IV-2C). With all the inactive analogs, we found that less compound is required to compete for HB (3) inhibition relative the amounts required to compete with PB (2) and SSA (1). Conversely, more of each inactive compound was required to compete with SSA (1) relative to HB (3) and PB (2). These data allows us to rank the affinity of the compounds relative to one another, with SSA (1) > PB (2) > HB (3) in SF3B1 binding affinity. In the same vein, the inactive analogs can also be ranked according to their affinities. Less of the iHB (6) was consistently required to compete with the active compounds, relative to iPB (5) and iSSA (4). In contrast, iSSA (4) was the worst competitor of the inactive compounds for all three active molecules. If competition between active and inactive compounds is mediated by affinity, these results indicate that the feature that is modified to inactivate SSA (1) is more important for binding than the features that are modified in PB (2) and HB (3), respectively.
Figure IV-2. SSA, PB, and HB bind to the same site on SF3B1. (A) Denaturing gel analysis of RNA isolated from splicing reactions incubated with no nuclear extract (NE), DMSO, or the indicated compounds. In lanes 3-8 compounds were added at the indicated concentration; in lanes 9-17 splicing reactions contained 1 µM active compound and 1, 10, or 100 µM inactive compound. Identities of bands are schematized to the left as (from top to bottom) lariat intermediate, free lariat, pre-mRNA, mRNA, free intron, and 5' exon intermediate. (B) Same as panel (A). (C) Columns show the concentration of inactive compounds that restores 50% splicing in the presence of 1 µM active compound (rows). Values were determined by quantification of the splicing reactions shown in (A) and (B).
3.3. SF3B1 inhibition is independent of branch point sequence *in vitro*

While each of the SF3B1 inhibitors have been shown to affect early spliceosome assembly *in vitro*, the assembly step most affected appears to vary (Folco et al. 2011; Roybal and Jurica 2010; Effenberger et al. 2014; Corrionero et al. 2011). For example, the Reed lab showed that that in nuclear extract the PB analog E7107 blocks a U2 snRNP interaction with an oligonucleotide containing the branch point sequence (Folco et al. 2011). Notably, the drug did not affect the interaction if the extract was pre-treated with ATP. They concluded that that PB interferes with an ATP-dependent conformational change in U2 snRNP that exposes the branch point binding sequence, which would preclude stable incorporation into the spliceosome. However, this model is inconsistent with data that show U2 snRNA / pre-mRNA base pairing both before ATP addition and in SSA-inhibited spliceosomes (Corrionero et al. 2011; Wassarman and Steitz 1992). The differences between these results could be attributed to differences between the compounds, the assay systems, the pre-mRNA substrates employed, and/or heparin concentration in native gels. Our results from Figure IV-2 strongly suggest that the three SF3B1 inhibitors are mechanistically interchangeable, which led us to look at the other factors.

One prediction of the Reed model is that if nuclear extract is pre-treated with ATP, U2 snRNP would be in a conformation competent to base pair with the branch point sequence and no longer sensitive to SF3B1 inhibitors. We tested that prediction by pre-incubating nuclear extract with ATP before and after PB (2) addition, and then used those extracts for *in vitro* splicing. In both cases, we observe a loss of splicing and a block in assembly at the same A-like complex (Figure IV-3A, B). This
result suggests that in the context of a complex assembly on an intact pre-mRNA, the conformational change in U2 proposed to be inhibited by PB (2) does more than just expose the branch binding sequence for base pairing, and that SF3B1 has an additional role in spliceosome assembly.

To look at the interplay between pre-mRNA sequence and susceptibility to SF3B1 inhibition, we tested the hypothesis that splicing substrates with a weak branch point sequence will be more sensitive to SF3B1 inhibitors relative to those with a strong branch point sequence. We based this hypothesis on the observation that alternative splicing changes caused by SSA (1) in cells occur with introns containing weak branch point sequences putatively because of less stable U2 snRNA base pairing (Corrionero et al. 2011). If the function of SF3B1 targeted by the inhibitors can be compensated by more stable U2 snRNA base pairing, we expected to see an increase in IC$_{50}$ value with branch sequence strength (i.e., more drug is required for inhibition). We generated pre-mRNA substrates for in vitro splicing with different branch point sequence strength, including those from introns that show drug sensitivity in cells (Effenberger et al. 2014; Corrionero et al. 2011) and measured splicing efficiency of these pre-mRNAs with increasing concentration of the drug SSA (1) (Figure IV-3C). As expected, we found that changes from consensus in branch point sequence result in a decreased splicing efficiency in the in vitro assay system (Supplementary Figure IV-1A). However, the concentration of drug that reduces splicing by 50% relative to the no-drug control was similar for each substrate. This result indicates that substrates with different branch point sequences have the same dependence on SF3B1 in nuclear extract. It also implies that U2 snRNA / pre-mRNA base pairing alone cannot explain the different sensitivity to SF3B1 inhibitors in cells.
Because SF3B1 interactions with the protein U2AF2 at the polypyrimidine tract (PYT) downstream of the branch point are also important for splicing (Gozani et al. 1998) we also examined the effect of PYT length on SF3B1 inhibition (Figure IV-3D, Supplementary Figure IV-1B). Again, the splicing efficiency is decreased with a shorter PYT, but the IC$_{50}$ value for SSA (1) is not affected. This result shows that in an in vitro context, the drug-targeted function of SF3B1 is not compensated by its interaction with U2AF2 bound to the PYT. Together these results show that splicing sequence strength alone does not confer increased sensitivity to SF3B1 inhibitors, and suggests that other factors and or sequence context play a role in mediating the differential splicing changes observed for transcripts with SF3B1 inhibitor in cells.
Figure IV-3. **Branch point region and polypyrimidine tract do not correlate with compound-sensitivity.** (A) Native gel analysis of spliceosome assembly. Results from time-course analysis of splicing reactions in HeLa nuclear extract (NE) incubated for indicated time (minutes). No ATP indicates ATP-depleted NE; +ATP indicates ATP-depleted extract with added ATP; in lanes 3-6 NE was pre-incubated with 1 µM PB (2) followed by addition of ATP and pre-mRNA substrate; in lanes 7-10 NE was pre-incubated with ATP followed by addition of 1 µM PB (2) and pre-mRNA substrate. The identities of spliceosome complexes are denoted with assembly occurring in the following order: H/E → A → B → C. (B) Denaturing gel analysis of the same in vitro splicing reactions shown in (A). Band identities are indicated on the left as in Figure IV-2. (C) In vitro splicing efficiency of pre-mRNA substrates with different branch point regions normalized to DMSO control reactions are shown plotted against SSA (1) concentration. The branch point A is underlined. (D) Same as (C) using pre-mRNA substrates with different polypyrimidine tract length.
3.4. **SF3B1 activity is required after stable A complex formation**

Previous studies have shown that the A-like splicing complex that accumulates in the presence of SF3B1 inhibitors SSA and E7107 is sensitive to heparin concentration (Folco et al. 2011; Corrionero et al. 2011). Based on these observations, it has been proposed that the drug blocks an SF3B1 conformation required for stable U2 snRNP association with the spliceosome. We also observe by native gel analysis that the A-like complex that also forms in the presence of PB (2) disappears with increasing concentrations of heparin (Figure IV-4A, lanes 1-5). Previous studies point to an ATP-dependent step in the formation of stable A complex (Brosi et al. 1993), but it is not clear whether this is the same step that is disrupted by the drug. If they are the same, then the drugs should not inhibit splicing following stable U2 incorporation into the spliceosome.

To test whether SF3B1 inhibitors can affect spliceosome assembly after stable A complex formation, we needed to bypass the first block caused by the drugs. To do this we assembled splicing complexes in extracts depleted of U4/U6 snRNAs. We achieved depletion by using the endogenous RNase H present in splicing extracts in combination with short DNA oligonucleotides complementary to U4 and U6 snRNAs (Blencowe et al. 1989) (Supplementary Figure IV-2A). The A complex that accumulates in this extract is much more stable to heparin concentration relative to the complexes that form in the presence of SF3B1 inhibitors (Figure IV-4A, lanes 6-10). We used these stable A complexes to test whether SF3B1 inhibitors would affect the formation of mature spliceosomes when chased with new extract containing U4/U6 snRNAs. To protect the U4/U6 snRNAs in the chase extract, we
used DNase I digestion to destroy the RNase H oligos in the ∆U4/6 extracts. To prevent any new A complex assembly, we also depleted U2 snRNA in the chase extract by the same RNase H digestion followed by DNase I digestion protocol (Supplementary Figure IV-2A). As expected, no complexes form past E/H in the ∆U2 extract (Figure IV-4B, lanes 5 and 7), and when ∆U4/6 and ∆U2 extracts are mixed, normal complex formation occurs (Figure IV-4B, lane 3).

To test whether SF3B1 inhibitors block splicing after stable A complex formation, we added drugs to the ∆U2 chase extract. With DMSO, spliceosome assembly is rescued as observed by the appearance of robust B/C complex (Figure IV-4B, lane 11). In contrast, little to no higher order spliceosome complex formation occurs in the presence of HB (3) and SSA (1) (Figure IV-4B, lanes 12, 16); and although a small amount of B/C complex may still be forming, we do not detect any splicing chemistry (Supplementary Figure IV-2B, C). This result shows for the first time that SF3B1 inhibitors affect in vitro splicing after stable A complex formation.

Given the high specificity of the inhibitors, it is likely that the compounds interfere with the same or a very similar conformational change in SF3B1 affect initially by the inhibitors. If this hypothesis is true, then the inactive inhibitor analogs should rescue spliceosome assembly at the next block as well. Using the same competition approach described for Figure I-1, we indeed found that increasing amounts of inactive compounds restore normal spliceosome assembly. In parallel to our previous results, the identity of the inactive analog was not important (Figure IV-4B, lanes 12-15 and lanes 16-19), which further suggests that the mechanism of the inhibitors is the same both before and after stable A complex formation.
Figure IV-4. **SF3B1 inhibitors interfere with spliceosome assembly after stable A complex formation.** (A) Native gel analysis of A complex spliceosomes assembled in the presence of PB (2) (lanes 1-5) or in nuclear extract (NE) depleted of U4/6 snRNAs (∆U4/6) for 30 minutes at 30°C and challenged with increasing heparin concentration (0.5, 1.25, 2.5, 3.75, 5 mg/ml). (B) Native gel analysis of spliceosome complexes assembled in snRNA-depleted NE in the presence of the indicated compounds. Control reactions in lanes 1-10 were incubated for 30 minutes at 30°C unless otherwise indicated. Mock indicates mock-depleted NE; mix indicates combined ∆U2 NE and ∆U4/6 NE; active compounds are at 5 µM and inactivate HB is at 200 µM. In lanes 11-19 stable A complexes were accumulated in ∆U4/6 NE for 10 minutes before the indicated compounds and ∆U2 NE were added and incubated for additional 20 minutes. Where added active compound are at 5 µM and inactive HB at the indicated ratio. Complex identities are indicated on the left as in Figure IV-3.
3.5. SF3B1 inhibitors block exon ligation

Given that SF3B1 is present throughout the spliceosome cycle (Ilagan et al. 2013; Agafonov et al. 2011), we hypothesized that in addition to its roles in A complex, it also functions at later stages of spliceosome assembly. We decided to examine catalytic spliceosomes in this context, specifically after 1st step chemistry, using the bimolecular exon ligation reaction (Konforti and Konarska 1995; Anderson and Moore 1997) (Figure IV-5A). If the conformational change in SF3B1 suggested by our previous experiments also has a role in the spliceosome after 1st step chemistry, then exon ligation should be affected by SF3B1 inhibitors. To by-pass the early points of PB (2) inhibition, we incubated an unlabeled pre-mRNA 5' substrate that contains a 5' exon and an intron in nuclear extract. This substrate will complete first step chemistry to produce a free 5' exon and lariat intron intermediate. We then added a labeled 3' substrate consisting of a 3' exon preceded by a 3' splice site with and without drug. In this assay with just DMSO added, we detected a labeled mRNA band that is a result of exon ligation (Figure IV-5B, lane 3). However, the mRNA band nearly disappears with increasing concentration of PB (2) (Figure IV-5B, lanes 4-9), indicating that PB (2) still binds its target, SF3B1, to inhibit exon ligation, and is also the first demonstration of a functional role for SF3B1 after 1st step chemistry.

We also tested whether inactive analogs could compete with PB (2) inhibition of exon ligation. As with earlier inhibited steps, increasing concentrations of both inactive PB (5) and inactive HB (6) restore mRNA production (Figure IV-5B, lanes 10-17). This result supports the idea that a conformational change in SF3B1 is required at multiple steps of the spliceosome cycle, including after 1st step chemistry.
Figure IV-5. **Bimolecular assay shows that PB inhibits exon ligation.** (A) Schematic of the bimolecular exon ligation reaction. The 5' substrate consists of a 5' exon and intron containing the branch point and polypyrimidine tract and is capable of 1\textsuperscript{st} step splicing chemistry to produce splicing intermediates. The 3' substrate consists of a 3' splice site and 3' exon and is capable of 2\textsuperscript{nd} step chemistry to produce mRNA when added in trans. To test for inhibition, PB is added with the 3' substrate. (B) Denaturing gel analysis of in vitro bimolecular exon ligation reactions. Identities of bands are schematized to the left as (from top to bottom) lariat intermediate, 5' substrate, mRNA, 5' exon and 3' exon. A three-day exposure is shown for the top region of the gel, while the bottom region is a one-day exposure. In lanes 1 and 2 both 5' and 3' substrates are radiolabeled. In lanes 3-17 only the 3' substrate is radiolabeled. In lanes 4-9 increasing concentration of PB (2) (0.1, 1, 2, 5, 10, 100 µM) were included with the 3' substrate. In lanes 10-12 and 14-16 active compound was added at 2 µM and increasing concentration of inactive compound was added at the indicated ratio. In lanes 13 and 17 inactive compound is at 200 µM.
4. Discussion

The goal of our studies was to understand the mechanisms of splicing inhibition by three structurally distinct SF3B1 inhibitors. This understanding should in turn provide insight into the role of SF3B1 in the spliceosome. We succeeded in both aspects. First, we made the remarkable discovery that inactivating changes to SF3B1 inhibitors do not necessarily abrogate compound binding. This means binding alone is not sufficient to confer SF3B1 inhibition, and lead us to speculate that the inhibitors prevent a conformation change in SF3B1 that is required for its role in splicing (Figure IV-6).

Figure IV-6. Model of SF3B1 function in the spliceosome cycle. Spliceosomes assemble on a pre-mRNA substrate through a series of intermediate splicing complexes (E → A → B → C → P). We propose that a conformational change in SF3B1 is required at multiple transitions, and that SF3B1 inhibitors bind the same site to interfere with the switch.
We also found that SSA (1), PB (2) and HB (3) bind to the same site on SF3B1, which strongly supports the presence of a common pharmacophore between the compounds, although the features of the pharmacophore are still not readily apparent. PB (2) and HB (3) resemble each other with a side chain linked to a ring structure. The side chains are similar, and the positions that differ do not affect PB (2) activity as evidenced by analogs that also differ in those positions and still retain activity (Effenberger et al. 2014; Ghosh et al. 2014b; Sakai et al. 2002a; Mizui et al. 2004). In our study, the modification that inactivates PB (5) is the loss of two methyl groups in the side chain; a change has never been present in active HB analogs (Effenberger et al. 2014; Sakai et al. 2002a). In terms of the rings, the PB (2) macrolide ring is more complex than the tetrohydropyran of HB (3), and the positions that are altered in inactive HB (6) do not have an obvious equivalent in PB (2). Previous SAR data for PB indicates that an acetyl group on the macrolide ring is important (Mizui et al. 2004). SSA (1) has a very different architecture, and the shared characteristics with PB (2) and HB (3) are not obvious. The inactivating change in SSA (4) is localized to the ring containing an epoxide (note that iSSA is equivalent to SSE) (Ghosh et al. 2014c). Although it is clear that our data indicate a shared binding site, it is difficult to imagine how these three compounds with very different structures interact with SF3B1. It is also remarkable that changes to distinct chemical features inactivate them and yet do not abrogate binding. Nonetheless, these data represent an excellent starting point for SAR studies to differentiate the features of the common pharmacophore that confer binding from those that directly confer inhibition.
Even though these compounds behave similarly *in vitro*, their structural differences could affect their stability, permeability, *etc.* in cells. However, all three drugs have been tested in a cytological profiling assay in HeLa cells with nearly identical outcomes. The three compounds also confer similar "mega-speckle" phenotypes in HeLa cells as determined by immunofluorescence staining with an antibody to the splicing factor SRSF2 (SC-35) (Kaida et al. 2007; Effenberger et al. 2014). The Koide lab showed that HB (3) and SSA (1) have similar IC$_{50}$ values for splicing of a minigene reporter in HEK293-II cells, although meamycin B, another FR901464 derivative, is more potent purportedly because it is more stable (Gao et al. 2013). For splicing of endogenous genes, SSA (1) and HB (3) both cause retention of an intron in the p27 transcript (Hasegawa et al. 2011; Kaida et al. 2007), and SSA (1) and PB (2) both promote exon 16 skipping in the RBM5 transcript (Effenberger et al. 2014; Corrionero et al. 2011). The compounds have also each been assayed for cytotoxicity in a variety of cancer cell lines, however with little overlap (Sakai et al. 2002a; Mizui et al. 2004; Gao et al. 2013; Sakai et al. 2004; Nakajima et al. 1996). The reported IC$_{50}$ values are in the low nanomolar range for all three compounds, although in some cases, HB (3) seems to be a somewhat less potent growth inhibitor (Gao et al. 2013). Therefore, most indications suggest that as *in vitro* the compounds behave similarly in cells. Still, whether they all affect the same splicing events and can be used interchangeably needs to be tested directly.
In using the drugs to investigate SF3B1 function, we were surprised that \textit{in vitro} the requirement for SF3B1 activity is independent of branch point sequence and PYT strength. This finding contrasts with the effect of SF3B1 inhibitors in cells, which appear to preferentially affect splicing of introns with weaker branch point sequences. We conclude that in cells other factors play a role in specifying the alternative splicing changes induced by SF3B1 inhibitors. Identifying those factors will be key to understanding how cancer cells are sensitized to SF3B1 inhibitors.

The remarkable observation that SF3B1 inhibitors affect the spliceosome cycle from early assembly to splicing catalysis represents a new and important addition to our understanding of SF3B1 function in the spliceosome. Inhibition of SF3B1 at all steps is rescued by inactive analogs, suggesting that the same conformational change in SF3B1 that we inferred from our competition data is involved. We propose that SF3B1 cycles between conformational states to progress the spliceosome to the next stage, and that the inhibitors interfere with this switch (Figure IV-6). This model is consistent with observations from the Reed lab that a PB analog inhibits a conformational switch in SF3B1 that allows U2 snRNP to stably bind an oligonucleotide containing the branch point sequence (Folco et al. 2011). It also is consistent with the observation, that SSA prevents a contact between SF3B1 and the pre-mRNA that normally forms during A complex stabilization (Corrionero et al. 2011; Gozani et al. 1998; 1996). We speculate that the changes in stability of the association of the SF3B complex with the spliceosome, which have been observed before catalytic activation and after exon ligation, result from the same conformational switch (Lardelli et al. 2010; Coltri et al. 2011; Ilagan et al. 2013). Finally, it is possible that changes in SF3B1 conformation are linked to the
rearrangements in U2 snRNA structure that also cycle during spliceosome assembly and which clearly have roles in branch point recognition, A complex stabilization, and catalytic activation of the spliceosome (Perriman et al. 2003; Perlman et al. 2004; Hilliker et al. 2007; Perriman and Ares 2007; 2010; Ghosh and Chen 2013; Ghosh and Anderson 2012). The SF3B1 inhibitors open the door to investigating these possibilities in the future.
5. Supplemental Material

5.1. Supplementary Figure 1

Supplementary Figure IV-1. **Splicing efficiency decreases with weak branch point region or shorter polypyrimidine tract.** Denaturing gel analysis of RNA isolated from splicing reactions incubated with DMSO or the indicated concentration of SSA (1). Identities of bands are schematized to the left as (from top to bottom) lariat intermediate, free lariat, pre-mRNA, mRNA, and 5′ exon intermediate. The branch point A is underlined. (A) Pre-mRNA substrates with different branch point regions and 28 nt polypyrimidine tract. (B) Pre-mRNA substrates with a consensus branch point region and varying polypyrimidine tract length.
Supplementary Figure IV-2. **Splicing and rescue in snRNA-depleted nuclear extracts.** (A) Denaturing gel analysis of RNA isolated from 5 µl of nuclear extract (NE), stained for total RNA with SYBR Gold™. Lane 1: untreated NE; lane 2: mock-treaded NE; lane 3: U4/U6 snRNA-depleted NE; lane 4: U2 snRNA-depleted NE. (B) and (C) Denaturing gel analysis of the same reactions shown in Figure IV-4B. (B) snRNA-depleted NE. Lane 1: no splicing control; lane 2: mock-depleted NE; lane 3: mixture of ∆U2 and ∆U4/6 NE; lanes 4: U4/6 snRNA-depleted NE; lane 5: U2 snRNA-depleted NE. (C) Lanes 1-4: Mock-depleted NE with indicated compounds (200 µM for inactive compound, 5 µM for active compound). Lanes 5-10: Stable A complexes were accumulated in ∆U4/6 NE for 10 minutes before the indicated compounds and ∆U2 NE were added and incubated for additional 20 minutes. Reactions contain 5 µM active compound and 5 µM, 50 µM, or 200 µM inactive compound, resulting in the indicated 1:1, 1:10, or 1:40 ratios. Identities of bands are schematized to the left as (from top to bottom) lariat intermediate, free lariat (in (C) only), pre-mRNA, mRNA, free intron, and 5 exon intermediate.
Chapter V: Summary, impact, and future directions

My dissertation work had two related goals: (1) stall spliceosomes using new small-molecule inhibitors, and (2) use these inhibitors to understand the function of individual spliceosome proteins. With my results, I wanted to add new tools and increase the currently limited mechanistic understanding of the spliceosome.

I reached the first goal of stalling spliceosomes with new small-molecule tools by identifying three new splicing inhibitors in a large compound library through high-throughput screening (Chapter II). I transformed an established in vitro splicing assay so that reactions can be set up in 384-well plates using liquid handling robots. I also developed a pipeline to verify potential hits, manage and visualize the screening data, and determine which library compounds are dose-dependent splicing inhibitors. Using this system, I identified three compounds that block splicing at different assembly stages. I further characterized where hit compounds stall spliceosomes with respect to chemistry and complex assembly using standard in vitro assays. To test whether splicing of endogenous pre-mRNA transcripts in HeLa cells is altered, I developed an RT-PCR assay for endogenous pre-mRNA transcripts. Because I also wanted to determine the effect of splicing inhibitors on cells, I collaborated with the UCSC Screening Center and co-workers to establish phenotypic microscopy-based assays. This series of assays and protocols facilitated and streamlined the initial characterization of small molecules and is readily available for any new compound to be tested in the future.
In addition to characterizing the three hits from my screen, I used the *in vitro* and *in vivo* pipeline for extensive structure-activity relationship analyses on a class of compounds called SF3B1 inhibitors (Chapter III). This class consists of three main members (SSA, PB, and HB), along with several closely related compounds and a large number of chemically synthesized structural analogs. For all three compounds, I determined functional groups that are required for splicing inhibition and other features that are dispensable. The results showed ways to simplify the complex synthesis and pointed out positions that can be modified to generate biological probes, for example with a cross-linker or fluorescence moiety.

I reached the second goal of using inhibitors as tools for functional studies when I determined that the three structurally distinct inhibitors SSA, PB, and HB all bind to the same site on the core spliceosome protein SF3B1 (Chapter IV). With a combination of active and inactive compounds from my structure-function studies and a competition/rescue approach I also showed that the molecules can be used interchangeably, but vary in potency relative to each other. Furthermore, I manipulated the assay system to bypass the first block caused by the inhibitors and identified two additional assembly stages in which SF3B1 function was required, including a later role before exon-exon ligation.

Taken together, my work expanded our mechanistic understanding of the spliceosome by adding new tools and defining the function of a core protein. While my research focused on biochemistry, the results are also beneficial for several other fields including medicinal chemistry, cancer biology, and drug development. Additionally, the data opened the door for several follow-up experiments. For
example, if drug-stalled complexes are stable enough for affinity purification, a combination of mass spectrometry and electron microscopy can identify their protein composition and overall shape, respectively (Jurica et al. 2002; Ilagan et al. 2013). This information will add more detail to the spliceosome cycle (Figure I-1D) and might help to determine how the spliceosome transitions from one intermediate to the next.

The SF3B1 inhibitors are currently of particular interest because of their direct connection to cancer, but their relevance and their effect on splicing in tumors is not well understood. My results expand the functional role of SF3B1, which opens the road to determine how changes in those functions relate to cancer. For example, a possible experiment would be to test whether the inhibitors are still active when SF3B1 is mutated, which would be a requirement for their use as potential new chemotherapeutics. Another experiment could focus on determining where exactly SSA, PB, and HB bind on SF3B1, and where the binding site is relative to the region that is mutated in cancer. My structure-activity relationship analyses pointed out features on the inhibitors that can be modified with a cross-linker, which in combination with mass spectrometry will pinpoint the binding site. Previous data suggest that the inhibitor interaction site is on the opposite end of SF3B1 relative to the cancer mutations (Yokoi et al. 2011), and that likely the inhibitors will still work.

My results will also help to interpret genome-wide studies measuring splicing changes under disease conditions, because SF3B1 inhibitors are useful tools to determine which gene transcripts are critical for tumor survival. Comparing RNA sequencing data from untreated cells to cells treated with SSA, PB, or HB will show
which splicing events are the most sensitive, and whether the three different inhibitors affect the same transcripts. It would also be interesting to identify how splicing, especially of critical transcripts, changes in tumors with SF3B1 mutations. These data would shed light on the relevance of SF3B1 mutations for cancer development and progression and their influence on disease prognosis.

The connection between splicing and cancer is interesting and the potential for novel chemotherapeutics should not be dismissed, but it is even more important to understand how the different parts of the spliceosome functions to precisely remove introns on most human transcripts under normal, healthy conditions. My work shows that it is possible to find new inhibitors, and that these compounds lead to new mechanistic insights, but it is also clear that we are still lacking tools. The high-throughput assay and follow-up pipeline makes it straightforward to screen more compound libraries for tools that block spliceosome assembly at new assembly stages. Since the most potent splicing inhibitors are natural products, the next library to screen would likely be a natural product library with a great diversity of chemical scaffolds. Highly potent hits might represent new drug lead compounds, but, more importantly, they will help to build a more complete mechanistic understanding of the spliceosome.
Chapter VI: Methods and materials

1. *In Vitro* splicing reactions

Pre-mRNA substrate was derived from the adenovirus major late transcript. A $^{32}$P-UTP body-labeled G(5')ppp(5')G-capped substrate was generated by T7 run-off transcription followed by gel purification. Where indicated, additional pre-mRNA substrates with varying branch point region or polypyrimidine tract length were used. Nuclear extract was prepared as previously described (Dignam et al. 1983) from HeLa cells grown in DMEM/F-12 1:1 and 5% (v/v) newborn calf serum. For splicing reactions, 10 nM pre-mRNA substrate was incubated with 60 mM potassium glutamate, 2 mM magnesium acetate, 2 mM ATP, 5 mM creatine phosphate, 0.05 mg ml$^{-1}$ tRNA, and 50% (v/v) HeLa nuclear extract at 30 °C. For yeast splicing reactions, extracts were prepared according to Yan et al. (Yan et al. 1998) and assayed using RP51A pre-mRNA at 4 nM as previously described (Perriman and Ares 2000).

2. Bi-molecular exon ligation

Templates for 5' and 3' substrates were generated by PCR of same AdML construct using primers that added a 5' T7 promoter sequence. $^{32}$P-UTP body-labeled G(5')ppp(5')G-capped 5' substrate and 32P-UTP body-labeled GMP-capped 3' substrate were generated by T7 run-off transcription followed by gel purification. Unlabeled G(5')ppp(5')G-capped 5' substrate was generated by T7 run-off transcription followed size exclusion chromatography. For bimolecular exon ligation reactions, 5 nM 5' substrate was incubated under standard *in vitro* splicing conditions.
After 30 minutes, 3’ substrate in splicing buffer with and without SF3B1 inhibitors was added to the reaction and incubated for an additional 60 minutes.

3. Denaturing gel analysis

RNA was extracted from in vitro splicing reaction and separated on a 15% (v/v) denaturing polyacrylamide gel. $^{32}$P-labeled RNA species were visualized by phosphorimaging and quantified with ImageQuant software (Molecular Dynamics). Splicing efficiency is the amount of mRNA relative to total RNA and normalized to a dimethyl sulfoxide (DMSO) control reaction. IC$_{50}$ values for inhibitors are the concentrations of inhibitor that cause 50% decrease of splicing efficiency, which were derived from averaged plots of splicing efficiency vs. compound concentration from 2–6 independent assays.

4. Native gel analysis

Splicing reactions were set up as described above and incubated 30 °C for 4–30 min. Time point samples were kept on ice until all samples were ready for analysis. 10 µL of splicing reactions were mixed with 10 µL of native gel loading buffer (20 mM Trizma base, 20 mM glycine, 25% (v/v) glycerol, 0.1% (w/v) cyan blue, 0.1% (w/v) bromphenol blue, 1 mg mL$^{-1}$ heparin sulfate) and incubated at room temperature for 5 min before loading onto a 2.1% (w/v) low melting temperature agarose gel. Gels were run at 72 V for 3.5 h, dried onto Whatman paper, and exposed to phosphorimaging screens, which were digitized with a Typhoon Scanner (Molecular Dynamics). For heparin stability experiments, the native gel loading buffer had heparin concentrations between 1 to 10 mg mL$^{-1}$. For yeast splicing complexes,
5 μL of yeast splicing reactions were mixed with 5 μL complex buffer, and yeast splicing complexes were separated using 0.5% (w/v) agarose, 3% 80:1 acrylamide:bis (v/v) gel runs for 16h at 85 V (Perriman and Ares 2000).

5. snRNA-depletion of HeLa nuclear extracts

HeLa nuclear extracts were depleted of U2, U4, and U6 snRNAs using a combination of RNase H and DNase I digestion. First, nuclear extracts were incubated in the presence of endogenous RNase H, 2 mM magnesium acetate, 2 mM ATP, and 10 μM DNA oligonucleotides complementary to the snRNA to be depleted for 60 min at 30 °C. Then, DNase I was added and incubated for 10 min at 30°C to digest the DNA oligonucleotides. The following DNA oligonucleotides were used for depletion: U2, 5'-ATCGCTTCTCGGCCT-3' (Black et al. 1985); U4, 5'-AGCTTTGCGCAGTGG-3' and 5'-CTAATTGAAAACTTT TCC-3' (Black and Steitz 1986); U6, 5'-ACGCAAAT CGTGAAGCG-3' (Black and Steitz 1986). To confirm depletion, nucleic acids were isolated from 5 μl of nuclear extracts, separated on a 7% (v/v) denaturing polyacrylamide gel, and directly stained with SYBR Gold™.

6. High-throughput screening

6.1. RT-qPCR reagents

RT-qPCR reactions were carried out using the TaqMan® One-Step RT-PCR kit (Applied Biosystems) with the following primers and TaqMan probe: 5'-TCTCTTCCGCATCGCTGTCT-3' (forward primer) directed to the 5’ exon; 5'-GCGAAGAGTTTGTCCTCAACGT-3' (reverse primer) directed to the 3’ exon; 5'FAM-
6-AGCTGGTGCGTCGAG SPC3-BH13’(TaqMan probe) directed to the exon junction. We determined the qPCR efficiency for these primers as \[10^{(-1/\text{slope})} - 1\] where slope was derived from the linear regression analysis from a standard curve of values for cDNA containing spliced mRNA.

6.2. High-throughput splicing assay

Pre-mRNA substrate was derived from the adenovirus major late transcript. A G(5')ppp(5')G-capped substrate was generated by T7 run-off transcription followed by G50 gel filtration. A cDNA copy of spliced mRNA was used in some experiments as a control. In vitro splicing reactions were prepared in 384-well plates by dispensing 5 µL of nuclear extract by a liquid handling robot (Perkin Elmer Janus). A second robot equipped with a 384-pin tool (Perkin Elmer Janus MDT) transferred 200 nL of library or control compounds into the nuclear extract, then 5µL splicing mix containing pre-mRNA substrate and buffer were added for final concentrations of: 50% (v/v) nuclear extract, 200 µM library compound, 10 nM substrate RNA, 60 mM KGlu, 2 mM MgAc, 2 mM ATP, 5 mM creatine phosphate, 0.05 mg ml\(^{-1}\) yeast tRNA. Plates were sealed and incubated for 60 min at 30°C. After incubation, the splicing reaction was diluted 1:2 with water using a peristaltic dispenser (Matrix WellMate).

6.3. RT-qPCR analysis

15 nL of diluted splicing reaction were transferred by pin robot to a new 384-well plate containing 5 µl of RT-qPCR premix (1x TaqMan master mix, 0.8 µM reverse primer, 0.4 µM forward primer, 0.5 µM TaqMan probe). RT-qPCR plates were analyzed with an ABI PRISM 7900HT Sequence Detection System under the
following conditions: RT-step: 30 min 48°C, 10 min 95°C; qPCR: 40 cycles of 30 sec 95°C, 50 sec 50°C, 50 sec 72°C followed by 7 min 72°C. Threshold cycle values (C<sub>T</sub>) for individual wells were normalized on a plate-to-plate basis to uninhibited control reactions.

6.4. Z' calculation

We calculated the Z' value for the high-throughput assay using the following equation: $Z' = 1 - \left[ (3\sigma_{\text{inhibited}} + 3\sigma_{\text{uninhibited}}) / (\mu_{\text{inhibited}} - \mu_{\text{uninhibited}}) \right]$ (Zhang et al. 1999). Inhibited values were derived from splicing reactions containing 200 nM SSA and uninhibited values were derived from splicing reactions containing 2% (v/v) DMSO alone.

7. Synthesis of SSA, BP, and HB structural analogs

The synthesis of the natural products and their structural analogs used in this dissertation was previously described. For details about the synthesis of FR901464, SSA, and the structural analogs, see (Ghosh et al. 2014a). The synthesis of PB structural analogs was based on a previously published total synthesis (Ghosh and Anderson 2012). See Chapter III for NMR analysis of structural analogs. The synthesis of HB was described in (Ghosh and Li 2011), and the synthesis for GEX1Q1 and its structural analogs was described in (Ghosh et al. 2014b). The manuscript that will describe the synthesis of iHB is in preparation.
8. Cytological profiling

For the cytological profiling data, HeLa cells were treated with 2 nM to 67 µM of PB, PB analogs, or herboxidiene for 20 h, followed by staining, automated imaging, and processing as described by Schulze et al. 2013.

9. Immunostaining for nuclear speckle analysis

1 x 10^5 HeLa cells were cultured with 0.01–1 µM of drug for 4 h in chamber slides, acetone-fixed, and blocked with 1% BSA. The cells were then DAPI-stained, incubated with anti-SC35 (1:500; BD Biosciences), and labeled with Alexa Fluor®568-conjugated anti-mouse IgG (1:500; Invitrogen). Images were acquired with a Leica EPI fluorescence microscope.

Relative speckle sizes were obtained in an unbiased manner by importing cell images into ImageJ and running a script that used the auto default threshold algorithm and “analyze particles” routine to select DAPI-stained nuclei in the blue channel. For each nucleus, the script then used the auto MaxEntropy threshold and “analyze particles” routine to select speckles in the red channel and calculate the speckle area. The distribution of speckle sizes from 20 to 40 nuclei from each condition was plotted with the Acula.com box plot generator and compares to DMSO control by a nonparametric Mann-Whitney U value test.
10. Monitoring endogenous splicing changes by semiquantitative RT-PCR

HeLa cells were treated for 4 h with DMSO or 0.01–1 µM drug. Total RNA was isolated from 1–2 x 10^6 HeLa cells with TRI reagent LS (Molecular Research Center, Inc.) according to the manufacturer’s instructions. After DNase treatment, 1 µg of total RNA was reverse-transcribed by MMLV reverse transcriptase in a 20-µL reaction. 10-µL PCRs were performed with a reaction mixture containing 1 µL of the RT reaction, 200 µM dNTPs, 0.2 µM of each forward and reverse primer, ~1 unit Taq polymerase, and 1x Taq buffer. PCR conditions were 2 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 55-60°C, 1 min at 60°C, and a final elongation of 10 min at 68°C. PCR products were separated on a 6% native polyacrylamide gel, stained with ethidium bromide, imaged, and quantified using the Quantity One software (Bio-Rad). Primer sequences are as follows: RBM5ex17: forward, 5’-CGGCTGTAGTGCAGAGT-3’, and reverse, 5’-TTGCGAGTTGGGTCATAAT-3’, 58 °C annealing temperature (Corrionero et al. 2011); CCNA2: forward, 5’-AACCTGTAGTG GCCAGAAGT-3’, and reverse, 5’-AAAGGCAGCTCCAGCAATAA-3’, 60 °C annealing temperature; SF3A1: forward, 5’-CCAAATTCCAGGAACGTG-3’, and reverse, 5’-AGCTCCTCTGGCGTG-3’, 55 °C annealing temperature (Tanackovic and Krämer 2005); U6: forward, 5’-CGCTTCGGCAGCACATATAC-3’, and reverse, 5’-GAATTTGCCTCAGCATCCTT-3’, 60 °C annealing temperature (O’Brien et al. 2008).
Appendix

1. Detailed NMR data for different PB analogs

The following NMR data were the basis to determine the structures shown in Supplementary Figure III-1.

Analog 1: C3 hydroxy epimer. \(^1\)H NMR (CD\(_3\)OD, 800 MHz): δ 6.36 (dd, J = 11.0, 15.0, 1H), 6.11 (d, J = 10.6 Hz, 1H), 5.74 (dd, J = 10.8, 15.2 Hz, 1H), 5.70 (dd, J = 8.2, 15.0 Hz, 1H), 5.52 (dd, J = 9.9, 15.4 Hz, 1H), 5.07 (d, J = 14.1, 1H), 5.04 (d, J = 9.8 Hz, 1H), 4.26 (m, 1H), 3.54 (quint, J = 4.2 Hz, 1H), 2.76 (dt, J = 0.7, 7.5 Hz, 1H), 2.69 (dd, J = 1.9, 7.9 Hz, 1H), 2.62 (m, 1H), 2.56 (dd, J = 5.2, 13.5 Hz, 1H), 2.51 (m, 1H), 2.48 (dd, J = 12.1, 13.0 Hz, 1H), 2.09 (s, 3H), 1.79 (s, 3H), 1.75-1.37 (m, 8H), 1.23 (m, 1H), 1.19 (s, 3H), 1.12 (d, J = 7.8 Hz, 3H), 0.97 (t, J = 7.4 Hz, 3H), 0.93 (d, J = 6.5 Hz, 3H), 0.90 (d, J = 6.8 Hz, 3H). \(^{13}\)C NMR (CD\(_3\)OD, 125 MHz): δ 172.2, 171.7, 142.5, 141.3, 132.3, 127.3, 125.8, 83.9, 80.3, 75.3, 74.2, 68.3, 63.0, 58.5, 42.8, 42.0, 40.7, 40.4, 36.7, 32.2, 28.6, 28.2, 24.1, 21.7, 21.1, 16.9, 11.8, 10.9. FTIR (thin film): \(3436, 2925, 2854, 1730, 15556, 1460, 1377, 1238, 1143, 1101, 1020, 975, 910 \text{cm}^{-1}\). [\(\alpha\)]\(_D\)\(^{20}\) = 10.0 (c 0.25, MeOH). LR-ESI (+) m/z (relative intensity), ion: 559.2 (100%), [M+Na]\(^+\). HR-ESI (+) m/z: [M+Na]\(^+\) calculated for C\(_{30}\)H\(_{48}\)O\(_8\), 559.3247; found, 559.3253.

Analog 2: C10-C11 anti-diastereomer. \(^1\)H NMR (CD\(_3\)OD, 500 MHz): δ 6.30 (dd, J = 10.8, 14.9 Hz, 1H), 6.05 (d, J = 10.9 Hz, 1H), 5.68 (dd, J = 10.0, 15.4 Hz, 1H), 5.63 (dd, J = 7.2, 13.9 Hz, 1H), 5.45 (dd, J = 10.0, 15.1 Hz, 1H), 4.99 (dd, J = 10.7, 18.6 Hz, 2H), 4.23-4.17 (m, 1H), 3.50-3.46 (m, 1H), 2.69 (td, J = 2.2, 5.9 Hz, 1H), 2.63 (dd, J = 2.3, 8.2 Hz, 1H), 2.60-2.38 (m, 4H), 2.04-2.03 (m, 1H), 2.03 (s, 3H), 1.72 (s, 3H), 1.68-1.40 (m, 8H), 1.13 (s, 3H), 1.05 (d, J = 6.8 Hz, 3H), 0.91 (t, J = 7.3 Hz, 3H), 0.87 (d, J = 7.1 Hz, 3H), 0.83 (d, J = 6.7 Hz, 3H). FTIR (thin film): \(3436, 2925, 2854, 1730, 15556, 1460, 1377, 1238, 1143, 1101, 1020, 975, 910 \text{cm}^{-1}\). [\(\alpha\)]\(_D\)\(^{20}\) = 21.1 (c 0.09, CHCl\(_3\)). LR-ESI (+) m/z (relative intensity), ion: 559.4 (100%), [M+Na]\(^+\). HR-ESI (+) m/z: [M+Na]\(^+\) calculated for C\(_{30}\)H\(_{48}\)O\(_6\), 559.3247; found, 559.3252.

Analog 3: C18-C19 Deoxy derivative. \(^1\)H NMR (CD\(_3\)OD, 500 MHz): δ 6.24-6.16 (m, 1H), 6.04 (d, J = 12.3 Hz, 1H), 5.70-5.60 (m, 2H), 5.54 (dd, J = 9.9, 15.1 Hz, 1H), 5.40-5.26 (m, 2H), 5.02 (d, J = 10.0 Hz, 2H), 3.78-3.73 (m, 1H), 3.20-3.13 (m, 1H), 2.58-2.50 (m, 1H), 2.50 (d, J = 3.7 Hz, 2H), 2.28-2.22 (m, 1H), 2.12-2.06 (m, 1H), 2.03 (s, 3H), 2.03-1.99 (m, 2H), 1.70 (m, 3H), 1.62-1.50 (m, 3H), 1.37-1.22 (m, 3H), 1.16 (s, 3H), 0.99 (d, J = 6.8 Hz, 3H), 0.96 (d, J = 6.7 Hz, 3H), 0.90 (t, J = 7.4 Hz, 3H), 0.85 (d, J = 6.8 Hz, 3H). \(^{13}\)C NMR (CD\(_3\)OD, 125 MHz): δ 172.1, 171.7, 143.1, 141.7, 136.2, 132.5, 131.7, 129.3, 127.0, 125.1, 84.3, 80.3, 77.9, 74.1, 70.4, 44.5, 41.8, 41.2, 40.1, 38.7, 37.5, 30.4, 28.5, 24.2, 21.1, 20.4, 17.2, 16.9, 11.8, 10.6. FTIR (thin film): \(3468, 2960, 2926, 1736, 1459, 1370, 1243, 1177, 1021, 972, 754 \text{cm}^{-1}\). [\(\alpha\)]\(_D\)\(^{20}\) = 3.9 (c 0.36, CHCl\(_3\)). LR-ESI (+) m/z (relative intensity), ion: 543.4 (100%), [M+Na]\(^+\). HR-ESI (+) m/z: [M+Na]\(^+\) calculated for C\(_{30}\)H\(_{48}\)O\(_7\), 543.3298; found, 543.3293.
Analog 4: C18-C19 Desoxy derivative with C10-C11 anti-diastereomer. $^1$H NMR (CD$_3$OD, 500 MHz): δ 6.19 (dd, J = 10.6, 15 Hz, 1H), 6.02 (d, J = 10.8 Hz, 1H), 5.68 (dd, J = 9.8, 15.2 Hz, 1H), 5.63 (dd, J = 7.8, 15.1 Hz, 1H), 5.46 (dd, J = 10.0, 15.2 Hz, 1H), 5.40-5.26 (m, 2H), 5.01 (d, J = 10.6 Hz, 1H), 4.98 (d, J = 9.8 Hz, 1H), 4.24-4.18 (m, 1H), 3.19-3.13 (m, 1H), 2.60-2.40 (m, 3H), 2.30-2.05 (m, 3H), 2.05-2.05 (m, 1H), 2.03 (s, 3H), 1.71 (s, 3H), 1.67-1.20 (6H), 1.13 (s, 3H), 0.99 (d, J = 6.8 Hz, 3H), 0.96 (d, J = 6.8Hz, 3H), 0.90(t, J = 7.4Hz, 3H), 0.84 (d, J = 6.8Hz, 3H). $^{13}$C NMR (CD$_3$OD, 125MHz): δ 172.2, 171.7, 143.2, 141.3, 136.2, 132.5, 131.6, 129.3, 127.3, 125.0, 84.0, 80.4, 77.9, 74.1, 68.3, 44.5, 42.0, 41.2, 40.4, 38.7, 32.2, 28.5, 28.2, 24.1, 21.1, 20.4, 17.2, 16.9, 11.8, 10.6. FTIR (thin film)$_{max}$: 3436, 2924, 2853, 1726, 1560, 1459, 1376, 1237, 1100, 971 cm$^{-1}$. [α]$^0_{D}$ 10.9 (c 0.11, CHCl$_3$). LR-ESI (+) m/z (relative intensity), ion: 543.4 (100%), [M+Na]$^+$. HR-ESI (+) m/z: [M+Na]$^+$ calculated for C$_{39}$H$_{48}$O$_7$, 543.3298; found, 543.3306.

Analog 5: C16,C20-Didesmethyl derivative-isomer 1. $^1$H NMR (CD$_3$OD, 800 MHz): δ 6.33 (dd, J = 10.7, 15.0 Hz, 1H), 6.08 (d, J = 10.7 Hz, 1H), 5.80 (dt, J = 7.0, 15.0 Hz, 1H), 5.69 (dd, J = 9.8, 15.2 Hz, 1H), 5.56 (dd, J = 9.9, 15.2 Hz, 1H), 5.05 (d, J = 10.1 Hz, 2H), 3.80-3.76 (m, 1H), 3.67-3.63 (m, 1H), 2.90-2.88 (m, 1H), 2.79-2.76 (m, 1H), 2.59-2.50 (m, 3H), 2.31-2.24 (m, 2H), 2.06 (s, 3H), 1.74 (s, 3H), 1.70-1.26 (m, 10H), 1.19 (s, 3H), 0.94 (t, J = 7.4 Hz, 1H), 0.88 (d, J = 6.7 Hz, 1H). LR-ESI (+) m/z (relative intensity), ion: 531.69 (100%), [M+Na]$^+$.  

Analog 6: C16,C20-Didesmethyl derivative with C21-epimer-isomer 2. $^1$H NMR (CD$_3$OD, 800 MHz): δ 6.33 (dd, J = 11.1, 15.1 Hz, 1H), 6.10 (d, J = 10.8 Hz, 1H), 5.80 (dt, J = 7.4, 14.6 Hz, 1H), 5.69 (dd, J = 9.8, 15.2 Hz, 1H), 5.57 (dd, J = 9.8, 15.1 Hz, 1H), 5.05 (d, J = 9.6 Hz, 1H), 3.80-3.76 (m, 1H), 3.65-3.20 (m, 1H), 2.88-2.85 (m, 1H), 2.77-2.74 (m, 1H), 2.58-2.51 (m, 3H), 2.32-2.26 (m, 2H), 2.06 (s, 3H), 1.74 (s, 3H), 1.70-1.26 (m, 10H), 1.19 (s, 3H), 0.94 (t, J = 7.4 Hz, 3H), 0.88 (d, J = 6.7 Hz, 3H). LR-ESI (+) m/z (relative intensity), ion: 531.69 (100%), [M+Na]$^+$.  

Analog 7: C16,C20-Didesmethyl derivative with C10-C11 anti-diastereomer-isomer 1. $^1$H NMR (CD$_3$OD, 800 MHz): δ 6.33 (ddt, J = 1.5, 10.8, 15.0, 1H), 6.08 (d, J = 10.8 Hz, 1H), 5.80 (dt, J = 7.0, 15.1 Hz, 1H), 5.71 (dd, J = 9.8, 15.2 Hz, 1H), 5.48 (dd, J = 9.9, 15.1 Hz, 1H), 5.04 (d, J = 10.6 Hz, 1H), 5.01 (d, J = 9.8 Hz, 1H), 4.25-4.21 (m, 1H), 3.65 (sept, J = 3.7 Hz, 1H), 2.91-2.88 (s, 1H), 2.78-2.76 (s, 1H), 2.60-2.56 (m, 1H), 2.52 (dd, J = 5.3, 13.4 Hz, 1H), 2.45 (dd, J = 11.4, 13.5 Hz, 1H), 2.31-2.36 (m, 2H), 2.06 (s, 3H), 1.75 (s, 3H), 1.71-1.26 (m, 10H), 1.16 (s, 3H), 0.94 (t, J = 7.5 Hz, 3H), 0.86 (d, J = 6.7 Hz, 3H). LR-ESI (+) m/z (relative intensity), ion: 531.67 (100%), [M+Na]$^+$.  

111
**Analog 8: C16,C20-Didesmethyl derivative with C10-C11 anti-diastereomer and C-21 epimer-isomer 2.**  
$^1$H NMR (CD$_3$OD, 800 MHz): δ 6.33 (dd, $J = 10.8, 15.0$ Hz, 1H), 6.08 (d, $J = 10.8$ Hz, 1H), 5.80 (dt, $J = 7.0, 14.5$ Hz, 1H), 5.71 (dd, $J = 9.8, 15.2$ Hz, 1H), 5.49 (dd, $J = 9.9, 15.2$ Hz, 1H), 5.04 (d, $J = 10.6$ Hz, 1H), 5.00 (d, $J = 9.8$ Hz, 1H), 4.25-4.21 (m, 1H), 3.66-3.62 (m, 1H), 2.88-2.85 (m, 1H), 2.77-2.74 (m, 1H), 2.60-2.56 (m, 1H), 2.52 (dd, $J = 5.1, 13.4$ Hz, 1H), 2.45 (dd, $J = 11.3, 13.5$ Hz, 1H), 2.31-2.26 (m, 2H), 2.06 (s, 3H), 1.75 (s, 3H), 1.71-1.26 (m, 10H), 1.16 (s, 3H), 0.95 (t, $J = 7.4$ Hz, 3H), 0.86 (d, $J = 6.7$ Hz, 3H). LR-ESI (+) m/z (relative intensity), ion: 531.67 (100%), [M+Na]$^+$.  

2. **Notes Chapter II: A high-throughput splicing assay identifies new classes of inhibitors of human and yeast spliceosomes**

Chapter II is a modified version of the following publication:


Statement of contributions: Conceived and designed experiments: KE, MJ, WB, RP; performed the experiments: KE, WB, RP; analyzed the data: KE, MJ, RP; wrote the paper: KE, MJ, RP; discussion and editing: KE, MJ, MA, RP, SL

Approval to use the publications as part of the dissertation was obtained from the Journal of Biomolecular Screening. Before publication, all authors agreed to the journal's author information (see excerpt below), which includes consent to use the article as part of my thesis.

TO: sagepub-feedback+jbx@highwire.stanford.edu  
NAME: Kerstin Effenberger  
EMAIL: keffenbe@ucsc.edu

Dear madam or sir,
Thanks for you time,  
Kerstin Effenberger
Re: Permission to use my article as part of my Ph.D. dissertation (JBX Feedback Form) [ref:_00DA0CN1j._500A0Q3O6O:ref ]

Dear Kerstin Effenberger,
Thank you for contacting SAGE Journals. Author information can be downloaded at:
http://www.sagepub.com/repository/binaries/journals/permissions/author_use.doc.
See excerpt below for relevant details in your case.
Regards,
Dave Vernon

Excerpt from the author information. The following SAGE’s Journal Author Reuse Policy, effective as of March 20, 2013:

- You may use the published article (version 3) for your own teaching needs or to supply on an individual basis to research colleagues, provided that such supply is not for commercial purposes.
- You may use the article (version 3) in a book authored or edited by you at any time after publication in the journal.
- When posting or re-using the article please provide a link to the appropriate DOI for the published version of the article on SAGE Journals (http://online.sagepub.com)

3. Notes Chapter III: Coherence between cellular responses and in vitro splicing inhibition for the anti-tumor drug pladienolide B and its analogs

Chapter III is a modified version of the following publication:


Statement of contributions: Conceived and designed experiments: KE, MJ, DA, AG; performed the experiments: KE, DA, WB, BP, NA, MA; analyzed the data: KE, MJ, DA; wrote the paper: KE, MJ, AG.

Approval to use the publications as part of the dissertation was obtained from the Journal of Biological Chemistry. Before publication, all authors agreed to the journal’s guidelines (see excerpt below), which includes consent to use the article as part of my thesis.
Dear madam or sir,


Thank you for your time,

Kerstin Effenberger

RE: Permission to use my article as part of my Ph.D. dissertation (JBC Feedback Form)

Hi Dr. Effenberger,

According to the Journal of Biological Chemistry copyright permission policy below and online: http://www.jbc.org/site/misc/Copyright_Permission.xhtml you are allowed to use the article for your thesis and dissertation. Please be sure to cite the manuscript in your dissertation.

If you have any additional questions please let me know.

Regards,

Ed Marklin

Excerpt from the guidelines. These guidelines apply to the reuse of articles, figures, charts and photos in the Journal of Biological Chemistry. For authors reusing their own material: Authors need NOT to contact the journal to obtain rights to reuse their own material. They are automatically granted permission to do the following:

- Reuse the article in print collections of their own writing.
- Present a work orally in its entirety.
- Use an article in a thesis and/or dissertation.
The chapter contains additional supplemental material that was not part of the original publication. This material represents KE’s contribution (designing and performing the experiments, analyzing the data, making figures, discussion and editing the manuscript) to the following publications:


References


