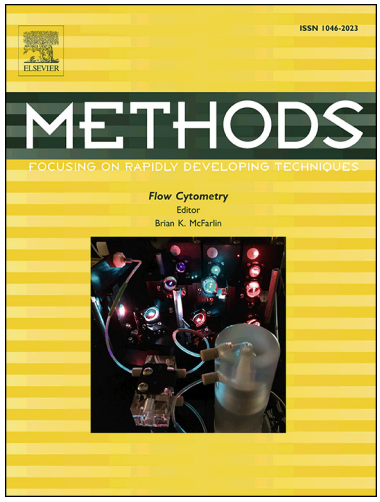
Accepted Manuscript



Splice-switching small molecules: a new therapeutic approach to modulate gene expression

Andrea Taladriz-Sender, Emma Campbell, Glenn A. Burley

PII: S1046-2023(18)30387-6
DOI: https://doi.org/10.1016/j.ymeth.2019.06.011
Reference: YMETH 4735

To appear in: *Methods*

Received Date: 25 February 2019
Revised Date: 10 June 2019
Accepted Date: 11 June 2019

Please cite this article as: A. Taladriz-Sender, E. Campbell, G.A. Burley, Splice-switching small molecules: a new therapeutic approach to modulate gene expression, *Methods* (2019), doi: https://doi.org/10.1016/j.ymeth.2019.06.011

Splice-switching small molecules: a new therapeutic approach to modulate gene expression

Andrea Taladriz-Sender,[§1] Emma Campbell,[§1] Glenn A. Burley[1*]

[1] Department of Pure and Applied Chemistry, University of Strathclyde. Thomas Graham Building, 295 Cathedral Street, Glasgow, United Kingdom. G1 1XL. Email: glenn.burley@strath.ac.uk

[§] Authors contributed equally.

ABSTRACT

Manipulating alternative RNA splicing events with small molecules is emerging as a viable mechanism for the development of therapeutics. A salient challenge in the field is understanding the molecular determinants defining the selectivity of splice-switching events and their mechanisms of action. In this review, the current state-of-the-art in splice-switching small molecules is described. Three examples of splice-switching small molecules are presented, and the differences in their modes of action compared.

INTRODUCTION

For more than half a century, small molecule drug discovery has been very much a protein-centric endeavor. Although great strides have been made in 'rational design' of small molecule inhibitors of target protein classes [1], combined with chemical biological [2] and –omic-based [3] approaches to enhance our understanding of target engagement *in vivo*, the ability to identify new druggable targets is increasingly challenging. At present, ~15% of the proteome is considered 'druggable' by traditional small molecule-protein interactions and new strategies are now needed to target biomolecules traditionally considered intractable or 'undruggable'[4].

Developing small molecules, which target RNA or RNA-protein interfaces, is now emerging as a potential alternative to traditional targets exclusively involving the proteogenic

components [5, 6]. Sitting upstream to translation in the gene expression hierarchy, the processes of transcription and alternative splicing of specific RNA molecules is a highly regulated process [7]. By virtue of the complexity and means of regulation, alternative RNA splicing holds particular promise as a drug target [8]. Approximately 95% of genes are alternatively spliced, resulting in a rich source of protein diversity from a single gene. The timing and location of splicing reactions is critical, which is exemplified when mutations in *cis*-acting (*i.e.,* RNA sequences) and *trans*-acting (*e.g.,* RNA-binding proteins involved in the regulation of splicing) factors arise, resulting in the onset of a range of diseases [9-11]. The differential impact of mutation on alternative RNA splicing is exemplified by ~15% of *cis*-acting mutations located at the site of transesterification or in regulatory regions remote from these splice sites can influence splicing outcomes. The design of small molecules, which interact with pre-mRNA sequences or RNA-protein complexes and correct aberrant splicing pathways, potentially offers a new platform for therapeutic intervention. The purpose of this review is two-fold: firstly, we will summarise the current workflows used to identify splice-switching small molecules. Secondly, we highlight current limitations in existing approaches and suggest future opportunities ripe for therapeutic development. For the latest developments in the modulation of alternative RNA splicing using oligonucleotide-based strategies [12, 13] or general spliceosomal inhibitors [14, 15], the reader is directed to several recent reviews.

Splice-site selection and its dysregulation in disease

Around 95% of genes in higher eukaryotes have their protein-coding sequences (exons) of genes disrupted by long non-protein coding regions known as introns [16-18]. Pre-mRNA splicing involves the removal of introns and ligates the remaining exonic regions by a series of transesterification reactions catalyzed by the spliceosome (Figure 1) [19, 20]. A sophisticated suite of non-covalent interactions between pre-mRNA sequences and spliceosomal protein and ribonucleoprotein (RNP) components define the sites of

transesterification (*i.e.,* the splice sites, SS) which can be separated by up to several thousand nucleotides [16]. The selection of a particular SS defines primary sequence of the mature mRNA isoform and is regulated by a swarm of competing interactions influenced by the pre-mRNA sequence (*cis*-acting) or RNA-protein (*trans*-acting) factors. *Cis*-acting factors present within pre-mRNA sequences can either encourage (*e.g.,* Exonic Splicing Enhancer, ESE; Intronic Splicing Enhancer, ISE) or perturb (*e.g.,* Exonic Splicing Silencer, ESS; Intronic Splicing Silencer, ISS) the use of a particular splice site [21]. Single nucleotide changes in these enhancer/silencer sequences regulate SS usage can have a significant impact on splicing by, for example, promoting the inclusion of exons or utilizing cryptic sites, to produce different mature mRNA sequences in different amounts. Therefore, mutations can result in the over or under-representation of particular mature mRNA isoforms, which can lead to the onset of a range of diseases [9, 11, 22, 23].

Strategies for the development of splice-switching small molecules

Strategies towards the identification of small molecules which induce a switch in the ratio of mature mRNA isoforms conceptually differs from conventional medicinal chemistry approaches, which are typically focused towards inhibiting a target protein with a small molecule [8]. Examples of therapeutic modulation of alternative splicing include:

(a) Point mutations resulting in abnormal exclusion or inclusion of a particular exon leading to the overexpression of an undesirable mature mRNA isoform (Figure 2a). A prominent example of this type of event is spinal muscular atrophy (SMA), where an alternative sequence in one of the SMN genes results in exon skipping.[24, 25]

(b) The overexpression of an undesirable mature mRNA isoform, which is deleterious to homoeostatic function of a cell. Anti-apoptotic isoforms of Bcl-x and Mcl-1 are over-expressed in breast, lung and prostate cancers and leukemia [26, 27]. Both of these apoptotic check-point genes have splicing isoforms with antagonistic functions (Figure 2b).

(c) A small molecule inducing a switch from the expression of an undesirable mature mRNA isoform towards a truncated isoform, which is translated into a "frame-shifted protein". This leads to the introduction of a premature stop codon, which in turn results in the degradation of the corresponding mRNA by Nonsense Mediated Decay (NMD) (Figure 2c) [28, 29].

For each of the scenarios proposed in Figure 2, the mechanisms of action of splice-switching small molecules could involve *(i)* directly binding to an RNA sequence or RNA-RNP complex which sequesters or promotes the usage of a particular SS; or *(ii)* binding to a structural protein involved in spliceosomal assembly or by inhibiting splicing factor regulators.

Splice-switching small molecules which stimulate exon 7 inclusion in SMN2 mRNA

SMA is a deleterious neuromuscular disease, which is caused by the reduced expression of the Survival Motor Neuron (SMN) protein. This is due to the deletion of *SMN1* in patients leaving only *SMN2* to produce SMN protein [13]. *SMN2* is a paralog of *SMN1* which differs by a single C to T substitution at position 6 in exon 7. The alternative splicing of the pre-mRNA of *SMN2* results in exon 7 skipping in ~90% of the corresponding mRNA, which produces a truncated, non-functional SMN protein.[24, 30-32] Consequently, the expression of *SMN2* produces ~ 10-20% SMN protein relative to *SMN1*. However, since increasing *SMN* copy number is known to reduce the severity of SMA,[24] developing therapies which can increase *SMN2* expression is a viable therapeutic strategy. The development of splice-switching oligonucleotides (SSOs; *e.g.,* Spinraza®), which induces the inclusion of exon 7 in *SMN2* [13], provided the first clinical example of splice-switching therapeutics, which could enhance *SMN2* expression.

The ability to enhance *SMN2* expression has now evolved to the identification of small molecules which induce a Exon 7 inclusion in *SMN2*.[33, 34] The PTC/Roche team has established a high-throughput screening platform using a HEK293H cell line containing an

SMN2 minigene construct linked to a firefly luciferase coding sequence (Figure 3). Exclusion of exon 7 induces a frameshift in the luciferase initiation codon to produce *SMN2*-Δ7, whereas Exon 7 inclusion results in the production of *SMN2*-FL (FL = full length), keeping the initiation codon 'in-frame' and as a result, producing a chemoluminescent readout in the presence of luciferin. A 200 000 small molecule screen identified lead compound **4**, which induced a 7-fold increase in luciferase-mediated luminescence at 10μM (Figure 4) [35]. However, compound **4** did not induce a measurable increase in *SMN2* production in patient-derived fibroblasts. In order to increase the potency, the PTC/Roche team systematically modified the basic head unit, heterocyclic core, head group (**5-7**, Figure 4), which resulted in an increase in SMN protein *in vivo*. Further SAR-based optimization [36, 37] led to **1** (Risdiplam or RG-7916), which is currently in Phase 2 clinical trials for adult and pediatric patients with type II and III forms of SMA [37-39].

In a parallel study led by Novartis, compounds **2** and **8** were identified from a similar high-throughput screen (~ 1.4 x 10^6 compound library) conducted in fibroblasts using a *SMN2* luciferase minigene construct and subsequent SAR-based optimization of splice-switching potency (Figure 5) [34]. A dose-dependent elevation of SMN protein in a C/+ SMA mouse model was observed using compound **2**, which resulted in a 62% survival rate after 35 days using a dose of 3 mg/kg/day.

A key aspect of this work was the use of a luciferase-based high-throughput assays developed by both teams. This target agnostic approach lead to the identification of lead compounds **1** and **2**. However, identifying the mechanisms of action of these chemotypes such as **2** and **9** (Figure 6) has been a challenging. The utilization of a suite of chemical biological, biophysical, structural biological and –omics-based approaches has been essential to establish how these compounds interact with ternary complexes of small molecule-RNA-RNP complexes. A recent study by Sivaramakrishnan *et al.* has

identified the SMN-C class of molecules which putatively stabilize a quaternary RNA-RNP complex formed within the *SMN2* pre-mRNA [40]. Whilst both chemotypes bind to the 5′ SS of exon 7, the exact site of RNA binding was slightly different, with **10** binding a few nucleotides upstream relative to compound **2** [40]. More in-depth analysis of the binding landscape of **5** using the photocrosslinking probe (**11**) [41] and RNA mapping (SHAPE) [42] revealed preferential binding to a 5′-AGGAAG sequence present in the +24 to +29 region of exon 7 [40]. Small molecule binding induces a conformation change in this pre-mRNA sequence, which promotes the recruitment of splicing activators such as far upstream element binding protein 1 (FUBP1) and its homolog, KH-type splicing regulatory protein (KHSRP) to form a higher order multi-protein complex.[41] The working hypothesis is the splicing activation effect is due to small molecule (*e.g.,* **5** or **9**) binding to the pre-mRNA enhances recognition of FUBP1 and KHSRP. This in turn displaces heterogeneous nuclear ribonucleoprotein G (hnRNP G),. The collective works from both industrial teams highlights the clinical potential in developing small molecules, which selectively modulate splicing by targeting the interface of RNA and RNP complexes.

Splice-switching small molecules which induce apoptosis

BCL2-like 1 (or more commonly known as Bcl-x) is a key checkpoint apoptotic gene which is alternatively spliced to produce splicing isoforms that have antagonistic functions [43]. The anti-apoptotic Bcl-x_L isoform, which when translated, preserves mitochondrial membrane integrity by binding to and antagonizing the function of pro-apoptotic effector proteins *via* BCL-2 homology domains [44]. Overexpression of the Bcl-x_L isoform has been linked to a suite of haematological and solid tumours [45]. The pro-apoptotic protein isoform Bcl-x_S is produced by an alternative 5′-SS in exon 2 (Figure 7), resulting in a protein which lacks the BH3 domain. This induces mitochondrial membrane

permeabilization by pro-apoptotic effector proteins, triggering caspase-mediated apoptosis [46]. The antagonistic properties of the isoforms of *Bcl-x* at the apoptotic gateway could provide a novel anti-cancer target for small molecule drug development.

The pre-mRNA transcript of *Bcl-x* is rich in G-tracts (*i.e.,* short sequences of G doublets and G triplets) close to both 5′ Bcl-x_L and Bcl-x_S splice sites, which could suggest the ability of these sequences to fold into an RNA G-quadruplex (G4) [47]. The formation of G4s require hydrogen bonding from a Watson-Crick base-pairing face to a Hoogsteen pairing face of another G nucleotide to form a G tetrad (Figure 8) [48]. An essential requirement for the formation of G4s is the N7 atom within this purine nucleobase, which enables hydrogen-bonding *via* its Hoogsteen pairing face. To assess the global impact of G nucleotide structure and to map the propensity of G4s to form in the Bcl-x pre-mRNA, Weldon *et al.* developed the footprinting technique termed FOLDeR (footprinting of long 7-deazaguanine-substituted RNAs) to probe for G4s in long RNA sequences. Two Bcl-x pre-mRNA transcripts were prepared, one using GTP and the other where GTP was replaced with 7-deazaGTP. The transcript containing 7-deazaG retains the capacity of Bcl-x pre-mRNA to Watson-Crick pair but perturbs G4 formation by blocking Hoogsteen pairing [49]. Structural difference mapping of Bcl-x pre-mRNA *versus* pre-mRNA containing 7-deazaG was used to the identify G tracts close to the 5′-SS which have the potential to form G4. With G4 sites putatively identified, Weldon *et al.* probed the effects of stabilizing these G4s using an established suite of DNA G4 stabilizers (Figure 9) [50-53]. SAR-analysis revealed the optimal structure to induce a switch towards the expression of Bcl-x_S *in vitro* and in HeLa cells was the ellipticine analogue (**3,** GQC-05). The working hypothesis of how compound **3** induces this splice-switch is *via* differential stabilization of RNA G4s and, to increase the availability of the Bcl-x_S 5′-SS.

A completely unexpected observation was a quindoline analogue **14** induced the opposite splice-switching effect *in vitro* (*i.e.,* increased expression of Bcl-x_L, which suggests that

splice-switching can be controlled in both directions by structural changes to the G4 stabilizer. Interestingly, compound **14** reduced the stimulatory effect of a SSO to induce exon 7 inclusion in SMN2, by binding to an intermolecular G4 [54]. This holds up the possibility that the modes of action of other types of neoplastic agents such as ellipticine acetate (*e.g.,* Celitpium®) could also be working *via* splice-switching in addition to their previously identified mechanisms of cytotoxicity such as topoisomerise inhibition and the formation of DNA bulky adducts [55].

Protein kinase inhibitors as modulators of angiogenesis

Based on existing data, the above examples highlight the potential of small molecules influencing alternative splicing by interacting with RNA motifs or RNA-RNP complexes. Small molecules can also induce a switch in alternative splicing indirectly by altering the function of *trans*-acting factors such as SR proteins [8, 23].

One of the most promising family of splice-switching small molecules that have emerged in recent years are inhibitors of the protein kinase SRPK1 [56, 57]. SRPK1 (along with CLK1) mediates nuclear localization and spliceosomal function of SRSF1 by phosphorylation of the RS domain [58, 59]. Once phosphorylated and imported into the nucleus, SRSF1 is a key protein in defining the usage of particular splice sites by interacting with RNPs U1-70K and U2AF and ESE sequences [60-63]. SRSF1 plays a crucial role in the alternative splicing of vascular endothelial growth factor (VEGF) pre-mRNA. The VEGF-A_{165}a isoform promotes angiogenesis and has been implicated as a key driver in cancer progression [64], whereas the VEGF-A_{165}b isoform is anti-angiogenic but cytoprotective (Figure 10). Inhibiting SRSF1 phosphorylation by blocking SRPK1 activity has the potential to restore the balance of VEGF-A isoforms, which has therapeutic potential for treatment of angiogenic-related diseases and development of anticancer agents [65, 66].

A high-throughput screen identified compound (**15**, Figure 11), which exhibited an IC_{50} of 31 nM for the inhibition of SRPK1 [67]. The potency of SRPK1 inhibition was improved even further when the piperazine ring was extended with a pyridine (Figure 11, **16**, IC_{50} of 5.9 nM).[65, 66] X-ray crystal structural analysis of **16** in complex with SRPK1 revealed that this compound is a competitive inhibitor of SRPK1 with selectivity arising from the trifluoromethyl group interacting with a unique hydrophobic domain (Figure 12). Selective inhibition of SRPK1 activity was confirmed using PC3 cells incubated with compound **16** (EC_{50} of 360 nM). In addition, compound **16** exhibited anti-angiogenic activity in a mouse model (C57/Bl6) used for age-related macular degeneration. Compound **16** was effective at inhibiting blood vessel growth *via* an increase in the expression of VEGF-A_{165}b, thus providing a promising lead for the development of anti-angiogenic therapeutics.

Challenges and opportunities in the development of splice-switching small molecules

The exciting clinical developments that have emerged from Risdiplam (**1**) and Branaplam (**2**) entering clinical trials for the treatment of SMA highlight the potential to develop small molecule therapies *via* altering the outcome of alternative RNA splicing events.

Critical for the future development of splice-switching therapeutics is an understanding of their complex mode(s) of action, particularly when these compounds exert their effect at RNA-protein interfaces [6]. This opens up considerable opportunities to develop techniques and new chemistry biological tools that will have to be integrated with bioinformatics, biochemistry and molecular and cellular biology to define these interactions [68]. Chemical biology will play a key role in these developments, where photo-cross-linking probes and tagging technologies [69, 70] combined with –omics-based approaches will provide fine annotation of interacting partners influenced by these small molecules and the downstream changes induced by such splice-switching events [71].

The splicing field is reaching an exciting tipping point with structural biology providing insight into the molecular determinants defining splice-site selection by the spliceosome [72-77]. In addition, innovations in the design of RNA-selective small molecules [68] and the continuing clinical development of SSO therapeutics [13, 78, 79] both enhance our understanding of how to induce splice-switching for therapeutic applications.

ACKNOWLEDGEMENTS

E.C. thanks the GlaxoSmithKline-University of Strathclyde for an industrial PhD studentship. A.T-S. and GA.B. thank the Biotechnology and Biological Sciences Research council (BBSRC; BB/R006857/1) for funding this work.

REFERENCES

[1] K.D. Barnash, L.I. James, S.V. Frye, Target class drug discovery, Nat. Chem. Biol. 13 (2017) 1053-1056.
[2] M. Schürmann, P. Janning, S. Ziegler, H. Waldmann, Small-Molecule Target Engagement in Cells, Cell Chem. Biol. 23 (2016) 435-441.
[3] A. Wafi, R. Mirnezami, Translational –omics: Future potential and current challenges in precision medicine, Methods 151 (2018) 3-11.
[4] A.L. Hopkins, C.R. Groom, The druggable genome, Nat. Rev. Drug Discov. 1 (2002) 727-730.
[5] A. Donlic, A.E. Hargrove, Targeting RNA in mammalian systems with small molecules, Wiley Interdisciplinary Reviews: RNA 9 (2018) e1477.
[6] M.D. Disney, B.G. Dwyer, J.L. Childs-Disney, Drugging the RNA World, Cold Spring Harb. Perspect. Biol. 10 (2018) a034769.
[7] T.A. Cooper, L.L. Wan, G. Dreyfuss, RNA and Disease, Cell 136 (2009) 777-793.
[8] M.G. Woll, N.A. Naryshkin, G.M. Karp, Drugging Pre-mRNA Splicing, in: A.L. Garner (Ed.), RNA Therapeutics, Springer International Publishing, Cham, 2018, pp. 135-176.
[9] E. Antonopoulou, M. Ladomery, Targeting splicing in prostate cancer, Int. J. Mol. Sci. 19 (2018) 1287.
[10] F. Muntoni, M.J.A. Wood, Targeting RNA to treat neuromuscular disease, Nat. Rev. Drug Discov. 10 (2011) 621-637.
[11] R.K. Singh, T.A. Cooper, Pre-mRNA splicing in disease and therapeutics, Trends Mol. Med. 18 (2012) 472-482.
[12] M.A. Havens, M.L. Hastings, Splice-switching antisense oligonucleotides as therapeutic drugs, Nucleic Acids Res. 44 (2016) 6549-6563.
[13] C. Rinaldi, M.J.A. Wood, Antisense oligonucleotides: the next frontier for treatment of neurological disorders, Nat. Rev. Neurol. 14 (2017) 9-21.
[14] A.A. Agrawal, L.H. Yu, P.G. Smith, S. Buonamici, Targeting splicing abnormalities in cancer, Curr. Opin. Genet. Dev. 48 (2018) 67-74.
[15] B. Leon, M.K. Kashyap, W.C. Chan, K.A. Krug, J.E. Castro, J.J. La Clair, M.D. Burkart, A Challenging Pie to Splice: Drugging the Spliceosome, Angew. Chem. Int. Ed. 56 (2017) 12052-12063.
[16] R.A. Padgett, P.J. Grabowski, M.M. Konarska, S. Seiler, P.A. Sharp, Splicing of Messenger RNA Precursors, Annu. Rev. Biochem. 55 (1986) 1119-1150.
[17] A.J. Ward, T.A. Cooper, The pathobiology of splicing, J. Pathol. 220 (2010) 152-163.
[18] Q. Pan, O. Shai, L.J. Lee, B.J. Frey, B.J. Blencowe, Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing, Nat. Genet. 40 (2008) 1413-1415.
[19] S.M. Fica, K. Nagai, Cryo-electron microscopy snapshots of the spliceosome: structural insights into a dynamic ribonucleoprotein machine, Nat. Struct. Mol. Biol. 24 (2017) 791.
[20] M.C. Wahl, C.L. Will, R. Luhrmann, The Spliceosome: Design Principles of a Dynamic RNP Machine, Cell 136 (2009) 701-718.
[21] X. Roca, A.R. Krainer, I.C. Eperon, Pick one, but be quick: 5 ' splice sites and the problems of too many choices, Genes Dev. 27 (2013) 129-144.
[22] M. Montes, B.L. Sanford, D.F. Comiskey, D.S. Chandler, RNA Splicing and Disease: Animal Models to Therapies, Trends Genet. 35 (2019) 68-87.
[23] D.O. Bates, J.C. Morris, S. Oltean, L.F. Donaldson, Pharmacology of Modulators of Alternative Splicing, Pharmacol. Rev. 69 (2017) 63-79.
[24] A.H.M. Burghes, C.E. Beattie, Spinal muscular atrophy: why do low levels of survival motor neuron protein make motor neurons sick?, Nat. Rev. Neurosci. 10 (2009) 597.
[25] M. Bowerman, C.G. Becker, R.J. Yáñez-Muñoz, K. Ning, M.J.A. Wood, T.H. Gillingwater, K. Talbot, Therapeutic strategies for spinal muscular atrophy: SMN and beyond, Dis. Model Mech. 10 (2017) 943-954.
[26] R. Beroukhim, C.H. Mermel, D. Porter, G. Wei, S. Raychaudhuri, J. Donovan, J. Barretina, J.S. Boehm, J. Dobson, M. Urashima, K.T. Mc Henry, R.M. Pinchback, A.H. Ligon, Y.-J. Cho, L. Haery, H. Greulich, M. Reich, W. Winckler, M.S. Lawrence, B.A. Weir, K.E. Tanaka, D.Y. Chiang, A.J. Bass, A. Loo, C. Hoffman, J. Prensner, T. Liefeld, Q. Gao, D. Yecies, S. Signoretti, E. Maher, F.J. Kaye, H. Sasaki, J.E. Tepper, J.A. Fletcher, J. Tabernero, J. Baselga, M.-S. Tsao, F. Demichelis, M.A. Rubin, P.A. Janne, M.J. Daly, C. Nucera, R.L. Levine, B.L. Ebert, S. Gabriel, A.K. Rustgi, C.R. Antonescu, M. Ladanyi, A. Letai, L.A. Garraway, M. Loda, D.G. Beer, L.D. True, A. Okamoto, S.L. Pomeroy, S. Singer, T.R. Golub, E.S. Lander, G. Getz, W.R. Sellers, M. Meyerson, The landscape of somatic copy-number alteration across human cancers, Nature 463 (2010) 899-905.

[27] A.N. Hata, J.A. Engelman, A.C. Faber, The BCL2 Family: Key Mediators of the Apoptotic Response to Targeted Anticancer Therapeutics, Cancer Discov. 5 (2015) 475-487.
[28] T. Shiraishi, J. Eysturskarð, P.E. Nielsen, Modulation of mdm2 pre-mRNA splicing by 9-aminoacridine-PNA (peptide nucleic acid) conjugates targeting intron-exon junctions, BMC Cancer 10 (2010) 342.
[29] H.S. Sekhon, C.A. London, M. Sekhon, P.L. Iversen, G.R. Devi, c-MYC antisense phosphosphorodiamidate morpholino oligomer inhibits lung metastasis in a murine tumor model, Lung Cancer 60 (2008) 347-354.
[30] L.A. Skordis, M.G. Dunckley, B. Yue, I.C. Eperon, F. Muntoni, Bifunctional antisense oligonucleotides provide a trans-acting splicing enhancer that stimulates SMN2 gene expression in patient fibroblasts, Proc. Nat. Acad. Sci. 100 (2003) 4114-4119.
[31] Y. Hua, Y.H. Liu, K. Sahashi, F. Rigo, C.F. Bennett, A.R. Krainer, Motor neuron cell-nonautonomous rescue of spinal muscular atrophy phenotypes in mild and severe transgenic mouse models, Genes Dev. 29 (2015) 288-297.
[32] E.J.N. Groen, K. Talbot, T.H. Gillingwater, Advances in therapy for spinal muscular atrophy: promises and challenges, Nat. Rev. Neurol. 14 (2018) 214-224.
[33] N.A. Naryshkin, M. Weetall, A. Dakka, J. Narasimhan, X. Zhao, Z. Feng, K.K.Y. Ling, G.M. Karp, H. Qi, M.G. Woll, G. Chen, N. Zhang, V. Gabbeta, P. Vazirani, A. Bhattacharyya, B. Furia, N. Risher, J. Sheedy, R. Kong, J. Ma, A. Turpoff, C.-S. Lee, X. Zhang, Y.-C. Moon, P. Trifillis, E.M. Welch, J.M. Colacino, J. Babiak, N.G. Almstead, S.W. Peltz, L.A. Eng, K.S. Chen, J.L. Mull, M.S. Lynes, L.L. Rubin, P. Fontoura, L. Santarelli, D. Haehnke, K.D. McCarthy, R. Schmucki, M. Ebeling, M. Sivaramakrishnan, C.-P. Ko, S.V. Paushkin, H. Ratni, I. Gerlach, A. Ghosh, F. Metzger, SMN2 splicing modifiers improve motor function and longevity in mice with spinal muscular atrophy, Science 345 (2014) 688-693.
[34] J. Palacino, S.E. Swalley, C. Song, A.K. Cheung, L. Shu, X. Zhang, M. Van Hoosear, Y. Shin, D.N. Chin, C.G. Keller, M. Beibel, N.A. Renaud, T.M. Smith, M. Salcius, X. Shi, M. Hild, R. Servais, M. Jain, L. Deng, C. Bullock, M. McLellan, S. Schuierer, L. Murphy, M.J.J. Blommers, C. Blaustein, F. Berenshteyn, A. Lacoste, J.R. Thomas, G. Roma, G.A. Michaud, B.S. Tseng, J.A. Porter, V.E. Myer, J.A. Tallarico, L.G. Hamann, D. Curtis, M.C. Fishman, W.F. Dietrich, N.A. Dales, R. Sivasankaran, SMN2 splice modulators enhance U1–pre-mRNA association and rescue SMA mice, Nat. Chem. Biol. 11 (2015) 511-517.
[35] M.G. Woll, H. Qi, A. Turpoff, N. Zhang, X. Zhang, G. Chen, C. Li, S. Huang, T. Yang, Y.-C. Moon, C.-S. Lee, S. Choi, N.G. Almstead, N.A. Naryshkin, A. Dakka, J. Narasimhan, V. Gabbeta, E. Welch, X. Zhao, N. Risher, J. Sheedy, M. Weetall, G.M. Karp, Discovery and Optimization of Small Molecule Splicing Modifiers of Survival Motor Neuron 2 as a Treatment for Spinal Muscular Atrophy, J. Med. Chem. 59 (2016) 6070-6085.
[36] E. Pinard, L. Green, M. Reutlinger, M. Weetall, N.A. Naryshkin, J. Baird, K.S. Chen, S.V. Paushkin, F. Metzger, H. Ratni, Discovery of a Novel Class of Survival Motor Neuron 2 Splicing Modifiers for the Treatment of Spinal Muscular Atrophy, J. Med. Chem. 60 (2017) 4444-4457.
[37] H. Ratni, M. Ebeling, J. Baird, S. Bendels, J. Bylund, K.S. Chen, N. Denk, Z. Feng, L. Green, M. Guerard, P. Jablonski, B. Jacobsen, O. Khwaja, H. Kletzl, C.-P. Ko, S. Kustermann, A. Marquet, F. Metzger, B. Mueller, N.A. Naryshkin, S.V. Paushkin, E. Pinard, A. Poirier, M. Reutlinger, M. Weetall, A. Zeller, X. Zhao, L. Mueller, Discovery of Risdiplam, a Selective Survival of Motor Neuron-2 (SMN2) Gene Splicing Modifier for the Treatment of Spinal Muscular Atrophy (SMA), J. Med. Chem. 61 (2018) 6501-6517.
[38] S. Sturm, A. Günther, B. Jaber, P. Jordan, N. Al Kotbi, N. Parkar, Y. Cleary, N. Frances, T. Bergauer, K. Heinig, H. Kletzl, A. Marquet, H. Ratni, A. Poirier, L. Müller, C. Czech, O. Khwaja, A phase 1 healthy male volunteer single escalating dose study of the pharmacokinetics and pharmacodynamics of risdiplam (RG7916, RO7034067), a SMN2 splicing modifier, Br. J. Clin. Pharmacol. 85 (2019) 181-193.
[39] M.A. Farrar, S.B. Park, S. Vucic, K.A. Carey, B.J. Turner, T.H. Gillingwater, K.J. Swoboda, M.C. Kiernan, Emerging therapies and challenges in spinal muscular atrophy, Ann. Neurol. 81 (2017) 355-368.
[40] M. Sivaramakrishnan, K.D. McCarthy, S. Campagne, S. Huber, S. Meier, A. Augustin, T. Heckel, H. Meistermann, M.N. Hug, P. Birrer, A. Moursy, S. Khawaja, R. Schmucki, N. Berntenis, N. Giroud, S. Golling, M. Tzouros, B. Banfai, G. Duran-Pacheco, J. Lamerz, Y. Hsiu Liu, T. Luebbers, H. Ratni, M. Ebeling, A. Cléry, S. Paushkin, A.R. Krainer, F.H.T. Allain, F. Metzger, Binding to SMN2 pre-mRNA-protein complex elicits specificity for small molecule splicing modifiers, Nat. Commun. 8 (2017) 1476.
[41] J. Wang, P.G. Schultz, K.A. Johnson, Mechanistic studies of a small-molecule modulator of SMN2 splicing, Proc. Nat. Acad. Sci. 115 (2018) E4604-E4612.
[42] N.A. Siegfried, S. Busan, G.M. Rice, J.A.E. Nelson, K.M. Weeks, RNA motif discovery by SHAPE and mutational profiling (SHAPE-MaP), Nat. Methods 11 (2014) 959-965.

[43] L.H. Boise, M. González-García, C.E. Postema, L. Ding, T. Lindsten, L.A. Turka, X. Mao, G. Nuñez, C.B. Thompson, bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death, Cell 74 (1993) 597-608.
[44] J.T. Opferman, A. Kothari, Anti-apoptotic BCL-2 family members in development, Cell Death Differ. 25 (2017) 37-45.
[45] K. Papadopoulos, Targeting the Bcl-2 Family in Cancer Therapy, Semin. Oncol. 33 (2006) 449-456.
[46] L. Lindenboim, C. Borner, R. Stein, Bcl-xS can form homodimers and heterodimers and its apoptotic activity requires localization of Bcl-xS to the mitochondria and its BH3 and loop domains, Cell Death Differ. 8 (2001) 933-942.
[47] M.M. Fay, S.M. Lyons, P. Ivanov, RNA G-Quadruplexes in Biology: Principles and Molecular Mechanisms, J. Mol. Biol. 429 (2017) 2127-2147.
[48] S. Neidle, Quadruplex Nucleic Acids as Novel Therapeutic Targets, J. Med. Chem. 59 (2016) 5987-6011.
[49] C. Weldon, I. Behm-Ansmant, L.H. Hurley, G.A. Burley, C. Branlant, I.C. Eperon, C. Dominguez, Identification of G-quadruplexes in long functional RNAs using 7-deazaguanine RNA, Nat. Chem. Biol. 13 (2017) 18-20.
[50] G. Biffi, M. Di Antonio, D. Tannahill, S. Balasubramanian, Visualization and selective chemical targeting of RNA G-quadruplex structures in the cytoplasm of human cells, Nat. Chem. 6 (2013) 75-80.
[51] S. Kendrick, A. Muranyi, V. Gokhale, L.H. Hurley, L.M. Rimsza, Simultaneous Drug Targeting of the Promoter MYC G-Quadruplex and BCL2 i-Motif in Diffuse Large B-Cell Lymphoma Delays Tumor Growth, J. Med. Chem. 60 (2017) 6587-6597.
[52] R.V. Brown, F.L. Danford, V. Gokhale, L.H. Hurley, T.A. Brooks, Demonstration that Drug-targeted Down-regulation of MYC in Non-Hodgkins Lymphoma Is Directly Mediated through the Promoter G-quadruplex, J. Biol. Chem. 286 (2011) 41018-41027.
[53] R.V. Brown, T. Wang, V.R. Chappeta, G. Wu, B. Onel, R. Chawla, H. Quijada, S.M. Camp, E.T. Chiang, Q.R. Lassiter, C. Lee, S. Phanse, M.A. Turnidge, P. Zhao, J.G.N. Garcia, V. Gokhale, D. Yang, L.H. Hurley, The Consequences of Overlapping G-Quadruplexes and i-Motifs in the Platelet-Derived Growth Factor Receptor β Core Promoter Nuclease Hypersensitive Element Can Explain the Unexpected Effects of Mutations and Provide Opportunities for Selective Targeting of Both Structures by Small Molecules To Downregulate Gene Expression, J. Am. Chem. Soc. 139 (2017) 7456-7475.
[54] L.D. Smith, R.L. Dickinson, C.M. Lucas, A. Cousins, A.A. Malygin, C. Weldon, A.J. Perrett, A.R. Bottrill, M.S. Searle, G.A. Burley, I.C. Eperon, A Targeted Oligonucleotide Enhancer of SMN2 Exon 7 Splicing Forms Competing Quadruplex and Protein Complexes in Functional Conditions, Cell Rep. 9 (2014) 193-205.
[55] B. Dugue, C. Auclair, B. Meunier, Covalent Binding of Elliptinium Acetate (NSC 264137) to Nucleic Acids of L1210 Cells in Culture, Cancer Research 46 (1986) 3828-3833.
[56] G. Ghosh, J.A. Adams, Phosphorylation mechanism and structure of serine-arginine protein kinases, FEBS J. 278 (2011) 587-597.
[57] A. Mavrou, K. Brakspear, M. Hamdollah-Zadeh, G. Damodaran, R. Babaei-Jadidi, J. Oxley, D.A. Gillatt, M.R. Ladomery, S.J. Harper, D.O. Bates, S. Oltean, Serine–arginine protein kinase 1 (SRPK1) inhibition as a potential novel targeted therapeutic strategy in prostate cancer, Oncogene 34 (2014) 4311-4319.
[58] X.-Y. Zhong, J.-H. Ding, J.A. Adams, G. Ghosh, X.-D. Fu, Regulation of SR protein phosphorylation and alternative splicing by modulating kinetic interactions of SRPK1 with molecular chaperones, Genes Dev. 23 (2009) 482-495.
[59] Brandon E. Aubol, G. Wu, Malik M. Keshwani, M. Movassat, L. Fattet, Klemens J. Hertel, X.-D. Fu, Joseph A. Adams, Release of SR Proteins from CLK1 by SRPK1: A Symbiotic Kinase System for Phosphorylation Control of Pre-mRNA Splicing, Mol. Cell 63 (2016) 218-228.
[60] J.Y. Wu, T. Maniatis, Specific interactions between proteins implicated in splice site selection and regulated alternative splicing, Cell 75 (1993) 1061-1070.
[61] J.D. Kohtz, S.F. Jamison, C.L. Will, P. Zuo, R. Lührmann, M.A. Garcia-Blanco, J.L. Manley, Protein–protein interactions and 5'-splice-site recognition in mammalian mRNA precursors, Nature 368 (1994) 119-124.
[62] A. Lavigueur, H. La Branche, A.R. Kornblihtt, B. Chabot, A splicing enhancer in the human fibronectin alternate ED1 exon interacts with SR proteins and stimulates U2 snRNP binding, Genes Dev. 7 (1993) 2405-2417.

[63] A.M. Jobbins, L.F. Reichenbach, C.M. Lucas, A.J. Hudson, G.A. Burley, I.C. Eperon, NAR Breakthrough Article The mechanisms of a mammalian splicing enhancer, Nucleic Acids Res. 46 (2018) 2145-2158.
[64] Elianna M. Amin, S. Oltean, J. Hua, Melissa V.R. Gammons, M. Hamdollah-Zadeh, Gavin I. Welsh, M.-K. Cheung, L. Ni, S. Kase, Emma S. Rennel, Kirsty E. Symonds, Dawid G. Nowak, B. Royer-Pokora, Moin A. Saleem, M. Hagiwara, Valérie A. Schumacher, Steven J. Harper, David R. Hinton, David O. Bates, Michael R. Ladomery, WT1 Mutants Reveal SRPK1 to Be a Downstream Angiogenesis Target by Altering VEGF Splicing, Cancer Cell 20 (2011) 768-780.
[65] D.G. Nowak, J. Woolard, E.M. Amin, O. Konopatskaya, M.A. Saleem, A.J. Churchill, M.R. Ladomery, S.J. Harper, D.O. Bates, Expression of pro- and anti-angiogenic isoforms of VEGF is differentially regulated by splicing and growth factors, J. Cell Sci. 121 (2008) 3487-3495.
[66] K. Tzelepis, E. De Braekeleer, D. Aspris, I. Barbieri, M.S. Vijayabaskar, W.-H. Liu, M. Gozdecka, E. Metzakopian, H.D. Toop, M. Dudek, S.C. Robson, F. Hermida-Prado, Y.H. Yang, R. Babaei-Jadidi, D.A. Garyfallos, H. Ponstingl, J.M.L. Dias, P. Gallipoli, M. Seiler, S. Buonamici, B. Vick, A.J. Bannister, R. Rad, R.K. Prinjha, J.C. Marioni, B. Huntly, J. Batson, J.C. Morris, C. Pina, A. Bradley, I. Jeremias, D.O. Bates, K. Yusa, T. Kouzarides, G.S. Vassiliou, SRPK1 maintains acute myeloid leukemia through effects on isoform usage of epigenetic regulators including BRD4, Nat. Commun. 9 (2018) 5378.
[67] J. Batson, H.D. Toop, C. Redondo, R. Babaei-Jadidi, A. Chaikuad, S.F. Wearmouth, B. Gibbons, C. Allen, C. Tallant, J. Zhang, C. Du, J.C. Hancox, T. Hawtrey, J. Da Rocha, R. Griffith, S. Knapp, D.O. Bates, J.C. Morris, Development of Potent, Selective SRPK1 Inhibitors as Potential Topical Therapeutics for Neovascular Eye Disease, ACS Chem. Biol. 12 (2017) 825-832.
[68] C.S. Eubanks, A.E. Hargrove, RNA Structural Differentiation: Opportunities with Pattern Recognition, Biochemistry 58 (2019) 199-213.
[69] C. Feng, D. Chan, J. Joseph, M. Muuronen, W.H. Coldren, N. Dai, I.R. Corrêa Jr, F. Furche, C.M. Hadad, R.C. Spitale, Light-activated chemical probing of nucleobase solvent accessibility inside cells, Nat. Chem. Biol. 14 (2018) 276–283.
[70] R.C. Spitale, P. Crisalli, R.A. Flynn, E.A. Torre, E.T. Kool, H.Y. Chang, RNA SHAPE analysis in living cells, Nat. Chem. Biol. 9 (2013) 18–20.
[71] S.P. Velagapudi, M.D. Cameron, C.L. Haga, L.H. Rosenberg, M. Lafitte, D.R. Duckett, D.G. Phinney, M.D. Disney, Design of a small molecule against an oncogenic noncoding RNA, Proc. Nat. Acad. Sci. 113 (2016) 5898-5903.
[72] C. Plaschka, P.-C. Lin, K. Nagai, Structure of a pre-catalytic spliceosome, Nature 546 (2017) 617.
[73] X. Zhang, C. Yan, J. Hang, L.I. Finci, J. Lei, Y. Shi, An Atomic Structure of the Human Spliceosome, Cell 169 (2017) 918-929.
[74] C. Yan, R. Wan, R. Bai, G. Huang, Y. Shi, Structure of a yeast step II catalytically activated spliceosome, Science 355 (2017) 149-155.
[75] K. Bertram, D.E. Agafonov, W.-T. Liu, O. Dybkov, C.L. Will, K. Hartmuth, H. Urlaub, B. Kastner, H. Stark, R. Luehrmann, Cryo-EM structure of a human spliceosome activated for step 2 of splicing, Nature 542 (2017) 318-323.
[76] C. Yan, R. Wan, R. Bai, G. Huang, Y. Shi, Structure of a yeast activated spliceosome at 3.5 angstrom resolution, Science 353 (2016) 904-911.
[77] N. Thi Hoang Duong, W.P. Galej, X.-c. Bai, C. Oubridge, A.J. Newman, S.H.W. Scheres, K. Nagai, Cryo-EM structure of the yeast U4/U6.U5 tri-snRNP at 3.7 angstrom resolution, Nature 530 (2016) 298-302.
[78] D.J. Foster, C.R. Brown, S. Shaikh, C. Trapp, M.K. Schlegel, K. Qian, A. Sehgal, K.G. Rajeev, V. Jadhav, M. Manoharan, S. Kuchimanchi, M.A. Maier, S. Milstein, Advanced siRNA Designs Further Improve In Vivo Performance of GaINAc-siRNA Conjugates, Mol. Ther. 26 (2018) 708-717.
[79] S.T. Crooke, S.Y. Wang, T.A. Vickers, W. Shen, X.H. Liang, Cellular uptake and trafficking of antisense oligonucleotides, Nat. Biotech. 35 (2017) 230-237.

Figure Captions

Figure 1 – Schematic diagram of the two sequential transphosphoesterification splicing reactions. In the first step of pre-mRNA splicing (branching), the 2′ hydroxyl group of a conserved RNA adenine in the branch point sequence (BPS) of an intron attacks a phosphate of the guanine at the 5′ end of the intron (5′ splice site, 5′-SS), to produce a free 5′ exon and a lariat intron–3′ exon intermediate. In the second step (exon ligation), the new 3′hydroxyl group at the 3′end of the 5′-exon attacks a phosphate at the 3′ splice site (3′-SS) to ligate the exons and release the lariat intron. Intron sequences are indicated with grey lines. Nucleophilic attacks are indicated with red arrows.

Figure 2 – Examples of druggable splicing events. Splice switching molecules can restore a desired splicing event by (a) inclusion or exclusion of a cassette exon, (b) disruption of alternative splicing by switching mutually exclusive exons, and (c) disruption of the corresponding reading frame. Semicircles represent the disrupted coding sequence.

Figure 3 – SMN2 minigene reporter splicing in HEK293H cell line. Exon 7 exclusion results in the formation of SMN2-Δ7 where the exporession luciferase is out of frame. Exon 7 inclusion results in the expression of luciferase in-frame, and the subsequent production of a fluorescent output in the presence of luciferin.

Figure 4 – Lead optimisation of PTC/Roche splice-switching compounds. Lead compound **4** was optimised through sequential SAR of the basic head unit (grey), heterocyclic core (blue) and the head group (red).

Figure 5 – Splicing of *SMN* minigene constructs. (a) Full length (FL) construct expresses luciferase when exon 7 is retained in the transcript. (b) Δ7 construct expresses luciferase when exon 7 is excluded from the transcript. Red bars show position of C nucleotide insertions to adjust sequence to ensure the luciferase is in-frame. Oral dosing of compounds **2** (NVS-SM1) and **8** (NVS-SM1) induced elevated levels of *SMN*-FL transcript in C/+ mouse model. (c) Structures of **2** (NVS-SM1) and **8** (NVS-SM1)

Figure 6 – SMN-C class splice switching molecules. (a) Structures of SMN -C3 (**9**), SMN-C5 (**10**), SMN-C2 (**5**) (b) Structure of SMN-C2-BD (**11**), an SMN-C2 derived photo crosslinker probe.

Figure 7 – Alternative splicing of Bcl-x. Splicing at the x_S splice site (SS) produces the shorter pro-apoptotic isoform, Bcl-x_S. Splicing at the x_L SS produces the longer anti-apoptotic isoform, Bcl-x_L.

Figure 8 – G-Quadruplex (G4) formation. (a) Representation of G-quartet formation through Hydrogen-bonding of the Hoogsteen face and the monovalent potassium cation (K^+) which

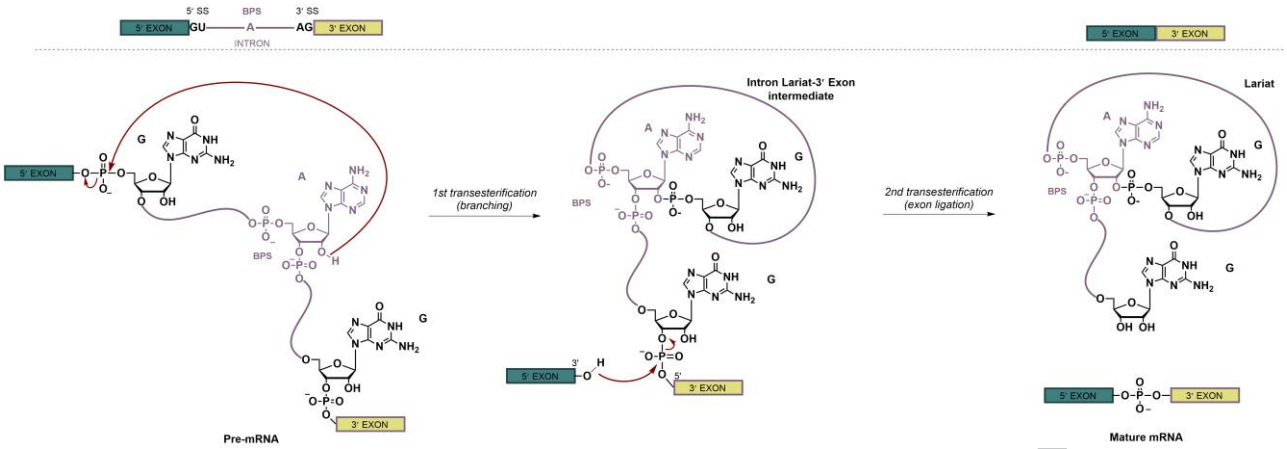
coordinates with the Oxygen atoms. (b) Disruption of Hoogsteen Hydrogen-bonding by substitution with 7-deazaguanine.

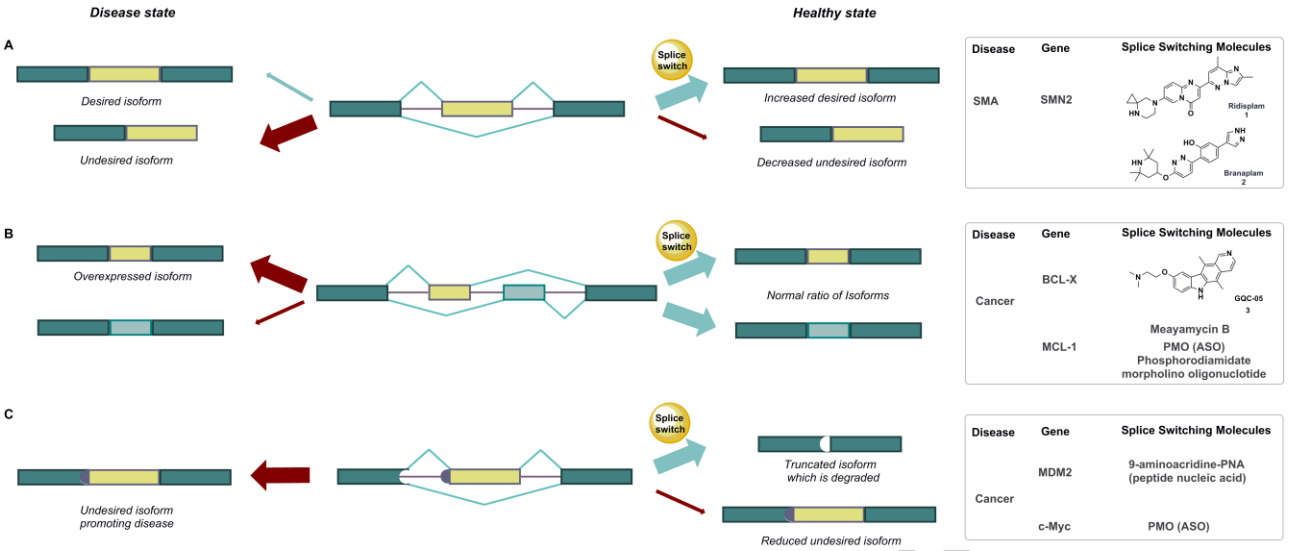
Figure 9 – *In vitro* Bcl-x-681 Splicing Assays with G4 stabilising ligands. (a) Splicing assays were done in the presence of G4 ligands at the concentrations shown (μM) GQC-05 showed an increased expression of Bcl-x_S (pro-apoptotic form), highlighted in red. (b) Proportion of mRNA spliced into Bcl-x_S isoform, error bars depict standard deviation. (c) Structures of GQC-05 (**3**), Quarfloxin (**12**), TmPyP4 (**13**) and GSA-0902 (**14**).

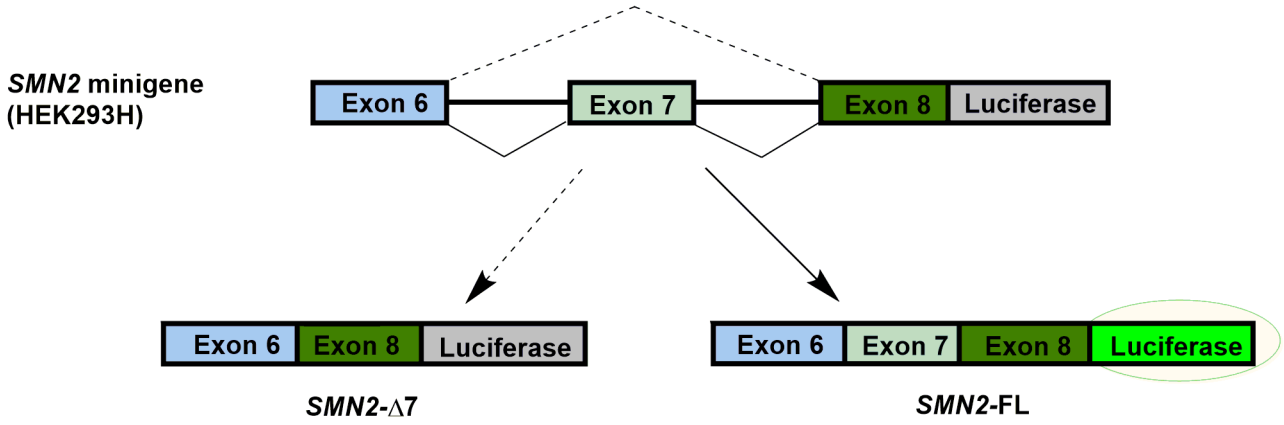
Figure 10 – Schematic diagram of a potential splicing pathway of VEGF-A. Exclusion of exons 6a-b and 8b produce the angiogenic isoform, VEGF-165a. Exclusion of exons 6a-b and 8a produce the anti-angiogenic isoform, VEGF-165b.

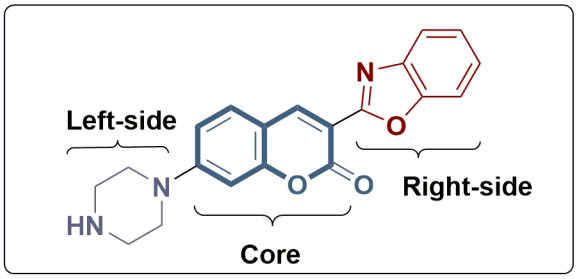
Figure 11 – SRPK1 Inhibitors. Structures of (**15**) and SPHINX31 (**16**).

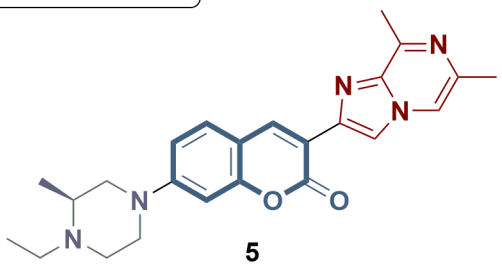
Figure 12 – X-ray crystallography structure of SRPK1 with bound SPHINX31. CF_3 group interacts with the deep hydrophobic cavity formed by the inset side chains of Tyr227, Ile228 and Leu231 (PDB ID = 5MY8).



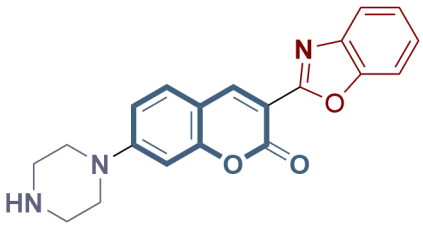




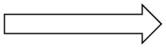


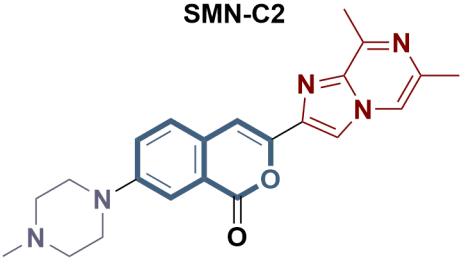


5
SMN-C2

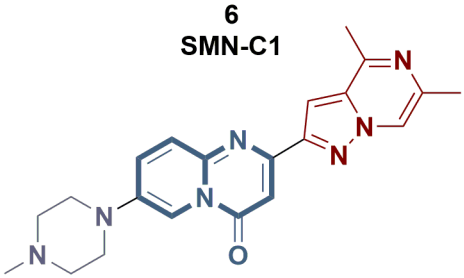


4

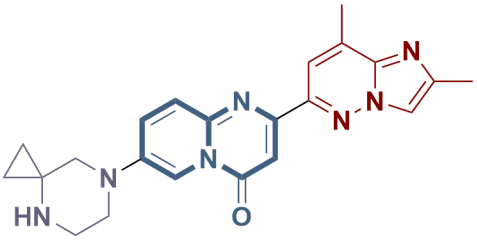




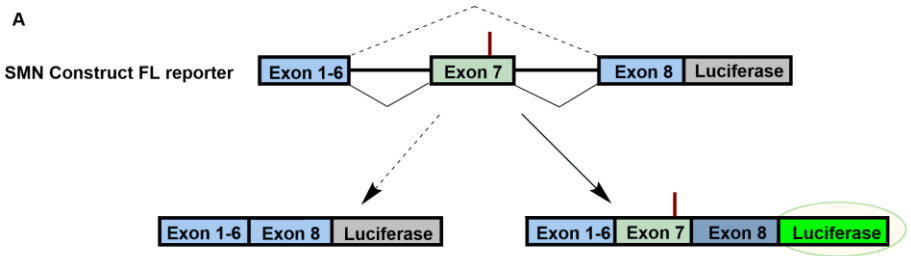
6
SMN-C1

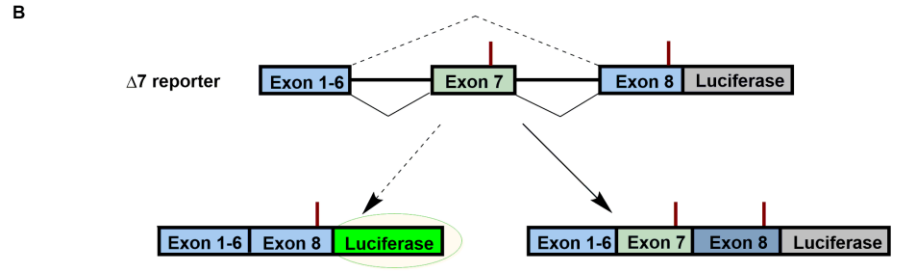


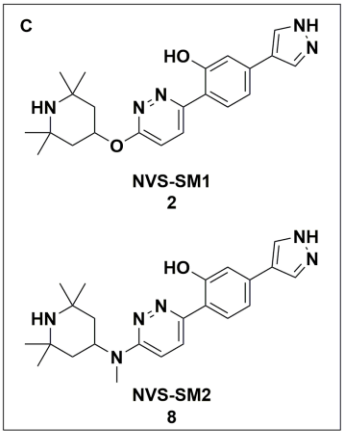
7

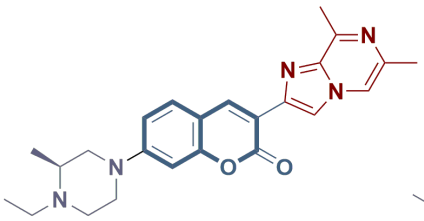


1
(Ridisplam or RG7916)

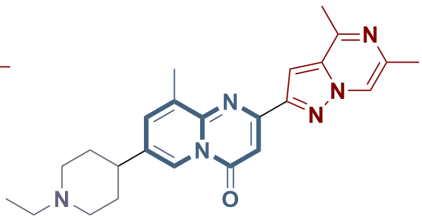




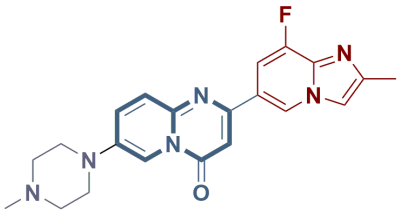




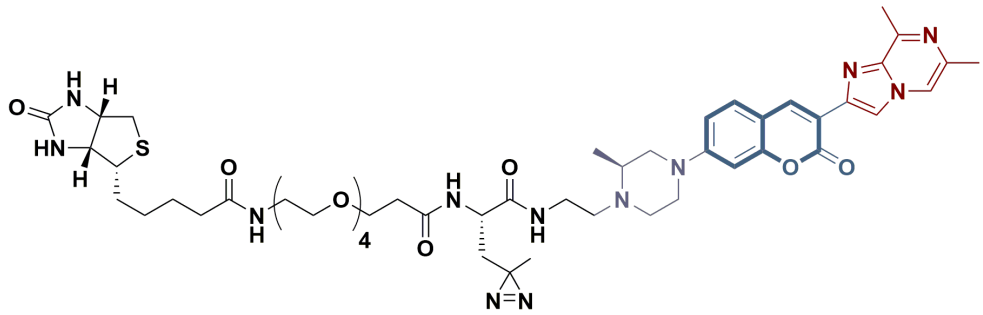
5
SMN-C2



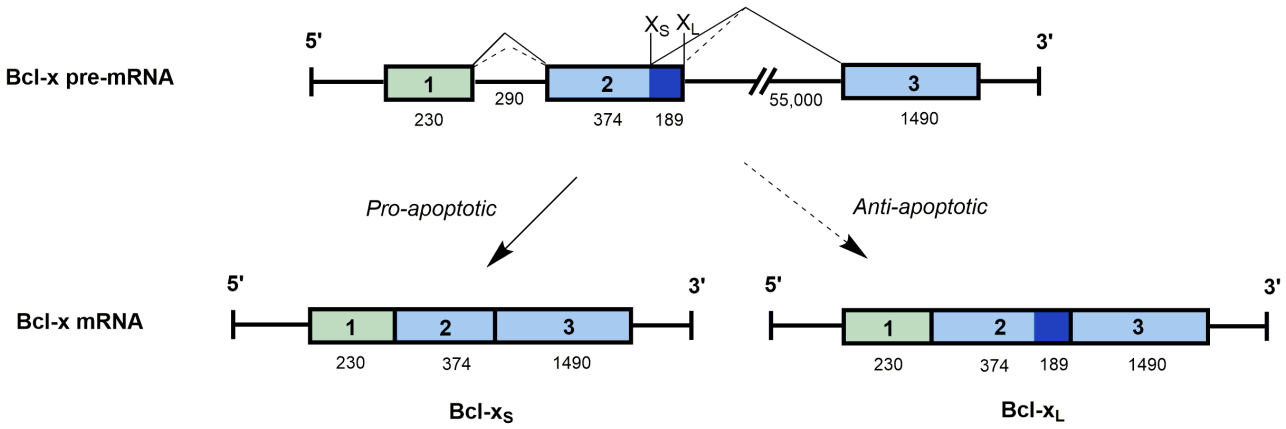
9
SMN-C3

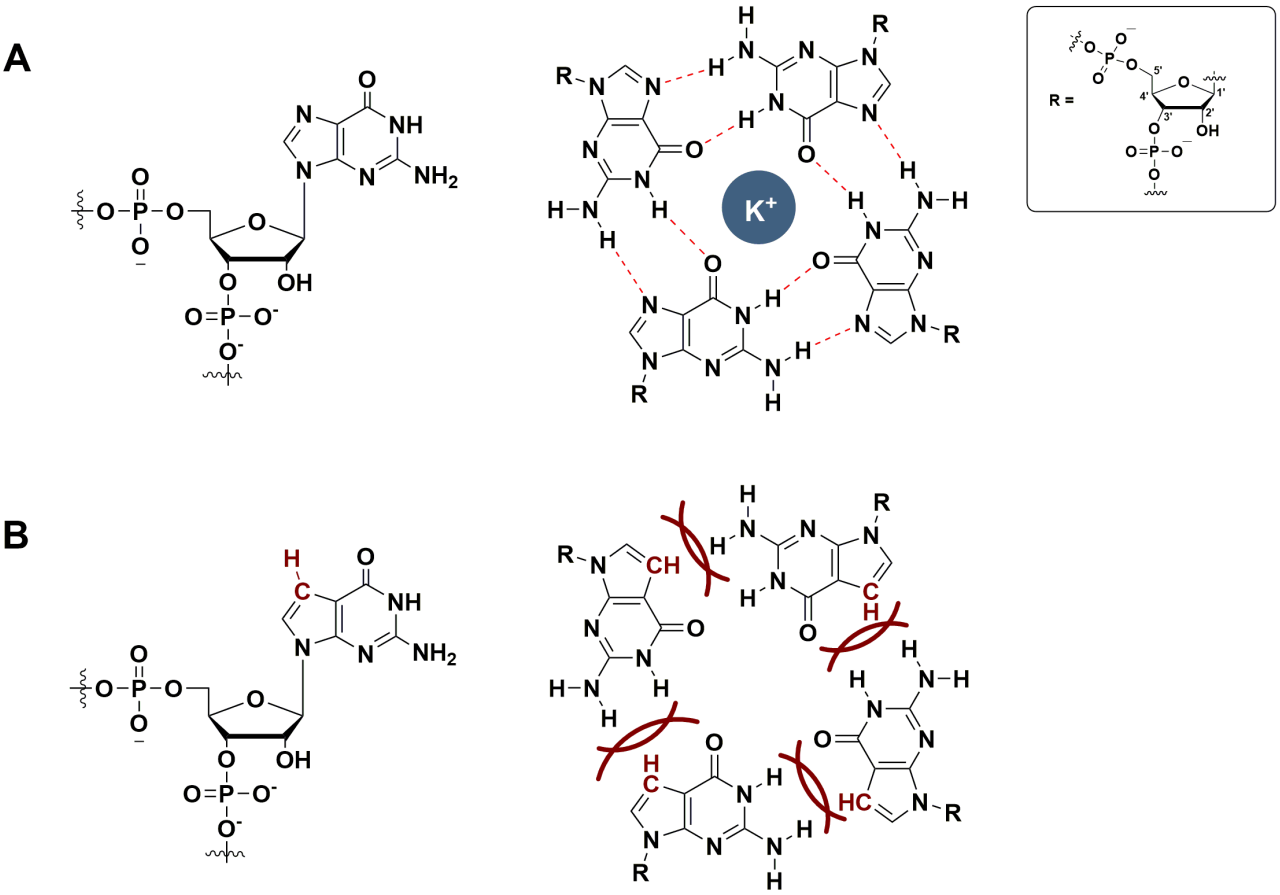


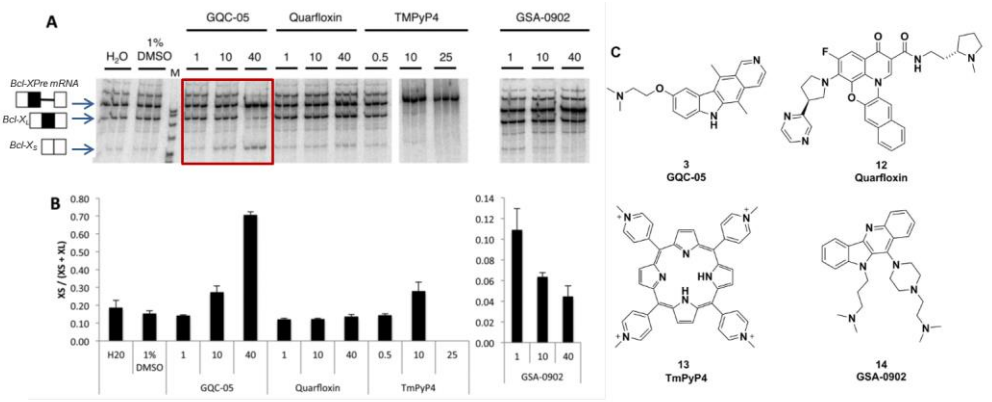
10
SMN-C5

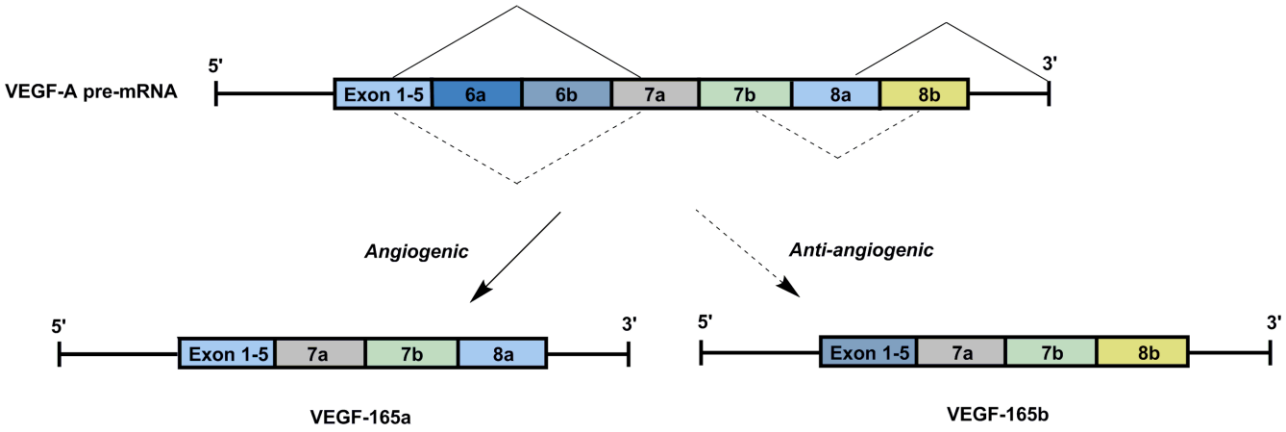


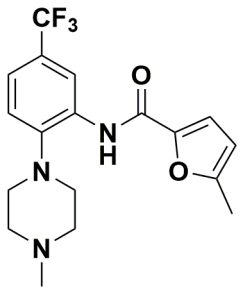
11
SMN-C2-BD



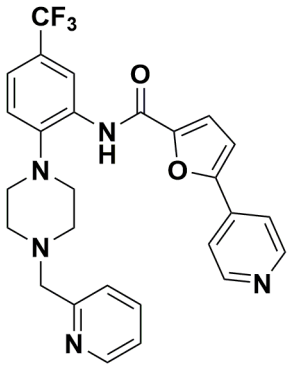




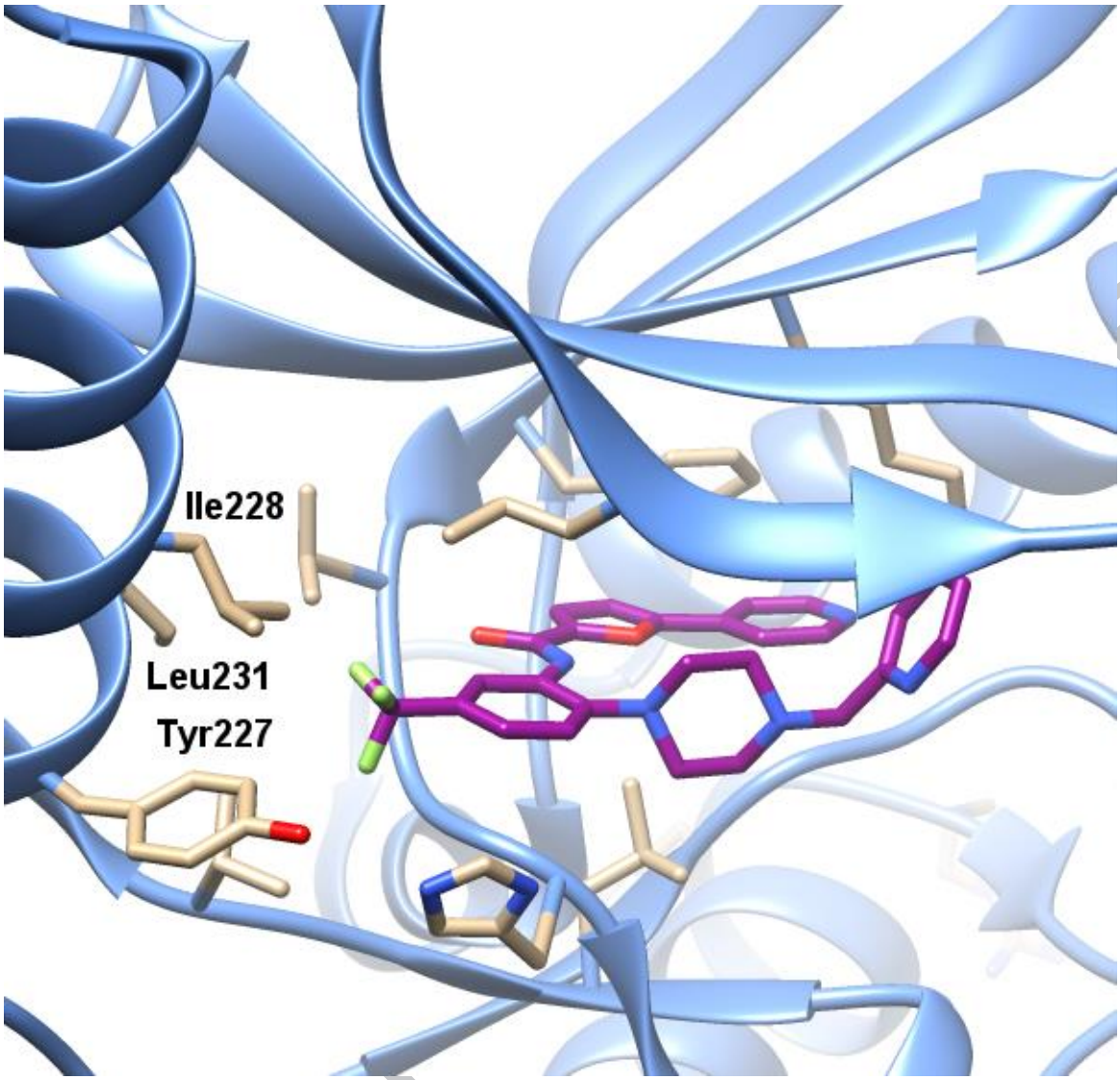




15



16
SPHINX 31



Highlights

- Alternative RNA splicing is a source of protein diversity in higher eukaryotes.
- Small molecules can manipulate alternative RNA splicing for therapeutic purposes.
- Splice-switching small molecules can exert their effect by binding RNA, RNA-protein interfaces or *via* inhibition of enzymes indirectly involved with spliceosomal function.