Mini-review

mRNA Transcript Diversity Creates New Opportunities for Pharmacological Intervention

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Abbreviations: UTR, untranslated region; IRES, internal ribosome entry sites; miR, microRNA; CETP, cholesterylester transfer protein; DRD3, dopamine receptor D3; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CAD, coronary artery disease; NAT1, N-acetyltransferase 1; CAR, constitutive androstane receptor; PXR, pregnane X receptor; CYP, cytochrome P450; MECP2, methyl CpG binding protein 2; FOX1, RNA binding protein, fox-1 homologue (C. elegans); CACNA1C, voltage dependent L-type calcium channel α-subunit 1c; UGT, UDP-glucuronosyltransferases; CNS, central nervous system; SCN1A, neuronal sodium channel; MOR, mu opioid receptor; M6G, morphine-6β-glucuronide; GPCR, G protein-coupled receptor; DRD2, dopamine receptor D2; FD, Familial dysautonomia; NSAID, non-steroidal anti-inflammatory drug; COX, cyclooxygenase; SSO, splice-switching oligonucleotides; UsnRNA, uridine-rich small nuclear RNA; RNAi, RNA interference; siRNA, small interfering RNA; RISC, RNA induced silencing complex; SMA, spinal muscular atrophy; SMN1, Survival of Motor Neuron; snRNP, small nuclear ribonucleoproteins; ASO, antisense oligonucleotides; EIPA, 5-(N-Ethyl-N-isopropyl)amiloride; HDAC, histone deacetylase; DMD, Duchenne muscular dystrophy; VEGF, vascular endothelial growth factor; Bcl-x, B-cell lymphoma 2-like 1; MSTR1, macrophage stimulating 1 receptor; MyD88, myeloid differentiation primary response; TLR, toll-like receptors; IL-1R, interleukin-1 receptor; NF-κB, nuclear factor kappa-light-chain enhancer of activated B cells; TNF, tumor necrosis factor; RA, rheumatoid arthritis
Abstract

Most protein coding genes generate multiple RNA transcripts through alternative splicing, variable 3' and 5'UTRs, and RNA editing. While drug design typically targets the main transcript, alternative transcripts can have profound physiological effects, encoding proteins with distinct functions or regulatory properties. Formation of these alternative transcripts is tissue-selective and context-dependent, creating opportunities for more effective and targeted therapies with reduced adverse effects. Moreover, genetic variation can tilt the balance of alternative versus constitutive transcripts or generate aberrant transcripts that contribute to disease risk. Additionally, environmental factors and drugs modulate RNA splicing, affording new opportunities for the treatment of splicing disorders. For example, therapies targeting specific mRNA transcripts with splice-site directed oligonucleotides that correct aberrant splicing are already in clinical trials for genetic disorders such as Duchenne muscular dystrophy. High-throughput sequencing technologies facilitate discovery of novel RNA transcripts and protein isoforms, with applications ranging from neuromuscular disorders to cancer. Consideration of a gene’s transcript diversity should become an integral part of drug design, development, and therapy.
Introduction

The human genome consists of 20,000-25,000 protein-coding genes, but the repertoire of mRNA sequences and encoded proteins is far greater due to multiple RNA isoforms generated from each gene. RNA transcript diversity evolves from several mechanisms, including alternative splicing, alternative transcription initiation and polyadenylation site usage, RNA editing, and trans-splicing over long distances from different gene loci. RNA splicing, in particular, is a major factor driving phenotypic diversity in higher eukaryotes. Alternative transcripts are created by a series of splicing events: exon skipping, intron retention, alternative 5' and 3' splice sites, alternative last exons, tandem 3' untranslated regions, alternative first exons, and mutually exclusive exons. In addition, translational control elements in mRNA isoforms can affect protein levels, including internal ribosome entry sites (IRES) in the 5' UTR or degradation signals and microRNA (miR) binding sites in the 3'UTR (Figure 1). All RNA processing events follow a tissue-specific expression pattern that varies less across individuals than between different tissues in the same individual, enabling selective drug targeting of RNA and protein isoforms to the site of action. Tissue-specific alternative splicing is greatly affected by splicing factors present in that tissue. These may be ubiquitously expressed, but present at different levels in different tissues, or the factors may be preferentially expressed in certain tissues. For example, NOVA-1 is only expressed in neurons, and is present at different levels, depending on the brain region studied. While the target sequences for many of these splicing factors are available, much is still unknown about their mechanisms. Whole transcript screening has elucidated some tissue-specific regulatory motifs and detected patterns that cluster by tissue (Castle et al., 2008) but this area is still under investigation.

RNA splicing and human transcriptome diversity: relevance to disease and drug design

Splicing events are highly prevalent, estimated to occur for 95% of all multi-exon genes (Pan et al., 2008), 86% of which express a minor isoform abundance of 15% or more of the total gene expression (Wang et al., 2008). The majority of splicing events (70-88%) alter the encoded protein (Kan et al., 2001; Modrek et al., 2001), with more than half causing a shift in the mRNA reading frame. Using high-
throughput sequencing technologies to accurately predict transcript isoforms remains challenging because of short and less than parsimonious sequence motifs directing processing, complex effects of tertiary structure, and tissue-specific assembly of the multi-component spliceosome (Modrek and Lee, 2002). In addition to genetically determined splicing differences, environmental factors such as diet or toxins can alter splicing patterns. Even environmental regulation of epigenetic factors typically associated with transcription can regulate splicing, with the same proteins involved in both events (Young et al., 2005). Consequently, transcript variability resulting from both genetic and environmental factors can adversely affect drug efficacy or enhance toxicity, but can also be exploited for therapeutic purposes.

Common genetic variants can afford changes in alternative splicing within a ‘normal’ physiological range. However, mutations causing aberrant splicing typically result in non-functional protein or nonsense mediated RNA decay, if a codon phase shift introduces premature termination signals (Cooper et al., 2009; Lareau et al., 2007; Wang and Cooper, 2007). Up to 50% of diseases with genetic components involve splicing mutations (Faustino and Cooper, 2003; Wang and Cooper, 2007). One approach to effective therapy is to reduce formation of disease-associated aberrant RNA isoforms using oligonucleotides to suppress splicing defects (Wilton and Fletcher, 2005), further discussed below.

Alternatively, targeting a specific protein or RNA isoform can increase efficacy or reduce off-target adverse effects. A drug typically binds to multiple proteins in the body involving many genes, refuting the idea of one drug/one protein target. However, the concept of multiple proteins generated from a single gene locus has not systematically impacted drug design, even though protein isoforms can have distinct functions. For example, the exon 9-lacking (Δ9) cholesteryl ester transfer protein (CETP) (Inazu et al., 1992; Lira et al., 2008) and dopamine receptor DRD3 (Elmhurst et al., 2000; Karpa et al., 2000) act in a dominant-negative manner with the alternatively spliced transcript preventing the function of the normal transcript. CETP transfers cholesterol esters from HDL to LDL, thus decreasing the ratio of HDL to overall cholesterol. In an effort to protect against coronary artery disease (CAD) by boosting HDL levels at the expense of LDL, several CETP inhibitors are currently under development. The first drug candidate acting as a CETP inhibitor (torcetrapib) was withdrawn in late Phase III trials because of an
unexpected rise in CAD incidence (Barter et al., 2007), suggesting a complex relationship between HDL, cardiovascular disease, and underlying genetic factors. A better understanding of the physiological regulation of CETP activity by Δ9-CETP as an ‘internal’ inhibitor, and genetic variants potentially affecting this process, will prove critical in guiding optimal therapy with CETP inhibitors.

While it is important to consider transcript diversity exhibited by drug targets, the enzymes that metabolize these drugs can also be affected by both normal and aberrant splicing. Variation in drug metabolizing enzyme activity, as observed with cytochrome P450s, introduces variability in drug response, leading to increased incidence of toxicity, poor efficacy, or both (Phillips et al., 2001). A common variant allele, CYP2D6*4, introduces a mutation in the splice site of intron 3, causing the pre-mRNA of this gene to be incorrectly spliced, resulting in a non-functional enzyme (Kagimoto et al., 1990; Marez et al., 1997). Patients homozygous with this variant are poor metabolizers of numerous drugs; therefore, current drug design aims at avoiding CYP2D6 substrates. Another metabolizing enzyme, N-acetyltransferase 1 (NAT1) processes endogenous and exogenous compounds including drugs and environmental carcinogens. NAT1 RNA transcripts arise from one coding exon (exon 9) with three possible upstream promoter/transcription start sites, several splice variants, and three polyadenylation sites resulting in different translation yields (Boukouvala and Sim, 2005; Butcher et al., 2005; Wang et al., 2011). In contrast to the mostly hepatic expression of NAT2, NAT1 is ubiquitously expressed, but enzyme activities are differentially regulated across tissues with pharmacological and toxicological implications resulting from drug or toxin acetylation. Another example of pharmacological significance, the nuclear receptors constitutive androstane receptor (CAR) and pregnane X receptor (PXR), regulators of drug metabolizing enzyme expression with a key role in defense against xenobiotic exposure, are alternatively spliced, increasing the range of recognized ligands and downstream target genes. Phthalates, chemicals used in commercial manufacturing, and other endocrine-disrupting compounds have differential selectivity for CAR isoforms (DeKeyser et al., 2011), an issue relevant to toxicology.

*In vitro and in silico screening for drugs that alter splicing*
General splicing defects occur in several complex disorders, including cancer and psychiatric disorders (Cooper et al., 2009; Wang and Cooper, 2007). For example, mutations in genes encoding auxiliary splicing factors such as MECP2 and FOX-1 affect multiple downstream targets in autism spectrum disorders and schizophrenia (Smith and Sadee, 2011; Voineagu et al., 2011). Discovery of drugs broadly targeting splicing has accelerated with rapid screening techniques. For example, a splicing reporter assay allows for high-throughput screening of splicing modulators (Stoilov et al., 2008). Using splicing reporter constructs, 4,000 compounds were screened for their ability to inhibit spliceosome assembly (Soret et al., 2005). In silico approaches can assist in predicting the impact of sequence variation on splicing enhancer/suppressor sites (Human Splicing Finder) (Desmet et al., 2009), or assessing secondary RNA structure with programs such as Mfold (Zuker, 2003) to reveal sequence-independent structural motifs crucial for protein-RNA binding (G-quartets, Kissing Complexes, etc.). These tools can guide in designing and screening compounds in drug development.

Below, we discuss pharmacologically relevant examples of transcript complexity as demonstrated by the calcium channel CACNA1C and the drug-metabolizing enzyme UGT1A. Additionally, we provide examples where transcript isoforms are relevant to drug response and drug design (Table 1). Finally, we review approaches to altering specific RNA processing as a means for treating genetic disorders.

A voltage dependent L-type calcium channel: example of extreme splicing diversity

The L-type calcium channel α-subunit 1c (Cav1.2, CACNA1C) regulates blood pressure and cardiac function, serving as a target of Ca^{2+} channel blockers. An extraordinary example of transcript diversity, at least 10 of 55 exons undergo alternative splicing, with several sites yielding multiple isoforms, leading to over 10,000 possible splice variants (Tang et al., 2004; Welling et al., 1997). In addition to displaying profound differences in channel functions, these splice variants have been associated with disease susceptibility and variable response to drugs used to treat hypertension and arrhythmias. CACNA1C is expressed in cardiac and smooth muscle, brain, and other tissues. Mutually exclusive exons E8a/8 encode part of the dihydropyridine drug binding site. E8 containing channels
expressed in smooth muscle display 10-fold greater affinity for dihydropyridine channel blockers than channels containing E8a, the cardiac form (Welling et al., 1997). We have studied interindividual variability in mRNA expression and splicing of CACNA1C, in 65 heart tissue samples from heart transplant recipients (Wang et al., 2006), demonstrating dramatic differences in the expression pattern of exon 8/8a between individuals, ranging from 9 to 87% for exon 8a usage. Two heart tissues predominantly contained the smooth muscle-specific form, raising the question as to the pharmacological response to dihydropyridines in individuals with this expression pattern, likely to experience strong effects in the heart (Wang et al., 2006). This example highlights interindividual variability in alternative splicing, which drastically modulates pharmacological effects. We failed to detect any genetic factors accounting for these differences, suggesting trans-effects from the splicing machinery play a key role. We propose that this type of variability should be considered in drug design, potentially targeting channel blockers to binding pockets unaffected by the 8/8a splice variation.

**Transcript diversity affecting multiple drugs: UGT1A**

UDP-glucuronosyltransferases (UGT) are a family of enzymes responsible for the detoxification of endogenous and exogenous compounds through the formation of hydrophilic glucuronides excreted through urine, feces or bile. Metabolic targets include bilirubin, steroids, and drugs such as irinotecan and clozapine. The UGT1A gene locus is under the control of tissue-specific promoters, which results in expression of unique multiple first exons paired with four constitutively expressed downstream exons via alternative splicing (Gong et al., 2001). The alternative first exons encode the N-terminal 280 residues, which are responsible for substrate specificity (Ritter et al., 1992). To date, 13 UGT1A alternative first exons have been identified, with varying substrate selectivity and tissue expression. This transcript diversity has physiological and pharmacological relevance. For example, the primary function of UGT1A1, mainly expressed in the liver, is the glucuronidation of bilirubin (Ritter et al., 1992).

Alternative splicing also occurs at the 3’ end of the UGT1A locus. Originally identified in UGT1A1, alternative splicing of exon 5 (Levesque et al., 2007) yields two splice variants, the active enzyme UGT1A1_i1 and a truncated isoform UGT1A1_i2, illustrated in Figure 2A. Upon
heterodimerization, UGT1A_i2 inhibits UGT1A_i1-mediated glucuronidation, another example where a splice isoform exerts dominant-negative effects (Levesque et al., 2007). Alternative splicing of exon 5 extends through all active UGT1A genes (UGT1A1, IA3-10) in a tissue-specific manner, presumably creating inhibitory isoforms for each (Girard et al., 2007).

Genetic variation contributes an additional layer of complexity to the relationship between UGT1A expression and substrate metabolism. Located in the promoter region of an alternative exon 1, a dinucleotide repeat allele, termed *28, alters UGT1A1 expression. Homozygous carriers of the minor allele have Gilbert’s syndrome (hyperbilirubinemia) (Bosma et al., 1995) and reduced irinotecan inactivation, associated with irinotecan toxicity (Marsh and McLeod, 2004), making *28 a potential biomarker for guiding irinotecan therapy and reducing toxicity. Non-synonymous mutations in UGT1A4, such as P24T or L48V can alter metabolism of xenobiotics, steroids (Ehmer et al., 2004), and clozapine (Mori et al., 2005).

The ability of the UGT1A locus to impact endogenous and exogenous drug metabolism strongly depends on transcript variation across the many tissues expressing UGT1A. In a similar fashion, UGT1A transcript complexity via alternative first exon usage and exon 5 splicing, together with genetic factors, will have significant impact on drug efficacy in target tissues. Certainly, tissue selective isoform expression bears upon drug design, considering the site of pharmacological action or toxicity.

**Splicing in the CNS affects drug targets**

Nowhere is mRNA transcript diversity more prevalent than in the brain (de la Grange et al., 2010; Modrek et al., 2001; Yeo et al., 2004), offering opportunities for generating selective pharmacological profiles where alternative splicing results in unique functional proteins. Less obvious effects on protein expression also arise from alternative UTR usage. For example, greater length and complexity of the 5' UTR generally correlates with reduced protein translation efficiency (Gray and Hentze, 1994; Kozak, 1989). In heteromeric receptor complexes, receptor stoichiometry can depend on the availability of a particular subunit (Moroni et al., 2006), thus resulting in unique pharmacological profiles where tissue-specific 5'UTRs differ in translation efficiency. A similar case can be made for polyadenylation site
usage, which determines 3’ UTR length, potentially increasing the number of regulatory elements in longer UTRs that are subject to trans-acting factors (miRs, translational control proteins, etc.). Discussed below are select examples of transcript diversity creating distinct pharmacological profiles.

The neuronal Nav1.1 sodium channel (SCN1A)

Voltage-gated sodium channels are heteromultimeric complexes involved in generating and propagating action potentials. Type I channels transmit signals between neurons in the brain. Defects in SCN1A cause certain types of epilepsy and migraines. Encoding Nav1.1, a large alpha subunit, SCN1A is alternatively spliced to include either the canonical 5A or alternative (5N) exon 5. Channels with the alternative exon are more sensitive to the effects of phenytoin and lamotrigine, common anti-epileptic medications (Thompson et al., 2011). These drugs interact differently with spliced targets, thus affecting their efficacy. Epileptic patients with SNP rs3812718, located in a splice donor site (Tate et al., 2005) have increased expression of the 5N form of the transcript, requiring lower doses of phenytoin.

The mu opioid receptor (OPRM1)

The mu opioid receptor (MOR, encoded by OPRM1) is the crucial target for analgesic compounds and narcotics, including morphine, codeine, and heroin. OPRM1 transcripts originate from two distinct first exon promoters (Pan et al., 2001; Xu et al., 2009; Xu et al., 2011), while the 3’ end undergoes extensive splicing to produce at least 19 distinct isoforms in humans (20 isoforms at AceView and 23 at Ensembl, 4 of which do not encode protein.) These different mRNA transcripts are regionally distributed throughout the brain (Pan et al., 2001).

Ligand-dependent MOR signaling varies depending on alternative promoter usage at the OPRM1 gene. OPRM1 transcription can initiate approximately 28 kilobases upstream of the annotated exon 1, to transcribe an alternative first exon (known as exon 11) (Xu et al., 2009). Disruption of exon 11-derived variants markedly reduces analgesic effects of heroin and morphine-6β-glucuronide (M6G), while not affecting morphine and methadone analgesia (Pan et al., 2009). Conversely, disruption of exon-1 derived spliced isoforms in mice causes ligand-dependent differences in analgesia whereby heroin and M6G
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retain residual analgesic properties, while morphine does not (Schuller et al., 1999). The mechanisms underlying differential opioid effects on OPRM1 isoforms remain controversial.

A second critical element of MOR signaling is protein diversity generated at the carboxyl terminus through alternative splicing at the 3’ ends of OPRM1 transcripts, with significant influence on ligand-binding affinity. For example, the human MOR transcript variant hMOR-1B2 is alternatively spliced at exon 5, binding most opioids and opioid peptides with lower affinity than other C-terminus variants (Pan et al., 2005a). C-terminus splicing of OPRM1 RNA in humans (Pan et al., 2005a) and rodents (Pan et al., 2005b) also affects potency and efficacy for a range of opioid compounds. An additional layer of complexity among all G protein-coupled receptors (GPCRs), of which OPRM1 is a member, is receptor oligomerization (Bouvier, 2001; Wang et al., 2005). As it pertains to transcript splicing, ligand affinity differences for hetero-oligomers of MORs and delta opioid receptors are dependent upon carboxyl tail length (Fan et al., 2005). While GPCRs appear to dimerize and polymerize readily, the promiscuity of GPCR heteropolymerization is still under debate, as is its role in pharmacology and drug design.

The dopamine D2 receptor (DRD2)

The D2 receptor serves as a primary target for antipsychotic compounds, with a finite window of D2 receptor occupancy linked to clinical efficacy (Kapur et al., 2000; Nordstrom et al., 1993) or adverse effects (Farde et al., 1992; Kapur et al., 2000). DRD2 is spliced to produce functionally distinct proteins with unique pharmacological profiles. Alternative splicing of exon 6 produces either the short (D2S) or long (D2L) protein isoforms (Giros et al., 1989) (Figure 2B) that differ in their subcellular localization (Khan et al., 1998). D2S predominately localizes on the presynaptic terminal, acting as an autoreceptor to inhibit synaptic dopamine release, whereas D2L acts primarily as a postsynaptic target for dopamine transmission (Khan et al., 1998; Usiello et al., 2000). We have identified two SNPs in introns 5 and 6 that significantly shift splicing towards inclusion of exon 6, thereby reducing autoreceptor activity of D2S in the striatum and prefrontal cortex, significantly affecting cognition, neural activity (Bertolino et al., 2009;
Bertolino et al., 2010; Blasi et al., 2009; Zhang et al., 2007) and risk of cocaine abuse/overdose (Moyer et al., 2011).

Differential pharmacological modulation of D2S versus D2L signaling offers opportunity for correcting imbalanced $\text{DRD}_2$ signaling, with drugs that display differential activity for the long versus short protein (Castro and Strange, 1993; Malmberg et al., 1993; Usiello et al., 2000; Xu et al., 2002). In a neuroendocrine cell line, sarizotan acts as a full agonist at D2L, but as a partial agonist at D2S (Kuzhikandathil and Bartoszyk, 2006). As another example, glutamate-mediated spontaneous excitatory postsynaptic currents are inhibited by quinpirole when the D2 ratio was altered in favor of D2S (Centonze et al., 2004). Using D2L-specific knockout mice, Xu et al. indirectly demonstrated higher D2L affinity compared to D2S for haloperidol and clozapine, while they observed the opposite for raclopride (Xu et al., 2002). Malmberg et al. also found greater D2L affinity for remoxipride, sulpiride, chlorpromazine, clozapine, and thioridazine, but greater D2S affinity for raclopride (Castro and Strange, 1993; Malmberg et al., 1993). However, owing to the propensity of antipsychotics to interact with multiple receptors, most notably serotonin receptor 2A (Borroto-Escuela et al., 2010), it remains uncertain whether targeting D2S or D2L conveys higher efficacy or toxicity. Yet, treatment success may depend on such subtype selectivity, an area in need of further exploration.

*Non-steroidal anti-inflammatory drugs (NSAIDs)*

Drug design and development benefit from knowing the molecular mechanism of action and intended target, especially when represented by multiple isoforms. Currently, neither of these issues is clear for NSAIDs, thought to act in part by inhibiting cyclooxygenases (COX). Originally, two cyclooxygenases derived from separate genes were described, COX-1 and COX-2. Varying affinities for COX-1 or COX-2 define pharmacological and toxicological NSAID properties (Chandrasekharan et al., 2002). A third isoform, COX-3, created by retention of intron 1 following alternative splicing of $COX\,-\,1$, was identified in canine tissues. However, the proposed selectivity of acetaminophen for COX-3 did not translate to humans (Kis et al., 2005). Alternate splicing of $COX\,-\,1$ actually produces at least three COX-1 isoforms in humans that differ from the canine isoforms. The most prevalent isoform, COX-1b1,
encodes a truncated and non-functional protein. COX-1b2, the isoform most similar to COX-3, did not differ in its response to NSAIDs compared to COX-1 (Qin et al., 2005). Targeting of alternate transcripts from COX genes stimulated short-lived excitement, but failure to produce a breakthrough unfortunately quenched enthusiasm for drug design targeting splice variants.

**Drug design directly targeting aberrant splicing or suppressing pathogenic RNA transcripts**

Drugs that target RNA processing to treat disorders involving aberrant isoforms are an alternative approach to current therapies. On one hand, small molecules can modulate *trans*-acting splice proteins, thereby changing spliced isoform expression profiles across many genes. On the other hand, splice-switching oligonucleotides (SSOs) can be deployed to correct aberrant splicing or downregulate unwanted RNA transcripts, as discussed below. The technique of using SSOs to correct splicing defects was originally developed by the Kole group to treat β-thalassemia (Schmajuk et al., 1999; Sierakowska et al., 1996). This disorder is caused by mutations creating aberrant splicing sites, leading to the inclusion of a stop codon and the creation of a truncated β-globin protein. In this case, the SSOs bind and cover the mutated splice site to prevent inclusion of the intron; however, SSOs can affect splicing along several mechanisms.

A component of the spliceosome required for splice donor site recognition, uridine-rich small nuclear RNAs (U1 snRNA), can be modified to correct specific splicing defects (Gorman et al., 2000; Gorman et al., 1998). Similarly, spliceosome-adapted U7 snRNA is needed to process histone mRNA. Modified synthetic U1 and U7 RNAs were shown to be effective in cell culture at inducing exon skipping or inclusion (Geib and Hertel, 2009; Hartmann et al., 2010; Incitti et al., 2010; Pinotti et al., 2008). A means of targeting specific mRNA transcript isoforms relies on complementary antisense oligonucleotides that can restore the normal splicing process disrupted by a mutation, skip disease-associated exons (Skordis et al., 2003), degrade unwanted transcripts, or increase production of a desired transcript. RNAi therapies employ small interfering RNA (siRNA) duplexes of 21-23 nucleotides. By incorporation into the RNA induced silencing complex (RISC), siRNAs promote degradation of mRNA
containing matched complementary sequences. siRNAs, such as miRs and their antisense counterparts, can regulate splicing, but the numerous other mechanisms by which they impart therapeutic potential goes beyond the scope of this review.

Abundant delivery of oligonucleotides to the target tissue and into the cell interior presents a major challenge for developing effective therapeutics. Slow penetration of lipid bilayers and rapid degradation \textit{in vivo} require chemical modifications and specific delivery strategies. Oligonucleotides can be delivered by liposomes, conjugates with cell surface recognition factors, viral vectors or biolistic injection (plasmid DNA affixed to heavy metal coated particles, delivered through the skin by particle bombardment); however, successful delivery remains a challenge, with only a few target tissues highly susceptible to intracellular drug delivery methods.

\textit{Small molecules to treat familial dysautonomia}

Compounds consumed in our daily diet can affect splicing, as observed with \(\Delta 9\)-CETP upregulation in response to dietary components (Dessi et al., 1997; Yang et al., 1996). One can take advantage of this insight to search for drugs that modify splicing. Familial dysautonomia (FD) is caused by a splice site mutation in the \textit{IKBKAP} gene excluding exon 20. Treatment with phosphatidylserine, an FDA-approved supplement, increases the wild-type form of \textit{IKBKAP} mRNA and IKAP protein levels in a patient cell line (Keren et al., 2010). A second therapeutic option for FD treatment is the plant-derived kinetin, which has the ability to cross the blood-brain barrier. In a transgenic mouse model, kinetin corrected \textit{IKBKAP} splicing defects in the brain and across tissues, resulting in increased IKAP protein levels (Shetty et al., 2011). These results were replicated in humans, with patients receiving one month of oral kinetin treatment demonstrating increased levels of wild-type \textit{IKBKAP} mRNA (Axelrod et al., 2011). Kinetin’s effect is not specific for the \textit{IKBAP} gene as it also modulates and partially corrects aberrant splicing of neurofibromatosis type 1 (\textit{NF-1}) (Pros et al., 2010). The use of amiloride in the treatment of a neuromuscular disorder and cancer to modulate splicing events is discussed further below. The number of candidate drugs in this category is still small, but it holds promise in the therapy of diseases involving aberrant splicing.
Applications in neuromuscular disorders

Spinal muscular atrophy (SMA) is a neuromuscular disease caused by a mutational splicing defect in the SMN1 gene. This deficiency is only partially compensated for by the homologous SMN2 gene, as exon 7 is readily spliced out of SMN2 nascent RNA, producing an unstable protein, resulting in inadequate SMN activity (Vitte et al., 2007). Fitting to the theme of this review, mature SMN plays a role in snRNP assembly essential to spliceosome function (Gabanella et al., 2007) so that deficiencies lead to widespread splicing defects (Zhang et al., 2008). Modifying SMN2 splicing to include exon 7 restores the fully active protein, achievable with use of short antisense oligonucleotides (ASOs). In cells taken from an SMA patient, an 8-mer ASO restored exon 7 inclusion and mature SMA formation, a first step towards effective therapy (Singh et al., 2009). While encouraging, appropriate drug delivery into the CNS is particularly challenging because oligonucleotides cannot readily cross the blood-brain barrier. Getting the drug to the target tissues requires an intracranial injection with use of an osmotic pump. This approach effectively increased expression of SMN in the brain and spinal cord, in a mouse model of SMA (Hua et al., 2010; Williams et al., 2009).

Alternative therapies have also shown promise for modifying SMN splicing. The antihypertensive drug amiloride and its analogue EIPA increase expression of SMN2 protein in SMA cells by increasing inclusion of exon 7 (Yuo et al., 2008), as does valproic acid (Harahap et al., 2011). A variety of HDAC inhibitors increase the lifespan of SMA mice (Wirth et al., 2006). Modulating extracellular pH also modifies splicing of SMN2, with high pH increasing exon 7 inclusion, while low pH increases skipping of exon 7 (Chen et al., 2008). These observations demonstrate the sensitivity of RNA splicing to environmental factors that could either aggravate disease symptoms or lead to palliative therapies.

Fukuyama-type congenital muscular dystrophy is a common recessive disorder in Japan, leading to disability and premature death. A retrotransposon insertion disrupts the fukutin gene activating a previously inaccessible donor splice site and creating a new splice acceptor site. To correct this defect, an ASO was developed which targets the defect and blocks the detrimental splicing. It has been effective in
expressing normal protein in a patient cell line, and was also able to restore partial fukutin protein expression in a mouse model following intramuscular injection (Taniguchi-Ikeda et al., 2011).

Duchenne muscular dystrophy (DMD) is a lethal disorder caused by mutations in the dystrophin gene that interrupt the open reading frame. A variety of mutations can lead to DMD; here we discuss deletion of exon 50, alone or in combination with other adjacent deletions, present in 13% of patients, with potential treatments in Phase 2 clinical trials. In these patients, a new stop codon leads to a truncated, non-functional protein. Two oligonucleotide agents are being investigated, aimed at splicing out exon 51 to restore the open reading frame, thereby producing functional dystrophin. Although the dystrophin gene is not alternatively spliced, and DMD is not caused by a splicing defect, the oligonucleotides are effective without targeting the splice site, as this therapy introduces a splicing event to overcome the genetic aberration. These oligonucleotides are complementary to a portion of the exon 51 pre-mRNA sequence and block it from being included in the processed transcript. The shift restores the reading frame, allowing a shorter, yet partially functional protein to be translated. Administration of ASO PRO051 enhanced dystrophin activity in patients following either intramuscular injection (van Deutekom et al., 2007) or systemic administration (Goemans et al., 2011). Cirak et al. demonstrated the safety and efficacy of AVI-4658, a phosphorodiamidate morpholino oligomer, with increased dystrophin protein expression (Cirak et al., 2011). These therapies all hold significant promise for treatment of neuromuscular diseases, a proof of principle indicating that correcting aberrant RNA processing can be applied to other diseases as well.

Applications in cancer therapy

Both alternative and aberrant splicing play a role in oncogenesis and treatment response. Anti-angiogenesis compounds that reduce the activity of vascular endothelial growth factor (VEGF) or its receptors (VEGFRs) can be effective at slowing tumor growth (Hurwitz et al., 2004). However, alternative splicing of VEGF and VEGFRs can result in transcripts with opposite effects to those traditionally attributed to VEGF signaling, inhibiting angiogenesis and slowing the growth of a wide
range of cancers (Varey et al., 2008; Woolard et al., 2004). As a consequence, nonspecifically targeting VEGF signaling potentially promotes angiogenesis or cell survival; therefore, anti-VEGF drugs should target specific spliced isoforms (reviewed in: (Harper and Bates, 2008)) or trans-acting splice proteins that favor expression of anti-angiogenic splice variants. As an example of the latter, spliceostatin A blocks the ability of the endogenous splicing machinery to process VEGF, thereby reducing angiogenesis in vivo (Furumai et al., 2010).

Vemurafenib, a relatively new and promising treatment for metastatic melanoma, acts by inhibiting an oncogenic form of BRAF. Normally involved in cell replication and survival, BRAF is mutated in approximately half of melanoma patients, causing increased cell proliferation via constitutive activity of BRAF(V600E). However, most patients develop resistance to the drug within a year of use, making it vital to determine the causes of drug resistance. A novel mechanism was recently discovered in vemurafenib-resistant cells, containing a truncated form of BRAF, p61BRAF(V600E). Monomeric p61BRAF(V600E) is as sensitive as the full length form, yet upon dimerization, the cells are no longer sensitive to vemurafenib. The mRNA is missing exons 4-8, which normally encode a region that suppresses dimerization; therefore, the deletion allows increased dimerization and bypasses the drug inhibition. BRAF variants lacking this domain were found in a subset of patient samples, confirming its clinical relevance (Poulikakos et al., 2011). Aberrant splicing of the target renders this drug ineffective, highlighting our central thesis, that by understanding alternative and aberrant splicing, one can develop therapeutics tailored to the patient’s genetic and biochemical characteristics.

The antihypertensive drug amiloride can regulate alternative splicing of oncogenic fusion genes, showing therapeutic promise in the treatment of chronic myelogenous leukemia by increasing the sensitivity of cancerous cells to imatinib (Chang et al., 2011). Targeting trans-acting splice proteins, however, lacks specificity, as these proteins direct alternative splicing of many genes and can lead to non-specific effects. Natural compounds with anti-tumor activity have been discovered, and bind to components of the spliceosome, inhibiting pre-mRNA splicing (Albert et al., 2007; Kaida et al., 2007; Kotake et al., 2007; O’Brien et al., 2008). While the development of new chemotherapeutics is
promising, it is important to understand the implications of blocking splicing in general. A more direct and specific effect can be achieved with SSOs, an approach used for treatment of the neuromuscular disorders described above. Bauman et al. demonstrated successful reduction in tumor growth through lipid nanoparticle delivery of an SSO that increases splicing of the anti-apoptotic Bcl-\(x_L\) transcript towards formation of the shorter pro-apoptotic Bcl-\(x_S\) isoform (Bauman et al., 2010). SSOs can also be used to produce longer alternatively spliced transcripts through the inclusion of alternative exons, as demonstrated for the proto-oncogene Ron (\(MSTR1\)) (Ghigna et al., 2010). These examples demonstrate potential utility but also reveal that the field is still in its infancy.

**Applications in immune disorders**

Novel approaches modulating RNA processes have also emerged in therapy of immune disorders. Facilitated drug delivery to immune cells ranks as a key advantage for this approach over targeting CNS disorders.

MyD88 is an adapter molecule involved in signal transduction by toll-like receptor (TLR) (Akira et al., 2001) and interleukin-1 receptor (IL-1R) family members (Mitcham et al., 1996). By activating the NF-\(\kappa B\) signaling cascade, MyD88 leads to activation of inflammatory cytokines, such as tumor necrosis factor (TNF). A naturally occurring spliced isoform lacking exon 2 (MyD88\(_S\)) is unable to activate the NF-\(\kappa B\) pathway (Janssens et al., 2002) and acts as a dominant-negative regulator of TLR and IL-1R activity (Burns et al., 2003). This alternate isoform is primarily expressed in spleen and weakly in brain, whereas MyD88\(_L\) is broadly expressed in most tissues (Janssens et al., 2002). To ameliorate inflammatory diseases associated with overactive IL-1R signaling, directing MyD88 splicing from MyD88\(_L\) towards MyD88\(_S\) represents a promising therapeutic approach. Screening different ASOs revealed that the RNA target sequence has a significant effect on their activity: those targeted to the exon 2 donor splice site were more effective than ASOs targeted to the acceptor site (Vickers et al., 2006). The 20-mer antisense oligonucleotide ISIS337846 was shown to induce switching to MyD88\(_S\) in both murine and human liver, intestine and adipose cells, but clinical implementation is still distant.
TNF is released by activated immune cells in response to infection. This cytokine can be pathogenic when overactive in autoimmune conditions, where high concentrations can be found in the joints of rheumatoid arthritis (RA) patients (Palladino et al., 2003) or the cerebral spinal fluid of multiple sclerosis patients (Sharief and Hentges, 1991). Drugs that block TNF or signaling at TNF-2 receptors (TNFR2) such as etanercept, infliximab, and adalimumab are currently used to treat these conditions. TNFR2 is membrane bound, and when activated by TNF, leads to downstream gene expression and apoptosis. The gene encoding TNFR2 (TNFRSF1B) is alternatively spliced to exclude exons 7 and 8 (Δ7/8TNFR2), resulting in a protein lacking the transmembrane domain (Lainez et al., 2004). An artificially generated isoform, Δ7TNFR2 (only lacking exon 7) can act as a soluble decoy receptor by binding and inactivating TNFα, thereby antagonizing TNFα signaling (Graziewicz et al., 2008). Splice-switching oligonucleotide (SSO) 3274 acts by blocking the 5' splice site of exon 7 to encourage formation of the Δ7TNFR2 isoform in an attempt to prevent TNFα damage. In a mouse model of collagen-induced RA, SSO3274 treatment delayed disease progression and produced detectable Δ7TNFR2 protein levels in serum. The SSO effects were sequence specific, dose-dependent, and persistent, lasting up to 35 days (Graziewicz et al., 2008). Treatment with SSO3274, also prevented TNFα-induced liver damage in mice, proving more effective than etanercept (Graziewicz et al., 2008).

Interindividual differences in these splicing events can affect anti-TNF therapy. In a clinical trial, RA patients with high levels of soluble protein derived from an alternatively spliced TNFR2 transcript demonstrated prolonged responsiveness and sensitivity to anti-TNF therapy (Canete et al., 2010). Anti-inflammatory SSO treatments may allow for less frequent dosing and avoid immune resistance observed with other currently prescribed anti-TNF medications.

**Summary**

Functionally relevant splicing events impact disease risk or treatment outcomes conditional on internal and external factors. The CACNA1C and UGT1A gene loci exemplify how transcriptional regulation and alternative splicing/RNA processing can generate diversity in protein structure and function, with pharmacological consequences. Differential tissue distribution and functional variations
are hallmarks of this transcript diversity, with relevance to drug design and clinical application. Examples in the brain include DRD2 and OPRM1 spliced isoforms with unique pharmacological properties that should be considered in drug design. Targeting of RNA processing is another therapeutic approach. Small molecules that produce global splicing effects or oligonucleotides that directly block or redirect splicing from one form to another are promising therapies. Preliminary studies have shown efficacy of these molecules in therapy of inflammatory diseases. Targeting specific RNA and protein isoforms has the potential to improve drug efficacy and reduce side effects. Moreover, environmental and genetic contributions affecting the spectrum of transcripts expressed must be considered for achieving optimal therapeutic benefits. Applications in personalized medicine may include measuring a patient’s transcriptome in target tissues, thereby optimizing individual therapies against cancer, immune disorders, and more. With genomics concepts and tools moving into mainstream biomedical sciences, drug design and therapy must reflect not only multiple protein targets, but also multiple protein isoforms at each gene locus.

Authorship Contributions

Participated in research design: n/a
Conducted experiments: n/a
Contributed new reagents or analytic tools: n/a
Performed data analysis: n/a
Wrote or contributed to writing of the manuscript: Barrie, Smith, Sanford and Sadee.
References


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Footnotes

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MOL #76604

Figure 1. Schematics of a protein coding gene locus, showing consensus binding motifs in genomic DNA: transcription factor complexes (e.g., C/EBP); in RNA exons and introns: alternative splicing enhancers/silencers (NOVA-1 & FOX-1); and in untranslated regions (hatched): internal ribosome entry sites (60S/40s), alternative polyadenylation signals (CPSF), and microRNA sites (miR).

Figure 2. A. Splice isoforms of UGT1A1. Alternative splicing of exon 5 at UGT1A gene locus creates protein isoforms capable of modulating UGT1A activity. Usage of exon 5a creates UGT1A1_i1, responsible for enzymatic activity, while usage of exon 5b truncates the C-terminus creating UGT1A1_i2. Multiple alternative exon 1 transcripts with separate promoter regions are not shown. B. Alternative splicing of DRD2 at exon 6 produces distinct protein isoforms D2S (short) and D2L (long) with varying pharmacological profiles.
### Table 1. Biological functions of transcript isoforms and pharmacological implications

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function/Role</th>
<th>Features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CETP</td>
<td>Lipid transport protein</td>
<td>Splicing affected by diet, dominant-negative</td>
<td>Lira et al., 2008; Dessi et al., 1997; Yang et al., 1996</td>
</tr>
<tr>
<td>UGT1A</td>
<td>Drug metabolism</td>
<td>Alternative first exon usage and exon 5 splicing</td>
<td>Gong et al., 2001; Levesque et al., 2007</td>
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<tr>
<td>CACNA1C</td>
<td>Target in hypertension, arrhythmia treatment</td>
<td>Splice variants have different expression patterns and dihydropyridines sensitivity</td>
<td>Tang et al., 2004; Welling et al., 1997; Wang et al., 2006</td>
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<tr>
<td>DRD2</td>
<td>Antipsychotic drug target</td>
<td>Splice forms have different drug sensitivity</td>
<td>Malmberg et al., 1993; Usiello et al., 2000; Xu et al., 2002</td>
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<tr>
<td>OPRM1</td>
<td>Analgesics and narcotics receptor</td>
<td>Alternate exon 1 affects opioid analgesic effects</td>
<td>Pan et al., 2009; Pan et al., 2005a,b; Schuller et al., 1999</td>
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<td>COX-1</td>
<td>NSAID target</td>
<td>3 splice isoforms, relevance in humans</td>
<td>Chandrasekharan et al., 2002; Kis et al., 2005; Qin et al., 2005</td>
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<td>MYD88</td>
<td>Inflammation</td>
<td>Splice form lacking exon 2 (MyD88S) decreases inflammation</td>
<td>Janssens et al., 2002; Vickers et al., 2006; Burns et al., 2003</td>
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<tr>
<td>TNFR</td>
<td>Inflammation</td>
<td>Exon exclusion produces soluble TNF receptor</td>
<td>Graziewicz et al., 2008</td>
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<td>NAT1</td>
<td>Drug detoxification</td>
<td>Isoforms with different translation efficiencies</td>
<td>Boukouvala and Sim, 2005; Butcher et al., 2005; Wang et al., 2011</td>
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<tr>
<td>CYP2D6</td>
<td>Drug metabolism</td>
<td>*4 allele alters splicing, no enzyme activity</td>
<td>Kagimoto et al., 1990; Marez et al., 1997</td>
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<tr>
<td>SCN1A</td>
<td>Drug target in epilepsy treatment</td>
<td>Splice isoforms have different sensitivity to phenytoin and lamotrigine</td>
<td>Thompson et al., 2011</td>
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<tr>
<td>CAR</td>
<td>Nuclear receptor, xenobiotic</td>
<td>Alternate splicing alters ligands recognized by receptor</td>
<td>DeKeyser et al., 2011</td>
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<td>Gene</td>
<td>Disease from splicing defect</td>
<td>Mutation/Correction</td>
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<td><strong>VEGF</strong></td>
<td>Growth factor</td>
<td>Splice isoforms can have opposite effect</td>
<td>Harper and Bates, 2008</td>
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<td><strong>BCL2L</strong></td>
<td>Apoptosis regulator</td>
<td>Splice isoforms are anti- or pro-apoptotic</td>
<td>Bauman et al., 2010</td>
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<td><strong>Gene</strong></td>
<td><strong>Gene</strong></td>
<td><strong>Gene</strong></td>
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<td><strong>MSTR1</strong></td>
<td>Oncogene</td>
<td>SSO can increase transcript length</td>
<td>Ghigna et al., 2010</td>
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<td><strong>SMN</strong></td>
<td>Spinal muscular atrophy</td>
<td>Exon 7 skipped. Multiple approaches for inclusion</td>
<td>Hua et al., 2010; Singh et al., 2009; Vitte et al., 2007; Wirth et al., 2006</td>
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<td><strong>DMD</strong></td>
<td>Duchenne muscular dystrophy</td>
<td>Exon 51 mutation and frameshift</td>
<td>Cirak et al., 2011; Goemans et al., 2011; van Deutekom et al., 2007</td>
</tr>
<tr>
<td><strong>IKBKAP</strong></td>
<td>Familial dysautonomia</td>
<td>Phosphatidylserine and kinetin correct splicing defect</td>
<td>Axelrod et al., 2011; Keren et al., 2010; Shetty et al., 2011</td>
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<tr>
<td><strong>NF-1</strong></td>
<td>Neurofibromatosis</td>
<td>Kinetin corrects splicing defect</td>
<td>Pros et al., 2010</td>
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