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MINIREVIEW

mRNA Transcript Diversity Creates New Opportunities for Pharmacological Intervention

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ABSTRACT

Most protein coding genes generate multiple RNA transcripts through alternative splicing, variable 3′ and 5′UTRs, and RNA editing. Although 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. In addition, 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, 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 to 25,000 protein-coding genes, but the repertoire of mRNA sequences and encoded proteins is far greater as a result of 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 in the 5′ UTR or degradation signals and microRNA (miR) binding sites in the 3′UTR (Fig. 1). All RNA processing events follow a tissue-specific expression pattern that varies less across individuals than among 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,

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ABBREVIATIONS: Δ9, exon 9-lacking; ASO, antisense oligonucleotide; CACNA1C, voltage dependent L-type calcium channel α-subunit 1c; CETP, cholesterylester transfer protein; CNS, central nervous system; COX, cyclooxygenase; D2L, dopamine D2 receptor long form; D2S, dopamine D2 receptor short form; DMD, Duchenne muscular dystrophy; DRD2, dopamine D2 receptor; FD, familial dysautonomia; HDL, high-density lipoprotein; IL, interleukin; IL-1R, interleukin-1 receptor; miR, microRNA; MOR, μ opioid receptor; MyD88, myeloid differentiation primary response; NAT1, *N*-acetyltransferase 1; NF-κB, nuclear factor κ-light-chain enhancer of activated B cells; NSAID, nonsteroidal anti-inflammatory drug; OPRM1, μ opioid receptor 1; RA, rheumatoid arthritis; SCN1A, neuronal Nav1.1 sodium channel; SMA, spinal muscular atrophy; SMN, survival of motor neuron; snRNA, small nuclear RNA; SSO, splice-switching oligonucleotide; TNF, tumor necrosis factor; UGT, UDP-glucuronosyltransferase; UTR, untranslated region; VEGF, vascular endothelial growth factor.

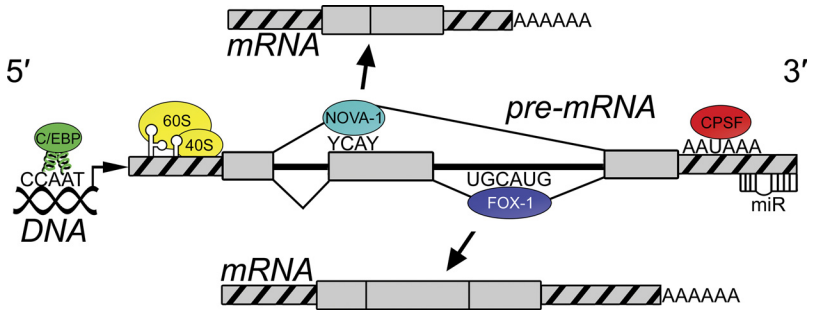

Fig. 1. Schematics of a protein coding gene locus, showing consensus binding motifs in genomic DNA: transcription factor complexes (e.g., C/EBP); in RNA exons and introns: alternative splicing enhancers/silencers (NOVA-1 and FOX-1); and in untranslated regions (hatched): internal ribosome entry sites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

and is present at different levels, depending on the brain region studied. Although 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 multiexon 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), 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 multicomponent 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 nonfunctional protein or nonsense-mediated RNA decay, if a codon phase shift introduces premature termination signals (Lareau et al., 2007; Wang and Cooper, 2007; Cooper et al., 2009). 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 affected 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 D3 (Elmhurst et al., 2000; Karpa et al., 2000) act in a dominant-negative manner; the alternatively spliced transcript prevents the function of the normal transcript. CETP transfers cholesterol esters from HDL to low-density lipoprotein, thus decreasing the ratio of HDL to overall cholesterol. In an effort to protect against coronary artery disease by boosting HDL levels at the expense of low-density lipoprotein, 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 coronary artery disease 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.

Although 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 nonfunctional 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, pharmacological and toxicological implications resulting from drug or toxin acetylation. Another example of pharmacological significance, the nuclear receptors constitutive androstane receptor and pregnane X receptor, 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 differen-

tial selectivity for constitutive androstane receptor 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 (Wang and Cooper, 2007; Cooper et al., 2009). For example, mutations in genes encoding auxiliary splicing factors such as methyl CpG binding protein 2 and RNA binding protein fox-1 homolog (*Caenorhabditis elegans*) 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, 4000 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 UDP-glucuronosyltransferase (UGT) 1A. In addition, 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 more than 10,000 possible splice variants (Welling et al., 1997; Tang et al., 2004). 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,

TABLE 1
Biological functions of transcript isoforms and pharmacological implications

Gene	Function/Role	Features	References
CETP	Lipid transport protein	Splicing affected by diet, dominant-negative	Yang et al., 1996; Dessi et al., 1997; Lira et al., 2008
UGT1A	Drug metabolism	Alternative first exon usage and exon 5 splicing	Levesque et al., 2007; Gong et al., 2001
CACNA1C	Target in hypertension, arrhythmia treatment	Splice variants have different expression patterns and dihydropyridines sensitivity	Welling et al., 1997Tang et al., 2004; ; Wang et al., 2006
DRD2	Antipsychotic drug target	Splice forms have different drug sensitivity	Malmberg et al., 1993; Usiello et al., 2000; Xu et al., 2002
OPRM1	Analgesics and narcotics receptor	Alternate exon 1 affects opioid analgesic effects	Schuller et al., 1999; Pan et al., 2005a,b, 2009
COX-1	NSAID target	Three splice isoforms, relevance in humans	Chandrasekharan et al., 2002; Kis et al., 2005; Qin et al., 2005
MYD88	Inflammation	Splice form lacking exon 2 ($MyD88_S$) decreases inflammation	Janssens et al., 2002; Burns et al., 2003; Vickers et al., 2006
TNFR	Inflammation	Exon exclusion produces soluble TNF receptor	Graziewicz et al., 2008
NAT1	Drug detoxification	Isoforms with different translation efficiencies	Boukouvala and Sim, 2005; Butcher et al., 2005; Wang et al., 2011
CYP2D6	Drug metabolism	**4* allele alters splicing, no enzyme activity	Marez et al., 1997; Kagimoto et al., 1990
SCN1A	Drug target in epilepsy treatment	Splice isoforms have different sensitivity to phenytoin and lamotrigine	Thompson et al., 2011
CAR	Nuclear receptor, xenobiotic sensing/processing	Alternate splicing alters ligands recognized by receptor	DeKeyser et al., 2011
VEGF	Growth factor	Splice isoforms can have opposite effect	Harper and Bates, 2008
BCL2L	Apoptosis regulator	Splice isoforms are anti- or pro-apoptotic	Bauman et al., 2010
Disease-causing genes			
MSTR1	Oncogene	SSO can increase transcript length	Ghigna et al., 2010
SMN	Spinal muscular atrophy	Exon 7 skipped. Multiple approaches for inclusion	Wirth et al., 2006; Vitte et al., 2007; Singh et al., 2009; Hua et al., 2010
DMD	Duchenne muscular dystrophy	Exon 51 mutation and frameshift	van Deutekom et al., 2007, Goemans et al., 2011; Cirak et al., 2011
IKBKAP	Familial dysautonomia	Phosphatidylserine and kinetin correct splicing defect	Keren et al., 2010; Axelrod et al., 2011; Shetty et al., 2011
NF-1	Neurofibromatosis	Kinetin corrects splicing defect	Pros et al., 2010

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 recipients of heart transplants (Wang et al., 2006), demonstrating dramatic differences in the expression pattern of exon 8/8a among 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 persons 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 that *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

UGTs 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 that have various degrees of 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 (Lévesque et al., 2007) yields two splice variants, the active enzyme *UGT1A1_i1* and a truncated isoform *UGT1A1_i2*, illustrated in Fig. 2A. Upon heterodimerization, UGT1A_i2 inhibits UGT1A_i1-mediated glucuronidation, another example in which a splice isoform exerts dominant-negative effects (Lévesque et al., 2007). Alternative splicing of exon 5 extends through all active *UGT1A* genes (*UGT1A1*, *1A3-10*) 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. Nonsynonymous 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 affect 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 (Modrek et al., 2001; Yeo et al., 2004; de la Grange et al., 2010), 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 effi-

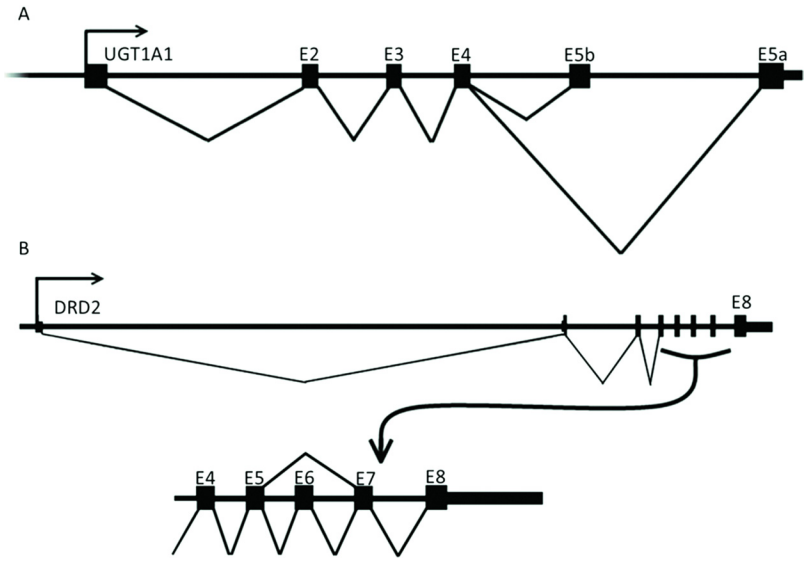

Fig. 2. A, splice isoforms of UGT1A1. Alternative splicing of exon 5 at UGT1A gene locus creates protein isoforms capable of modulating UGT1A activity. Usage of exon 5a creates *UGT1A1_i1*, responsible for enzymatic activity, whereas usage of exon 5b truncates the C terminus, creating *UGT1A1_i2*. Multiple 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.

ciency (Kozak, 1989; Gray and Hentze, 1994). 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 in which 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. 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 α 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 antiepileptic medications (Thompson et al., 2011). These drugs interact differently with spliced targets, thus affecting their efficacy. Epileptic patients with single-nucleotide polymorphism 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 μ Opioid Receptor. The μ 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), whereas 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, but does not affect 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 morphine-6β-glucuronide retain residual analgesic properties, whereas 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-terminal variants (Pan et al., 2005a). C-terminal 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 δ opioid receptors are dependent upon carboxyl tail length (Fan et al., 2005). Although GPCRs seem to dimerize and polymerize readily, the promiscuity of G protein-coupled receptor heteropolymerization is still under debate, as is its role in pharmacology and drug design.

The Dopamine D2 Receptor. The D2 receptor serves as a primary target for antipsychotic compounds, with a finite window of D2 receptor occupancy linked to clinical efficacy (Nordström et al., 1993; Kapur et al., 2000) 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) (Fig. 2B) that differ in their subcellular localization (Khan et al., 1998). D2S predominantly 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 single-nucleotide polymorphisms in introns 5 and 6 that significantly shift splicing toward inclusion of exon 6, thereby reducing autoreceptor activity of D2S in the striatum and prefrontal cortex, significantly affecting cognition, neural activity (Zhang et al., 2007; Bertolino et al., 2009, 2010; Blasi et al., 2009) and risk of cocaine abuse/overdose (Moyer et al., 2011).

Differential pharmacological modulation of D2S versus D2L signaling offers an opportunity for correcting imbalanced *DRD2* 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. (2002) indirectly demonstrated higher D2L affinity compared with D2S for haloperidol and clozapine, whereas they observed the opposite for raclopride. Malmberg et al. (1993) 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. However, treatment success may depend on such subtype selectivity, an area in need of further exploration.

Nonsteroidal Anti-Inflammatory Drugs. 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 after 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-$1b_1$, encodes a truncated and nonfunctional protein. COX-$1b_2$, the isoform most similar to COX-3, did not differ in its response to NSAIDs compared with 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 down-regulate 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 (Sierakowska et al., 1996; Schmajuk et al., 1999). 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., 1998, 2000). Likewise, 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 (Pinotti et al., 2008; Geib and Hertel, 2009; Hartmann et al., 2010; Incitti et al., 2010). 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. RNA interference therapies employ small interfering RNA duplexes of 21 to 23 nucleotides. By incorporation into the RNA-induced silencing complex, 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 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 particles coated with heavy metal, 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.

Small Molecules to Treat Familial Dysautonomia. Compounds consumed in our daily diet can affect splicing, as observed with Δ9-CETP up-regulation in response to dietary components (Yang et al., 1996; Dessí et al., 1997). 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 *IKBKAP* gene, excluding exon 20. Treatment with phosphatidylserine, an FDA-approved supplement, increases the wild-type form of 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 *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 *IKBKAP* mRNA (Axelrod et al., 2011). Kinetin's effect is not specific for the *IKBAP* gene; it also modulates and partially corrects aberrant splicing of neurofibromatosis type 1 (*NF-1*) (Pros et al., 2010). The use of amiloride in the treatment of a neuromuscular disorder and cancer to modulate splicing events is further discussed below. The number of candidate drugs in this category is still small, but they hold 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, because 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 small nuclear ribonucleoprotein 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 a patient with SMA, an 8-mer ASO restored exon 7 inclusion and mature SMA formation, a first step toward effective therapy (Singh et al., 2009). Although 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 (Williams et al., 2009; Hua et al., 2010).

Alternative therapies have also shown promise for modifying *SMN* splicing. The antihypertensive drug amiloride and its analog 5-(*N*-ethyl-*N*-isopropyl)amiloride 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., 2012). A number of histone deacetylase inhibitors increase the lifespan of SMA mice (Wirth et al., 2006). Modulating extracellular pH also modifies splicing of *SMN2*; high

pH increases exon 7 inclusion and 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 that 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 after 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, nonfunctional protein. Two oligonucleotide agents under investigation are 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, because 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 after either intramuscular injection (van Deutekom et al., 2007) or systemic administration (Goemans et al., 2011). Cirak et al. (2011) demonstrated the safety and efficacy of AVI-4658, a phosphorodiamidate morpholino oligomer, with increased dystrophin protein expression. 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. Antiangiogenesis compounds that reduce the activity of vascular endothelial growth factor (VEGF) or its receptors can be effective at slowing tumor growth (Hurwitz et al., 2004). However, alternative splicing of *VEGF* and *VEGF* receptors can result in transcripts with effects opposite those traditionally attributed to VEGF signaling, inhibiting angiogenesis and slowing the growth of a wide range of cancers (Woolard et al., 2004; Varey et al., 2008). As a consequence, nonspecifically targeting VEGF signaling potentially promotes angiogenesis or cell survival; therefore, anti-VEGF drugs should target-specific spliced isoforms (for review, see Harper and Bates, 2008) or *trans*-acting splice proteins that favor expression of antiangiogenic 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 patients with melanoma, 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 to 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: 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, because these proteins direct alternative splicing of many genes and can lead to nonspecific effects. Natural compounds with antitumor 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). Although 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., 2010 demonstrated successful reduction in tumor growth through lipid nanoparticle delivery of an SSO that increases splicing of the antiapoptotic B-cell lymphoma 2-like 1_L transcript toward formation of the shorter proapoptotic B-cell lymphoma 2-like 1_S isoform. SSOs can also be used to produce longer alternatively spliced transcripts through the inclusion of alternative exons, as demonstrated for the protooncogene Ron (*MSTR1*, macrophage stimulating 1 receptor) (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 receptors (Akira et al., 2001) and interleukin-1 receptor (IL-1R) family members (Mitcham et

al., 1996). By activating the NF-κB 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-κB pathway (Janssens et al., 2002) and acts as a dominant-negative regulator of toll-like receptor and IL-1R activity (Burns et al., 2003). This alternate isoform is expressed primarily 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 $MyD88_L$ toward $MyD88_S$ 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). A 20-mer antisense oligonucleotide 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 patients with rheumatoid arthritis (RA) (Palladino et al., 2003) or the cerebrospinal fluid of patients with multiple sclerosis (Sharief and Hentges, 1991). Drugs that block TNF or signaling at TNFR2 receptors, 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 (lacking only exon 7), can act as a soluble decoy receptor by binding and inactivating TNFα, thereby antagonizing TNFα signaling (Graziewicz et al., 2008). 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, SSO 3274 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 SSO 3274, 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, patients with RA who have high levels of soluble protein derived from an alternatively spliced TNFR2 transcript demonstrated prolonged responsiveness and sensitivity to anti-TNF therapy (Cañete et al., 2011). 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 affect 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

Wrote or contributed to the writing of the manuscript: Barrie, Smith, Sanford and Sadee.

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