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Antisense Modulation of RNA Processing as a Therapeutic Approach in Cancer Therapy

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Abstract

Next-generation antisense technologies are re-emerging as viable and powerful approaches to the treatment of several genetic diseases. Similar strategies are also being applied to cancer therapy. Re-programming of the expression of endogenous oncogenic products to replace them with functional antagonists, by interfering with alternative splicing or polyadenylation, provides a promising novel approach to address acquired drug resistance and previously undruggable targets.

Introduction

Cancer develops when a series of genetic changes allows a cell to break free from the normal regulatory pathways governing cell division and proliferation. Its progression is characterized by the appearance of common steps, which include sustained proliferative signaling, evasion of growth suppressors, resistance to apoptosis, activation of replicative immortality, angiogenesis, evasion from host immune response, reprogramming of metabolic pathways, genomic instability, tumor-promoting inflammation, invasion and metastasis [1, 2]. These “hallmarks of cancer” are typically driven by genetic alterations that involve gain-of-function mutations and/or over-expression of oncogenes on one hand, and loss-of-function mutations and/or silencing of tumor suppressors on the other.

Cancer therapy has traditionally been based upon the combination of chemotherapy and radiation. In recent years, the molecular characterization of cancer through genomic, transcriptome and proteomic technologies has led to the development of “targeted therapies” that specifically hit pathways essential for cancer growth and progression [3, 4]. These treatments, usually in the form of small molecule inhibitors or antibodies, have revolutionized cancer therapy and dramatically impacted survival rates [5]. However, because of the consistent emergence of resistance to conventional and targeted therapies in

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tumor cells, and due to the “undruggability” of many promising putative therapeutic targets, there is a persisting requirement for novel alternative drug discovery strategies.

Recent advances in nucleic acid antisense-based technology, comprising improved chemistry, pharmacology and delivery, have led to the development of a new generation of antisense oligonucleotides compounds (ASOs) with greatly improved pharmacokinetic and pharmacodynamic properties [6]. ASOs are emerging as a powerful class of drugs that can be generated against virtually any RNA-coding or non-coding- and their potential thus surpasses traditional drug discovery based on small molecule inhibitors or monoclonal antibodies. These features have led to the FDA approval of the first antisense-based drug in two decades (Mipomersen [7]), with many more currently in clinical trial for the treatment of numerous diseases [8, 9], including several for cancer (Table 1).

“Antisense technology” is a broad umbrella term to indicate any approach that involves base-pairing of a compound to a target nucleic acid. It can involve diverse mechanisms of action, ranging from multiple ways to promote target RNA degradation (for example by RNAi), to translation interference, to modulation of non-coding RNA activities and to various pre-mRNA re-programming strategies (Box 1). In this review, we will focus on the use of synthetic ASOs to force specific changes in endogenous alternative splicing (AS) and alternative polyadenylation (APA) events and their application as a therapeutic avenue for cancer therapy.

Antisense oligonucleotides: Knock-down vs. re-programming

In the discussion of antisense-based compounds, it is important to distinguish whether the primary goal of the treatment is to down-regulate the target gene or to alter its coding potential.

Target knock-down can be achieved by various mechanisms. The most commonly used one exploits the RNA interference (RNAi) pathway, which is activated by small interfering RNAs (siRNAs), or short hairpin RNA (shRNAs). These double-stranded RNAs are loaded into the RNA-Induced Silencing Complex (RISC) and repress expression by base-pairing with the target mRNAs and directing their cleavage and degradation [10]. SiRNAs and shRNAs have been utilized *in vivo* with some success, and several RNAi-based compounds are in clinical development [9, 11]. However, their current therapeutic appeal is still somewhat hindered by the requirement for complex formulations and unsatisfactory delivery *in vivo*, particularly to tumors.

While the lion's share of antisense applications (especially *in vitro*) is currently held by RNAi technologies, the use of ASOs to inhibit gene expression was pioneered in 1978 by Zamecnik and Stephenson-long before the discovery of RNAi- when ASOs complementary to a Rous sarcoma virus RNA target sequence were shown to repress viral replication [12]. Since then, multiple classes of ASOs have been developed, and while they share the defining targeting by complementary Watson-Crick base-pairing and an average length of ~20 nucleotides, they involve very different chemistries which exploit multiple underlying biological mechanisms [13] (Box 1).

“Classical” ASOs down-regulate target RNA by recruiting ribonuclease H to the ASO:RNA duplex, resulting in RNaseH-mediated cleavage of the RNA moiety. They typically carry phosphorothioate (PS) backbones, the most widely used chemistry of the initial antisense technologies, and the basis for *Fomivirsen*, the first and-until the approval of *Mipomersen*-the only FDA-approved ASO drug [14]. Additional modifications coupled and/or alternative to PS have resulted in second and third generation compounds with reduced toxicity, enhanced stability *in vivo*, better tissue distribution and higher targeting efficiency [6, 13]. These modifications include substitutions of the ribose groups with 2'-O-Alkyl groups, (2'-O-methyl [2'-OME] or 2'-O-methoxyethyl [2'-MOE]), and 2'Fluoro (2'F) or Lock Nucleic Acid (LNA) modifications, which can reduce or prevent the recruitment of RNase H. Therefore, when the desirable outcome is the knock-down of the target gene, they are typically used in a chimeric “gapmer” context as protective wings at the sides of a core region utilizing simple DNA or other RNase H-activating chemistries [6, 13] (Box 1). Other common non RNaseH-activating modifications include replacing the sugar backbone with morpholino rings (phosphorodiamidate morpholino [PMO] oligonucleotides, or with peptoid-like chemistry (peptide nucleic acid [PNA] oligonucleotides) (reviewed in Kole et al., 2012).

Importantly, the same modifications that do not trigger RNA degradation also allow for their usage in applications where the primary goal is not knock-down, but rather the re-programming of target pre-mRNAs to express alternative, more desirable variants by AS or APA.

Alternative splicing and polyadenylation: One gene, many proteins

Processing of pre-mRNAs to translation-competent mRNAs is a complex and tightly regulated multi-step process that results in the accurate removal of intronic sequences by splicing and the addition of a 5' cap and a 3' polyadenylation sequence [15].

The choice and usage of splice sites in the pre-mRNA can be differentially selected by AS, depending on the developmental state, tissue and cell type or in response to a variety of physiological stimuli or pathological conditions [16]. AS occurs in more than 90% of multi-exonic pre-mRNA transcripts and provides much of the proteomic diversity observed in mammalian cells [17]. AS can also down-regulate expression through the introduction of premature termination codons (PTCs) by inducing frame shifts in the open reading frame. PTCs trigger the nonsense mediated decay (NMD) pathway thus marking a transcript for degradation [18].

Polyadenylation is a co-transcriptional nuclear process essential for efficient nuclear export, stability and translation of mRNAs [19, 20]. The process involves the endonucleolytic cleavage of the pre-mRNA, followed by the addition of a polyA tail. The specific cleavage location is controlled by cis-acting motifs, including a hexamer polyadenylation signal (PAS) and a downstream UG-rich element that aids in the recruitment of the polyadenylation machinery [20].

Like AS, APA is prevalent and affects over 50% of human genes. APA is commonly assumed to occur ‘in tandem’ within a given UTR, but recent analysis has shown that

alternative PAS are also frequently present in 'upstream' intronic regions [21, 22]. While tandem APA contributes mainly to the regulation of the *levels* of expression of one encoded product, intronic polyadenylation (IPA) also adds to the *diversity* of protein isoforms generated from a single gene. For example, tandem APA motifs can affect gene expression levels or transcript stability by removing/adding regulatory sequences, such as micro-RNA binding sites and/or AU-rich elements (AREs) [23]. On the other hand, the usage of IPA motifs will generate truncated mRNAs that could produce protein isoforms with potentially vastly different functions than their full-length counterparts, including dominant-negative characteristics.

Collectively AS and IPA are key molecular mechanisms for increasing the functional diversity of the human proteome, allowing the relatively small human genome (<25,000 genes) to generate an excess of 100,000 different protein isoforms [17].

Alternative Splicing and Polyadenylation in Cancer

The alternative splicing pattern of individual pre-mRNAs is a complex process primarily controlled by the intrinsic strength of the splice sites, by the combinational effects of competitive *cis*-regulatory elements within the pre-mRNA (intronic or exonic enhancers or silencers) and by the relative abundance of the trans-acting factors that recognize them (such as SR and hnRNP proteins) [24, 25].

Given AS complexity and its pivotal role in the control of gene expression and functions, it is not surprising that many human genetic diseases are a direct consequence of aberrant splicing events. Indeed, approximately 50% of disease-associated point mutations directly affect splicing [15, 26]. These typically result from mutations that impair specific splice sites or other regulatory *cis*-acting elements, or that activate cryptic sites, as it can occur, for example, in β -Thalassaemia [27].

A growing body of evidence suggests that deregulated AS is also extensively linked to the development of cancer. Cancer cells show aberrant splicing profiles, expressing isoforms that can stimulate cell proliferation, enhance survival, promote migration and invasion, influence metabolism and in general affect any aspect of tumor progression and maintenance [28]. As in genetic diseases, this can be sometimes gained through acquisition of mutations in *cis*-acting AS elements, like for example with splice-site mutations identified in the BRAC1 and APC tumor suppressor transcripts [29, 30]. More commonly however, the pathological splicing patterns in tumors are due to broader changes in the expression, activity and possibly specificity of regulatory trans-acting RNA binding proteins which might in part reflect regulated AS programs that are improperly activated [28, 31] or to mutations in splicing factor or core spliceosomal components [32].

Indeed, many genes that play a key role in tumor development, invasiveness or chemo-resistance express multiple splicing isoforms, with very different and often antagonistic functions. The idea of a more direct role of splicing in tumorigenesis is also supported by the observation of oncogene-like behaviour for some splicing factors, which can act as very early markers of cancer (e.g.: hnRNP A2/B21 in lung tumors [33]), can carry frequent activating mutations (e.g.: SRSF2, SF3b1 and others in Myelodysplasia [32, 34]) or, like

SRSF1, can directly behave as powerful single-agent oncogenes in mouse models and are found to be amplified in some tumors [35, 36]. Similarly, the splicing factor hnRNP-H is up-regulated and contributes to the pathogenesis and progression of glioblastoma multiforme (GBM) by inducing oncogenic alternative splicing isoforms of Receptor d'origine nantais (RON) and Insuloma-glucagonoma protein 20 (IG20) which promote migration and survival, respectively, a switch that might reflect the re-activation of a stem-like gene expression program in the GBM cells [37].

Although not as well characterized as AS, recent genome-wide analysis of APA also points to its de-regulation as being linked to tumorigenesis by increasing cellular proliferation and transformation. Cancer cells preferentially express oncogenes with shorter 3'UTRs, resulting in higher expression due to the loss of destabilizing and/or repressive elements from the UTR [38, 39]. Alternatively, the modulation of upstream IPA sites can generate truncated variants with tumorigenic capabilities. For example, the usage of an IPA site in Cyclin D1, which normally controls progression through the cell cycle, results in a constitutively active truncated Cyclin D1 isoform (Cyclin D1) that can lead to cellular transformation [40, 41].

Therefore, deregulation of AS and APA/IPA can contribute to tumorigenesis due to inactivation of tumor suppressors, activation of oncogenes and generation of novel pathogenic cancer-specific isoforms.

Splice-switching oligonucleotides

Since aberrant AS and APA contribute to a multitude of different human diseases, correction of such defective pre-mRNA processing by ASOs could open an avenue for therapeutic intervention. The feasibility of this approach has been elegantly demonstrated in the development of ASO compounds for the treatment of β -Thalassaemia [42] Spinal Muscular Atrophy (SMA) [43, 44], and Duchenne Muscular Dystrophy (DMD) [45]. Systemic delivery of splice-switching oligonucleotides (SSOs) have proven to be highly effective in pre-clinical mouse models of these genetic disorders, where a shift in endogenous splicing has been induced to correct an aberrant splicing pattern or induce expression of therapeutic splice variants, with significant physiological responses [42–44]. Furthermore, exciting early results from clinical trials of compounds directed at the treatment of DMD have shown benefit in patients, indicating that the technology is finally maturing for clinical applications [46, 47].

The principle of inducing therapeutic variants whilst reducing deleterious ones can also be applied as a cancer therapy approach, especially since many key 'cancer genes' can generate antagonistic isoforms, which often have dominant-negative characteristics. In other words, the dual role of these proteins can be exploited by splicing re-direction approaches to manipulate their expression, in order to simultaneously eliminate the deleterious isoforms and substitute them with therapeutically desirable ones. This would be particularly effective when the targets are essential to survival of cancer, as in the case of "oncogene addiction", where cancers remain dependent upon a primary oncogenic driver activity for their continued survival and malignant progression [48].

Here we describe some examples using this rationale to modulate AS and IPA and redirect splicing to generate antagonist or therapeutic mRNA variants that function as potential novel cancer therapies.

The anti-Warburg effect: Switching off PK-M2

Cancer cells exhibit altered metabolic pathways, to derive energy to sustain their aberrant physiology. Normally, differentiated and non-proliferating cells generate the bulk of their energy requirements from mitochondrial oxidative phosphorylation. However, in cancer cells, energy is mainly generated through aerobic glycolysis, converting glucose to lactate, in a phenomenon known as *the Warburg effect* [49].

To achieve this metabolic switch, cancer cells reprogram their energy metabolism pathways by multiple mechanisms, including by changing the splicing pattern of pyruvate kinase M (PK-M). PK-M functions as the rate limiting final step in the glycolysis pathway, converting phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP [50]. AS of the PK-M gene generates two isoforms, PK-M1 and PK-M2, through the mutually exclusive usage of Exon 9 and Exon 10, respectively [51]. PK-M1 is found principally in differentiated cells, while PK-M2 is expressed in normal proliferating tissues (e.g. during embryonic development), but it is also expressed in tumor cells [52]. The regulatory role of PK-M2 in metabolism is complex, but its general role is to alter cellular glucose metabolism, increasing the availability of glycolytic metabolites that are then utilized to support proliferation and growth. In tumors, these features are essential for cancer survival and progression [52].

The splicing regulation of the PK-M1/M2 switch involves both exonic splicing silencers (ESSs) in exon 9 and exonic splicing enhancers (ESEs) in exon 10, in conjunction with trans-acting RNA binding proteins SRSF3, PTB/nPTB, and hnRNPA1/A2, partially controlled by oncoprotein MYC [53–55]. In combination, they repress exon 9 inclusion and promote expression of the PK-M2 splice variant. 2'-MOE ASOs directed against an identified exonic splicing enhancer in exon 10 of the PK-M2 pre-mRNA were shown to inhibit exon 10 usage and re-activate PK-M1 expression by promoting exon 9 inclusion (Figure 1a). This resulted in down-regulation of PK-M2 protein expression, with a concurrent increase of the PK-M1 isoform in a panel of glioblastoma cell lines. The switch was associated with reduced viability and increased apoptosis, thus validating this approach [55]. Although further *in vivo* work is required, the PK-M2 to PK-M1 switchback provides a therapeutic strategy to specifically target cancer cells based on their unique metabolic signature.

From survival to apoptosis: Switching between Bcl-XL and Bcl-XS

The ability of cancer cells to escape apoptotic programmed cell death is also a key aspect of the oncogenic process and of the appearance of resistance to drugs in tumors [2]. Central to the activation of the apoptotic program are the Bcl2 family of survival proteins, which includes Bcl-X [56]. Alternative 5'ss splicing in Bcl-X exon 2 generates two isoforms with opposite functions: Bcl-XL, a well-characterized survival protein and the smaller Bcl-XS, a pro-apoptotic variant [57].

Bcl-XL is up-regulated in a broad range of cancers and its over-expression is strongly associated with resistance to chemotherapy and poor clinical outcome. While Bcl-XL inhibits apoptosis through neutralizing hetero-dimerization with pro-apoptotic proteins Bak and Bax [58], its alternative isoform, Bcl-XS induces apoptosis by directly inhibiting Bcl-XL and Bcl-2, and its levels are low in cancer cells despite the high expression of the Bcl-X gene [59]. The Bcl-XL/XS splicing regulation has been extensively investigated, and multiple controlling *cis*-elements and their cognate *trans*-acting splicing factors have been identified [60, 61]. The long recognized antagonistic functions of Bcl-XL and Bcl-XS and their central role in controlling survival of cancer cells have made them a prime target of splicing re-programming approaches.

Several studies have employed various ASO strategies to modulate the endogenous Bcl-X AS, to effectively activate the upstream Bcl-XS 5'ss at the expense of the downstream Bcl-XL one in exon (Figure 1b). This switch in Bcl-X pre-mRNA processing induces apoptosis and increased *in vitro* chemosensitivity in lung, prostate and breast cancer cells [62–64]. Furthermore, systemic delivery of such ASOs demonstrates that switching of Bcl-X pre-mRNA can be obtained *in vivo*, where it reduces tumor load and metastases in a mouse model of metastatic melanoma [64].

STAT3: the alpha to beta anti-tumorigenic switch

Signal transducer and activator of transcription 3 (STAT3) is a member of the STAT family of transcription factors, which are key down-stream effectors of gene expression following cytokine, hormone or growth factor stimulation [65]. STAT3 is persistently activated in a broad range of tumors, where it promotes growth and survival of tumor cells, induces tumor angiogenesis and suppresses anti-tumor immune responses [66]. Abnormal cytokine or growth factor signaling through the Janus kinases (JAK) and receptor tyrosine kinases (RTKs) such as EGFR, led to its aberrant hyper-phosphorylation, and its enhanced activation is often associated with acquired therapeutic resistance to chemotherapy and radiation therapy. Furthermore, STAT3 promotes resistance to targeted EGFR therapies in non-small cell lung carcinoma (NSCLC) and head and neck squamous cell carcinoma (HNSCC) [67, 68]. Because of this position at the center of many converging signaling and oncogenic pathways, STAT3 has become an attractive target for cancer therapy. Several strategies have been developed to target the STAT3 pathway *in vivo*, including small drug inhibitors, peptide analogues, decoy binding-site oligonucleotides and over-expression of STAT3 β , an alternative isoform of STAT3 that acts as a dominant negative/antagonist variant of STAT3 [69, 70].

The truncated STAT3 β isoform is generated from usage of an alternative 3'ss of exon 23, 50 nucleotides downstream of the normal 3'ss [71]. The resulting stop codon, causes STAT3 β to lack 55 residues of the C-terminal transactivation domain (TAD). STAT3 β can still be phosphorylated at the Tyr705 position, essential for dimerization and nuclear translocation and thus it retains the ability to bind target nuclear DNA sequences as a homo-dimer or hetero-dimer (with full length STAT3 β , or other transcription factors). As a consequence, STAT3 β can act as a dominant negative regulator as well as mediate additional distinctive features that add to the complex STAT3 signaling cascade [70, 72, 73].

Phosphorodiamidate morpholinos (PMOs) targeting an identified ESE in Exon 23 of STAT3 can quantitatively switch the endogenous mRNA from the alpha to the beta form in multiple cell types (Figure 1c) [73]. *In vitro*, antisense modulation of STAT3 β / β ratios results in a reduction in cell viability and an increase in apoptosis, higher than those observed in a parallel total STAT3 knock-down experiment [73]. The enhanced biological effect of the alpha to beta switch compared to the knock-down appears to be due to the activation of a unique STAT3 β -specific regulation program with anti-tumorigenic characteristics, rather than a direct block of canonical STAT3 targets. Conjugation of the STAT3 β -inducing PMOs to a cell-penetrating dendrimer (vivo-morpholinos [74]), allows redirection of STAT3 splicing *in vivo* and concomitant tumor regression in a breast cancer xenograft model [73]. Remarkably, under comparable experimental conditions, effective knockdown of STAT3 has no significant effect on tumor growth, underscoring the qualitative difference in re-programming versus knock-down approaches and the vast, untapped potential of splicing redirection in cancer therapy.

Activation of intronic polyadenylation (IPA): Induction of secreted decoy RTKs

Activation of the RTK signaling pathways represents a key aspect of tumorigenesis in a broad range of human cancers [75, 76]. The targeting of oncogenic RTKs provides a classic example of molecular targeted therapeutic approach in modern cancer therapy. Effective targeted therapies to RTKs include small molecule tyrosine kinase inhibitors and monoclonal antibodies [77]. However, resistance invariably emerges over time, and secondary lines of treatment need to be developed for relapsing patients.

An alternative approach to target RTK receptor pathways has been the delivery of recombinant soluble, dominant-negative RTKs variants (secreted decoy RTKs, sdRTKs) comprising the sole ligand-binding, extracellular domain (ECD), by either direct intravenous administration or using gene-therapy viral expression vectors. SdRTKs antagonize RTK signaling by binding and sequestering the activating ligand and/or by engaging the endogenous receptor in non-productive dimers (Figure 2). An example of this approach is Aflibercept, a VEGF trap that suppresses tumor growth and vascularization *in vivo* and is currently in Phase III clinical trials [78].

A recent study demonstrated the existence of tens of natural endogenous sdRTKs, which are expressed from most RTK genes, where they are generated by the activation of IPA sites in introns upstream of the transmembrane domain-encoding exons [79]. The controlled and specific induction of such sdRTKs can be obtained by ASO targeting the 5' splice site immediately upstream of an IPA signal [79]. Normally, directing an ASOs to the 5'ss would interfere with splicing, typically resulting in exon skipping. However, if a *bona fide* PAS is present just downstream in the intron, targeting the 5'ss activates the intronic PAS to generate a truncated mRNA by a U1snRNP-dependent mechanism (Figure 2).

U1snRNP plays an essential role in pre-mRNA splicing, where it is needed to select and define 5' splice sites by base-pairing with its own snRNA [80]. In addition, U1snRNP can also directly inhibit polyadenylation when tethered to the 3'UTR of a gene [81, 82]. This

suppression by U1 is not limited to the 'normal' 3' UTR, but is in fact a general non-splicing function of U1, possibly as part of the RNA surveillance machinery [79, 83]. Since in any pre-mRNA, every intron 5'ss is bound by U1 during splicing, downstream IPA sites are silenced as a consequence. Hence, preventing U1 binding at the 5'ss would relieve suppression and allows IPA to occur, in a splicing-independent mode.

Based on these observations, ASOs were designed to specifically block U1snRNP binding to individual 5'ss sites upstream of the IPA sites corresponding to various natural sdRTKS (EGFR, MET, HER2, VEGFR1 and VEGFR2), resulting in their activation in place of the endogenous full length RTKs [79]. This was exemplified by targeting of VEGFR2, the main mediator of VEGF signaling. Dysregulation of signaling via this receptor is common in tumorigenesis with mitogenic and angiogenic consequences. Vivo-morpholinos ASOs were used to induce endogenous sVEGFR2 by IPA activation [79] (Figure 1d) and to suppress VEGF signaling and angiogenesis in a paracrine and autocrine fashion *in vitro* and *in vivo* [79, 84], reproducing the inhibitory activity of the natural sVEGFR2 variant [85].

A similar approach has been applied to HER2/ErbB2, a member of the EGFR family of receptor kinases, which is over-expressed in many human malignancies and is prevalent in breast cancer [86]. To activate endogenous soluble HER2 receptors, a 2'-MOE ASO was directed against the 5'ss of exon 15 of HER2, inducing exon skipping and leading to a frameshift and the putative use of a premature stop codon upstream of the transmembrane domain [87]. The ASO treatment was associated with an increase in apoptosis and inhibition of proliferation in breast cancer cell lines. Interestingly, intron 15 of HER2 also contains an intronic PAS, which can be activated by targeted PMOs [79], suggesting that the observed truncated version and associated biology may be generated by IPA activation rather than (or in addition to) NMD-escaping exon-skipping.

In general, the generation of sdRTK is a potent approach to target oncogenic signaling pathways via three potential scenarios (Figure 2) that occur simultaneously upon addition of a single ASO compound: 1) concomitant down-regulation of full-length oncogenic RTK; 2) secretion of a ligand-binding soluble receptor leading to its sequestration; 3) non-productive dimerization with residual receptors or co-receptors.

The result is a powerful approach that recapitulates, by use of a single compound, the application of multiple inhibitory strategies: knock-down of the gene's expression, trapping of the ligand and trans-inactivation of the receptor, all of which are widely used to block signaling RTK activities in different experimental systems and therapeutic scenarios.

Overall, given the essential and central role of aberrant RTK signaling in a broad range of cancers and the existence of sdRTK variants for most of them, ASO-mediated activation of IPA and the induction of endogenous antagonistic RTK isoforms holds a tremendous therapeutic potential in cancer therapy.

Conclusion

The application of large scale cancer genome sequencing and molecular profiling has opened the door to tackling cancer through the identification of genes and networks essential

to the survival of a tumor and/or its malignant progression. It has also permitted the development of highly specific molecular targeted therapies, traditionally based on small molecule compounds or monoclonal antibodies. Advances in nucleic acid chemistry and pharmacology, indicate that antisense-based therapies may finally fulfill their large potential as a *bona-fide* alternative drug discovery platform to develop novel anti-cancer agents.

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Box 1. Antisense-based strategies

Expression of any typical multi-exon gene, represented above, can be controlled by antisense compounds by a multiplicity of mechanisms. Some target pre-mRNA processing in the nucleus, like splicing or polyadenylation, affecting both levels and structure of the protein products. Others lead to mRNA degradation by various nucleases or can modulate translation, both negatively and positively.

<p>a. Alternative Splicing re-direction</p> <p>RNAseH-independent ASOs targeted to splice sites or regulatory elements prevent snRNPs or RNA-binding proteins from recognizing them. Splicing is thus re-directed to the alternative variant. The effect is typically splicing inhibition of the selected event, but it can be enhancement if a silencer is targeted.</p>	<p>f. RNAi</p> <p>Short double stranded RNAs (siRNAs or shRNAs) are processed and incorporated into the RNA-induced silencing complex (RISC) as templates to recognize target mRNAs, which are then degraded by the nuclease activity.</p>
<p>b. ESSENCE</p> <p>Exon-Specific Silencing Enhancement by small Chimeric Effectors (ESSENCE). ASOs are coupled to an RS peptide to mimic the 'splicing activation domain' of an SR protein. The RS peptide recruits spliceosomal components by protein:protein interactions to activate splicing of nearby splice sites. (Cartegni et al., <i>Nat SMB</i>, 2003).</p>	<p>g. RNase H-dependent degradation</p> <p>DNA:RNA heteroduplexes are recognized by RNaseH and the RNA is degraded. RNaseH-dependent ASOs recruit RNaseH to the target mRNAs and direct their degradation. Modern compounds use chimeric gapmers, with a RNaseH-triggering core surrounded by protective wings.</p>
<p>c. TOES</p> <p>Targeted Oligonucleotide Enhancers of Splicing (TOES). Bifunctional ASOs are used. One part works as a targeting moiety, the other as a high-affinity binding site for</p>	<p>h. U1-mediated PA suppression</p> <p>U1 snRNP can inhibit polyadenylation when bound upstream of a PAS. Bifunctional U1 adaptors can be used to tether U1 upstream of any given PAS,</p>

	splicing factors (e.g. SR proteins). The SR protein is recruited by the ASO and activates splicing. (Owen et al., <i>NAR</i> , 2011)		blocking polyadenylation and thus silencing the gene. (Goraczniak et al. <i>Nat Biotech</i> , 2009)
d. IPA Activation		i. Translation interference
U1 snRNP normally inhibits use of downstream intronic polyadenylation sites from its position at the 5'ss. Targeting ASOs to the 5'ss prevent U1 binding and releases IPA, leading to a truncated mRNA. (Vorlova et al. <i>Mol Cell</i> , 2011)		Morpholinos or other RNaseH-independent ASOs are directed to the ATG region of an mRNA to prevent its recognition by the ribosome and inhibit translation. (Summerton J, <i>BBA</i> , 1999)
e. FSD-NMD		j. miRNA block
Forced Splice-Dependent Nonsense Mediated Decay (FSD-NMD). ASOs targeted to an early out-of-frame exon induce exon skipping, causing a frameshift. The resulting PTC triggers NMD and thus mRNA degradation. (Zammarchi et al., <i>PNAS</i> , 2011)		MicroRNAs bind to sites on mRNAs and silence genes by repressing translation. Their activity can be blocked by complementary ASOs or RNAs that directly bind miRNAs (anti-miRNAs or miRNA sponges), or by ASO that compete for their binding sites (miRNA masks). The treatment results in activation of protein expression. (Ebert et al., <i>Nat Methods</i> , 2007)

Figure 1. Examples of Therapeutic Potential of Modulating AS and APA Events in Cancer
SSO can be employed to redirect pre-mRNA processing to induce therapeutic anti-tumorigenic isoforms. (a) SSO targeting an ESE in exon 10 of PK-M produces an isoform (PK-M1) which antagonizes the Warburg effect. (b) SSOs targeting the alternative downstream 5' ss in exon 2 of the Bcl-X pre-mRNA, modulates AS to produce Bcl-XS, a pro-apoptotic isoform. (c) SSO targeting a ESE in exon 23 of STAT3 shifts AS to produce STAT3β, an anti-tumorigenic isoform. (d) SSO targeting the 5' SS of exon 13 in

VEGFR/KDR activates IPA, producing a soluble VEGFR/KDR isoform with anti-angiogenic and anti-mitogenic properties.

Figure 2. Effects of IPA activation on RTK signaling

Splicing re-direction compounds block U1 snRNP suppression of an intronic PAS upstream of the exon encoding the transmembrane (TM) domain, and activate IPA. This inhibits signaling by three simultaneous mechanisms: 1) direct reduction of the levels of active FL-RTK; 2) sequestration of ligand; 3) inhibition of residual FL-RTK or other compatible RTKs by engaging them in non functional hetero-dimers. Adapted from Vorlova et al, Mol Cell, 2011.

Table 1

Antisense Oligonucleotides in Clinical Trials for Cancer

Name	Target	ASO	Phase	Cancer Types
Custirsén	Clusterin	2'-MOE	III	Prostate and NSCLC
ISIS EIF-4ERX	EIF-4E	2'-MOE	II	Prostate and NSCLC
OGX-427	HSP-27	2'-MOE	II	Bladder and NSCLC
ISIS-STAT3RX	STAT3	2'-MOE	I/II	Advanced Tumors
AEG3516	XIAP	2'-MOE	I/II	Advanced Tumors
Oblimersen	BCL-2	PS	I/II/III	Lymphoma, AML, CLL, SCLC, RCC
AP12009	TGF β 2	PS	I/IIb/III	Glioblastoma
Cenersen	P53	PS	II	CLL

2'MOE= 2'-O-methoxyethyl, PS=Phosphorothioate