Oligonucleotide Therapeutics in Cancer

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Abstract

Alterations in pre-mRNA splicing can have profound effects on gene expression and lead to cellular transformation. Oligonucleotide therapeutics are drugs that manipulate gene expression and improve the disease state. Antisense oligonucleotides hybridize with a target mRNA to downregulate gene expression via an RNase H-dependent mechanism. Additionally, RNase H-independent splice switching oligonucleotides (SSO) modulate alternative or aberrant splicing, to favor the therapeutically relevant splicing product. This chapter summarizes the progress made in the application of these oligonucleotide drugs in the treatment of cancer.

Keywords

Alternative RNA splicing · Modulation of RNA splicing · Bcl-x (bcl2l1) · Splice switching oligonucleotides (sso)

Contents

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1 Introduction

Recent estimates state that over 90 % of the approximately 26,000 human genes undergo alternative splicing [1–3], yielding a vast protein diversity from a limited number of genes. However, dysregulation of this process can affect cellular events such as induction of apoptosis and proliferation, which may contribute to the development of cancer. In addition, several cancer-associated genes are naturally alternatively spliced and, in cancer, splicing is shifted in favor of the anti-apoptotic form [4, 5]. Antisense oligonucleotides can be used to target the transcripts generated from oncogenes for destruction via RNase H, constituting a gene-specific treatment for cancer. In a more powerful approach, alternative or aberrant splicing can be manipulated by RNase H-independent, steric blocking antisense molecules, termed splice switching oligonucleotides (SSO), resulting in the upregulation of favorable splice variants, and the simultaneous downregulation of the detrimental forms, thus providing potentially greater clinical benefits [6–10]. In this chapter, we review oligonucleotide drugs, used for the manipulation of alternative/aberrant splicing and gene downregulation, as cancer therapeutics.

2 Splice Switching Oligonucleotides

SSO are a class of antisense compounds that are used to manipulate alternative splicing to yield a therapeutic benefit. In general, SSOs can: (1) restore correct splicing of an aberrantly spliced transcript, (2) manipulate alternative splicing, yielding upregulation of one splicing isoform, with simultaneous downregulation of another, and (3) generate a novel splice variant protein that is not naturally present, but has therapeutic value in a disorder, including malignancy.

Alternative splicing and its regulation have been extensively reviewed elsewhere [11–14]. In general, splice site selection is guided by loosely conserved sequences, namely 5′ and 3′ splice sites, branch points and polypyrimidine tracts as well as sequence elements within exons and introns such as exonic splicing

enhancers (ESE), exonic splicing silencers (ESS), intronic splicing enhancers (ISE), and intronic splicing silencers (ISS) [15–17]. Selection of alternative splice sites is determined by competition between the above splice elements for splicing factors during assembly of spliceosome, a splicing complex composed of up to 100 proteins, and five small nuclear RNAs [18–20].

SSOs bind with high affinity to specific splice elements within the pre-mRNA (e.g., splice site or ESE) and therefore compete with splicing factors for access to elements, that help define exons and introns. Thus, the spliceosome is redirected to the other accessible splice elements and in consequence, the manner in which the pre-mRNA is spliced is altered. Upon blocking of a splice element by SSOs, several alternative splicing events may take place, including exon skipping, exon inclusion, intron retention, or selection of alternative 5′ or 3′ splice sites (see Chap. 3).

Early work by Dominski and Kole on aberrant splicing of human thalassemic pre-mRNA led to initial discovery that blocking an active splice site with an SSO does not lead to the complete shutdown of splicing in that transcript, but simply forces the spliceosome to select an alternative splicing pathway [6].

The SSO technology has been applied to a number of pre-mRNAs in cell culture, in animal models, and most recently in patients. Non-cancer genes that have been targeted, included β-globin, for the treatment of β-thalassemia [7, 8], SMN2 for the treatment of spinal muscular atrophy [21], tau for the treatment of dementia [22, 23], and dystrophin for the treatment of muscular dystrophy [24, 25]. In addition, morpholino (PMO) SSO targeted to the dystrophin pre-mRNA was tested in a clinical trial for the treatment of patients with Duchenne Muscular Dystrophy [26].

As described in the following section, to be useful as an SSO, an oligonucleotide must be composed of highly modified nucleotides, with biochemical characteristics distinct from classical antisense compounds.

3 Oligonucleotide Chemistry

3.1 RNAse H-Competent Chemistries (First Generation)

Classical antisense oligonucleotides (ASO) form double-stranded hybrids in which RNA is digested by RNase H (Fig. 1b*1*, an enzyme that catalyses endonucleolytic breakdown of the RNA strand in an RNA/DNA heteroduplex [27–30]. A typical antisense oligonucleotide molecule is 17–25 nucleotides (nt) in length [31–35], and since a specific 17nt sequence occurs only once within the human genome, exhibits exquisite target specificity [36]. Natural phosphodiester oligonucleotides have poor biodistribution, are quickly degraded by endogenous nucleases, and are of limited clinical use. Therefore, the "1st generation" phosphorothioate backbone modification (Fig. 1a*1*, in which one of non-bridging oxygen atoms of the phosphodiester linkage is replaced with a sulfur atom, was introduced to improve the therapeutic efficacy of antisense compounds [30, 32].

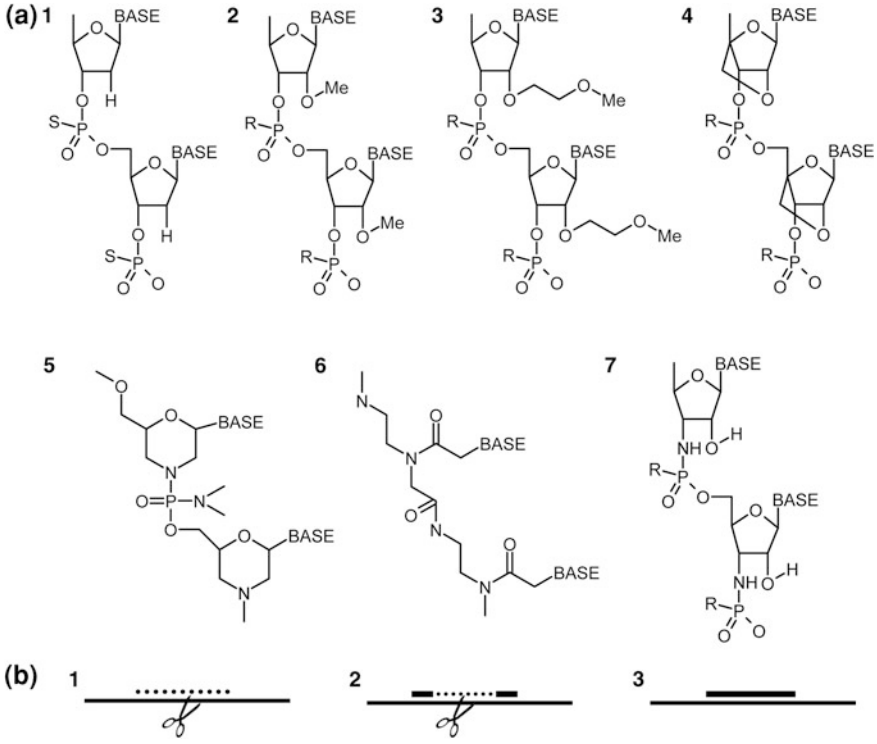

Fig. 1 **a** Oligonucleotide structures: *1* DNA phosphorothioate, *2* 2'-O-methyl-oligoribonucleotide (*2'OMe*), *3* 2′-O-methoxyethyl oligoribonucleotide (*2′MOE*), *4* locked nucleic acid (*LNA*), *5* phosphorodiamidate morpholino oligomer (*PMO*), *6* peptide nucleic acid (*PNA*), 7 phosphoroamidate oligoribonucleotide. R = O or S. **b** Antisense oligonucleotide strategies. *1* Traditional antisense oligonucleotides utilize RNase H-competent chemistries to mediate degradation of the RNA in oligonucleotide-mRNA duplex, *2* antisense gapmer oligonucleotides utilize an RNase H-competent core flanked by more potent and stable RNase H non-competent ends, *3* potent RNase H-resistant oligonucleotide chemistries are used to sterically block access to RNA without leading to its degradation. Compounds that block access to pre-mRNA by splicing factors modulate splicing as splice switching oligomers (*SSO*). Compounds that block access of ribosomes to mRNA inhibit translation as translation suppressing oligomers (*TSO*)

3.2 RNAse H Non-Competent Chemistries (Second Generation)

Other modifications that improve oligonucleotide performance in vivo, but that do not support RNase H activation, may also be used as so-called "2nd generation" gapmers, in which a core of RNase H competent DNA-phosphorothioates are flanked by more highly modified nucleotides (Figs. 1b*2* and 1a*2–4*). Importantly, fully modified second generation oligonucleotides are ideal for use as SSO (Fig. 1b*3*). Thus, these second generation chemistries, developed over the past 20–30 years benefit both the antisense and SSO fields.

In contrast to ASOs that downregulate expression of the genes by leading to target RNA degradation, the SSOs must be sufficiently modified, such that RNase H does not recognize the RNA:SSO duplex and destroy the pre-mRNA before it can be spliced. To ensure RNase H non-competence, modified nucleotides must be included such that not more than three contiguous deoxynucleotides are present in the SSO; this is in contrast to gapmers, which use modified nucleotides to provide stability and high target affinity by flanking up to 10 nucleotide long RNase H competent core.

The SSO must also form very stable duplexes with its pre-mRNA targets, a feature that allows successful competition with natural splicing factors for specific binding sequences. It must also have a significant therapeutic window, be resistant to enzymatic degradation in the cellular environment and demonstrate preferable tissue distribution and pharmacodynamics in the organism [37]. The following second generation chemistries have thus far shown the above characteristics.

3.2.1 Locked Nucleic Acids

Locked nucleic acids (LNA) contain a methylene group that links the 2′-O and 4′ position of the ribose ring (Fig. 1a*4*). The furanose ring is locked into the N-type conformation, which favors RNA binding, and importantly LNA:RNA duplexes do not activate RNase H [38, 39]. SSOs containing a uniform phosphorothioate backbone and alternating LNA/DNA sugars have been shown to be both potent and persistent modulators of splicing in vivo, especially in the liver, small intestine, and colon [40]. LNA are sometimes referred to as third generation modifications.

3.2.2 Substitutions at 2′ Carbohydrate

Substitutions at the 2′-posititon of deoxyribose with an O-alkyl groups or Fluorine renders RNA-oligonucleotide duplexes RNase H non-competent [41]. 2′-O-alkylated oligonucleotides, such as the common 2′-O-methyl (2′OMe) (Fig. 1a*2*), have higher affinity toward their targets and are resistant to nuclease degradation [41]. The 2′-O-methoxyethyl (MOE) oligonucleotides (Fig. 1a*3*) have demonstrated significant target affinity and high efficiency as ASO and SSO in cell culture [21, 42], in mice [43, 44], and in patients with familial cholesterolemia [45].

3.2.3 Morpholino Oligomers

Morpholino oligomers (PMO) are RNA-based molecules, in which the ribose is replaced with a 6-membered morpholine ring, and which contain phosphorodiamidate linkages that eliminate backbone charge [46] (Fig. 1a*5*). PMOs are highly nuclease resistant and have good affinity, which results in effective blocking of AUG start codons in eukaryotic cells [47], in zebrafish [48], in bacteria-infected mice [49, 50], and in cynomolgus monkeys where PMO-based drugs eliminated infection by deadly hemorrhagic Ebola and Marburg viruses [51]. PMOs also act as potent SSOs, yielding significant increases in β-globin production in erythropoietic progenitor cells in thalassemic mice in vivo [8], and restoring dystrophin in humans [26] and muscle function and strength in a mouse model of muscular dystrophy [25].

3.2.4 Peptide Nucleic Acids

The bases of peptide nucleic acids (PNAs) are connected by a non-cyclic peptide-like backbone (Fig. 1a*6*). They have high affinity toward their mRNA targets, are neutral, do not activate RNase H and are both peptidase and nuclease resistant. PNAs can be used both as gene expression inhibitors, as transcription and translation blocking agents, and as SSOs; their splice correction efficiency was significantly improved by placing positively charged amino acid modifications at the 3′ end [44, 52].

3.2.5 Phosphoramidates

In a N3′-P5′ phosphoramidate oligonucleotide the 3′ oxygen linking the ribose to the phosphorus is replaced by a nitrogen [53] (Fig. 1a*7*). This modification renders the compound RNase H and nuclease resistant [54] and allows formation of stable duplexes with target RNA [53]. Oligonucleotides were shown to have a good bioavailability and biodistribution, while being non-toxic in mice [55]. However, there is no evidence that they are effective in modulating splicing in vivo.

4 Positive Readout Assay for Antisense Oligonucleotide Activity

The assay, developed in Kole laboratory, is based on a transgenic mouse that ubiquitously expresses the coding sequence for enhanced green fluorescence protein (EGFP) interrupted by an aberrantly spliced intron 2 of human β-globin, such that basal expression of EGFP protein is essentially zero [44]. The aberrant splicing is induced by a thalassemic IVS2-654 mutation. Delivery to the nuclei of the cells of SSO targeting the 654 mutation (SSO-654) results in correctly spliced mRNA with restored EGFP reading frame, which renders the cells fluorescent and provides a positive readout of nuclear oligonucleotide activity.

Development of EGFP-654 transgenic mouse allowed efficacy comparison in vivo of SSO-654 with different oligonucleotide chemistries: 2′OMe, MOE, morpholino, PNA, and LNA [40, 44]. We found that MOE and PNA were active in vivo in several tissues, with highest activity in the liver and small intestine. LNA were potent in the liver, small intestine, and colon. Interestingly, in contrast to MOE and PNA, LNA functional distribution was limited to these organs.

5 Splice Switching Oligonucleotide Targets in Cancer

5.1 Bcl-x (BCL2L1)

Bcl-x is a member of the Bcl-2 family of genes involved in regulating apoptosis, which has been a major focus of cancer research [56]. The bcl-x pre-mRNA undergoes alternative splicing giving rise to two protein isoforms with opposing functions (Fig. 2a). The longer, anti-apoptotic bcl-xL, and the shorter,

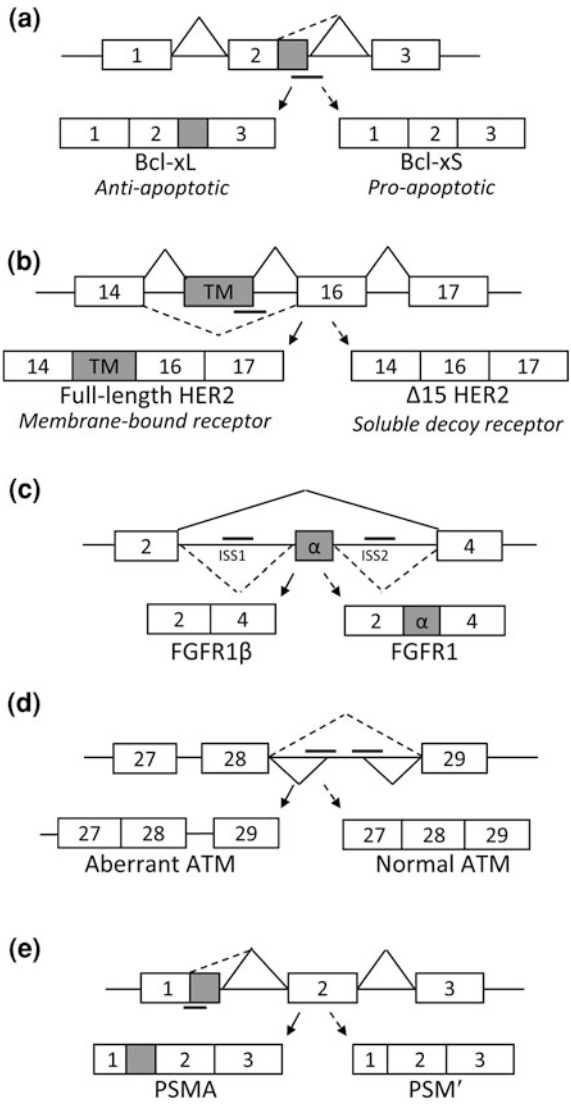

Fig. 2 Applications of SSOs in cancer. **a** Manipulation of Bcl-x alternative splicing switches production from anti-apoptotic Bcl-xL to pro-apoptotic Bcl-xS. **b** Modification of Her2 splicing leads to the production of a novel splice variant, Δ15HER2, that acts as a dominant-negative soluble decoy receptor. **c** Blocking intronic splicing silencer elements switches mRNA splicing from aberrant FGFR1β, a variant expressed predominantly in malignant tissues, to the normal FGFR variant that encodes the α exon. See text for details. **d** Correction of aberrant splicing in the ATM gene leads to production of functional protein ATM protein. **e** Modification of PSMA splicing switches production from membrane-bound PSMA protein, which is expressed predominantly in malignant prostate tissue, to PSM′, a variant that lacks the transmembrane domain but retains enzymatic activity and is expressed predominantly in normal prostate epithelium. See text for details

pro-apoptotic bcl-xS are produced by splicing at the proximal 5′ splice site of exon two, while bcl-xS is produced by splicing at the distal (upstream) 5′ splice site of exon 2 [57]. Bcl-xL promotes cell survival by sequestering the pro-apoptotic molecules Bak and Bax. Bcl-xS is thought to promote apoptosis by interacting with Bcl-xL, as well as the anti-apoptotic Bcl-2 protein, thereby freeing Bax and Bak to carry out their apoptotic function [58–60]. Overexpression of bcl-xL enables tumor cells to escape apoptosis and leads to resistance to chemotherapeutic agents [61]. Not surprisingly, bcl-xL overexpression is common in numerous cancers including multiple myeloma [62], small cell lung carcinoma

[63], breast cancer [64], prostate cancer [65], and hepatocellular carcinoma [66]. Recent data show that bcl-xL is essential for survival of a large fraction of all cancers [67]. Furthermore, bcl-xL expression has correlated with reduced sensitivity to chemotherapeutic agents [61] and in some cases is actually induced by chemotherapy, protecting residual cancer cells and setting the stage for re-emergence of metastatic cancer [68].

Bcl-x is an attractive target for SSOs because for each molecule of pre-mRNA that is affected, an anti-apoptotic Bcl-xL is replaced with a pro-apoptotic Bcl-xS. The resulting pool of bcl-xS protein is then capable of counteracting bcl-xL and bcl-2 [65, 69, 70]. Previous studies have validated bcl-x as an appropriate cancer target for ASOs that activate RNase H-mediated degradation [71–73]. However, by simultaneously upregulating one pro-apoptotic bcl-xS protein and downregulating one bcl-xL protein for each pre-mRNA affected, an SSO approach was predicted to be more potent than traditional downregulating ASOs. SSOs were designed to the proximal 5′ splice site of bcl-x pre-mRNA to block splicing at this site and induce splicing at the distal 5′ site. Treatment of various cancer cell lines with these SSOs caused a decrease in bcl-xL mRNA and protein, and a concomitant increase in bcl-xS mRNA and protein [65]. These effects were both dose-dependent and sequence specific.

Bcl-x SSO was also more effective than classical ASOs at inducing apoptosis, particularly in PC3 cells [65]. Similarly, bcl-x SSO sensitized MCF7 and A159 cells to apoptosis induced by chemotherapeutic agents or UV radiation [65, 69, 74]. Of note, the potency of bcl-x SSOs correlated with the level of bcl-xL expression, indicating that the therapeutic effects of the SSO are more profound in those cells overexpressing bcl-xL [69].

SSO-induced shift from Bcl-xL to Bcl-xS was recently shown to reduce tumor burden in vivo in a mouse model of melanoma lung metastases. Bcl-x SSO was systemically delivered using a lipid nanoparticles and was evidently taken up by B16F10 tumor xenografts that were lodged in the lungs after i.v. injection. Nanoparticle alone or formulated with a control SSO had no effect. This was the first demonstration of SSO efficacy in tumors in vivo [70].

5.2 HER2

HER2 is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases. EGFR family members are comprised of three functional domains: an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain. Ligand-binding to a monomeric receptor promotes homo- or hetero-dimerization, which leads to auto- or trans-phosphorylation in the cytoplasmic domain and downstream signaling, primarily through mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathways [75]. Therefore, EGFR receptors play critical roles in cell proliferation, differentiation and survival, and deregulation of EGFR receptors has been implicated in numerous types of tumors. HER2 is

amplified and/or overexpressed in many human malignancies, including 25–30 % breast cancers [76].

The transmembrane domain of HER2 is encoded by a single exon, exon 15 (TM, Fig. 2b). Screening a series of MOE SSOs identified SSO111 that induced exon 15 skipping in a sequence-specific, dose-dependent manner, thereby downregulating full-length HER2 while producing a novel splice variant lacking the transmembrane domain, Δ15HER2 [77]. In SK-BR-3 human breast cancer cells, which highly express HER2, SSO111-induced splice switching potently inhibited cell growth and induced apoptosis. This effect was less pronounced in MCF7 cells, in which HER2 expression is 100-fold lower, despite the fact that splice switching was still observed, confirming that SSO111-induced growth inhibition was HER2-dependent. This was consistent with the previously reported correlation of SSO potency with increased target gene expression [69]. It also indicates that tumors highly expressing HER2 would be more sensitive to SSO treatment than surrounding healthy tissue. It was further demonstrated that His-tagged Δ15HER2 protein potently downregulated HER2 protein expression and inhibited HER3 activation in dose-dependent manner in SK-BR-3 cells [77].

5.3 FGFR1

Fibroblast growth factor receptor 1 (FGFR1) belongs to a family of tyrosine kinases that bind FGFs play a role in cellular proliferation and differentiation. FGFR1 is one of the most frequently amplified genes in large number of cancers [67]. Alternative splicing of FGFR1 leads to numerous splicing isoforms with different ligand affinities. FGFR1β, produced by skipping of the α-exon, exhibits increased ligand affinity and altered subcellular localization. Upregulation of FGFR1β is associated with pancreatic cancer [78], breast cancer [79], and glioblastoma [80]. In FGFR1 pre-mRNA, the α-exon is flanked on either side by ISS sequences, ISS1 and ISS2 [81]. A recent report sought to block recruitment of *trans*-splicing factors. Morpholino SSOs targeted to either ISS resulted in α-exon inclusion in a dose-dependent, sequence-specific manner in various cell lines (Fig. 2c). Interestingly, simultaneous targeting of SSOs to ISS1 and ISS2 had no additive effect, suggesting these splicing elements contribute to a common mechanism. SSO treatment had no effect on cell viability of U251 glioblastoma cells although it did result in upregulation of activated caspase-3 and caspase-7 [82].

5.4 ATM

Ataxia telangiectasia (AT) is a progressive autosomal recessive disease resulting from mutations in the ATM gene. ATM encodes a serine-threonine kinase involved in cell cycle checkpoint control, DNA damage repair and apoptosis, and is a potent tumor suppressor [83]. In addition to neurological symptoms, AT patients are predisposed to leukemia and lymphomas [84]. Heterozygote carriers,

although generally asymptomatic, also have increased risk of breast cancer [85]. Most AT patients do not express detectable levels of active ATM protein, due to point mutations, half of which disrupt proper pre-mRNA splicing. A subset of patients with low levels (5–20 %) of wild type ATM exhibit milder phenotypes, suggesting that even a modest increase in ATM protein expression could have a therapeutic effect [86].

Approximately 50 % of ATM mutations affect splicing, usually altering less conserved sequences surrounding splice sites or creating novel splice sites [87]. A recent report utilized SSOs to correct splicing in cell lines carrying three types of splicing mutations: an exonic 5′ cryptic splice site in TAT[C] cells, an exonic 3′ splice site in IRAT9 cells, and a pseudoexon inclusion in AT203LA cells [88]. Treatment of each cell line with 25-mer morpholino SSOs targeted to mutation-activated cryptic splice sites restored correct splicing of ATM 20–55 % in a dose-dependent and sequence-specific manner (Fig. 2d). Furthermore, SSO treatment resulted in the expression of kinase-active ATM protein [88].

5.5 PSMA

Prostate-specific membrane antigen (PSMA) is a type II glycoprotein encoded by the folate hydrolase (FOLH1) gene. PSMA has been extensively studied because it is highly expressed in cancerous prostate tissue and non-prostate tumor neovasculature. SSOs targeted to *FOLH*1 pre-mRNA were used to shift splicing from the full-length PSMA to three different splice variants: the cytoplasmic PSM′ alternatively spliced at exon 1 (Fig. 2e), and two other isoforms, PSMAΔ6 and PSMAΔ18, which lack exons six and eight respectively [89]. While the PSM′ isoform was demonstrated to retain enzymatic activity, PSMAΔ6 and PSMAΔ18 were inactive. Although the role of PSMA in prostate cancer is unclear, the presence of the cytoplasmic MXXXL internalization motif and demonstrated ability to internalize in response to ligand binding suggest PSMA may act as a receptor or transport protein [90, 91].

6 Other Applications of SSO Technology

6.1 ESSENCE

Point mutations that affect splicing frequently result in exon exclusion, which is not always amenable to correction by antisense SSOs alone [92]. Exon-specific splicing enhancement by small chimeric effectors (ESSENCE) is a PNA oligonucleotide-based approach designed to enhance the inclusion of a targeted, alternatively or aberrantly spliced exon into the spliced mRNA. This technology is based on manipulating the function of ESEs [93]. An ESSENCE PNA oligonucleotide contains a covalently bound peptide RS domain at the 3′ end designed to mimic SR proteins known to bind ESEs. This oligonucleotide bound internally to

the exon targeted for inclusion is expected to promote SR-protein-mediated exon definition and enhance exon inclusion into mature mRNA.

ESSENCE was used to correct splicing BRCA1 exon 18, which requires recognition of an intact ESE by the SR protein SF2/ASF. A point mutation, present in some breast and ovarian cancers, disrupts the ESE in exon 18 of BRCA1, causing its exclusion from the mature mRNA transcript [94, 95]. Using ESSENCE, Cartegni and Kramer corrected splicing of BRCA1 exon 18 in a specific, dose-dependent manner, restoring splicing to wild type levels [96]. Interestingly, oligonucleotide alone also induced an intermediate level of exon inclusion, albeit at lower levels than when the tethered splicing enhancer was included.

ESSENCE has also been applied to modify the splicing of bcl-x [97]. A PNA oligonucleotide was conjugated to an eight-amino acid SR domain and targeted to a region of bcl-x pre-mRNA that does not encode splicing regulatory sequences. Bcl-x ESSENCE caused a dose-dependent increase in the bcl-xS:bcl-xL ratio in HeLa cells. In addition, treatment of HeLa cells with bcl-x ESSENCE triggered apoptosis as indicated by Annexin V staining.

6.2 TOSS

Targeted oligonucleotide silencers of splicing (TOSS) take advantage of the specificity of oligonucleotides-based approaches combined with the interfering capacity of protein or complexes bound near splice sites. This strategy employs an oligonucleotide with a 5′ or 3′ extension that is recognized by hnRNP A1/A2 proteins, which strongly inhibits splicing at the targeted splice site [98].

TOSS was first used to alter splicing of bcl-x in favor of the pro-apoptotic bcl-xS [98]. In cell culture, an oligonucleotide targeted just upstream of the proximal, -xL 5′ splice site, alone did not alter splicing. Delivery of TOSS, containing the consensus-binding sequence for hnRNP A1, resulted in a dose-dependent increase in the ratio of bcl-xS to bcl-xL. Of note, hnRNP A1/A2 proteins are typically highly expressed in tumor cells as opposed to normal cells, a feature that could be exploited by TOSS in cancer therapy [99].

7 RNA Interference

RNA interference (RNAi) is the oligonucleotide-based strategy of targeting mRNA for degradation with short double-stranded RNA molecules [100–102]. In this process exogenous double-stranded RNA molecules are processes by Dicer, a protein complex containing endonuclease, into 21–22 nucleotide double-stranded small interfering RNA (siRNA) [103–105]. The siRNAs themselves may also be directly delivered to the cytoplasm of cells, or expressed as short hairpin RNA, or shRNA that is processed to an siRNA and integrated into the RNA-induced silencing complex (RISC). Subsequently, RISC cleaves the target mRNA [102, 105, 106], which as a result, is silenced.

Numerous studies in cultured mammalian cell lines focused on the use of siRNA in treating cancer and other diseases [107–110]. Additionally, several pre-clinical studies using siRNA against cancer targets have been reported. ALN-VSP01 was developed for the treatment of liver cancers and potentially other solid tumors (www.alnylam.com). With a liposomal formulation, ALN-VSP01 is comprised of two siRNAs, targeting vascular endothelial growth factor (VEGF), and kinesin spindle protein (KSP), respectively. These two genes are involved in distinct pathways of tumor pathology, cell proliferation, and angiogenesis in a broad range of cancers. ALN-VSP01 delivered to the liver caused silencing of both VEGF and KSP expression in the liver, and stopped cancer cell growth in vitro. Importantly, RNAi is being used as a tool for the discovery and validation of cancer gene targets [111].

Because naked siRNA is quickly degraded by serum nucleases in vivo, many groups have focused on improving siRNA delivery [112]. CALAA-01 is an experimental therapeutic comprised of siRNA targeted to ribonucleotide reductase subunit 2 (RRM2) formulated in a targeted nanoparticle [113]. A Phase I clinical trial with CALAA-01 was initiated in 2008. An interim analysis of the trial indicated that CALAA-01 demonstrated a true RNAi mechanism in humans for the first time [114].

RNAi technology has also entered clinical trials for the treatment of Respiratory Syncytial Virus, and promising studies in non-human primates show that siRNAs may be effective cholesterol lowering agents, by targeting APOB100 [115].

8 Inhibition of Translation Initiation and Other Antisense Approaches

Several types of oligonucleotide analogs have been used to inhibit translation initiation, and thus downregulate expression of the target gene. PMO oligomers that span the AUG start codon have been effectively used to downregulate therapeutic gene targets, including c-myc [47] and TNF-α [116], by preventing ribosomal assembly on the mRNA. Phosphoramidates have also proven to be effective translation inhibitors, yielding downregulation of the cancer targets c-myc and bcl-2 in cell culture and in mice [55, 117–119].

Downregulation of gene expression via RNA degradation can be also achieved by direct application of protein ribonucleases, such as onconase and bovine seminal-RNase, or ribozymes and DNazymes. These strategies have been extensively reviewed by Tafech and colleagues [120].

9 Antisense-Based Therapeutics in Cancer Clinical Trials

To date, only RNase H competent first and second generation ASO have entered clinical trials for the treatment of cancer. Below is a summary of these compounds. Note that although the first generation oligonucleotides have reached Phase III clinical trials in some instances, it appears that the second generation molecules,

with their superior chemistry, are poised to outperform the first generation phosphorothioates as therapeutics.

9.1 First Generation: Phosphorothioate DNA Oligonucleotides

9.1.1 Oblimersen Sodium (G3139, Genasense, from Genta)

Oblimersen Sodium is a clinically advanced first generation 18-mer phosphorothioate oligonucleotide [121], that is complementary to the first six codons of bcl-2, a well-characterized oncogene [122, 123]. Between July 2000 and February 2003, the largest randomized phase III trial in advanced melanoma was conducted, in which 771 patients with irresectable stage III or stage IV melanoma were randomly assigned to receive dacarbazine alone or preceded by Oblimersen treatment [124]. The combination of Oblimersen/dacarbazine slightly improved survival compared to the dacarbazine alone (median, 9.0 *vs.* 7.8 months; $P = .077$), and improved secondary end points. Importantly, patients with normal LDH level exhibited significantly improved multiple clinical outcomes to the addition of Oblimersen compared to patients with elevated LDH.

Recently, results of a randomized phase III trial of chemotherapy (fludarabine plus cyclophosphamide) with or without Oblimersen in 241 patients with relapsed or refractory chronic lymphocytic leukemia (CLL) were reported [125]. Addition of Oblimersen to chemotherapy increased CR/nPR from 7 to 17 % ($P = .025$). Overall, this trial met its intent-to-treat primary endpoint, and demonstrated that Oblimersen can improve the outcomes of standard CLL chemotherapy.

9.1.2 Trabedersen (AP 12009, From Antisense Pharma)

Trabedersen is a PS ASO targeted to the cytokine transforming growth factor-β, the overexpression of which is a hallmark of various cancers, including pancreatic carcinoma, malignant glioma, metastatic melanoma, and metastatic colorectal carcinoma. In randomized, open-label, dose-finding phase IIb study, Trabedersen was administered intratumorally by convection-enhanced delivery in patients with recurrent/refractory high-grade glioma. Median survival of patients treated with 10 μM Trabedersen following recurrence was 39.1 months compared 21.7 months for those treated with chemotherapy standard of care; however, this difference was no statistically significant [126]. A randomized, controlled international Phase III study was initiated in March 2009 and will compare trabedersen 10 microM versus conventional alkylating chemotherapy in patients with recurrent or refractory anaplastic astrocytoma after standard radio- and chemotherapy [127]. (Table 1)

9.2 Second Generation: Phosphorothioate Gapmers

Antisense drugs that incorporate second generation chemistries have more promise of success than first generation compounds clinically. Below is a summary of these second generation drugs.

Table 1 Clinical evaluation of other first generation antisense oligonucleotides

Drug name	Drug target	Clinical phase	Company
Oblimersen	Bcl-2	III	Genta
Trabedersen	TGF-β	III	Antisense pharma
LOR-2040	Ribonucleotide reductase	II	Lorus therapeutics
Archexin	AKT-1	II	Rexahn pharmaceuticals

9.2.1 OGX-011 (from OncoGeneX/ISIS)

OGX-011 is a second generation (i.e. phosphorothioate backbone with 2′-MOE modifications on the four nucleotides at the 3′ and 5′ end) 21-mer gapmer ASO targeting the translation-initiation site of clusterin, a cell survival gene associated with the development of hormone and drug resistance [128]. In phase II study of OGX-011in combination with docetaxel/prednisone in patients with metastatic prostate cancer OGX-011 treatment resulted in 26 % decline in target clusterin versus 0.9 % increase without the drug. Although the treatment translated into significant reduction in PSA, the declines were similar in treatment and control groups (50 and 54 % respectively). More encouraging results were observed, however, in patient survival. Progression-free survival was extended by 20 % (6.1–7.3 months) and overall survival by 41 % (16.9–23.8 months). Overall, the treatment with OGX-011 and docetaxel was well-tolerated and sufficiently promising to warrant further studies [129].

9.2.2 SPC2996 (from Santaris Pharma)

SPC2996 is a second generation ASO gapmer with LNA modifications at the 3′ and 5′ end. By targeting Bcl-2, SPC2996 showed strong induction of apoptosis and inhibition of proliferation in vitro and exhibited tumor growth inhibition both as a single agent and in combination with chemotherapy in mouse models. SPC2996 is currently in phase I/II clinical study for CLL. Preliminary results have shown evidence of efficacy, including decreased lymphocyte count, decreased bcl-2 expression in lymphocytes, and a beneficial effect on lymph nodes and tumors (www.santaris.com).

9.2.3 LY2181308 (from Eli Lilly/ISIS)

LY2181308 is a second generation 2′-MOE ASO gapmer that potently downregulates Survivin, an inhibitor of apoptosis protein (IAP), in a broad range of human tumor cells [130]. Survivin is generally not expressed in normal tissues, but has been detected at a high level in a broad range of human cancers [131], and elevated Survivin expression level in cancer patients is associated with lower apoptotic index in tumor cells and poor prognosis [132]. Phase I trials for LY2181308 have been initiated against a broad range of human cancers. One phase IB/II study of

Table 2 Clinical evaluation of other second generation antisense oligonucleotides

Drug name	Drug target	Chemistry	Clinical phase	Company
Custirsen	Clusterin	2′-O-MOE gapmer	II	ISIS pharmaceuticals
LY2181308	Survivin	2′-O-MOE gapmer	II	Eli Lily/ISIS
AEG35156	X-IAP	2′-OMe gapmer	II	Aegera therapeutics
OGX-427	Heat shock protein 27	2′-O-MOE gapmer	I	OncoGeneX
LY2275796	eIF-4E	2′-O-MOE gapmer	I	Eli Lily/ISIS
SPC2996	Bcl-2	LNA gapmer	I	Santaris pharmaceuticals
EZN2968	Hypoxia inducing factor 1-α	LNA gapmer	I	Enzon pharmaceuticals
EZN3042	Survivin	LNA gapmer	I	Enzon pharmaceuticals
CALAA-01	Ribonucleotide reductase	siRNA/ nanoparticle	I	Colando pharmaceuticals

LY2181308 in patients with advanced hepatocellular carcinoma began in 2007. (Table 2)

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