J Inherit Metab Dis (2010) 33:397–403
DOI 10.1007/s10545-010-9135-1

REVIEW

Present and future of antisense therapy for splicing modulation in inherited metabolic disease

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Received: 4 February 2010 / Revised: 12 May 2010 / Accepted: 17 May 2010 / Published online: 25 June 2010

Abstract The number of mutations identified deep in introns which activate or create novel splice sites resulting in pathogenic pseudoexon inclusion in mRNA continues to grow for inherited metabolic disease (IMD) and other human genetic diseases. A common characteristic is that the native splice sites remain intact thus retaining the potential for normal splicing. Antisense oligonucleotides (AO) have been shown to modulate the splicing pattern by steric hindrance of the recognition and binding of the splicing apparatus to the selected sequences. In the case of pseudoexons, AO force the use of the natural splice sites, recovering normally spliced transcripts encoding functional protein. This review summarizes the present knowledge of antisense splicing modulation as a molecular therapy approach for pseudoexon-activating mutations, with a focus in IMD. Although the feasibility of treatment for patients with IMD has yet to be proven, it appears to be clinically promising, as positive results have been reported in cellular and animal models of disease, and antisense therapy for splicing modulation is currently in the clinical trials phase for Duchenne muscular dystrophy patients. Here, we review the most recent advances in AO stability, targeting and delivery, and other issues to be considered for an effective treatment in the clinical setting. Although the number of patients who can be potentially treated is low for each IMD, it represents an excellent therapeutical option as a type of personalized molecular medicine which is especially relevant for diseases for which there is, to date, no efficient treatment.

Communicated by: John Christodoulou

Competing interest: None declared

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Introduction

Splicing defects constitute a major cause for genetic disease, representing ~10% of the mutations reported to the Human Gene Mutation Database (HGMD® Professional Release 2009.3). Although most reported splicing mutations disrupt the conserved 3' and 5' splice sites at the exon–intron junctions, aberrant splicing may also be caused by mutations within introns that create or activate novel splice sites which are used in combination with opportunistic complementary sites, resulting in the inappropriate inclusion of intronic sequences, usually known as pseudoexons given their resemblance to true exons with potential 3' and 5' splice sites. Several examples of this pathogenic mechanism have been shown for diseases such as cystic fibrosis (OMIM 219700) (Friedman et al. 1999), ataxia telangiectasia (OMIM 208900) (Du et al. 2007), neurofibromatosis type

1 (OMIM 162200) (Pros et al. 2009), and many different IMD, including organic acidemias (Rincon et al. 2007; Tsuruta et al. 1998), lysosomal disorders (Rodriguez-Pascau et al. 2009; Vervoort et al. 1998), congenital disorders of glycosylation (Schollen et al. 2007), and tetrahydrobiopterin deficiencies (Ikeda et al. 1997; Meili et al. 2009). The frequency of this type of changes has been calculated in some diseases, ranging from 2–7% of the total alleles (Gurvich et al. 2008; Pros et al. 2009). In IMD, our laboratory data show a frequency of 1.5% for propionic acidemia and 3% for methylmalonic acidemia in a cohort of 160 and 43 genotyped patients, respectively. It should be noted that standard mutation detection techniques do not systematically scan deep intronic sequences and that the inclusion of intronic sequence may generate a transcript with a premature termination codon (PTC), thus being degraded by the nonsense-mediated decay mechanism (NMD) (Maquat 2004). Most frequently, transcript analysis is not performed for genotyping. All this hampers the detection and the identification of the pseudoexon-activating mutation. Therefore, the frequency of this type of mutations may remain underestimated.

In the past few years, antisense oligonucleotides (AO) have been used to redirect splicing restoring gene function (Fig. 1). In most cases, AO are designed to modulate splicing target the 3' or 5' splice sites (Rincon et al. 2007), although in other cases they target exonic splice enhancer motifs (Aartsma-Rus et al. 2003). The AO binds by complementarity to a selected site in the pre-mRNA and inhibits by steric hindrance the recognition of that region by the spliceosomal machinery. This was first reported for the beta-globin gene where AO were used to mask the activated intronic cryptic splice sites leading to abnormal splicing and causing beta-thalassemia (Dominski and Kole 1993). To date, there are many examples in the literature of the use of AO to exclude an intronic sequence (pseudoexon) activated by a point mutation in the final transcript (Fig. 1a), including several IMD (Du et al. 2007; Pros et al. 2009; Rincon et al. 2007; Rodriguez-Pascau et al. 2009; Vega et al. 2009) (Table 1). In addition, splicing intervention with AO has also been used to induce removal of in-frame exons containing a mutation (Fig. 1b) or to skip one or more exons flanking a frameshift deletion to restore the open reading frame in the dystrophin gene (Fig. 1c) (Aartsma-Rus et al. 2003, 2004), or to force the selection of an alternative splice site producing a non-malignant transcript in certain cancer-related genes (Mercatante et al. 2002) (Fig. 1d).

Some of these antisense approaches for splicing intervention are already in the clinical trials phase, namely for Duchenne muscular dystrophy (DMD) (OMIM 310200). The use of AO that induce the skipping of a specific exon allowing the restoration of the reading frame results in a partially functional dystrophin associated with a milder Becker muscular dystrophy phenotype. The feasibility of the approach was shown in cellular models for different individual DMD exons as well as for double and multiexon skipping to eliminate the mutated exon and/or restore the open reading frame, representing a basis for treatment for most patients with DMD (Aartsma-Rus et al. 2009). In the first animal model of muscular dystrophy, the *mdx* mouse with a nonsense mutation in the dystrophin gene, intramuscular injection of AO targeted to the mutant exon resulted in persistent production of dystrophin with a partial restoration of physiological function (Alter et al. 2006). In the more severe and progressive mouse model of DMD, the dKO mouse (utrophin/dystrophin double knockout) that die at 15 weeks, intraperitoneal injections of AO result in a near-normal level of dystrophin expression, improved phenotype, and extended life span (Goyenvalle et al. 2010). In humans, two clinical trials are ongoing with DMD patients, and intramuscular injection of an AO has been shown to be safe and to induce the expression of dystrophin locally within treated muscles (Kinali et al. 2009; van Deutekom et al. 2007).

Pseudoexon exclusion by antisense therapy in IMD

Most of the IMD are inherited in an autosomal recessive fashion, and in many of them, a small number of alleles remain uncharacterized to date after standard mutation detection techniques. A number of reports have described pathogenic pseudoexon insertions due to activating deep intronic mutations (Table 1). The pathogenic insertion was detected by cDNA analysis either in homozygous fashion or in heterozygous fashion after the use of translation inhibitors such as puromycin or cycloheximide to avoid NMD in patients' cells. Further genetic analysis pinpointed the defect to a deep intronic mutation which created or activated (increasing the splicing score) a 5' or 3' splice site causing the insertion. In all cases, functional analyses using minigenes of the deep intronic changes provide further evidence of their pathogenic nature confirming that they cause the pseudoexon insertion.

As the full intronic sequence is not routinely screened and cDNA analysis is not always possible, it is conceivable that some of the uncharacterized alleles in the different IMD may harbor this type of defects. In several cases, the pseudoexon was characterized as an Alu or a LINE element (Knebelmann et al. 1995; Meili et al. 2009; Mitchell et al. 1991; Perez et al. 2009; Vervoort et al. 1998). This is consistent with the hypothesis that exonization of intronic sequences associated to Alu repeats is a frequent event playing an important role in gene evolution. Alu elements contain potential splice sites and can evolve into exons requiring only one or a few mutational events (Sorek et al.

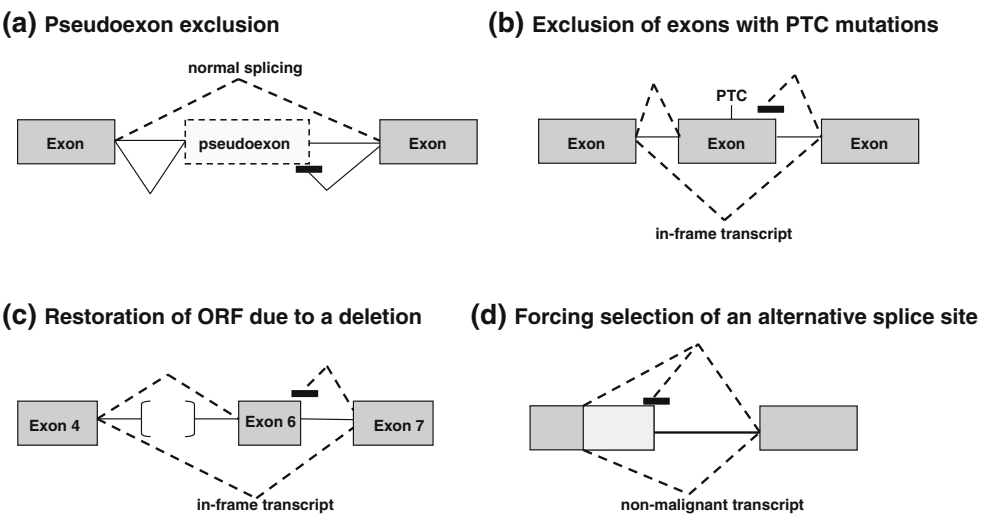

Fig 1 Overview of reported antisense applications for splicing modulation. AO (*thick black bars*) have been used to revert aberrant splicing including activated pseudoexons (**a**), to exclude mutated exons (**b**), to exclude one or more exons to restore the open reading frame caused by a frameshift deletion (**c**), or to force the use of alternative splice sites (**d**)

2004). In general, one should take into account the presence of such elements in the case of identification of intronic mutations of unpredictable effect if cDNA analysis is not possible.

Recently, antisense therapy has been applied successfully in cells from patients with different IMD caused by the mechanism described above. In propionic acidemia (OMIM 606054), both PCCA and PCCB pseudoexons were targeted with AO rescuing normal transcript and enzyme protein and activity within the normal range (Rincon et al. 2007). Similarly, in methylmalonic aciduria (OMIM 251000) due to a defect in the MUT gene encoding methylmalonylCoA-mutase, two different intronic mutations were shown to cause activation of the same pseudoexon, which was efficiently excluded from the mRNA by use of AO, recovering enzyme activity (Perez et al. 2009; Rincon et al. 2007). The same results have been obtained in cells from a patient with deficiency in phosphomannomutase (OMIM 212065) with a pseudoexon-activating mutation in heterozygous fashion in the PMM2 gene. In this case, the correction levels were 23 and 50%, for immunorreactive protein and activity, respectively (Vega et al. 2009). For Niemann-Pick type C (OMIM 257220), AO treatment also reversed the aberrant splicing with inclusion of a pseudoexon due to an intronic mutation (Rodriguez-Pascau et al. 2009).

In the pseudoexon activating mutations described, the change usually creates or activates the 5' splice site or the 3' splice site although in some cases the change creates a binding site for an auxiliary splice factor (Homolova et al. 2010; Rincon et al. 2007) (Table 1).

The fact that prevention of the aberrant inclusion of the pseudoexons by use of AO results in the recovery of normal splicing and enzymatic activity confirms that the insertions are the disease-causing mutations in the patients. The splicing correction is sequence-specific, as non-specific AO has no effect in each case. No obvious cytotoxicity has been observed. In one case examined, correctly spliced mRNA persisted for up to 15 days post-transfection (Rincon et al. 2007). Quantitative analysis of enzymatic activity has revealed that AO treatment results in a rescue of at least 30–40% of the normal level for heterozygous patients, which is therapeutically significant for autosomal recessive diseases. Interestingly, in both the heterozygous and homozygous patients control activity levels can be reached in some cases with AO treatment (Perez et al. 2009; Rincon et al. 2007). This suggests that in the treated cell population the amount of functional protein synthesized from the normally spliced mRNA is sufficient to correct the enzymatic defect. In any case, small increases in activity levels may be sufficient to achieve a therapeutic effect in a recessive disease as has been described in animal models modified with a liver-specific transgene (Miyazaki et al. 2001).

All these results reviewed here and others in different genetic diseases including data from animal models and clinical trials establish proof of principle for the application of AO to the correction of the mutant phenotype caused by pseudoexon-activating mutations in IMD.

Strategies for increased stability and efficient AO delivery. In vivo studies

The clinical potential of AO treatment for splicing intervention will depend on several factors, especially on the achievement of safe and effective delivery to target tissues and on the avoidance of unwanted side effects. To date, AO have been used in different animal models of human disease exploring the applicability of antisense therapeutics in vivo (Alter et al. 2006; Madsen et al. 2008; Sazani et al. 2002; Yokota et al. 2009). Several reviews have addressed the biological barriers that affect

Table 1 Overview of pseudoexon-activating mutations in IMD and antisense therapy applications

Disease (OMIM)	Gene	Activating mutation	AO used / target	Antisense effect	Reference
Ornithine aminotransferase deficiency (258870)	OAT	5′ ss creation	-	-	(Mitchell et al. 1991)
Dihydropteridine reductase deficiency (261630)	QDPR	5′ ss creation	-	-	(Ikeda et al. 1997)
Mucopolysaccharidosis type VII (253220)	GUSB	5′ ss creation	-	-	(Vervoort et al 1998)
Maple syrup urine disease (248600)	BCKDHB	5′ ss creation	-	-	(Tsuruta et al. 1998)
			-		
Ocular Albinism Type I (300500)	OA1	3′ ss creation	AMO / 3′ ss	Restoration of normal splicing and protein levels in patient' melanocytes	(Vetrini et al. 2006)
Ornithine transcarbamylase deficiency (311250)	OTC	3′ ss creation	-	-	(Ogino et al. 2007)
			-		
Mitochondrial trifunctional protein deficiency (609015)	HADHB	5′ ss creation	-	-	(Purevsuren et al. 2008)
			-		
6-pyruvoyl tetrahydropterin synthase deficiency (261640)	PTS	3′ ss activation	-	-	(Meili et al. 2009)
			-		
Methylmalonic aciduria (251000)	MUT	5′ ss activation	AMO / 3′ and 5′ ss	Restoration of normal splicing and enzyme activity in patients' fibroblasts	(Perez et al. 2009; Rincon et al. 2007)
Propionic acidemia (606054)	PCCA	Creation of an SRp40 binding site	AMO / 3′ ss	Restoration of normal splicing, protein and enzyme activity in patients' fibroblasts	(Rincon et al. 2007)
Propionic acidemia (606054)	PCCB	5′ ss activation	AMO / 5′ ss	Restoration of normal splicing and enzyme activity in patients' fibroblasts	(Rincon et al. 2007)
Deficiency of phosphomannomutase (212065)	PMM2	5′ ss creation	AMO / 3′ and 5′ ss	Restoration of normal splicing, protein and enzyme activity in patients' fibroblasts	(Vega et al. 2009)
Niemann-Pick type C (257220)	NPC1	5′ ss activation	AMO / 5′ ss	Restoration of normal splicing in patients' fibroblasts	(Rodriguez-Pascau et al. 2009)
Homocystinuria CblE type (236270)	MTRR	Creation of an SF2/ASF binding site	-	-	(Homolova et al. 2010)

ss Splice site, *AMO* antisense morholino oligonucleotide

biodistribution of AO and that must be overcome to attain in vivo efficacy (Juliano et al. 2009; White et al. 2009).

One of the major initial obstacles to the successful application of antisense therapeutics is the inherent instability of oligodeoxynucleotides in blood, tissues and cells due to degradation by nucleases. This has been overcome to a large extent with the development of new generation chemical modifications of AO, the most promising being morpholino oligomers, locked nucleic acids (LNA), and peptide nucleic acid (PNA) (Kurreck 2003). Morpholino oligomers contain a six-membered morpholine moiety instead of the sugar ribose and phosphordiamidate linkages. LNA are ribonucleotides containing a methylene bridge that connects the 2'oxygen of the ribose with the 4'-carbon. In PNA, the deoxyribose phosphate backbone is replaced by polyamide linkages. All three have been shown in vivo to be quite stable against nucleolytic degradation (although LNA are not as stable as morpholino oligomers), to have high target affinity, and potent biological activity (Kurreck 2003).

Once a superior chemistry is selected, the next step would be the strategy to use for efficient delivery. Various carrier systems have been developed and evaluated for intracellular delivery, such as cell-penetrating peptides, polymeric nanoparticles, or cationic lipids (Li and Morcos 2008; Thierry et al. 2006). The use of peptides has been

argued to risk a potential immune response to the conjugates, preventing necessary repeated administrations. Nanoparticles of various types offer many advantages as delivery agents as they can carry thousands of copies of the AO and can be conjugated to targeting ligands, thus providing high affinity for the target cells. The efficacy of an octa-guanidine dendrimer covalently linked to a morpholino oligo (Vivo-morpholino) has been evaluated and shown to be effective in cellular and animal models of disease (Li and Morcos 2008; Perez et al. 2009; Wu et al. 2009). Both local and systemic delivery methods have been tested in adult mice. By intravenous injection systemic delivery was achieved in most tissues except brain (Li and Morcos 2008; Moulton and Jiang 2009; Wu et al. 2009). This indicates that the Vivo-morpholino are able to achieve a high level of access to the cytosol of cells within tissues and to avoid the inhibitory effects of serum and other cellular components.

Biodistribution studies have shown that, after systemic delivery, the majority of the AO end up in the liver and kidney irrespective of the different chemistries (Sazani et al. 2002; White et al. 2009; Wu et al. 2009), which may be relevant for diseases where the responsible gene is mainly or highly expressed in the liver, as is the case for organic acidemias, some amino acid disorders, and lysosomal or peroxisomal disorders. The splice-modification levels in mice quantified in one study by qRT-PCR ranged from 40% in liver and kidney to 20% in other tissues (Sazani et al. 2002). The achievement of similar values in patients' tissues would be of clinical value in autosomal recessive diseases. For other target tissues, linking molecules or peptides to enhance tissue specific uptake can be envisaged. In a recent study, siRNA coupled to a cell-penetrating peptide traversed the blood–brain barrier after systemic delivery in adult mice (Kumar et al. 2007). In addition, efficient oral delivery of siRNA in mice has also been reported recently (Aouadi et al. 2009).

Final cellular uptake of the AO is mediated by some form of endocytosis and, once inside the cell, splice-modulating AO must enter the nucleus. Endosomal trapping may be an important barrier but apparently nuclear entry is not rate-limiting for free oligonucleotides that enter through the nuclear pore structures. However, accessing the nucleus for AO bound to a nanocarrier may constitute a major obstacle due to the significant increase in size (Juliano et al. 2009). Recently, the synthetic capping of oligos with a 2,2,7-trimethylguanosine cap (m_3G-CAP) has been shown to enhance nuclear delivery of AO and thus result in increased efficiency in a cell culture splice correction assay (Moreno et al. 2009). However, further studies in this area of intracellular trafficking are still needed.

Finally, another important issue to bear in mind is the possible toxic effects of delivering high doses of the AO repeatedly over years as patients will have to be treated repeatedly and chronically.

Conclusions

Regarding human diseases and more specifically IMD, pseudoexon inclusion events as a result of a deep intronic mutation are being increasingly described. The results reviewed here underscore the importance of looking for this type of mutations in IMD patients with incomplete genotypes after standard mutation detection analysis. Treatment of patients' cells with cycloheximide, emetine or other compounds to avoid NMD and subsequent cDNA analysis could help in the identification of these elusive mutations.

The activated pseudoexons can be targeted with AO that sterically hinders the recruitment of the spliceosomal machinery to the region preventing its inclusion in the mRNA. The effectiveness of AO for splicing modulation has been demonstrated in cell culture against several target genes and in different animal models of disease. The feasibility of its clinical application relies on the ongoing human clinical trials in Duchenne muscular dystrophy patients, although here the AO is applied intramuscularly. However, the resulting local restoration of dystrophin expression is insufficient for functional improvement, and it has been argued that the system must be scaled up to a systemic delivery system which in mouse models have proven effective. Clearly, systemic delivery would be the choice for IMD and final proof of clinical effectiveness for these diseases may rely on the results from such an approach in an appropriate in vivo model.

New generation AO have been developed, to enhance target affinity, biostabilty, and pharmacokinetics. However, as many potential applications of AO other than those reviewed here (e.g., knocking down gene expression for infectious diseases or certain cancers) are under development, it can be envisaged that the fields of AO chemistry, systemic delivery, enhanced intracellular stability, and nuclear delivery will continue to grow in the near future. Nevertheless, the potential off-target effects of splice-modifying AO must be considered and investigated, as well as secondary effects after long-term administration. To our knowledge, off-target effects arising from non-specific binding of the AO to different exons of a given gene thus modifying its splicing pattern has only been addressed in Perez et al. (2009) where gene expression profiling was analyzed by microarray technology after AO treatment, detecting no significant changes in any gene.

Antisense therapy has several advantages which include the fact that the corrected mRNA is transcribed in its natural context and under its native control and that it is easier to implement than gene therapy. On the other hand,

the use of splice-modulating AO has the limitation of being exclusive for each gene and mutation requiring individual development and optimization, as well as of having to comply with the corresponding drug regulations and going through all the clinical trial stages. Considering the mutation frequency, it may be impossible in some cases to recruit sufficient patients for phase III clinical trials. The approval of the AO by the FDA or EMEA as a class of drugs, rather than the specific sequences for each case, has been claimed as a possible solution to facilitate this (Hoffman 2007). In other personalized mutation-based molecular medicine approaches, there are similar limitations and, thus, drug regulatory processes may have to be adapted.

Acknowledgements This work was supported by grants from Comisión Interministerial de Ciencia y Tecnología (SAF2007-61350; SAF2006-12276), Fondo de Investigaciones Sanitarias (PI060512) and CIBERER (INTRA/07/720.1). An institutional grant from the Fundación Ramón Areces to the Centro de Biología Molecular Severo Ochoa and from the Fundació is gratefully acknowledged. The Human Molecular Genetics Group from the University of Barcelona also acknowledges the financial support received from the Generalitat de Catalunya (2009 SGR-971) and from the Fundación Niemann-Pick de España.

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