Published in final edited form as:

J Immunol Methods. 2011 February 28; 365(1-2): 1–7. doi:10.1016/j.jim.2010.12.001.

Potential therapeutic applications of antisense morpholino oligonucleotides in modulation of splicing in primary immunodeficiency diseases

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Abstract

Highly complementary antisense morpholino oligonucleotides (AMOs) can bind to pre-mRNA and modulate splicing site selection. This offers a powerful tool to regulate splicing process, such as correcting subtypes of splicing mutations and nonsense mutations and reprogramming alternative splicing processes. Therefore, AMO-mediated splicing modulation represents an attractive therapeutic strategy for genetic disorders. Primary immunodeficiency diseases (PIDs) are a heterogeneous group of genetic disorders that result from mutations in genes involved in development and maintenance of the immune system. Many of these mutations are splicing mutations and nonsense mutations that can be manipulated by AMOs. This review discusses AMO-mediated splicing modulation approaches and their potential applications in PIDs.

1. Introduction

Pre-mRNA splicing is an essential step in the expression of eukaryotic genes. Precise control of mRNA splicing is dependent on the correct recognition of exon-intron junctions and the assistance of regulatory sequence elements within exons and introns (Jurica and Moore, 2003; Sanford and Caceres, 2004; Sperling et al., 2008). Antisense oligonucleotides (AOs) can specifically bind to target sites in pre-mRNA and reprogram gene splicing by modulating splicing site (SS) selection, thereby offering potential therapeutic tools for genetic disorders (Sazani and Kole, 2003; Wilton and Fletcher, 2005; Du and Gatti, 2009). Antisense morpholino oligonucleotides (AMOs) are a new class of AOs with improved pharmaceutical properties. The chemistry of AMOs enhances their mRNA binding affinity and exquisite specificity; it also protects AMOs from destabilizing nucleases and proteases in cells and tissues. These properties improve their pharmaceutical potential in comparison with other AOs (Arora et al., 2004; Amantana and Iversen, 2005; Li and Morcos, 2008).

Primary immunodeficiency diseases (PIDs) are a heterogeneous group of disorders resulting from mutations in genes intrinsic to the development and maintenance of the immune system (Gatti and Seligmann, 1973; Fischer, 2007). Approximately 180 PIDs have been

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reported so far, and about 20 new ones are being discovered each year (Notarangelo et al., 2009). The number of genes related to PIDs keeps increasing (Geha et al., 2007) (<http://rapid.rcai.riken.jp/RAPID>). PID genes are involved in various cellular functions, such as signal transduction, transcription, nucleotide metabolism, gene diversification, phagocytosis, DNA repair, and apoptosis (Cunningham-Rundles and Ponda, 2005; Fischer, 2007; Oliveira and Gupta, 2008). Currently available treatments for PIDs include allogeneic bone marrow transplantation, enzyme replacement, and gene therapy; however, each approach has its drawbacks (Durandy et al., 2005; Ariga, 2006; Filipovich, 2008). Developing mutation-targeted therapeutic approaches will expand this armamentarium and benefit additional patients (Hu and Gatti, 2008; Du and Gatti, 2009).

2. AMO-mediated splicing modulation in genetic disorders

AOs have been used to restore gene function in genetic disorders by correcting aberrant splicing caused by mutations, removing nonsense mutations from mRNA by exon skipping, or regulating alternative splicing process (Du and Gatti, 2009). 2'-O-Methyl (2'-O-Me) and 2'-O-methoxyethyl (2'MOE) phosphorothioate oligomers are the two most commonly used early-generation AOs. New types of AOs have been developed by chemical modification to enhance target affinity, biostability and pharmacokinetics (Chan et al., 2006). So far, antisense phosphorodiamidate morpholino oligomers (AMOs) have proven to be amongst the most promising new-generation of AOs for splicing modulation (Arora et al., 2004; Amantana and Iversen, 2005).

2.1 Chemistry and pharmaceutical properties of AMOs

AMOs are synthetic DNA analogs with a redesign of natural nucleic acid structure. Compared to DNA, AMOs still have standard nucleic acid bases, but those bases are bound to morpholine rings instead of deoxyribose rings and linked through phosphorodiamidate groups instead of phosphates (Summerton and Weller, 1997) (Figure 1A). AMOs in organisms or cells are uncharged at physiological pH range because of the replacement of anionic phosphates with the uncharged phosphorodiamidates. Unlike many antisense molecules such as antisense phosphorothioates and siRNA, AMOs can bind to target sequences in mRNA by standard nucleic acid base-pairing without degrading their target RNAs. Instead, AMOs act by "steric blocking", binding to a target site and blocking the interaction of RNA interacting molecules with the RNA. Because of this aspect, AMOs have been used for translation blocking and splice modification. In the former case, AMOs are designed to block translation initiation by targeting sequence in the post-spliced mRNA around the AUG translational start site. In the latter case, AMOs target selected splicing sites or regulatory elements in pre-mRNAs to reprogram the splicing process. This review will focus on the AMO-mediated splicing modulation.

There are several advantages of using AMOs for splicing modulation. AMO has a high affinity for the target mRNA. The non-ionic character of AMOs avoids potential non-specific interactions with cellular components. The chemistry of AMOs also provides excellent resistance to nuclease and protease activity and improves their stability in cells, plasma and tissues. AMOs achieve exquisite sequence specificity, in large part because they must bind at least 14–15 contiguous bases to block RNA (Summerton, 2007). The efficiency in the antisense effect of AMOs has been demonstrated in cell cultures and in animal models (Arora et al., 2004b; Amantana and Iversen, 2005c; Morcos, 2007; Iversen et al., 2009). In vivo safety data on AMOs remains relatively sporadic. AMOs demonstrate a relative safety profile in several animals and clinical studies (Paessler et al., 2008; Kinali et al., 2009; Devi et al., 2005; Arora et al., 2004a; Iversen et al., 2003; Arora et al., 2002a; Arora et al., 2002b), while others have reported effects on cholesterol, hemoglobin and hematocrit levels as well as decreased weight gain in animals treated with AMOs (Stein et al., 2008; Deas et

al., 2007). Notably, each different AMO is customized to target a specific RNA site and may carry its own off-target effects, and will most likely require its own safety testing. Alternatively, some AMOs might be reserved only for use in otherwise terminal situation.

2.2 AMO-mediated splicing modulation

AMOs can be used to modulate pre-mRNA splicing in different ways. For example, AMOs against cryptic splicing sites that are activated by mutations can redirect splicing back to normal splice sites (Fig. 1B). Furthermore, AMOs can be used to mask exonic or intronic splicing silencer (ESS/ISS) sites to enhance exon inclusion during pre-mRNA splicing (Fig. 1C). AMOs can also be used to induce exon skipping, for example, to delete a region of mRNA that contains a mutation (Fig. 1D).

1) Correct splicing mutations—Certain subsets of splicing mutations generate new splice sites (SSs) or activate cryptic SSs in pre-mRNA. AMOs can be designed to block these SSs and restore normal splicing (Fig. 1B). This approach has been employed to correct aberrant splicing in various genes, such as *ATM*, *CIC-1*, *ATP7A*, *NF1*, *PCCA* and *PCC B* (Du et al., 2007; Wheeler et al., 2007; Ugarte et al., 2007; Pros et al., 2009); significant amounts of functional protein were restored after AMO treatment. The activity of AMOs appears to be highly dependent on the RNA sequence targeted. In some cases, AMOs targeting 5' SS seems more efficient than those targeting 3' SS (Du et al., 2007); this may reflect the accessibility of the AMOs to the pre-mRNA sites for hybridization. In addition to targeting mutation sites, AMOs can target splicing modulation elements to control splicing outcome. Targeting exonic/intronic splicing silencers (ESSs/ISSs) could enhance exon inclusion during pre-mRNA splicing (Fig. 1C). For example, *SMN2* is an almost identical copy of the *SMN1* gene, but is nonfunctional due to exon 7 skipping. Blocking ESS-containing regions that flank exon 7 promoted efficient exon 7 inclusion in the *SMN2* gene (Hua et al., 2007; Hua et al., 2008), implying the potential of targeting ESS/ISSs in correcting splice-junction mutations that cause exon skipping.

2) Correct nonsense mutations—Nonsense mutations can sometimes be corrected by AOs through exon skipping of a pre-mRNA region that contains the mutation. In this case, AMOs are used to target exon-intron boundary sequences and ESEs/ISEs to induce exon skipping (Fig. 1D). If removal of the mutation does not disrupt frame reading, protein translation can continue and a short form of the protein with partial function may be induced. This approach was validated in *DMD* gene and had been proven to be the most promising approach for treating Duchenne Muscular Dystrophy (Orengo and Cooper, 2007; Tazi et al., 2009). Combinations of AOs against different sites in pre-mRNA can enhance skipping efficiency (Adams et al., 2007; Wilton and Fletcher, 2009). The first clinical trial using a 2'-O-Me AOs targeted a nonsense mutation in exon 51 of *DMD* gene; it restored a modified dystrophin protein and improved the clinical phenotype (van Deutekom et al., 2007). A similar trial with an intramuscular AMO that also targeted exon 51 was also successful (Kinali et al., 2009). Besides the *DMD* gene, AO-based exon-skipping approach has been evaluated in the *COL7A1* gene to remove a nonsense mutation in exon 70 and restore expression of a shorter but partially functional Type VII collagen protein (Goto et al., 2006). In addition, this AO-mediated exon-skipping approach has been used to down-regulate the expression of *APOB* gene by disrupting normal pre-mRNA splicing (Khoo et al., 2007). This provides a tool to inhibit expression of disease-causing mRNA isoforms (Seeley et al., 2007).

3) Regulate alternative splicing—It is estimated that more than 80% of all human genes undergo alternative splicing (AS) (Lee and Wang, 2005). Alternative splicing allows the generation of functionally distinct isoforms from a single transcript and results in great

molecular diversity. AS also offers numerous opportunities for aberrant splicing events with pathological consequences. Advances in technologies such as the microarrays, new generation of sequencing and bioinformatics tools have allowed for the genome-wide identification and measurement of AS frequencies in different cell types and under various conditions (Kim and Lee, 2008). This is likely to revolutionize our knowledge about the roles of AS in diseases (Orengo and Cooper, 2007; Tazi et al., 2009).

AO-based approaches have proven effective in regulating alternative splicing in genes such as in *Bcl-x*, *IL-5* and *FGFR1* (Taylor et al., 1999; Karras et al., 2000; Mercatante et al., 2001; Mercatante et al., 2002). AMOs have recently been used to regulate AS of Myeloid cell leukemia-1 (Mcl-1, MCL-1L) to shift the splicing pattern from *Mcl-1L* (full length form) to *Mcl-1S* mRNA (short form, exon 2 spliced out) to regular apoptosis in cancer cell lines (Shieh et al., 2009). Mcl-1 is a critical anti-apoptotic protein of the Bcl-2 family. Mcl-1 pre-mRNA can undergo alternative splicing to yield the short isoform, Mcl-1S, which resembles BH3-only pro-apoptotic proteins and induces apoptosis. AMOs were designed to block SSs of exon 2 to shift splicing pattern from Mcl-1L to Mcl-1S. This shift increased the level of pro-apoptotic Mcl-1S and reduces the level of anti-apoptotic Mcl-1L and subsequently induced apoptosis in cancer cells, indicating the potential of apoptosis regulating AMOs in cancer therapy. Moreover, splicing regulatory sequences such as ESSs and ISSs may also be used as targets to regulate AS outcome. For example, AMOs were used to target ISSs in the *FGFR1* gene to enhance an alternative alpha-exon inclusion in an animal model from 10% up to 70% (Bruno et al., 2004).

2.3 Systemic delivery of AMOs

Systemic delivery is one of the major obstacles to the successful application of AO-based therapies. This is primarily due to the hydrophilic nature of biological membranes and the entrapment of AOs within endocytic vesicles and degradation in the lysosome. To enhance the efficiency of systemic delivery of AMOs, new carrier systems, such as cell-penetrating peptides (CPPs) and polymeric nanoparticles, are being developed.

CPPs are a class of small cationic peptides of ~10 to 30 amino acids that are used as transmembrane drug delivery agents for large molecules through endocytosis. Recent studies have demonstrated that arginine-rich CPPs significantly improved systemic delivery of AMOs (Abes et al., 2008). For example, a (RXR)4-CPP conjugated AMOs (CPP-AMOs) can be distributed to most tissues after intravenous (IV) administration in animals (Fletcher et al., 2007; Amantana et al., 2007). Moreover, a CPP derived from rabies virus glycoprotein RVG-9R has been described to deliver siRNA specifically to neuronal cells expressing the nicotinic ACh receptor in the brain (Kumar et al., 2007). These studies imply that CPPs have the potential to systemically deliver macromolecular oligonucleotides to the brain. However, chronic use of CPP-tagged AMOs may cause immunogenicity. For this reason, non-peptide moiety-conjugated AMOs are being evaluated for systemic delivery (Morcos et al., 2008; Moulton et al 2009). While their *in vivo* delivery efficiency is unclear, non-peptide moiety AMOs may reduce the immunogenicity of these cell penetrating molecules. Nanoparticles have also been applied to systemic delivery of AMOs into cells, such as when prepared in a streptavidin/tat nanoparticle (Wang et al., 2009).

3. Potential application of AMOs in PIDs

3.1 Mutation in PIDs and potential mutation-targeted approaches

Like other genetic disorders, genetic mutations in PIDs can be categorized into four general groups: nonsense, splicing, frameshift and missense mutations. Genotype-phenotype correlations in PIDs have been demonstrated to be highly variable (Notarangelo and Sorensen, 2008). The same clinical syndrome can result from mutations in different genes

(Villa et al., 2008). Conversely, different mutations in the same gene can lead to distinct phenotypes (Schuetz et al., 2008). Therefore, the unanticipated level of both phenotypic and genotypic heterogeneity has made mutation analysis especially important for PID diagnosis and prognosis. In the mutation databases for most PIDs, primary nonsense mutations and splicing mutations are two of major types. For PID genes such as ATM, WASP, ADA, CD40L and CYBB, splicing mutations account for approximately one quarter of unique mutations; primary nonsense mutations account for another quarter total mutations (Hu and Gatti, 2008b). The characterization of distinct genes and their mutations for each PID provide essential insights into molecular pathogenesis of each genetic disorder and opportunities for designing customized therapeutic approaches to target mutations and restore protein functions. For example, some compounds can induce readthrough of primary nonsense mutations and restore small amount of functional protein, which may have therapeutic benefit (Linde and Kerem, 2008; Du et al., 2009; Rosin-Arbesfeld et al., 2009), while AMO-mediated splicing modulation may represent a therapeutic strategy to correct splicing mutations.

3.2 AMO-based mutation targeting in PID genes

1) Block mutation-activated splicing sites—Studies with ataxia-telangiectasia (A-T) represent a good example for the application of AMOs. A-T results from autosomal recessive mutations in the *ATM* gene and is characterized by progressive neurodegeneration, immunodeficiency, elevated cancer risk and hypersensitivity to ionizing radiation (Chun and Gatti, 2004; Lavin and Kozlov, 2007). Approximately half of ATM mutations are splicing mutations. In A-T cell cultures, we used AMOs to correct three prototypic splicing mutations that activated 5' SS, 3' SS and pseudoexon formation, respectively. AMOs were able to correct aberrant splicing in each case, and restored up to 20% of ATM functional proteins in A-T cells (Du et al., 2007). Recently, we have been able to significantly improve correction efficiency by introducing CPP-conjugated AMOs). Compared to unconjugated AMOs, the CPP-AMOs almost fully corrected aberrant splicing and restored >80% of ATM protein expression (manuscript in preparation).

Similar splicing mutations have been reported in other PIDs genes. For example, a splicing mutation (c.2421-11G>A) in DNMT3B DNA methyltransferase gene (related to ICF immunodeficiency syndrome) activated a cryptic SS in an intron and resulted in a 9-nucleotide insertion in mRNA (Hansen et al., 1999). An IVS9-2 T>C mutation in WASP gene resulted in the activation of a cryptic splice site in intron 9 and the insertion of the 5' segment of intron 9 consisting of 114 bp into mRNA (Zhu et al., 1995). Specifically designed AMOs could be tested for these mutations.

2) Correct nonsense mutations—As previously discussed, AMOs have been used successfully to correct nonsense mutations in genes like *DMD* and *COL7A1*. This significantly extends the potential use of AOs. However, a protein with a region skipped is likely to be unstable in most cases even if it remains in frame. This exon-skipping may be useful for treating diseases involving structural proteins with repetitive stretches. Recently, Asai et al reported the application of AOs in correction of *parkin* gene (Asai et al., 2009). The *Parkin* gene encodes an E3 ubiquitin ligase protein, a component in the pathway that attaches ubiquitin to specific proteins for proteasome degradation. Mutations in the *parkin* gene cause autosomal recessive juvenile parkinsonism. AOs designed to induce exon 3 skipping resulted in a short form of functional parkin protein and restored the cellular phenotypes in the cells derived from a patient, indicating the potential of exon-skipping-based mutation correcting in genes other than structure proteins.

It still remains uncertain whether AMOs can restore nonsense mutations in PID genes by removing mutation-containing exons. Certainly, this approach will require that the resulting mRNA to remain in frame. The outcome will be likely to vary from gene to gene, even from mutation to mutation, depending on the location and local context of the mutation, as well as the importance of the affected region in the stability and function of the protein. Furthermore, nonsense-mediated mRNA decay (NMD) has to be considered because it can significantly degrade mRNAs containing nonsense mutations.

3) Alternative splicing (AS)—Considering the complexity of the immune system, it is not surprising that it utilizes AS to increase protein diversity and regulate the immune response (Mourich and Iversen, 2009). One recent study revealed that approximately 15% of the genes surveyed displayed detectable AS activity during T-cell activation (Ip et al., 2007). Some of the alternatively spliced isoforms of PID genes may have their own function. For example, the exon 7-skipped *lck* is most likely not an abnormal transcript because this isoform is also observed in normal peripheral blood lymphocytes. The exon 7-spliced Lck isoform may behave as a natural regulator, by limiting the extent of tyrosine phosphorylation, or may simply fulfill functions that do not require kinase activity (Chapgier et al., 2009). Moreover, Different alternative splicing pattern could be associated with distinct functions. For example, CD45 is expressed as five different isoforms, depending on the activation status of the T-cell. CD45 deficiency results in SCID (Dawes et al., 2006). Changes in combinations of CD45 isoforms alter the threshold for TCR signaling and cytokine production, resulting in altered susceptibility to infection and severity of disease.

So far there are no reports of using AOs to regulate disease-causing AS in PID genes. However, AOs have shown their ability to regulate AS in the *MyD88* gene. MyD88 is a key downstream adapter for most Toll-like receptors (TLRs) and interleukin-1 receptors (IL-1Rs). MyD88 deficiency causes recurrent life-threatening pyogenic bacterial infections, including invasive pneumococcal disease (Von et al., 2008). The *MyD88* gene has a splice variant lacking exon II, which behaves as a dominant-negative inhibitor of IL-1 - and NF- B activation by lipopolysaccharides. Treatment with 2'-MOE AOs selectively modified MyD88 alternative splicing in both cell culture and in animals, implying the potential use of AMOs in regulation of AS in PIDs (Vickers et al., 2006).

4. Conclusion

Taken together, AMOs have shown their potential in personalized treatment of genetic disorders. AMO-based approaches may have advantages over other therapies in certain PIDs such as severe combined immunodeficiency (SCID), in which corrected lymphocytes have selective advantages over uncorrected cells (Qasim et al., 2009). Advances in molecular genetics analyses of PIDs will identify more mutations and disease-related alternative splicing events, for which AMOs-based therapeutic strategy can be explored.

Some important issues have to be solved in order to move AMO-based approaches forward. Perhaps the most central limitation is that these approaches can only be applied to a small percentage of mutations. The majority of splicing mutations that disrupt normal SSs may not be correctable by AMOs because the normal SSs itself cannot be corrected by an AO. In this case, targeting nearby ESSs/ISSs may be helpful to enhance exon inclusion (Hua et al., 2008). For deleting nonsense mutations with AMOs, exon skipping may only work for certain mutations and for certain proteins. However, target-specific AMOs have to be designed for individual mutations or specific alternative spliced isoforms and the efficiency of each AMO needs to be assessed independently. This will significantly increase the workload and cost for each treatment. Notably, it is still very challenging to develop a safe and effective delivery system to specifically reach the target cells. For example, in A-T,

Purkinje cells in cerebellum play a key role in the pathogenesis and most likely will have to be targeted. Furthermore, because the splicing modulation mediated by AMOs occurs at the pre-mRNA level, the effects will most likely be temporary, requiring repeated administration of AMOs for treatment of a genetic disease. The potential long-term off-target effects caused by chronic use of AMOs will have to be documented. Potential immunogenicity of CPP-AMOs will also have to be investigated (Trehin and Merkle, 2004, Weert M).

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Figure 1. AMO structure and AMO-based splicing modulation approaches. **(A):** Structure of AMO. **(B-D):** AMO-based splicing targeting approaches. **(B):** AMO blocks mutation-generated SSs to restore normal splicing. AMO masks mutation-generated SSs to force the selection of normal SSs during pre-mRNA splicing. **(C):** AMO enhances exon inclusion by targeting ESSs or ISSs. **(D):** AMO induces exon-skipping by targeting splice-boundary or ESEs; this can be used either to correct pseudoexon inclusion, or to remove exons that contain nonsense mutations.