Preclinical Safety Assessment of Therapeutic Oligonucleotides

Patrik Andersson

Abstract

During the last decade, therapeutic oligonucleotide drugs (OND) have witnessed a tremendous development in chemistry and mechanistic understanding that have translated into successful clinical applications. Depending on the specific OND mechanism, chemistry, and design, the DMPK and toxicity properties can vary significantly between different OND classes and delivery approaches, the latter including lipid formulations or conjugation approaches to enhance productive OND uptake. At the same time, with the only difference between compounds being the nucleobase sequence, ONDs with same mechanism of action, chemistry, and design show relatively consistent behavior, allowing certain extrapolations between compounds within an OND class. This chapter provides a summary of the most common toxicities, the improved mechanistic understanding and the safety assessment activities performed for therapeutic oligonucleotides during the drug discovery and development process. Several of the considerations described for therapeutic applications should also be of value for the scientists mainly using oligonucleotides as research tools to explore various biological processes.

Keywords Oligonucleotide drugs, ASO, siRNA, Antisense, Non-clinical safety assessment, Preclinical safety assessment, Toxicity

1 Introduction: Oligo Classes, Chemistries, and Designs

Oligonucleotide drugs (OND) of different classes range from 10–12 up to 100 nucleotides in length, often with chemical modifications of the backbone and ribose sugar. The chemistry and design used are dictated by the desired mechanism of action, resulting in different classes of therapeutic oligos with specific properties. The most common classes in clinical studies target RNA and rely on Watson-Crick hybridization for selectivity and affinity, where antisense oligonucleotides (ASOs) and short interfering RNAs (siRNAs) are the most common, with several examples of approved products [1, 2].

ASOs are single-stranded ONDs of 12–20 nucleotides in length, often with a phosphorothioate (PS) backbone and 2′ribose

modifications like OMe, MOE, LNA, and cEt that improve drug properties like metabolic stability, tissue uptake, and increased affinity to the target transcript [3–5]. The ASO class can be further subdivided into ASO gapmers that trigger RNase H-mediated cleavage and degradation of target transcripts and steric blocking ASOs that modulate splicing events or inhibit activity of, e.g., microRNA (miR) [6–8]. For ASOs with a steric blocking activity, use of other chemistries resulting in neutral backbones like Phosphorodiamidate Morpholinos (PMO), Peptide nucleic acids (PNA) and tricyclo-DNA (tcDNA) is possible and quite common [9–13].

siRNAs and microRNA-mimics have a double-stranded design with each strand approximately 20–24 nucleotides in length and rely on loading of the antisense strand into RISC for activity [14, 15]. The design of siRNA results in cleavage and subsequent degradation of the target transcript, whereas miR mimics regulate gene expression by binding to miR sites in mRNA, inducing degradation and regulating protein translation [8].

In addition to hybridization dependent ASOs, miR mimics, and siRNA, there are several classes of hybridization independent ONDs including aptamers [16, 17] and immunostimulatory CpG oligos [18–21]. Rather than binding to RNA, the three-dimensional structure of folded RNA of a given sequence is combined with chemical modifications to achieve specific binding to proteins. Other therapeutic approaches such as mRNA therapy and the guide RNA in various gene editing approaches (e.g., CRISPR/CAS9) utilize nucleotides and but are commonly not classified as ONDs.

This chapter will focus on safety assessment of the hybridization dependent PS backbone ASOs and siRNA.

2 Delivery

The activity of ASOs, siRNA, and miR mimics rely on reaching the interior of the target cells. Uptake per se is not enough; the oligo needs to access the right subcellular compartments for activity, e.g., the cytosol for RISC loading or the nucleus for splice modulation or RNase H activity. Cellular uptake leading to pharmacodynamic effects are often referred to as “productive uptake” [22–24]. For ASOs, the best productive uptake after systemic delivery is often observed in the liver, in particular hepatocytes [24, 25]. Early siRNA candidates were delivered in protective formulations that also showed best uptake into hepatocytes [26]. Local delivery has successfully been used to bypass the blood–brain barrier, resulting in good therapeutic effects after local delivery to CNS [27–29], and the eye [30–33]. However, for use of therapeutic oligos beyond hepatocytes [3, 5, 34–37] and local delivery, achieving sufficient productive uptake has become one of the biggest challenges and

numerous ways to overcome this has been proposed [36, 38, 39]. One approach is delivery in different types of formulations like the lipid nanoparticle (LNP) used to deliver Onpattro [26], the first siRNA receiving regulatory approval by FDA in 2018 [40]. However, although efficient delivery at low doses can be achieved, the LNP triggers proinflammatory flu-like responses that are managed by pre-medication before administration [41]. Since discovery of this LNP, a number of alternative formulations have been presented with different tissue distribution and improved efficacy:safety relationship.

An alternative way to improve the productive uptake is conjugation of the oligo to a targeting ligand, utilizing binding to cell surface receptors that internalize the oligo conjugate. An excellent example of this strategy is conjugation of the GalNAc carbohydrate, resulting in significant improvements in productive uptake of both siRNA and ASOs [42, 43]. This GalNAc-mediated improvement in productive uptake is mediated by the binding of the conjugate to the asialoglycoprotein receptor (ASGR), which mainly is expressed on hepatocytes. Although non-conjugated ASOs show hepatocyte activity, adding GalNAc conjugates increased the clinical potency 20–30-fold for several re-formatted ASOs with hepatocyte targets [44]. Combining GalNAc conjugation with nuclease-resisting chemical stabilization has led to a tremendous increase in the utility of siRNA in the clinic, with several recent approvals with no need for the pre-medication required for LNP formulated Onpattro [2].

3 Safety Assessment of Therapeutic Oligos

The focus of this chapter is the preclinical safety assessment of therapeutic oligos intended to enter clinical trials to get regulatory approval for use in patients. With increasing experience and mechanistic understanding, screening cascades, study designs and data interpretations for ONDs are constantly improving, leading to more potent clinical candidates with better safety profiles. As described below, safety assessment of clinical OND candidates is a highly regulated process that at first sight could be of less interest for scientists primarily using oligonucleotides as tools to dissect and understand basic biological process. However, several of the considerations for bringing safe candidates to clinical trials could also be of value when developing optimal tool oligos and study designs for basic research. This includes cross species activity, restricted uptake distribution, long tissue half-life and effect duration, hybridization dependent off-target effects and the need to screen away from sequence dependent toxicities.

**3.1 Discovery Phase:
Selecting the Oligo
Candidate with the
Best Balance Between
Potency and Safety**

The potential safety concerns with therapeutic oligos can be divided into:

1. Sequence and hybridization dependent (Sect. 3.1.1).
2. Sequence and hybridization independent (Sect. 3.1.2).
3. Sequence dependent, but hybridization independent (Sect. 3.1.3).

**3.1.1 Sequence and
Hybridization Dependent
Effects: Assessing On- and
Off-Target Safety**

On-target toxicities, also known as exaggerated pharmacology, are dependent on sequence and RNA hybridization for ASOs, siRNA, and miR mimics. It can manifest in too strong intended effect or an adverse consequence of the pharmacological response in an unintended organ. A first assessment of potential on-target safety risks should be a theoretical exercise compiling available information on the biological role, tissue expression pattern, competitor information, etc. to assess the likelihood and potential adverse impact of the identified risks in the intended patient population. Considerations for assessing on-target toxicities for ONDs has been discussed by Kornbrust et al. [45].

Another safety concern dependent on Watson-Crick base pairing is hybridization dependent **off-target risks**. In contrast to the risk for on-target toxicity described above where the oligo has the intended, but exaggerated activity, this off-target risk relates to oligo activity on other transcripts than the intended target. Key features determining likelihood for hybridization dependent off-target effects have been discussed in depth elsewhere [46–50] and several recommendations [51] are summarized below:

1. Identify candidates for off-target hybridization by in silico screening of the entire transcriptome of the pre-mRNA.
2. In vitro confirmation: experimentally assess potency of sequences meeting the in silico criteria in vitro, establishing margins to activity on the primary target transcript.
3. For off-targets with insufficient in vitro margins, assess potential consequences using the principles for on-target safety assessment.

There are some specific considerations when assessing potential hybridization-dependent safety concerns. First, due to species differences in sequence, it is often difficult to achieve pharmacological activity with the same OND in other species and it is common practice to use surrogate molecules with sufficient potency in the model species of choice.

Second, the chemical modifications used in therapeutic oligos lead to slow tissue elimination and extended effect duration [52]. This is convenient from a delivery perspective, but the wash-out period needed for the adverse effects to cease would be equally long should on- or off-target toxicities be observed.

Third, the restricted productive uptake of ASOs and siRNA to many cell types needs to be considered, as there is a large difference in the uptake between different cells and tissues. The oligo distribution can change with other administration routes and delivery systems like conjugates or formulations, so understanding the productive uptake distribution for such new conditions is critical for proper risk assessment of potential on- and off- target toxicities.

3.1.2 Sequence and Hybridization Independent Effects: Coagulation Time and Complement Activation

A couple of toxicities that are dependent on plasma $C_{\max}$ but independent of both hybridization and sequence can be observed at relative high doses of PS backbone ASOs. This includes prolongation of coagulation time and activation of the alternative complement system. Acutely, activation of the alternative complement system can lead to significant drops in blood pressure. Repeated complement activation can result in “consumption” of complement factor C3 with impaired complement-mediated clearance of antibody aggregates resulting in vascular inflammation [53]. Data from in vitro, in vivo, and clinical studies clearly show that cynomolgus monkeys are significantly more sensitive than humans for this lowered threshold of complement activation [54, 55].

Both these effects are driven by the plasma $C_{\max}$ levels [56–60] and transient in nature. With increased potency of modern ASOs and adapted dosing in current clinical studies, plasma concentrations rarely exceed these activation thresholds [55, 61, 62].

3.1.3 Sequence Dependent, But Hybridization Independent: Inflammation, Liver, and Kidney Toxicities

In contrast to these plasma $C_{\max}$ -driven effects, other hybridization independent toxicities are highly dependent on the OND sequence. This includes proinflammatory manifestations and effects in high exposure organs such as liver and kidney that can sometimes be observed during the discovery phase. For siRNA, liver toxicity has been explained to be caused by off-target effects in the seed-region of the siRNA [48]. For PS backbone ASOs, liver toxicity is more frequently observed with higher affinity chemistry like LNA and cEt. It is clear from a number of published and unpublished observations that this liver toxicity observed during the screening process of PS backbone ASO gapmers is *not* caused by knockdown of the intended target transcript or liver concentration per se, see e.g. [63]. ASO sequence motifs associated with liver tox [64, 65] and different molecular mechanisms have been proposed, including cell death as a cellular consequence to exaggerated RNase H activity resulting from non-selective hybridization [66, 67]. An alternative mechanism proposed involves PS backbone-dependent binding to key intracellular proteins in a sequence and chemistry-dependent manner. Being more hydrophobic, the higher affinity modifications showing higher incidence of liver toxicity also show higher affinity to a number of intracellular proteins compared to the same sequence with, e.g., MOE chemistry [68–71]. Importantly,

predictive in vitro models for liver and kidney toxicity have been developed [67, 72, 73], and design modifications reducing ASO toxicity without compromising potency have been proposed [69, 74] demonstrating that highly potent ASO sequences that do not show liver toxicity can be identified and progressed to clinical trials.

Immune-stimulatory effects have long been a prominent feature of ONDs, where responses may vary widely between species and depend on oligonucleotide design and sequence, as well as chemical modifications [75–85]. The immunomodulatory potential can deliberately be used to design nucleotide-based immunotherapies and vaccine adjuvants, often harboring so-called CpG motifs [18–21], but for most other OND these effects are unwanted. Despite avoiding established CpG motifs in the design phase, some therapeutic oligos induce clear proinflammatory effects that can show in several different ways in the clinic, including injection site or infusion related reactions, flu-like symptoms and thrombocytopenia [86–90]. These effects are dose-dependent and can occur at different time points after first administration of the drug. Rodents are particularly sensitive to the immunostimulatory effects of ONDs [91, 92]. Similar to the liver toxicity described above, oligo sequence is a key parameter defining the proinflammatory property of therapeutic oligos and subtle and systematic sequence modifications to a proinflammatory ASO resulted in clear differences in proinflammatory potential [93]. Chemical modifications can modify the immune stimulatory potential of ONDs of a given sequence: PS modification of the backbone has long been known to increase the immune stimulatory properties of ONDs [80, 85, 94], whereas the neutral backbone in PMOs does not evoke an immune response [95]. 5'-methylation of cytosine is frequently used to suppress the immune stimulatory effect of CpG DNA sequences [76, 79]. 2'OMe modification of ssRNA or siRNA sequences inhibit immune stimulation, whereby even single modifications can significantly reduce the cytokine upregulation [78, 84]. Other 2'ribose modifications (2'F, 2'H, 2'MOE, LNA) have also been described to reduce proinflammatory effects [76, 83]. Therapeutic oligos administered in lipid formulations have been shown to induce inflammatory responses, and humans seem to be more sensitive to these effects than both rodents and NHPs [96–98].

Thrombocytopenia (TCP), i.e., low concentration of circulating platelets, has been observed in NHP toxicity studies with ASOs. In most cases, the platelet counts show around 30% reduction from baseline and then stabilize at a non-adverse level. However, in some drug programs, a few individual monkeys have experienced severe TCP [99]. Severe TCP was observed in the phase 3 studies for volanesorsen and inotersen as well as for drisapersen [86, 100, 101]. These TCP events occurred in the highest dose group, and

platelet counts increased after drug cessation. A combination of high dose of proinflammatory ASOs and possibly patient susceptibility factors seem to be the most likely cause: a high frequency of severe TCP in cynomolgus monkeys of Mauritian origin whereas no cases of severe TCP were observed when the same ASO was given to non-Mauritian cynomolgus monkeys in a follow-up study [102].

3.2 Development Phase: In-Depth Characterization and Documentation of the Oligo Candidate

3.2.1 Preclinical Safety Assessment During the Development Phase

Strategies for preclinical safety assessment of therapeutic oligos have been discussed elsewhere [22, 89]. Results from a survey across 23 companies developing therapeutic oligos performed in 2018 showed that most companies follow the two species small molecule approach as outlined by the ICH M3(R2) guideline [103]. Although a guideline recently was adopted by Japanese regulators, most health authorities lack formal regulatory guidelines for therapeutic oligos, so the expectations and experience may vary between regions and even within health authorities [103]. However, white papers published by cross-pharma groups like OSWG (Oligo Safety Working Group) on best practice recommendations are frequently used as informal guidelines [45, 51, 96, 104–110].

For the common situation with a human active candidate having limited cross-species activity for meaningful assessment of potential on-target toxicity, a surrogate molecule can be included in parallel to the clinical candidate. Such surrogate molecules should be of the same design and chemistry as the clinical candidate and have a good general safety profile to allow meaningful assessment and documentation of potential on-target toxicities. Such surrogate molecules are mostly designed to be active in the rodent species of choice.

Despite lack of positive results in regulatory genotoxicity studies as discussed in the OSWG white paper by Berman et al. [105], several health authorities still request in vitro and in vivo assessment of genotoxicity [103].

For small molecules, in vitro and in vivo safety pharmacology studies are important to rule out adverse functional effects on key organs such as the CNS, cardiovascular and respiratory systems. Safety pharmacology assessment has been discussed in another OSWG white paper [106]. To the knowledge of the author, there is no information on systemically administered ASOs or siRNA showing activity in vitro or in vivo safety pharmacology studies, including activity on the hERG channel or any other ion channels important for cardiovascular or neuronal function. However, direct delivery into heart and CNS is a different story and could result in functional effects.

Similar to small molecules, the general toxicity studies for ASOs and siRNA are performed in a rodent and a non-rodent species. The duration of these Good Laboratory Practice (GLP) studies range from 1 to 3 months in the beginning of a program to chronic

studies of 6 and 9 months in duration for rodents and non-rodents, respectively. For double-stranded siRNA and microRNA mimics, the rat is by far the most commonly used rodent species whereas the mouse is rodent species of choice for most single stranded PS backbone ASO candidates [103]. Although rat is the most common rodent for toxicity studies of small molecules, rat specific lesions like Chronic Progressive Nephropathy (CPN) [91, 92] are aggravated by the high kidney concentrations resulting from systemic delivery of PS backbone ASO. CPN is of no human relevance [111] but can become problematic in toxicity studies of long duration.

Due to the highest likelihood of sequence-dependent crossover on activity and a robust historical background record, the non-human primate (NHP), is by far the most common non-rodent species used for both ASO and siRNA candidates, but other non-rodents have been evaluated [103], including the pig [112]. Other in vivo studies required before regulatory approval include developmental and reproductive toxicology studies (DART) and carcinogenicity studies. Considerations for the DART studies are described in an OSWG white paper [108] and is normally run in mouse, rat or rabbit. Carcinogenicity studies are commonly run as a lifelong (2 years) studies in rat and mice or a 2-year study in rat combined with a 6-month study in a transgenic mouse model. The relevance of these carcinogenicity studies for ASOs and siRNA has been questioned, but most health authorities are likely waiting for more data before discussing whether these studies can get a waiver or not.

3.2.2 *Regulatory Perspective*

Several of the oligonucleotide products approved to date (e.g., eteplirsen, mipomersen, inotersen, volanesorsen) are aimed at treating rare, often genetic diseases for which no alternative treatment is available. In such cases, the presence of some safety signals has been judged to be acceptable. However, with some recent projects aiming at targeting significantly larger populations with more common disease, like the cholesterol-lowering siRNA inclisiran targeting PCSK9 and for which other treatments exist, the risk:benefit assessment will likely be different. At the same time as ONDs are considered for much larger and broader patient populations than before, exciting opportunities on the other end of the patient population spectrum are emerging. Milasen is a splice modulating ASO developed to treat a fatal neurodegenerative condition unique to a single patient [113]. With increasingly refined and optimized screening cascades, OND treatments for $N = 1$ and other ultrarare conditions will most likely become more common practice.

In summary, safety of ONDs depend on sequence, chemistry, design, and delivery approach. Several properties like limited species cross-reactivity, long tissue half-life and restricted productive

uptake distribution needs to be considered when designing interpreting results for hybridization dependent on – and off-target safety assessment studies. Together with liver and kidney toxicity, proinflammatory effects are the most commonly observed safety findings in preclinical studies. With improved mechanistic understanding and screening approaches, more potent OND candidates with better safety profile can be identified for treatment of an increasing range of diseases and patient populations.

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