OTS Rare Disease N-of-1+ Workshop Briefing Document

Preface

This briefing document aims to outline the current state of the art of oligonucleotide therapies for those planning to develop individualized therapies for patients with very rare diseases or genetic mutations. We would like to thank those who contributed to the document by providing text, edits and/or critical comments. The focus is on approved modalities and tissues for which good delivery of oligonucleotides has been confirmed in humans. We believe development of individualized therapies should build on those approaches.

As the oligonucleotide therapy field is dynamic, this document will be dynamic as well. Initially we had planned a meeting to discuss outstanding issues in spring 2020. Currently due to the COVID-19 pandemic, this is not feasible. However, we still believe that a discussion about this topic is timely. We therefore welcome input and comments from the oligonucleotide therapy field in the comment box. This will initiate an online discussion now, help us to update this document as needed, and assist planning for a stakeholder meeting to discuss outstanding issues with those involved (scientists, regulators, patients, and funders) in 2021.

Thank you for your input, on behalf of the OTS Rare Disease N-of-1+ task force,

Annemieke Aartsma-Rus, PhD, Leiden University Medical Center
Keith Gagnon, PhD, Southern Illinois University School of Medicine Art Krieg, MD, Checkmate Pharmaceuticals
Jon Watts, PhD, RNA Therapeutics Institute, UMass Medical School
Tim Yu, MD, PhD, Harvard/Boston Children's Hospital

1. Introduction

1.1. Rare Diseases and Their Impact on Patient Care

This N-of-1+ initiative is focused on the development of antisense oligonucleotide (ASO) therapeutics for patients with a serious, life-threatening rare disease where there are fewer than ~100 known patients worldwide. These individuals would be candidates for treatment with the specific ASO and would be expected to benefit from this treatment. However, due to low patient numbers there is little or no commercial incentive to develop these ASOs (Khvorova & Watts, 2017; Kim, 2019).

As defined by the US Congress in the Orphan Drug Act of 1983, a rare disease is a condition that affects fewer than 200,000 patients in the United States. The European Union defines a rare disease as a condition that affects less than 1 in 2,000 people. About 7,000 rare diseases have been described (Boycott et al., 2019). Due to the infrequency of these diseases, the variability in clinical presentation, the lack of diagnostic tests, as well as other clinical challenges, many patients must wait an average of 4-5 years before getting a correct diagnosis (Boycott et al., 2019). For patients with rare diseases, this time delay can cause irreparable damage before a diagnosis occurs, let alone before an effective treatment can be identified or developed. The phenotypes of rare diseases can become apparent anytime from pre-birth into adulthood. The impact these diseases have on patients, families and care givers can be devastating, affecting every aspect of their lives.

Approximately 95% of rare diseases have no FDA-approved therapeutic (Slade et al., 2018). An estimated 80% of rare diseases have a genetic origin, usually due to mutations in a single gene that alters the gene product's function. A single rare disease may be caused by many different mutations, some of which may be unique to a single individual, or to a very small number of patients, while other mutations may be shared among many patients with that particular disease. Some shared mutations causing rare and ultrarare genetic diseases can potentially be treated by synthetic nucleic acid-based therapeutic drugs like antisense oligonucleotides (ASOs). These ASOs are designed to specifically target the patients' disease-associated gene product, specifically its ribonucleic acid (RNA) transcript.

1.2. ASO: A Nucleic Acid Therapeutic Modality with Potential for Rapid Clinical Translation

ASOs are a group of therapeutic modalities with proven potential for rapid translatability to the clinic, especially for genetic disorders. This modality provides the possibility for rapid therapeutic development, from genetic diagnosis of an eligible patient, through ASO design and in vitro testing, preclinical safety tests, and finally to treatment of a patient (Kim et al., 2019). Many nucleic acid-based approaches are in development and success has been achieved with some, including single stranded ASOs, and double stranded small interfering RNAs (siRNAs) (Aartsma-Rus & Corey, 2020). Compared to siRNAs, single stranded ASOs are presently more straightforward in design and implementation for the immediate goals of rapid development of therapeutics for rare and ultrarare diseases. ASO designs and chemistries with a proven track record of generating clinical benefit in patients are the initial focus of the OTS N-of-1+ initiative. The focus will further be on tissues to which ASOs can be delivered efficiently after systemic treatment (liver) or after local treatment (nervous system and potentially the eye).

1.2.A. Single Stranded Antisense Oligonucleotides (ASOs)

Eight ASO drugs (fomivirsen, mipomersen, eteplirsen, nusinersen, inotersen, valonesorsen, golodirsen and viltolarsen) have been approved by the Food and Drug Administration (FDA), European Medicines Agency (EMA), or Japanese Ministry of Health, Labour and Welfare. Many more related compounds are in clinical development. The success of these therapeutics has stemmed in part from the development of chemical modifications to the natural DNA and RNA nucleotide structure, thus endowing

better drug-like properties. Natural DNA and RNA oligonucleotides have poor pharmacologic properties, including low biodistribution, low cellular uptake, and weak binding to target RNA. These limitations have handicapped their direct use as therapeutics.

Single Stranded ASOs are short nucleic acid polymers comprised of DNA and RNA building blocks called nucleotides. Nucleotides chemically consist of three parts, a nucleobase (for specifying Watson-Crick base-pairing), a ribose or deoxyribose sugar, and a phosphate group **(Figure 1)**.
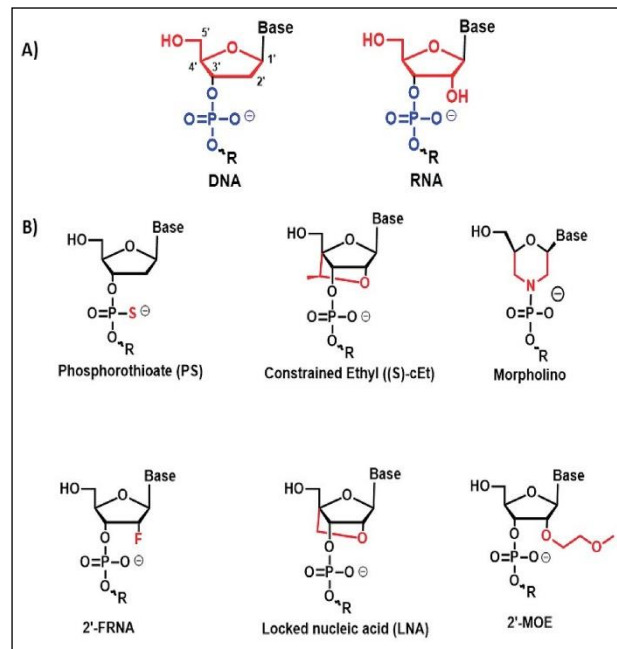

Figure 1: (**A**) Structures of deoxyribose (left) and ribose nucleotides (right). The phosphate is in blue, sugar in red and nucleobase in black. (**B**) Chemical modifications to DNA or RNA. From top left: phosphorothioate (PS), constrained ethyl ((S)-cEt) and morpholino. From bottom left: 2'fluororibonucleic acid (2'FRNA), locked nucleic acid (LNA), and 2'-O-(2-methoxyethyl)) (2'MOE). Red highlights the difference from the natural DNA or RNA structure (Wan & Seth, 2016).

Individual nucleotides of an oligonucleotide are strung together by phosphodiester backbone linkages. Chemical modifications to oligonucleotides that have enabled the greatest therapeutic success of ASOs include sugar and phosphodiester linkage modifications. Chemical modification of the phosphate usually involves replacing one or more of the non-bridging oxygen atoms. The most widely used is the phosphorothioate (PS) modification, where one non-bridging oxygen atom has been replaced with a sulphur atom. The PS modification provides enhanced stability, binding to serum proteins, and biodistribution. The ribose or deoxyribose sugar may be modified at specific positions around the ring or replaced altogether with a sugar ring mimic, such as a morpholino **(Figure 1)**. For ribose ring modifications, the 2'-OH of the ribose is most often replaced by 2'-O-(2-methoxyethyl) (2'MOE), 2'-fluororibose (2'FRNA), or 2'-O-methyl (2'OMe) groups, or the 2' carbon is bridged to the 4' carbon by a methylene (LNA) or constrained ethyl (cEt) linkage **(Figure 1)** (Deleavey & Damha, 2012). The nucleobase can also be modified, but only 5-methylcytidine is commonly incorporated into ASOs for its ability to reduce immune stimulation. Each of these modifications can provide one or more enhanced pharmacologic properties but also have distinct potential for unexpected or unwanted biological effects (Shen & Corey, 2018). These can include difficult-to-predict off-target effects as a result of binding to unintended cellular RNAs or to cellular proteins. Screening protocols for off-target effects are an important aspect of ASO design and development that is discussed later.

ASOs fall into a unique class of gene-targeting drugs due to their ability to recognize and bind to cellular RNA through predictable Watson-Crick base pairing. This makes it possible in principle to design

an ASO that is antisense to, and specific for, virtually any RNA transcript. In this manner ASOs can be employed to target and down-regulate RNA transcripts that directly cause disease, e.g. mutant messenger RNA (mRNA) transcripts that produce aberrant or toxic proteins. Alternatively, ASOs can modify disease outcomes by increasing the amounts of functional transcripts, which can produce (at least partially) a functional protein by modulating splicing. There are two common designs for therapeutic ASOs, steric-blocking (non-enzymatic) and gapmer (enzymatic).

Steric-blocking ASOs are designed to recognize an RNA without activating enzymatic cleavage of the RNA. Instead, they bind with high affinity and can block or disrupt RNA folding or protein binding that is critical for downstream cellular events like splicing (production of mature mRNA) or translation (production of proteins from mRNA) **(Figure 2)**.
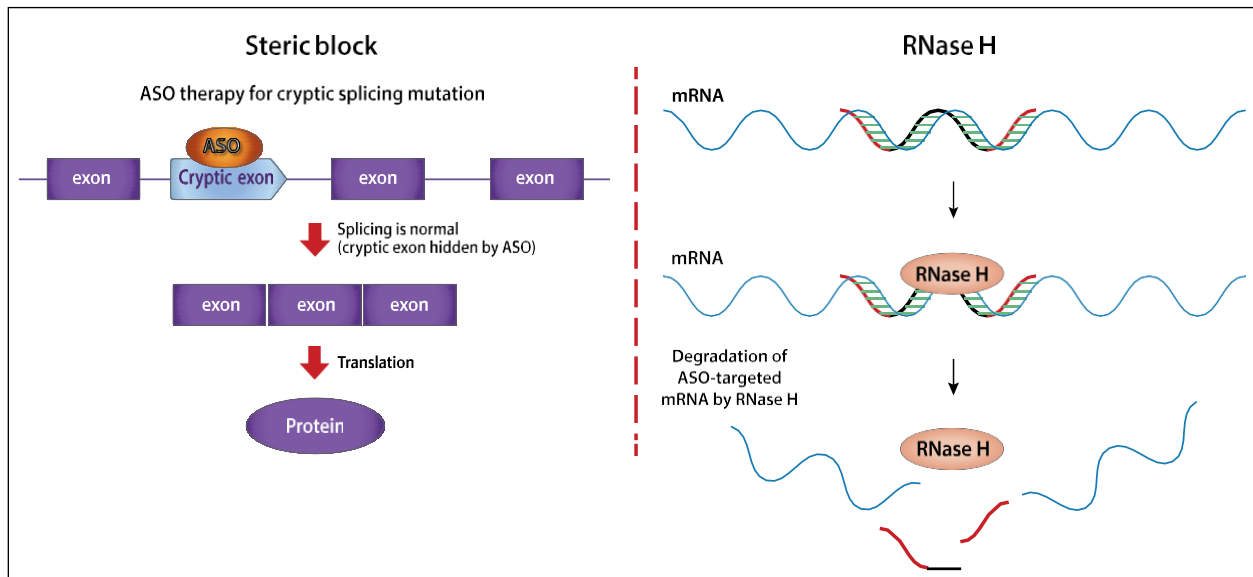
Figure 2: ASO mechanisms of action. (Left) ASO binds to pre-mRNA and acts as a steric block to modulate splicing of and result in a functional gene product, such as active protein. (Right) ASO binds to mRNA and recruits RNase H, which subsequently degrades the mRNA (Kole et al., 2012).

Steric-blocking ASOs range from 15-30 nucleotides (nt) in length and are heavily chemically modified in the sugar moiety as well as the backbone to increase stability (nuclease resistance), to avoid or reduce immune responses, and to increase binding affinity. Generally, splice modulation is used to revert an mRNA transcript that codes for a non-functional protein into an mRNA that codes for a functional or at least partially functional protein. When blocking splicing, ASOs bind to precursor mRNA (pre-mRNA) via Watson-Crick base-pairing to prevent interaction of splicing factors. Unformulated, or "naked," ASOs can be taken up by cells in the central nervous system and the eye after local injections and by the liver, kidney, and adrenal glands after systemic injections.

Gapmer ASOs also bind RNA target sequences with high affinity through Watson-Crick base-pairing. However, a "gap" in certain chemical modifications of about 10 nucleotides is maintained in the central portion of the RNA-ASO duplex to allow recruitment and enzymatic activation of the cellular nuclease RNase H **(Figure 2)**. The central gap portion is usually minimally modified with phosphorothioate backbone linkages to maintain DNA properties and support RNase H activity **(Figure 3)**. This results in cleavage of the RNA phosphodiester backbone at positions that are paired to the ASO in this central portion, which results in degradation and reduction of that RNA inside cells. Chemical modification of the two gapmer ASO termini, called the "wings," are designed to induce high binding affinity to the target **(Figure 3)**. Just like steric-blocking ASOs described above, gapmer chemical modifications increase nuclease resistance, reduce immune responses, and increase binding affinity to the target.
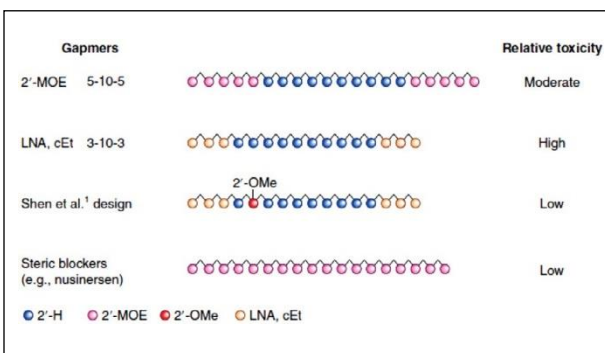
Figure 3: Common ASO designs. Gapmer ASOs comprise 5' and 3' "wings" typically three to five bases long in which the sugar is modified to improve hybridization affinity (for example, with 2'MOE, LNA or cEt), along with a central phosphorothioate (PS) DNA core that recruits RNase H1 to cleave a complementary target RNA. Compared with ASO gapmers using 2'MOE, the higher affinity of LNA or cEt bases makes it possible to reduce both the length of the wings and the length of the central gap. 2'OMe modification of the second position in the gap has been reported to reduce the frequency of toxicity (Shen et al., 2019). Steric blocking ASOs have no gap, and therefore do not recruit RNase H1 or cleave their targets. Steric blockers may be used therapeutically to alter the splicing, transcription or function of many different RNAs, including mRNA, microRNA and long noncoding RNA.

Efforts to target ASOs to specific tissues have succeeded in the case of the liver with their conjugation to N-acetylgalactosamine (GalNAc), a ligand for the asialoglycoprotein receptor on hepatocytes. This conjugation results in enhanced cellular uptake by approximately 10-fold (Springer & Dowdy, 2018; Shen & Corey, 2018). Research efforts focused on tissue-specific and cell-specific delivery of ASOs are ongoing.

1.2.B. Example of an Approved Steric-Blocking ASO Drug

Nusinersen (commercial name Spinraza) was approved by the FDA in December 2016 and by the EMA in 2017 (Stein & Castanotto, 2017). It was developed by Ionis Pharmaceuticals and Biogen and commercialized by Biogen to treat spinal muscular atrophy (SMA). SMA is caused by mutations in the *SMN1* gene. Its disease severity is modified by the copy number of a duplicate gene version called *SMN2*, which produces low levels of the SMN protein due to a sequence variation in exon 7. This variation severely decreases inclusion of exon 7 in the final mRNA and therefore only produces small amounts of functional SMN protein.

Nusinersen is an 18-nucleotide long ASO fully modified with 2'MOE and phosphorothioate (PS) bonds. The 2'MOE-PS chemistry and ASO sequence was designed to increase the level of properly spliced SMN2 mRNA to compensate for the loss of the *SMN1* gene. Nusinersen binds to intron 7 of SMN2 pre-mRNA and blocks binding of the splicing repressor protein hnRNPA1. This results in increased levels of SMN2 exon 7 inclusion, restoration of proper splicing, and increased levels of SMN protein. Spinraza is administered to patients via intrathecal injection by lumbar puncture at a dose of 12 mg in 5 mL every four months after an initial loading dose of 12 mg administered every month for the first four months. Spinraza is approved to treat infant through adult SMA. The following adverse reactions have been described: lower respiratory infection, upper respiratory infection, and constipation. The label has the following warnings: thrombocytopenia and coagulation abnormalities as well as renal toxicity. This warning relates to ASO drugs as a class and is not specific to Spinraza. Given Spinraza's efficacy, it has been tested in pre-symptomatic infants with mutations in SMN1 who possess 2-3 copies of the *SMN2* gene in the Nurture trial.
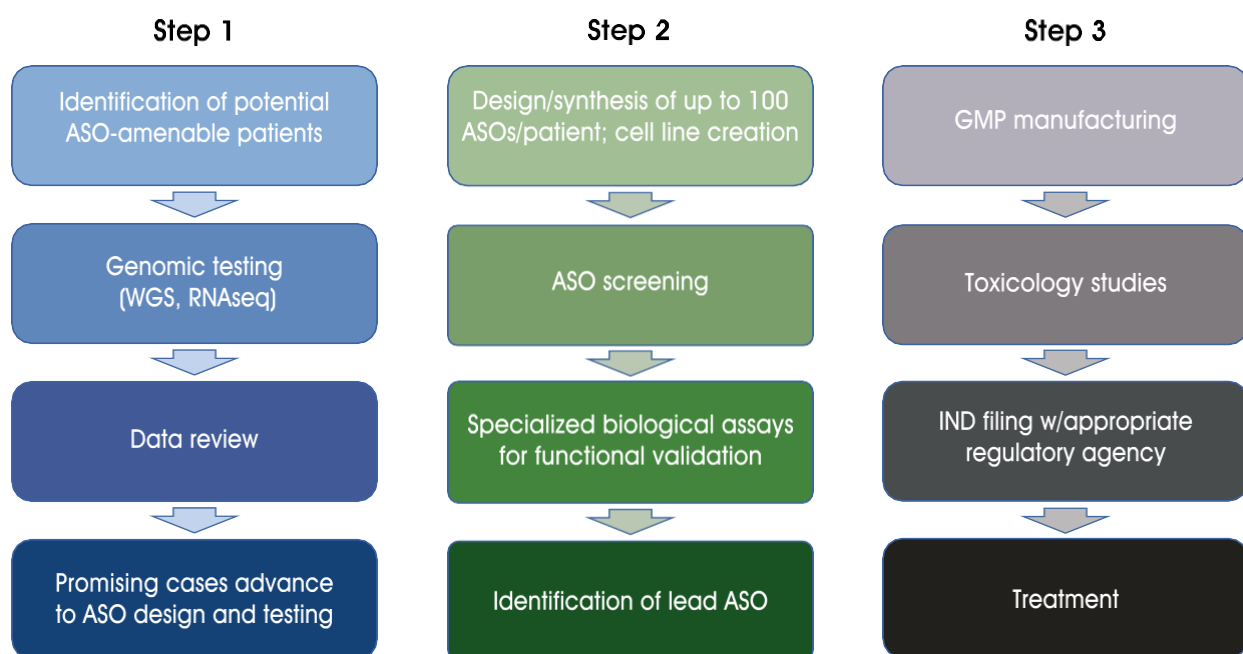
Mipomersen (commercial name Kynamro) was approved by the FDA in January 2013 and was developed by Ionis Pharmaceuticals and Genzyme and initially commercialized by Genzyme to treat homozygous familial hypercholesterolemia (Hair et al., 2013). In 2016, Ionis Pharmaceuticals and Kastle Therapeutics acquired the rights to Kynamro. In May 2018, the marketing of the drug was discontinued. Homozygous familial hypercholesterolemia is caused by homozygous or compound heterozygous mutations in either *APOB*, *LDLR*, or *PCSK9* genes, leading to severely elevated low-density lipoprotein-cholesterol (LDL-C) levels. Mipomersen is a 20-nucleotide long gapmer ASO with five 2'MOE nucleotides at each end flanking a stretch of 10 DNA nucleotides. It is completely modified with phosphorothioate bonds. Mipomersen was designed to bind to the coding region of the APOB-100 mRNA (an isoform encoded by the *APOB* gene), elicit RNase H-mediated degradation of the mRNA, and reduce ApoB protein levels. Kynamro is administered via weekly subcutaneous injections at a dose of 200 mg in 1 mL. Kynamro is indicated as an adjunct to lipid-lowering medications and diet to reduce LDL-C, Apo B protein, total cholesterol (TC), and non-high-density lipoprotein cholesterol (nonHDL-C) for patients with homozygous familial hypercholesterolemia. Kynamro has a black label warning that it can cause hepatotoxicity and increased levels of transaminases (ALT) and hepatic fat (hepatic steatosis). Because of the risk of hepatotoxicity, Kynamro is available only through a restricted program under a risk evaluation and mitigation strategy. Other adverse effects include injection site reaction, fever, flu-like symptoms, and fatigue. Mipomersen was not approved by the EMA citing safety concerns.

2. Road Map for Rare Disease N-of-1+ Success

In the N-of-1+ initiative **(Schematic 1)**, ASOs are assumed to be developed for patients with a serious, life-threatening (ultra)rare disease where there are fewer than about 100 known patients worldwide. ASO treatment may either apply to all patients with an ultrarare disease or a subgroup of patients with a rare or unique mutation, such as a cryptic splice site mutation. These represent diseases where there is currently no available effective therapy and no commercial incentive for therapeutic development, whereas there is a large unmet medical need.

Schematic 1: Work flow of recommended steps for N-of-1+ therapeutic ASO development

Step 1	Step 2	Step 3
Identification of potential ASO-amenable patients	Design/synthesis of up to 100 ASOs/patient; cell line creation	GMP manufacturing
Genomic testing (WGS, RNAseq)	ASO screening	Toxicology studies
Data review	Specialized biological assays for functional validation	IND filing w/appropriate regulatory agency
Promising cases advance to ASO design and testing	Identification of lead ASO	Treatment

2.1. Clinical and Practical Considerations and Expectations

2.1.A. Setting Appropriate Expectations and Managing Communication with Patients and Parents

It is anticipated that many patients will be children unable to provide informed consent or understand the potential impacts of participating in therapeutic development and testing. Thus, it is imperative that the parent(s) in these situations fully understand the experimental nature of this program, the low probability of success, the risks involved and the fact that functional loss is unlikely to be restored. There are currently no reliable estimates for the number of patients whose mutations may be amenable to ASO therapies. For the purpose of speaking to patients with a newly diagnosed rare disease, it should be assumed that the great majority of novel disease-causing mutations will not be amenable to an ASO therapeutic. Parents who expect a cure, or even a highly effective therapy that restores lost function, may unintentionally submit their child to medical procedures and discomfort that does not benefit them and may be painful. In addition, the burden on families to travel to medical appointments and potential delays in other treatment should be considered and explained before accepting a patient into this program.

2.2. STEP 1: Identifying Candidate Patients and Eligible Diseases

2.2.A. Is the Mutation or Disease Potentially Treatable with ASO Therapeutics?

The focus will primarily be on monogenetic diseases caused by specific mutations amenable to ASO therapy. Before a patient can be selected for this program, their disease-causing mutation(s) must therefore be identified. This is essential to assess whether the mutation is eligible for ASO design and therapy.

The biology of the candidate patient's disease should be understood sufficiently to provide a level of confidence that ASO treatment could potentially achieve a therapeutic effect and be of benefit to the patient. In order to assess therapeutic effects, the pathology and expected clinical course of the disease should ideally be understood.

The focus for N-of-1+ therapies should be on modalities where at least one drug has already been approved as a therapy for a disease in the same target tissue. For the present time, this would include liver diseases amenable to therapy with a GalNAc-conjugated ASO and central nervous system (CNS) diseases amenable to therapy with intrathecal ASOs.

Ultrarare diseases that can be attributed to a single mutation or specific biology that only requires reduction of a specific transcript would be examples of potential candidates for gapmer-based ASOs. This includes toxic RNA gain-of-function transcripts or toxic protein products. Alternatively, if a cellular pathway is perturbed and its biology is understood in sufficient detail, then reduction of expression of a specific protein in that pathway may also be a possible therapeutic approach for gapmer ASOs.

Mutations that impact splicing of an mRNA and result in aberrant or insufficient protein would be examples of candidates for steric-blocking ASOs. Generally, this involves diseases caused by loss of protein function. This may include instances where all or a part of an intron is aberrantly included in a mature mRNA transcript. This may also include mutations where an exon is omitted from the mature

mRNA transcript. Restoring normal splicing would be expected to restore normal protein production. Alternatively, as is the case for the action of Nusinersen (Spinraza), altered splicing of a gene (*SMN2*) that is well-understood to modify disease severity, may also serve as a target for steric-blocking ASOs.

For some diseases, patients may benefit from restoring the reading frame to produce shorter, partially functional proteins. Usually individuals with these mutations present with less severe disease, such as Becker muscular dystrophy versus Duchenne muscular dystrophy. Alternatively, skipping an in-frame exon that encodes the toxic gain of function, to produce shorter, partially functional non-toxic proteins could provide benefit. The opportunity to produce partially functional proteins applies mostly to modular, long proteins. Where possible, functionality of the shorter proteins should be shown.

The eligibility for individual patients or diseases to be treatable and benefit from N-of-1+ nucleic acid therapeutics will likely require a case-by-case evaluation. The examples above are not limiting but provide guidelines for how to assess a mutation or disease pathology. The leading ASO technologies currently amenable to N-of-1+ development are gapmer ASOs, which can reduce a targeted transcript, and steric blocking ASOs, which can change accessibility of proteins to a target RNA to modulate their activity, such as splicing. Simplistically, any patients with disease mutations that could be amenable to one of these basic mechanisms of drug action may be considered for further selection.

2.2.B. Is There an Expected Treatment Benefit for the Patient?

Antisense therapies should only be developed for patients for whom treatment is expected to result in benefit, such as diseases with a progressive nature where the intervention can stop or slow down progression. Assessment of patient benefit will involve consultation with the patient's physicians, and should include practical and clinical considerations specific to the patient that take into consideration disease progression at present, expected progression, and the timeline for expected development of a potential drug.

It will be crucial to also communicate well with patients and their families to ensure realistic expectations. The therapeutic effect will depend on many factors, especially the time of intervention – tissues and functions already degenerated should not be expected to recover. An appropriate goal of therapy is to delay disease progression. Patients and their family should be aware of the risks involved and not expect ASO therapy for N-of-1+ diseases to be a cure, or to restore lost function.

2.2.C. Can Treatment Effects or Outcomes be Measured in Patients?

When patients are treated with any therapeutic, it is desirable that a treatment effect, as well as potential side effects, can be measured. Treatment effects of ASOs should be monitored through a biomarker correlated to mechanism of drug action, such as restoration of a missing protein or reduction of a toxic gene product, in an accessible body fluid or sample. For some diseases, such as brain or eye diseases, this may be difficult or impossible. Treatment effects may be quantified by indirect methods like imaging or functional outcome measures. Ideally, whenever possible, treatment effects of an ASO monitored through a biomarker should be correlated to a clinically meaningful outcome.

For all diseases it is important to review natural history data from patients with biologically similar mutations (if possible) while developing the therapeutic approach. Ideally, the natural history of the patient to be treated is known before initiation of ASO treatment. Natural history data may include functional measurements, like visual acuity or cognition tests, as well as serial imaging. For diseases with an episodic component, the occurrence of disease episodes (e.g. frequency and duration of epileptic insults) should be monitored before and after initiation of treatment.

It is important to discuss possible treatment outcomes in advance with the patient(s) and family. The burden of treatment and therapeutic effects must be weighed and evaluated with the family during

treatment. Because there is no assurance that the treatment will have a beneficial effect, criteria for stopping treatment should be considered and discussed in advance with the patient and family.

2.2.D.   Final Patient Selection Considerations

Decisions regarding patient selection for N-of1+ therapies should be made by an institutional or inter-institutional Access to Treatment Committee made up of clinical experts working in direct patient care, basic scientists with disease-relevant biological expertise, genetic counselors, and medical ethicists, as well as representatives for other relevant stakeholders. Deliberations and decisions of the Access to Treatment Committee should be transparent, while maintaining patient confidentiality. The basic criteria to define access to treatment should focus on the needs of the patients and their families, and the feasibility of potential treatment with gene-targeted therapeutics like ASOs.

2.3.   STEP 2: Designing, Screening, and Identifying Lead Nucleic Acid Drug Candidates

2.3.A.   ASO Design and Functional Screening

ASO development programs start with the design of a candidate ASO to the target, generally with the aid of *in silico* tools to reduce the most common off-target effects. Both steric-blocking and gapmer ASO candidates can have off-target effects, such as hybridization-dependent interactions with unintended RNAs or nonspecific effects due to the PS backbone and other chemical modifications (Gagnon & Corey, 2019). Gapmer ASOs generally have a greater potential for off-target effects due to the catalytic nature of RNase H-mediated cleavage and the need for only partial complementarity to target RNA to activate cleavage (Kamola et al., 2015; Khvorova & Watts, 2017). As a result, gapmer ASO candidates may more frequently fail *in vitro* and/or *in vivo* safety screening compared to steric-blocking ASO candidates. Experts should be involved in ASO design to avoid selecting motifs known to be toxic and to help select the best chemistry to employ for a given modality and tissue.

An important paradigm during the design of both gapmer and steric-blocking ASO candidates is that chemical modifications to ASOs are not generically interchangeable: seemingly subtle changes in the position or nature of chemical modifications or the ASO sequence can dramatically and unpredictably change ASO safety and efficacy (Rigo et al., 2012; Jirka et al., 2015).

Identification and elimination of poorly tolerated ASOs is the main goal of screening ASO candidates prior to moving into formal toxicology studies or human clinical development. Gapmer screening programs may test >1000 candidate ASOs during preclinical development before selection of a lead molecule. Steric blocking ASOs generally have a more limited target space and have reduced potential for off-target effects, as noted above, so the number of candidates screened for steric blocking ASOs may be much lower.

Although *in silico* screens are routinely used for ASO design (Scharner et al., 2020), no *in silico* tools have been demonstrated to reliably predict the effects of chemical modifications or stereochemistry on ASO safety or efficacy (Scharner et al., 2020). An important element of screening is sufficient experience working with the class of ASO that is being considered for development. This is particularly important for ASOs that are intended for N-of-1+ Investigational New Drug (IND) filings where the overall nonclinical safety package is likely to be very small compared to the typical suite of IND-enabling toxicology studies. Thus, it is important to use screening protocols that have established utility in promoting the selection of well-tolerated ASOs within the chemical class that have successfully completed human clinical trials with a good safety record. Examples of design criteria and considerations are presented below for steric-blocking ASOs that can alter cryptic splicing mutations and gapmer ASOs that can reduce disease-associated RNAs and their protein products.

Steric-Blocking ASOs to Alter Cryptic Splicing Mutations. For splicing modulation, guides for

antisense oligonucleotide design have been published (Aartsma-Rus, 2012). Briefly, targeting predicted splicing enhancer/regulatory sites may be helpful in identifying effective splice modulators. In addition, a GC percentage of 40-60% appears most optimal. Finally, one should ensure that the target is unique in the human transcriptome to avoid off-target effects. Splice modulating ASOs can alter splicing at partially complementary non-target sites with up to 3 mismatches *in vitro* (Scharner et al., 2020), reinforcing the need for careful *in vitro* and *in vivo* screening of all ASO candidates. However, the potential *in vivo* relevance of this finding is uncertain.

The regulation of RNA splicing is very complex and is mediated by proteins that may be expressed in tissue-specific and cell-specific manners. In addition to confirming that a candidate steric-blocking ASO achieves the intended effect on RNA splicing, it is important to also confirm that the ASO restores adequate expression of the target protein (if appropriate) **(Figure 2)**. Ideally, the model system will allow assessment of downstream therapeutic effects beyond only protein restoration. For example, functional improvements at cellular level could include enzyme activity assays, restoration of a complex that the protein associates with, or correction of molecular pathology or disease phenotype.

Patient-derived, disease-appropriate cell models are preferred for screening and optimizing candidate ASOs that target splicing. If the target RNA transcript is expressed in fibroblasts, a skin biopsy from the patient can provide adequate cells for ASO screening. In addition to demonstrating the desired effect on RNA splicing, biologic characterization should be performed to demonstrate the intended phenotypic correction if possible. Alternatively, or in addition to screening in patient-derived fibroblasts, induced pluripotent stem cells (iPSCs) may be generated and differentiated into a disease-appropriate cell type, such as a neuronal sub-type, that expresses the transcript. This will enable cell-based assays to identify the most efficient ASOs in a disease-relevant cellular and genetic background. They can also facilitate measurement of the temporal dynamics of protein and functional restoration, such as how quickly protein levels increase after treatment.

When obtaining cells from a patient is not possible, but the disease biology can be replicated, it may be possible to generate the mutation in an iPSC line from a healthy individual and use this as an alternative screen. At present, CRISPR-based methods for precise genomic mutagenesis might be the most advanced technology to mimic a patient's mutation. While other approaches like mini-genes may recapitulate the cryptic splicing event, they take the mutation out of context and therefore should be used as a last resort.

Gapmer ASOs to Reduce Target RNA or Target Protein Levels. Gapmer ASOs are generally 16-20 nucleotides in length, in which the 5' and 3' ends are modified with 2'MOE or cEt/LNA modifications leaving an 8-10 base central core that is DNA (2'-deoxy) in nature to allow recruitment of RNAse H1 when bound to its cognate RNA sequence (Monia et al., 1993; Crooke, 2017). For proof-of-principle RNA or protein reduction, or knockdown, optimal gapmer ASOs can initially be screened in almost any cell-based model where the transcript is expressed. Cell models that recapitulate part of the molecular pathology, such as aggregate formation or reduced viability, can allow measurement of the level of protein knockdown required to minimize or halt the disease pathology. Since gapmer-based protein knockdown is often not mutation specific (i.e., both the toxic mutant and the normal wild-type allele might be targeted), it is important to establish whether this is tolerable for the particular disease biology. For example, the normal protein might be essential or play critical roles in metabolism or cellular health. Knock out models in animals may not be informative because of differences between a knockout from conception and post-natal knockdown. Thus, the biological roles of the candidate RNA or protein targets should be considered and, when possible, tested.

2.3.B. ASO Safety Screening.

As with all drugs, the safety of therapeutic ASO is determined by the inherent tolerability of the

xenobiotic and its pharmacology. Different ASO backbones, sugar modifications, and designs have strikingly different toxicology or pharmacology (Crooke, 2017).

ASOs modified with complete PS backbones have relatively high protein binding, which improves their systemic half-life and cellular uptake, but also results in a wide variety of biologic effects at active concentrations. These include several different categories of both sequence-specific and non-sequence-specific effects depending on whether it is a gapmer or steric-blocking ASO design **(Figure 2, Table 1)** (Crooke, 2017; Gagnon & Corey, 2019). Current ASO designs typically use modified sugars such as 2'-MOE, LNA, and cEt, which have improved specificity and potency by >10-fold compared to early ASO chemistries (Khvorova & Watts, 2017). Systemic potency against hepatocyte-expressed targets can be improved by up to an additional 10-fold by GalNAc conjugation, allowing lower ASO doses and therefore lower toxicity (Kinberger et al., 2016).

Table 1: Selected Potential Safety Effects of PS ASO to consider During ASO Development

Off-target Biologic Effects	Relevant ASO designs	In vitro assay to detect	References
Hybridization dependent			
Cleavage of partially complementary targets, (+/- protein binding)	RNAse H gapmers	In silico screens, and cytotoxicity	Burdick et al., 2014; Burel et al., 2015; Dieckmann et al., 2015; Kamola et al., 2015; Lindow et al., 2012; Shen et al., 2019;
Reversibly altering RNA splicing, function	Steric blockers	In silico screens, and cytotoxicity	Scharner et al., 2020
Hybridization independent			
Protein-binding causing toxicity	Any PS ASO		Burdick et al., 2014; Shen et al., 2019
Innate immune activation	Any PS ASO	Human PBMC	Vollmer et al., 2002; Hartmann, 2017; Senn et a 2005; Rodriguez et al., 2006, Toonen et al., 2018
Thrombocytopenia	Any PS ASO (systemic administration)	None (in vivo only)	Flierl, 2015; Crooke, 2016; Chi et al., 2017

When the ASO is to be administered intrathecally, the systemic exposure will be much lower and tolerability is generally good, based on thousands of SMA patients receiving Nusinersen and over 1000 patients who have received gapmer ASOs via intrathecal injection in clinical trials. While local deliver is delivery well-tolerated, there are also exceptions. Therefore, for ASOs that are to be administered intrathecally in patients, safety and tolerability of the intended ASO should be confirmed in animal models. It is known that certain ASOs can lead to activation of the innate immune response (Toonen et al., 2018) or possibly even death.

Preclinical Safety Screening In Vitro. The purpose of *in vitro* screens in ASO preclinical safety development is to eliminate toxic ASOs from the screening funnel that results in lead molecule selection. Based on the potential mechanisms of toxicity **(Table 1)**, these screens must be able to detect unexpected cytotoxicity and immune stimulation. Cytotoxicity is triggered by ASOs causing biologically significant off-target effects or nonspecific effects, and is therefore generally used to screen out likely poor or dangerous candidates (Burdick et al., 2014; Burel et al., 2015; Dieckmann et al., 2015; Kamola et al., 2015; Lindow et al., 2012; Shen et al., 2019). Because cytotoxic mechanisms resulting from off-

target RNA cleavage may be speciesand cell type-specific, this screen should be performed in clinically relevant human cell lines. For ASOs intended to be administered systemically, the primary *in vivo* organs of accumulation are the liver and kidney, making cell lines of this origin especially useful for screening. Cytotoxicity screening may be performed in patient-derived cells differentiated into iPSCs differentiated into a relevant cell type. For example, ASOs being developed for intrathecal administration could be screened using neuronal cells differentiated from patient-derived iPSCs or iPSCs engineered to carry the mutation of interest. ASO development candidates should not induce cytotoxicity in cell lines or human peripheral blood mononuclear cells (PBMCs) at active concentrations.

Screening for immune stimulation of ASO candidates is facilitated by the fact that all of the innate immune pathways known to be activated by PS-modified ASOs ultimately trigger one or more of three different categories of signaling pathways in PBMC. These are 1) interferon response factors leading to secretion of type I IFN and IFN-inducible chemokines; 2) NFkB leading to IL-6 and/or TNF-α secretion; and 3) inflammasome activation leading to IL-1b secretion **(Figure 4)**. Thus, by assaying PBMC supernatants for these products (typically performed after a 24-48 h incubation) the degree of innate immune activation by the ASO can be compared to that of positive controls. It is generally not necessary to determine the specific innate immune pathways that responded to any particular ASO. Human PBMC (or perhaps whole blood) assays should be optimized for the specific ASO design and chemistry (Forsbach et al., 2008). Incorporation of 2'-sugar modifications and 5-methyl cytosine can reduce but not eliminate innate immune activation by PS-modified ASO (Kandimalla et al., 2001).

Preclinical Safety Screening In Vivo. Depending on the chemical platform, protocols for *in vivo* screening are relatively well informed based on the available published literature. However, it is advised to carefully consider the particular sequence and chemical modifications for any given candidate. For ASOs containing 2'MOE, LNA, or cEt that are intended to be administered systemically, the primary *in vivo* organs of accumulation are the liver and kidney. The most common drug class effects include hepatotoxicity, renal toxicity or proinflammatory effects. A common *in vivo* approach to screen out toxic ASOs in mice is to dose the candidates at a total dose of 100 mg/kg and to evaluate kidney, lymphoid and liver histology (as well as other tissues) and function after 4 to 8 weeks (Berman et al., 2014). The dose should be at least 3 to 5 times higher than the expected human dose. There might be some signal of target organ effects, but this protocol should be sufficient to eliminate poorly tolerated sequences.

The optimal *in vivo* screening protocols for intrathecally administered drugs are not as far along in the process based on the relatively nascent stage of nonclinical and clinical safety testing. At this point, only single stranded 2'MOE and LNA phosphorothioate ASOs have advanced far enough in clinical trials for larger disease indications to even be considered for intrathecal N-of-1+ INDs. The technical challenges around the dose, route, species selection and endpoint design for intrathecal ASOs are still evolving. Preliminary experience does suggest that relatively acute rodent screening protocols can be developed to eliminate poorly tolerated sequences, but the validation with long-term human safety is still being acquired. The expectation is that similar protocols will be developed that can confidently translate a constellation of effects at high doses in rodents to acceptable human safety. It is advisable to involve experts in the field when designing and evaluating ASOs to be delivered via intrathecal injection.
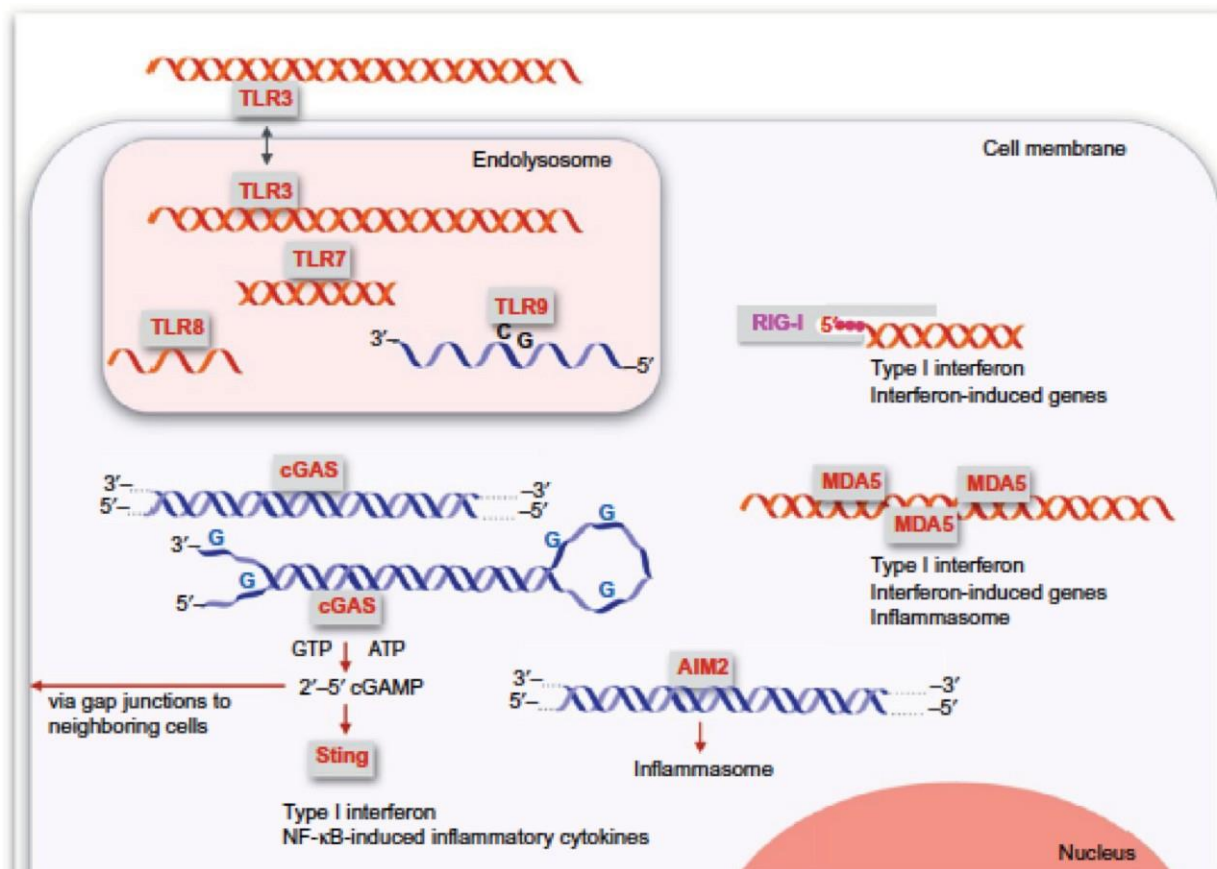
Figure 4: Immune-sensing receptors-detecting foreign nucleic acids and inducing indirect effector responses. TLR3 is the only receptor which, besides its endosomal localization, is also reported to be expressed on the cell membrane. TLR3 binds long double-stranded RNA which is not present in the cytosol of normal cells. TLR3 is expressed in myeloid immune cells and in a number of somatic cells including fibroblasts and endothelial cells. The other three TLRs expressed in the endolysosomal compartment of distinct immune cell subsets are TLR7, TLR8, and TLR9. TLR7 detects even short RNA, preferentially double-stranded, and containing G and U nucleotides. TLR8 detects single-stranded RNA. While TLR8 is expressed in human myeloid immune cells, TLR7 and TLR9 are predominantly expressed in human B cells and plasmacytoid dendritic cells. TLR9 detects single-stranded DNA containing unmethylated CpG dinucleotides. In the cytoplasm, RIG-I specifically detects RNA if it contains at least a short double strand with a blunt end and a 5'-triphosphate. The RIG-I-like receptor MDA5 detects long irregular forms of double-stranded RNA, but the exact definition of the ligand structure is unclear. Both RIG-I and MDA5 are widely expressed in immune cells and nonimmune cells, and induce a broad array of cell autonomous and extracellular antiviral responses including the production of type I interferon. MDA5 ligands also activate multiple other receptor pathways that depend on the detection of long double-stranded RNA, including PKR, ADAR1, and TLR3. The cytosolic receptor AIM2 detects long double-stranded DNA and activates the inflammasome. The other key receptor for the detection of DNA in the cytoplasm is cGAS. cGAS is activated by long double-stranded DNA and short forms of double-stranded DNA with single-stranded overhangs containing G nucleotides, a structure which was termed Y-form DNA and which is presented during retroviral infection or by endogenous retroelements. Upon activation, cGAS catalyzes the formation of 2'-5'-cGAMP from GTP and ATP. 2'-5'-cGAMP acts as a second messenger which binds to the downstream signaling protein Sting which induces type I interferon via TBK1 and IRF3. 2'-5'-cGAMP can travel alert neighboring cells via gap junctions. Sting also induces NF-κB activation and inflammatory cytokines (G. Hartmann, 2017).

2.4. STEP 3: Drug Manufacturing, Safety Testing, IND Preparation, and Patient Treatment

2.4.A. Production of Clinical Grade ASOs

When ASOs are to be used in humans, they must be prepared at clinical grade using good manufacturing practice (GMP). For the purpose of N-of-1+ applications, GMP should be at the level expected for phase 1 clinical testing. For more information, we refer the reader to the appendix and to

guidance documents produced by regulatory agencies for GMP production of ASOs and therapies for phase 1 clinical trials. The appendix presents a collection of proposed descriptions and tasks that could be required for an application. However, the specific requirements should be discussed with the appropriate regulatory agency and generally depend on the complexity of the API, and its manufacturing process or uniqueness in mode of action. For oligonucleotide candidates that do not fall within the designs and chemistries with a proven track record of adequate safety or generating clinical benefit in patients, additional requirements, e.g. residuals assessment, additional biochemistry, control strategies, etc., might be required.

2.4.B.   Safety Studies for Investigational New Drug (IND) Preparation

Once one or more lead candidates are selected for further development, the toxicology evaluation should be designed to suit the patient's needs, the nature of the target, and the chemical platform. For patients with a clear genetic basis for disease and urgent medical need, regulatory agencies have allowed quite minimalistic toxicology studies to support the initiation of patient treatment. An example is repeat-dose rodent toxicology studies that are initiated prior to the patient exposure. If justification can be made, the pharmacokinetics and supporting toxicology studies may be waived or minimized to save time and expense. However, the underlying foundation for such an approach is an established nonclinical and clinical safety experience that can be used to interpret the relevance of the single rodent toxicology studies. Tolerance for safety concerns and risks is directly related to the severity of the disease and the progression of the disease. For example, for very severe progressive diseases, treatment may initiate concurrently with repeat-dose rodent toxicology studies after safety has been established in single dose escalation studies. However, these decisions should be made jointly by the clinician and the patient or care takers, with input from regulatory authorities. Stopping criteria should be established before initiating treatment.

For now, the focus on our initiative is on developing ASOs for patients with a rapidly progressive disease (<12 months to severe outcome), since they have the largest unmet clinical need. For these patients, we propose that prior to the investigator filing an investigator IND for the experimental ASO, single dose four-week screening toxicity studies be performed on the lead ASOs. If the ASO is to be used to treat a neurological disorder intrathecally, an eight-week mouse study should be conducted after which the investigator IND will be filed on the chosen ASO with no additional toxicity data. During the clinical evaluation, 3-month studies of the human ASOs in mice would be conducted. For patients with a more slowly progressing disease the studies above would be performed prior to filing the investigator IND.

2.4.C.   Patient Treatment, Clinical Safety, and Patient Therapy Issues

Notably, there is as yet little expertise with treating very small groups of patients with individualized ASOs. As such, recommendations are general for now.

Patient Treatment. As mentioned throughout the document, patients to be treated (or their caretakers) should be actively involved in discussions about the potential risks, discomforts and benefits of the ASO therapy. Informed consent should be in place, and include assent, when appropriate. Further, during the informed consent discussion, it should be made clear the limits of the information known regarding foreseeable risks (and potential benefits, as well). The dialogue with the patient/ caretakers should start during ASO development and continue during treatment. Stopping criteria should be defined before starting the treatment and reevaluated during treatment. Patients should be closely monitored for potential beneficial and adverse effects of the treatment. Inclusion of a patient or medical advocate in the informed consent process would be optimal and well-advised.

Clinical Safety Issues. The most common adverse events seen in the clinical development of a

systemically administered ASO are transient, dose-dependent injection site reactions and flulike symptoms, most likely resulting from the inherent innate immune activation by PS-modified oligonucleotides (Levin, 1999; Bjersing et al., 2004). TLR-mediated innate immune activation in humans treated with a systemic ASO may be detected by elevation of serum cytokines and interferon-inducible chemokines such as CXCL10 at 24 h after each dose (Krieg et al., 2004). Elevation of liver enzymes or reduction of kidney function can occur and should be monitored but is rarely clinically significant (Berman et al., 2014). Chronic skin changes at sites of subcutaneous injection can also be seen (van Meer et al., 2016).

Intrathecal ASO administration can induce innate immune activation in rodents (Toonen et al., 2018) but the potential relevance of this observation to humans is unclear. Consideration should be given to monitoring the cerebrospinal fluid (CSF) in patients receiving intrathecal ASO for changes in the number and activation state of immune cells and the concentration of ASO-inducible cytokines and chemokines during therapy.

In general, innate immune activation by ASO occurs *in vivo* at lower concentrations than those required to achieve antisense effects, which is one reason why subcutaneous ASO delivery typically induces injection site reactions and flu-like symptoms even at subtherapeutic doses. All the major safety issues associated with ASOs appear to be dose-dependent, and so their frequency and severity is likely to fall as continued improvements in ASO technology provide reduced dosing.

3. Towards a platform for N-of-1+ therapy development

The aim of the briefing document is to provide the reader with the current state of the art to facilitate development of N-of-1+ therapy development and to initiate discussion around this topic. We are aware that oligonucleotide therapy development for N-of-1+ patients is in its infancy and there are many unresolved questions. These include but are not limited to:

- Identifying the bottlenecks in the key phases of therapy development which limit wider delivery for N-of-1+ cohorts.
- Identify opportunities for development of N-of-1+ cohort oligonucleotide therapies in the most safe and efficient manner.
- Optimal sharing of information to ensure alignment of efforts and sharing of expertise (i.e., a database of mutations for which oligonucleotide therapies are being developed or a database of sequences tested in safety studies).
- How to best facilitate and pay for the development of oligonucleotide therapies for N-of-1+ cohorts (i.e., in an academic setting through philanthropy funding, through initiatives like N-Lorem, or through national health agencies). Currently fundraising is often carried out by the families. However, this puts immense stress on caretakers of individuals with a rare progressive disease. The burden of trying to raise funds and coordinate the therapy development is added to the burden of caring for someone with a debilitating disease, as well as the financial and psychological implications and time constraints involved.
- How to assure fair access to N-of-1+ oligonucleotide therapies (i.e., establishing a database of available N-of-1+ therapies that are used in patients so that compounds or ASO sequences can be shared)

Input from all stakeholders (patients and patient advocates, academic and clinical experts, industry experts, regulators and payers) is required for this discussion. We welcome input to the briefing document and hope to gain insight into the outlined questions and other issues related to the topic of N-of-1+ cohort oligonucleotide therapy development through the comment box.

4. References

Aartsma-Rus, A. (2012) Overview of AON design.*Methods Mol. Biol.*, 867:1 7-129.

Aartsma-Rus, A., and Corey, D.R. (2020) The 10th Oligonucleotide Therapy Approved: Golodirsen for Duchenne Muscular Dystrophy. *Nucl. Acid Therap.*, 30:67-70.

Berman, C.L., Cannon, K., Cui, Y., Kornbrust, D.J.,
Lagrutta, A., Sun, S.Z., Tepper, J., Waldron, G., and Younis, H.S. (2014) Recommendations for Safety Pharmacology Evaluations of Oligonucleotide-Based Therapeutics. *Nucleic Acid Ther.*, 24:291-301.

Bjersing, J.L., Eriksson, K., Tarkowski, A., and Collins, L.V. (2004) The Arthritogenic and Immunostimulatory Properties of Phosphorothioate Oligodeoxynucleotides Rely on Synergy Between the Activities of the Nuclease-Resistant Backbone and CpG Motifs. *Inflammation*, 28:39-51.

Boycott, K.M., Lau, L.P., Cutillo, C.M., and Austin,C.P. (2019) International Collaborative Actions and Transparency to Understand, Diagnose, and Develop Therapies for Rare Diseases. *EMBO Mol. Med.*, 1 :e10468.

Burdick, A.D., Sciabola, S., Mantena, S.R., Hollingshead, B.D., Stanton, R., Warneke, J.A., Zeng, M., Martsen, E., Medvedev, A., Makarov, S.S., Reed, L.A., Davis, J.W. 2nd, and Whiteley, L.O. (2014) Sequence Motifs Associated with Hepatotoxicity of Locked Nucleic Acid -Modified Antisense Oligonucleotides. *Nucleic Acids Res.*, 42:4882-4891.

Burel, S.A., Hart, C.E., Cauntay, P., Hsiao, J.,Machemer, T., Katz, M., Watt, A., Bui, H.H., You-
nis, H., Sabripour, M., Freier, S.M., Hung, G., Dan,A., Prakash, T.P., Seth, P.P., Swayze, E.E., Bennett, C.F., Crooke, S.T., and Henry, S.P. (2015) Hepatotoxicity of High Affinity Gapmer Antisense Oligonucleotides is Mediated by RNase H1 Dependent

Promiscuous Reduction of Very Long Pre-mRNATranscripts. *Nucleic Acids Res.*, 44:2093-2109.

Chi, X., Gatti, P., and Papoian, T. (2017) Safety of Antisense Oligonucleotide and siRNA-Based Therapeutics. *Drug Discov. Today*, 22: 823-833.

Crooke, S.T., Baker, B.F., Kwoh, T.J., Cheng, W.,Schulz, D.J., Xia, S., Salgado, N., Bui, H.H., Hart,C.E., Burel, S.A., Younis, H.S., Geary, R.S., Henry, S.P., and Bhanot, S. (2016) Integrated Safety Assessment of 2'-O-Methoxyethyl Chimeric Antisense Oligonucleotides in Nonhuman Primates and Healthy Human Volunteers. *Mol. Ther.*, 24, 1771–1782.

Crooke, S.T. (2017) Molecular Mechanisms of Antisense Oligonucleotides. *Nucleic Acid Ther.*, 27:70-77.

Deleavey, G.F., and Damha, M.J. (2012) Designing Chemically Modified Oligonucleotides for Targeted Gene Silencing. *Chem. Biol.*, 19:937-954.

Dieckmann, A., Hagedorn, P.H., Burki, Y., Brügmann, C., Berrera, M., Ebeling, M., Singer, T., and Schuler, F. (2018) A Sensitive In Vitro Approach to Assess the Hybridization-Dependent Toxic Potential of High Affinity Gapmer Oligonucleotides." *Mol. Ther. Nucleic Acids*, 10:45-54.

Flierl, U., Nero, T.L., Lim, B., Arthur, J.F., Yao, Y.,Jung, S.M., Gitz, E., Pollitt, A.Y., Zaldivia, M.T., Jandrot-Perrus, M., Schäfer, A., Nieswandt, B., Andrews, R.K., Parker, M.W., Gardiner, E.E., and Peter,K. (2015) Phosphorothioate Backbone Modifications of Nucleotide-Based Drugs are Potent Platelet Activators. *J. Exp. Med.*, 212:129–137.

Forsbach, A., Nemorin, J.G., Montino, C., Müller, C., Samulowitz, U., Vicari, A.P., Jurk, M., Mutwiri, G.K., Krieg, A.M., Lipford, G.B., and Vollmer,

J. (2008) Identification of RNA Sequence Motifs Stimulating Sequence-Specific TLR8-Dependent Immune Responses. *J. Immunol.*, 180:3729-3738.Gagnon, K.T., and Corey, D.R. (2019) Guidelines for Experiments Using Antisense Oligonucleotides and Double-Stranded RNAs. *Nucleic Acid Ther.*, 29:116-122.

Hair, P., Cameron, F., and McKeage, K. (2013) Mipomersen Sodium: First Global Approval. *Drugs*, 73:487-493.

Hartmann, G. (2017) Nucleic acid immunity. *Adv. Immunol.*, 133:121-169.

Jirka, S.M., Tanganyika-de Winter, C.L., Boertje-van der Meulen, J.W., van Putten, M., Hiller, M., Vermue, R., de Visser, P.C., and Aartsma-Rus, A. (2015) Evaluation of 2'-Deoxy-2'-fluoro Antisense Oligonucleotides for Exon Skipping in Duchenne Muscular Dystrophy. *Mol. Ther. Nucleic Acids*, 4:e265.

Kamola, P.J., Kitson, J.D., Turner, G., Maratou, K., Eriksson, S., Panjwani, A., Warnock, L.C., Douillard Guilloux, G.A., Moores, K., Koppe, E.L., Wixted, W.E., Wilson, P.A., Gooderham, N.J., Gant, T.W., Clark, K.L., Hughes, S.A., Edbrooke, M.R., and Parry, J.D. (2015) In Silico and In Vitro Evaluation of Exonic and Intronic Off-Target Effects Form a Critical Element of Therapeutic ASO Gapmer Optimization. *Nucleic Acids Res.*, 43:8638-8650.

Kandimalla, E.R., Yu, D., Zhao, Q., Agrawal, S. (2001) Effect of Chemical Modifications of Cytosine and Guanine in a CpG-Motif of Oligonucleotides: Structure-Immunostimulatory Activity Relationships. *Bioorg. Med. Chem.*, 9:807-813.

Khvorova, A., and Watts, J.K. (2017) The Chemical Evolution of Oligonucleotide Therapies of Clinical Utility. *Nat. Biotechnol.*, 35:238-248.

Kim, J., Hu, C., Moufawad El Achkar, C., Black, L.E., Douville, J., Larson, A., Pendergast, M.K., Goldkind, S.F., Lee, E.A., Kuniholm, A., Soucy, A., Vaze, J., Belur, N.R., Fredriksen, K., Stojkovska, I., Tsytsykova, A., Armant, M., DiDonato, R.L.,

Choi, J., Cornelissen, L., Pereira, L.M., Augustine, E.F., Genetti, C.A., Dies, K., Barton, B., Williams, L., Goodlett, B.D., Riley, B.L., Pasternak, A., Berry, E.R., Pflock, K.A., Chu, S., Reed, C., Tyndall, K.,Agrawal, P.B., Beggs, A.H., Grant, P.E., Urion, D.K.,Snyder, R.O., Waisbren, S.E., Poduri, A., Park, P.J., Patterson, A., Biffi, A., Mazzulli, J.R., Bodamer, O., Berde, C.B., and Yu, T.W. (2019) Patient-Customized Oligonucleotide Therapy for a Rare Genetic Disease." *N. Engl. J. Med.*, 381:1644-1652.

Kinberger, G.A., Prakash, T.P., Yu, J., Vasquez, G.,Low, A., Chappell, A., Schmidt, K., Murray, H.M.,Gaus, H., Swayze, E.E., Seth, P.P. (2016) Conjugation of Mono and Di-GalNAc Sugars Enhances the Potency of Antisense Oligonucleotides via ASGR Mediated Delivery to Hepatocytes. *Bioorg. Med. Chem. Lett.*, 26:3690-3693.

Kole, R., Krainer, A. R., and Altman, S. (2012) RNA Therapeutics: Beyond RNA Interference and Antisense Oligonucleotides. *Nat. Rev. Drug Discov.*, 11:125-140.

Krieg, A.M., Efler, S.M., Wittpoth, M., Al Adhami, M.J., and Davis, H.L. (2004) Induction of Systemic TH1-Like Innate Immunity in Normal Volunteers Following Subcutaneous But not Intravenous

Administration of CPG 7909, a Synthetic B-Class CpG Oligodeoxynucleotide TLR9 Agonist. *J. Immunother.*, 27:460-471.

Monia, B.P., Lesnik, E.A., Gonzalez, C., Lima, W.F., McGee, D., Guinosso, C.J., Kawasaki, A.M., Cook, P.D., and Freier, S.M. (1993) Evaluation of 2'-Modified Oligonucleotides Containing 2'-Deoxy Gaps as Antisense Inhibitors of Gene Expression. *J. Biol. Chem.*, 268:14514-14522.

Levin, A.A. (1999) A Review of the Issues in the Pharmacokinetics and Toxicology of Phosphorothioate Antisense Oligonucleotides. *Biochim. Biophys. Acta*, 1489:69-84.

Lindow, M., Vornlocher, H.P., Riley, D., Kornbrust, D.J., Burchard, J., Whiteley, L.O., Kamens, J.,Thompson, J.D., Nochur, S., Younis, H., Bartz, S., Parry, J., Ferrari, N., Henry, S.P., and Levin, A.A. (2012) Assessing Unintended Hybridization-Induced Biological Effects of Oligonucleotides. *Nat. Biotechnol.*, 30:920-923.

Rigo, F., Hua, Y., Chun, S.J., Prakash, T.P., Krainer, A.R., and Bennett, C.F. (2012) Synthetic Oligonucleotides Recruit ILF2/3 to RNA Transcripts to Modulate Splicing. *Nat. Chem. Biol.*, 8:555-561.

Rodriguez, J.M., Elías, F., Fló, J., López, R.A., Zorzopulos, J., and Montaner, A.D. (2006) Immunostimulatory PyNTTTTGT Oligodeoxynucleotides: Structural Properties and Refinement of the Active Motif. *Oligonucleotides*, 16:275-285.

Scharner, J., Ma, W.K., Zhang, Q., Lin, K.T., Rigo, F., Bennett, C.F., and Krainer, A.R. (2020) Hybridization-Mediated Off-Target Effects of Splice-Switching Antisense Oligonucleotides. *Nucleic Acids Res.*, 48:802-816.

Senn, J.J., Burel, S., and Henry, S.P. (2005) Non-CpG-Containing Antisense 2'-Methoxyethyl Oligonucleotides Activate a Proinflammatory Response Independent of Toll-like Receptor 9 or Myeloid Differentiation Factor 88. *J. Pharmacol. Exp. Ther.*, 314:972-979.

Shen, X., and Corey, D. R. (2018) Chemistry, Mechanism and Clinical Status of Antisense Oligonucleotides and Duplex RNAs. *Nucleic Acids Res.*, 46:1584-1600.

Shen, W., De Hoyos, C.L., Migawa, M.T., Vickers, T.A., Sun, H., Low, A., Bell, T.A. 3rd, Rahdar, M.,Mukhopadhyay, S., Hart, C.E., Bell, M., Riney, S., Murray, S.F., Greenlee, S., Crooke, R.M., Liang, X.H., Seth, P.P., and Crooke, S.T. (2019) Chemical Modification of PS-ASO Therapeutics Reduces Cellular Protein-Binding and Improves the Therapeutic Index." *Nat. Biotechnol.*, 37:640-650.

Slade, A., Isa, F., Kyte, D., Pankhurst, T., Kerecuk, L., Ferguson, J., Lipkin, G, Calvert, M. (2018) Patient Reported Outcome Measures in Rare Diseases: A Narrative Review. *Orphanet J. Rare Dis.*, 13:61.

Springer, A.D., and Dowdy, S.F. (2018) GalNAc-siRNA Conjugates: Leading the Way for Delivery of RNAi Therapeutics. *Nucleic Acid Ther.*, 28:109-118.

Stein, C.A., and Castanotto, D. (2017) FDA-Approved Oligonucleotide Therapeies in 2017. *Mol. Ther.*, 25:1069-1075.

Toonen, L.J.A., Casaca-Carreira, J., Pellisé-Tintoré, M., Mei, H., Temel, Y., Jahanshahi, A., and van Roon-Mom, W.M.C. (2018) Intracerebroventricular Administration of a 2'-O-Methyl Phosphorothioate Antisense Oligonucleotide Results in Activation of the Innate Immune System in Mouse Brain." *Nucleic*

Acid Ther., 28:63-73.

Van Meer, L., Moerland, M., Gallagher, J., van Doorn, M.B., Prens, E.P., Cohen, A.F., Rissmann, R., and Burggraaf, J. (2016) Injection Site Reactions After Subcutaneous Oligonucleotide Therapy. *Br. J. Clin. Pharmacol.*, 82:340-351.

Vollmer, J., Janosch, A., Laucht, M., Ballas, Z.K., Schetter, C., and Krieg, A.M. (2002) Highly Immunostimulatory CpG-Free Oligodeoxynucleotides for Activation of Human Leukocytes. *Antisense Nucleic Acid Drug Dev.*, 12:165-175.

Wan, W.B., and Seth, P.P. (2016) The Medicinal Chemistry of Therapeutic Oligonucleotides. *J. Med. Chem.*, 59:9645-9667.

Appendix 1 – GMP guidelines for ASO production

32S part Active Substance

32S1 General information

The active substance, [VARIABLE DRUG SUBSTANCE], is a uniformly modified 2'-O-(2-methoxyethyl) or gapmer [to be adjusted according to the situation] phosphorothioate antisense oligonucleotide consisting of NN nucleotide residues with the sequence 5'-[VARIABLE DRUG SUBSTANCE]-3'.

The chemical name of VARIABLE DRUG SUBSTANCE sodium is [VARIABLE] corresponding to the molecular formula $C_{n1}H_{n2}N_{n3}O_{n4}P_{n5}S_{n6}Na_{n7}$ and has a relative molecular mass of TBC g/mol and the following structure:

Figure X1. Structural formula of [VARIABLE] sodium to be inserted.

The molecular weight, empirical formula, and molecular structure of [VARIABLE DRUG SUBSTANCE] will be confirmed using mass spectrometry (MS) and ^{31}P NMR.

The nucleotide sequence of [VARIABLE DRUG SUBSTANCE] will be determined by ESI MS/MS analysis – alternatively an IP-HPLC-TOF-MS failure sequence analysis method could be used.

[VARIABLE DRUG SUBSTANCE] is a mixture of multiple stereoisomers due to the presence of chiral centres at each phosphorothioate linkage.

The active substance is a white to yellow hygroscopic amorphous solid which is freely soluble in water.

32S Manufacture, characterisation and process controls

[VARIABLE DRUG SUBSTANCE] is a synthetic phosphorothioate oligoribonucleotide [to be adjusted depending on the nature of each individual product]. It is manufactured in one manufacturing site that is GMP compliant [to be defined in 32S21].
Drug substance will be released with a cGMP certificate of compliance.

Its manufacturing process will be following the state-of-the-art and best practices in the industry, similar to the process used for all approved oligonucleotide so far. It will consist of five process stages.

1. Solid-phase synthesis
2. Cleavage and deprotection (C&D)
3. Purification
4. Desalting [UF/DF]
5. Freeze drying (production of active substance): The drug substance solution obtained from Step 4 is freeze dried yielding a lyophilized solid [VARIABLE DRUG SUBSTANCE] active substance.

ALTERNATIVELY, the 5 steps in a "DMT-ON" method [Synthesis, C&D, Purification, Final detritylation and isolation, Freeze-drying] could be used.

Details of the different steps will be provided in each individual document in 32S22.

The chemistry used for the synthesis of the single strands is a standard solid-phase phosphoramidite technology. The product is synthesized on a controlled pore glass (CPG) or polymeric (Kinovate type) solid phase support. The first base of each sequence is attached to the support via a 3'-succinyl linkage and a proprietary long chain amino acid (LCAA) linker and is protected with a 5'-dimethoxytrityl group and a base-protecting group. Alternatively, universal support (like Unylinker) can be used.

Synthesis of the oligonucleotide is accomplished by using standard phosphoramidites with 5-

ethylthiotetrazole (ETT) or 4,5-Dicyanoimidazole (DCI) as the activator. For phosphorothioates, the well-known Xanthane hydride or PADS will be used.

If any alternate processing or reprocessing has to be performed in the production of [VARIABLE DRUG SUBSTANCE], a full risk assessment/mitigation, adherence to GMP and client approval would be required.

Control methods for reagents (32S23) will follow industry standard and will be sourced from vendors approved for existing commercial oligonucleotides. Pharma-grade reagents will be used. [To date, regulatory agencies have expected that the manufacturer apply Phase I manufacturing standards for N-of-1.]

Control method of critical steps (32S24) for intermediate isolated following cleavage and deprotection [CRUDE] and purification will be similar to those established for approved products to confirm the manufacturing step is successfully controlled and that the active substance with the intended quality is produced. The specifications and controlled methods for N-of-1 products will be based on those used for similar approved products.

Important critical quality attributes (CQAs) to be confirmed during release testing of [VARIABLE DRUG SUBSTANCE] are:

- appearance,
- identity,
- assay,
- purity, oligonucleotide impurities,
- residual solvents,
- elemental impurities,
- bacterial endotoxins and total aerobic microbial count [Bioburden].

USP methods already showed suitable for similar oligonucleotides will be used when available.

No process validation will be required for N-of-1[VARIABLE DRUG SUBSTANCE]

If some Process development studies were conducted to verify at small scale the validity of generic methods, the development information on those reports will be added to the application and batch details will be shown in 32S26.

We suggest that no experimental failure mode and effect analysis (FMEA) risk assessment should be done to the manufacturing process, procedures, and controls used in the synthesis of the active substance. However, we further recommend that a risk assessment be performed to evaluate how closely the proposed compound and proposed process falls within examples of approved products' manufacturing processes. If closely related, the risks at various control points across the active substance manufacturing process have been identified, evaluated and mitigated to control the risk to acceptable levels for approved products. The control strategy consists of control of material attributes, presumed critical process parameters, equipment including synthesizers, columns, synthesis solid support and column packing, and extractables of the solid support and the purification resin and synthesis parameters in process controls and release testing. The manufacturing process performance will be evaluated as out of trend results as part of the continued manufacture of the drug product.

32S3 – Characterization

The characterization of the active substance and its impurities are in accordance with the FDA/ICH/EU guidelines on chemistry of new active substances.

The potential impurities of [VARIABLE DRUG SUBSTANCE] consist of:

1. impurities derived from starting material and reagents;
2. process-related impurities;
3. degradation products.

Main impurities will be tentatively identified using LC-(ESI)-MS. An impurity profile reporting all impurities above 0.2% will be established on the basis of the TOX batch.

32S4 Control of drug substance – Specification

The active substance specification includes tests for appearance (visual inspection),

- identity (LC/MS; sequencing: see above; sodium counter ion: ICP-OES or ICP-MS),
- assay (UV or HPLC),
- purity (Full Length n) (LC-UV-MS),
- impurities (LC-UV-MS),
- residual solvents (GC),
- elemental impurities (ICP-MS),
- water content (Karl Fisher),
- bacterial endotoxins (USP/Ph. Eur.) and
- microbial enumeration test (USP/Ph. Eur.).

Analytical methods and those validated will be described [32S42, 32S43]

A justification for each attribute and the respective acceptance criteria in the active substance specification will be provided [32S45]. Specification limits should be based on ICH requirements, process capability and variability, considering the limited number of batches for such N-of-1 active substances.

Omission of a biological activity test will be based on antisense mechanism of action and the inclusion of a test to verify the correct nucleotide sequence.

Residual solvents included in the specification will be those used in the process.

For elemental impurities a risk assessment approach based on experience with multiple similar active products made using similar process lead us to suggest the following list: As, Cd, Cr, Cu, Fe, Hg, Mn, Mo, Ni, Pb.

The analytical methods will adequately be described and (non-compendial methods) appropriately qualified in accordance with the ICH guidelines. We suggest not to fully validate all the methods for N-of-1 but rather focus on specificity with limited forced degradation. The focus should be that the method is stability indicating to enable downstream stability laydowns.

32S6 Container – closure

The active substance will be stored in a multi-component container closure system. The bags will comply with the FDA and EU guidelines, and specifications as certificates of analysis for them were provided.

32S7 Stability

Stability data on a preliminary demonstration batch if available and on the clinical batch from the proposed manufacturer stored in a container closure system representative of that intended for the clinical use will be carried out for up to 36 months under long term conditions at -20 ± 5°C and for up to 6 months under accelerated conditions at 5 ± 3°C. Data from 3 months storage at 40 ± 2°C/65± 5% RH to simulate temperature excursions should also be provided.

The following parameters should be tested: appearance, assay, purity, impurities. Microbial enumeration test (TAMC and TYMC) and bacterial endotoxins should be tested every 12 months.

A forced degradation study should also be conducted on the demonstration batch if available and one production scale batch to demonstrate the stability indicating attributes of the analytical method used to test purity and impurities. [VARIABLE DRUG SUBSTANCE] would be exposed to light, acidic (HCl 1M), basic (0.1 N NaOH), oxidative (0.03% H_2O_2) and thermal (1 month at 80°C) stress conditions.

32P. Drug Product

Description of the product and Pharmaceutical development

The [VARIABLE] drug product should be a single use, unidose, sterile, preservative-free, white dry powder ready for injection and intended for parenteral [sub-cutaneous, iv, or intrathecal] administration containing N.X mg of [VARIABLE drug substance]. It is to be filled in aseptic conditions in single use vials that nominally containing an appropriate quantity of [VARIABLE drug substance].

The quality product profile (QTPP) should be defined as a single use, sterile, preservative free dry-powder for injection containing [VARIABLE drug substance], stable at -20 °C, packaged in glass vial, which meets pharmacopoeial requirements for parenteral dosage forms and product specific requirements.

All excipients used for reconstitution before use will be well known pharmaceutical ingredients and their quality is compliant with USP / Ph. Eur standards. That part of the process will be described in the "investigator brochure". There should be no new excipient used in the finished product formulation. None of the excipients will be of human or animal origin.

Due to the use of conditions and systems already used for approved oligonucleotides as well as to the simplicity of the process, we suggest that no formal pharmaceutical development of the [VARIABLE drug product] manufacturing should be done.

The manufacturing process will be described in sufficient detail. The use of a sterilisation by sterile filtration could be justified in line with the decision trees for the selection of sterilisation methods (CPMP/QWP/054/98).

Leachables and extractables studies on the container closure systems used for holding the bulk product at the proposed manufacturing sites should be omitted as already approved for similar product.

[VARIABLE drug product] dry-powder for injection should be packaged in an ISO 6R (or smaller, or larger, but similar in nature] type I, clear single-use vial, sealed with a 20-mm fluorinated polymer coated bromobutyl rubber stopper, and capped with a 20-mm aluminium over seal with a plastic flip off cap.

The vial contains a nominal quantity of drug substance depending on application.

The specifications, technical drawings, quality control information and certificates of analysis for the proposed container closure system will be provided. Confirmation will be provided that the rubber stopper material complies with current requirements of applicable regulations. Details of depyrogenation and sterilization cycles employed for the primary containers and the vials together with the respective validation reports were submitted. Confirmation will be provided that the sterilization of the rubber stoppers and sterilisation/ depyrogenation of the glass vials meet applicable regulatory requirements.

Manufacture of the product and process controls

The manufacturing process will consist of the following main steps:

1. receipt and storage of the drug substance at manufacturing site,
2. temperature equilibration of the drug substance,
3. compounding
4. sterilizing filtration
5. vial filling
6. stoppering
7. lyophilization
8. crimping
9. 100% visual inspection of filled vials

Details regarding the description, duration and holding times of different steps of the manufacturing process may vary for different products. The process for N-of-1 drug products is a non-standard manufacturing process by definition as no large batches will be provided. There will likely be no validation protocol of the manufacturing process of the finished product. Nevertheless, adequate in-process controls for this type of manufacturing process will be provided.

Product specification

The finished product specifications will include appropriate tests for this kind of dosage form but maybe not all the traditional ones:

1. appearance
2. identification,
3. assay,
4. purity and impurities,
5. moisture
6. bacterial endotoxins
7. sterility (? Require a large number of vials),
8. container closure integrity

The proposed limits for purity, specified, unspecified and total degradation products in the finished product specification are the same as those proposed for the active substance since no finished product degradation was observed in the long term stability studies for similar products.

A risk assessment for elemental impurities in the finished product was conducted as per ICH Q3D for other similar product. It did confirm that elemental impurities testing does not need to be included in the finished product specification. We suggest extending that to N-of-1

The analytical methods used will mostly be the same as the ones used for the drug substance and will be described and appropriately qualified in accordance with the ICH guidelines with a recommended focus on specificity and other attributes as needed.

A batch analysis results will be provided.

Stability of the product

Stability data of the initial production scale batches of finished product manufactured at one of the proposed manufacturing sites stored under long term conditions for up to 36 months at -20±5°C; and for up to 3 months under accelerated conditions at 25±2°C / 60±5%RH according to the ICH guidelines were provided.

Samples will be tested for appearance, assay, purity, total impurities (coming from API or degradation products possibly generated during manufacturing of drug substance), bacterial endotoxins, sterility, container closure integrity testing – microbiological test will be performed every 12M only.

Adventitious agents

No excipients derived from animal or human origin will be used.