Oligo Safety Working Group Exaggerated Pharmacology Subcommittee Consensus Document

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This document summarizes the current consensus opinion of the Exaggerated Pharmacology (EP) Subcommittee of the Oligonucleotide Safety Working Group on the appropriate strategies to assess potential adverse effects caused by an “exaggerated” degree of the intended pharmacologic activity of an oligonucleotide (ON). The Subcommittee focused its discussions primarily on the ON subclasses that impact expression of “host” (i.e., human gene products—antisense, small interfering RNAs, and related ONs that target messenger RNA), with later and more limited discussions on aptamer, immunostimulatory, and microRNA subclasses. It is expected that many of these principles will be relevant to other subclasses but will need to be carefully considered as those development programs advance towards clinical trials. The recommendations may also serve as a frame of reference when designing Good Laboratory Practice safety studies with ONs, with regard to the study design elements that address assessment of EP. It is also hoped that these recommendations will establish a foundation for discussion with regulatory agencies on this subject.

Objectives

THE PRIMARY GOALS OF THE Exaggerated Pharmacology (EP) Subcommittee of the Oligo-nucleotide Safety Working Group (OSWG) were to discuss the relevance, scope, and strategies for assessing EP with oligonucleotide therapeutics and to disseminate information on this subject by way of conference presentations and publications. The ultimate aim was to develop a consensus between the industry and regulatory agencies as to the best practices to assess the activity and safety of novel oligonucleotide (ON) products once sufficient data have accumulated for the product class. The EP Subcommittee held teleconferences on a monthly basis between July 2008 and September 2010 and sought input from those more familiar with certain classes of ONs. The positions represented in this summary document reflect the consensus from these deliberations.

General Discussion of Strategy

The primary focus of discussions was on the assessment of potential adverse effects caused by an “exaggerated” degree

of the intended pharmacologic activity of an ON. These effects can be differentiated from those stemming from the mechanism of action against unintended targets (i.e., off-target effects), which are the focus of another OSWG Subcommittee.

It was recognized initially that considerations about the necessity and value of assessing EP could differ among the various subclasses of ONs and that it may be logical to discuss the subclasses separately. Four major subclasses of ONs were distinguished by their mechanisms of action: (1) antisense ONs (ASOs); (2) small interfering RNAs (siRNAs) [RNA interference (RNAi) mechanism]; (3) aptamers; and (4) immunostimulatory (IS) ONs. Other types of ONs (e.g., ribozymes, microRNA, hairpins, decoys, etc.) may be the subject of future discussions as more of these products enter development. It is expected, however, that information gained from the initial product subclasses may be applicable to the other types of ONs. Based in large part upon the availability of data, it was agreed that the initial focus of discussions would be on those subclasses of ONs that effect gene expression [i.e., antisense, siRNAs, and other subclasses that inhibit the production of a target gene product, typically via interaction

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with a specific region of the target messenger RNA (mRNA)]. The largest volume of information (and the predominant experience of the Subcommittee members) on this subject was with the ASO subclass, with a growing body of data for siRNAs.

Among those ON subclasses that are intended to inhibit specific gene expression, the need for assessment of EP was considered relevant only for those directed against “host targets” (i.e., human gene products). ONs directed towards non-human targets (e.g., antimicrobial applications) do not require assessment of EP, as there would be no concern regarding human safety for an exaggerated effect on a non-host target. For the IS subclass (i.e., those ONs optimized for that activity), manifestations of intended pharmacologic activity (e.g., lymphoid tissue reactivity, mononuclear cell infiltration, and other types of proinflammatory effects) are typically observed across all commonly used rodent and non-rodent animal species, albeit with well-recognized species differences in sensitivity (see Immunostimulatory ONs section). Therefore, the IS subclass typically does not present any major challenges to demonstrating EP. Similarly, aptamers were considered as a different class for assessment, because of their mechanism of action (i.e., direct binding to protein) and greater propensity to exhibit cross-species activity, as compared with ASOs and siRNA molecules.

Historical Perspective on Regulatory Expectations

The absence of clear regulatory guidance for the preclinical safety evaluation of ONs has made for considerable uncertainty about how to properly address this area of investigation.

Most ONs are regulated as drugs in the Center for Drug Evaluation and Research because they are chemically synthesized molecules. After 2003, all ONs (even those conjugated to proteins) were submitted to the Center for Drug Evaluation and Research. During the early to mid 1990s, most ON development programs were based on the ASO subclass, and the majority of the first-generation ASO drug candidates were directed against viral targets (e.g., human immunodeficiency virus, herpes, papillomavirus, etc.), for which assessment of EP was irrelevant. For the first several ONs directed against host targets, such as intracellular adhesion molecule 1 (ICAM-1) and specific kinases, the scope of the EP investigations undertaken by different sponsors was highly variable, mainly owing to the uncertainty about what would be required by different Food and Drug Administration reviewing divisions or by international regulatory agencies. The absence of guidance during this period is presumably attributable to the fact that this was a novel class of drugs for which the safety issues were not well defined and, for EP assessment in particular, the considerations are complex. In addition, the early ASO programs for which EP was assessed (often using rodent-active analogues) did not reveal dramatic or consistent toxicity stemming from EP. Hence, the initial level of regulatory attention directed towards EP assessment for ONs appeared to be less than that applied to biotherapeutics, for which most adverse effects are related to EP.

Over time, an increasing degree of scrutiny was directed to the assessment of EP for ONs, and questions were typically posed during early-stage regulatory interactions (e.g., pre-IND meetings) regarding cross-species activity and the

sponsor's plans for investigation of EP (i.e., for those ONs targeting human gene products). Particularly for sponsors advancing antisense ONs, the angst about properly discerning the regulatory expectations regarding the scope of EP assessment has been high. The nature of the selection process for identifying antisense ONs with optimal activity in a human system often yields a lead molecule with little or no cross-species activity. One reason for this human specificity is that optimally active antisense ONs often target untranslated regions of the mRNA or other regions that are not well conserved across species. Further, the effectiveness of the hybridization-based destruction of the antisense target mRNA can be substantially undermined by minor non-homology (i.e., “mismatches”) in the mRNA target sequence between the animal species and humans. Depending on the length of the ON, even 1 mismatch can reduce pharmacologic potency, and two or more mismatches can reduce hybridization to an extent that antisense activity is nearly or entirely absent. For many of the early antisense programs, it was not uncommon for the target sequence in non-human primates (i.e., cynomolgus or rhesus monkeys) to differ from that in humans by 1 or 2 nucleotides. Because monkeys are commonly used as the non-rodent species for ON safety evaluation, this circumstance presented resource issues in terms of documenting pharmacologic activity of the ON in monkeys or identifying an analogue that would be more active in monkeys. In some cases, the nucleotide sequence for the target gene product in humans had not been elucidated in the animal species that were chosen for toxicity studies, which raised the daunting prospect of having to perform gene sequencing in an animal species for some molecular targets. In addition to these challenges, questions have been raised about the validity of using animal-active analogues (see the section on the use of animal active analogues below).

Consensus Opinions

This document is intended to serve as a focal point of discussions between industry and regulatory agency representatives, to attempt to identify best practices for assessing EP with oligonucleotides due to uncertainties in discerning regulatory expectations regarding the scope of EP assessment.

The primary focus was on the ON subclasses that impact expression of “host” gene products (antisense, siRNAs, and related ONs that target mRNA), with later and more limited discussions on the aptamer and IS subclasses. Therefore, the outcome of the discussions is presented for each of these types of ONs. Many of the principles are expected to be relevant to other subclasses but will need to be carefully considered as those development programs advance toward clinical trials.

ONs affecting gene product expression

Species selection. A key question initially addressed was how many and which animal species should be used for assessment of EP. Characterization of cross-species pharmacologic activity is important, particularly among the species commonly used for toxicity studies, and with an eye to those species that have been used historically for safety assessment of ONs, such as non-human primates, to enable selection of pharmacologically relevant species. However, the above-described species specificity of ONs often precludes ubiquitous activity in the commonly used animal species. In some

cases, activity can be documented or predicted (based on sequence homology) for 1 species (e.g., monkey) but may be lacking in other common laboratory species. Or, there may be no cross-species activity. Under such circumstances, concerns may arise about the validity of employing animal-active analogues (see below). In order to make judgments about the appropriate scope of EP assessment, the likelihood of encountering adverse effects stemming from EP should be considered. In this regard, only a few examples were identified where EP was expressed in animals that translated into significant toxicity and the Subcommittee could not readily identify ONs for which such expression of EP was a key safety issue that impacted the selection of the starting clinical dose. The absence of numerous examples of serious toxicity attributable to EP with ONs may be related, at least in part, to the often incomplete or modest impact on gene expression from ASOs, which is the subclass that has been most widely investigated. In addition, the toxicity profile of many types of ONs is often dominated by nonspecific “class effects,” particularly for ONs that contain chemically modified backbones that impart a strong anionic character, most notably, those with a phosphorothioate modification. The phosphorothioate modification has been widely employed to confer nuclease resistance and promote longer *in vivo* tissue persistence. For such molecules, the class effects typically manifest at lower dose levels than those required to produce complete inhibition of gene product expression, thereby obscuring or precluding any adverse effects stemming from EP.

More intensive investigation of EP might be justified based on the nature of the target and from well-founded concern over the consequences of knockdown of the specific target, or from the complete absence of information of the consequences of such knockdown. The newer generations of ASOs, as well as the newer subclasses targeting gene product expression (such as siRNAs and microRNAs) appear to be generally more pharmacologically potent, owing to greater *in vivo* stability and/or to their mechanism of action. For these newer types of ONs, greater attention to assessment of EP may be warranted. In general, the level of effort directed towards assessing the safety implications of EP with ONs should be based on the available body of information (case by case), and there should not be rigid requirements about the number of species and conditions for testing. The types of information to be considered in making a judgment about the scope of EP assessment should include: (1) the role of the target gene product and what is known about loss of its function (e.g., from knockout models); (2) the potency and persistence of the ON-induced inactivation; (3) the route of administration (and likelihood of extensive systemic exposure); (4) the expression profile of the specific target; (5) the dosing frequency and duration; and (6) the clinical indication (risk-benefit considerations).

As an example, the level of concern about possible EP-related toxicity may be elevated when the ON is targeting a ubiquitous key regulatory protein, whereas there may be less concern when the ON is directed against a protein that is expressed only in diseased tissue or abnormal cells (e.g., cancer cells). Similarly, there may be more concern about the consequences of EP when the ON is delivered systemically at relatively high doses and is widely distributed into tissues, as opposed to an ON that is administered topically to a discrete

area of skin and is not absorbed systemically. Obviously, the number of possible scenarios is quite large, and more extensive discussion of specific circumstances and considerations is beyond the scope of this document.

In general, it may be appropriate to consider the options for addressing EP in 2 species (i.e., both the rodent and non-rodent species) for cases where ONs act by novel mechanisms and/or where the toxicity profile of the subclass is not well characterized. However, for those ON subclasses under discussion, investigation of EP in 1 species should suffice, unless further investigation is warranted by compelling theoretical concerns or by the results of general toxicity studies that revealed a novel toxicity apparently stemming from EP. Apart from those reservations, assessment in 1 species is consistent with International Conference on Harmonisation (ICH) S6 (R1), where similar limitations in cross-species activity may exist, especially considering the fact that the dose-limiting toxicity of biopharmaceuticals is most often an extension of pharmacologic activity, whereas this has rarely been the case for the ON subclasses under discussion.

Determination of pharmacologic relevance. Another topic of extensive discussion was the level of information that is needed to “validate” a species for investigation of EP. If there is 100% sequence homology between the human and animal target mRNA sequences, the human ON will most likely be active in the animal species, particularly if the human ON had been screened for optimal activity. Under such circumstances, documentation of activity of the ON in the animal species is probably not needed. However when the degree of non-homology is moderate (e.g., 1 or 2 mismatches), the animal species may still be a valid model for assessment of EP (with the human ON), but the uncertainty warrants investigations to substantiate pharmacologic activity, either by demonstrating decreased target gene expression or some other measure of intended activity reflecting decreased gene expression (e.g., efficacy in a relevant animal model). Pharmacologic activity could be confirmed by a relatively simple *in vitro* assay, such as demonstration of reduced target mRNA or protein expression following incubation of the ON in a relevant *in vitro* system that contains the target gene (e.g., a monkey peripheral blood leukocyte preparation). The potency of the human ON in the whole animal or *in vitro* system need not precisely match that of the ON in a human system to be able to extrapolate the findings. This view is supported by the fact that the ON will usually be tested at high clinical-multiple doses in toxicity studies, such that target gene product inhibition will likely be achieved. However, for those programs in which a high clinical-multiple dose level cannot be evaluated in the toxicity studies (e.g., ocular studies), the pharmacologic potency of the human ON in the animal species should be considered in making a judgment about whether EP can be adequately assessed in that species.

When no activity can be documented for a human ON in any of the animal species that are commonly used for toxicity studies, the use of animal-active analogues (surrogates) for assessment of EP should be considered in 1 species (see below).

Case studies. Many cases are intermediate between the extremes of ubiquitous cross-species activity for the human ON and no cross-species activity, and several of these were

discussed. One scenario commonly encountered is when the human ON has 100% sequence homology (i.e., complementarity) with the target mRNA region for 1 animal species (e.g., monkey) and/or documentation of pharmacologic activity in the non-rodent species to be used for toxicity investigations is adequate, and the sponsor additionally has utilized a rodent analogue for pharmacology investigations. In this circumstance, documentation of EP in a single species (e.g., non-human primate) was considered sufficient. Inclusion of the rodent analogue in the good laboratory practice (GLP) toxicity study should not be needed, as EP of the clinical candidate should be adequately assessed in the non-rodent study. This position is also consistent with ICH S6 (R1). However, several developers have set a precedent for investigating the toxicity of a rodent analogue in such circumstances, either because such testing was requested by a Food and Drug Administration reviewing division, or because dialogue with the Agency could not be accomplished prior to initiating toxicity studies and the developer was concerned that assessment of EP in only 1 species (non-rodent) might be perceived as inadequate by the Agency. Importantly, in all of these cases the additional testing with the rodent analogue did not inform about the risks of EP (i.e., no new manifestations of toxicity stemming from EP were identified with the rodent analogue), and in some instances, unclear results were obtained that were difficult to interpret for clinical decision-making (e.g., no adverse findings with the clinical candidate in a relevant non-human primate study and toxicity with the rodent analogue in a rodent study).

In some development programs, the human ON has been inactive in the non-rodent species but active in rodents. In general, the same considerations about the potential adequacy of a 1-species assessment of EP would be applicable, such that EP investigation in a non-rodent species should not absolutely be required. However, in such cases where a non-rodent species analogue is available (e.g., because it had been developed for use in pharmacology studies), the inclusion of that analogue in the non-rodent toxicity study may be considered.

Another scenario is the case of an ON that is not pharmacologically active in the non-rodent species (e.g., non-human primate) or in rodents, but a rodent analogue is available. This circumstance may warrant utilization of the rodent analogue in the GLP toxicity study to address EP (i.e., tested in parallel with the human sequence), but developers should give careful consideration to the concerns outlined below regarding the use of analogues.

Concerns about the use of animal-active analogues. There have been numerous programs with ONs for which an animal-active analogue was developed as a pharmacology tool and was ultimately tested for safety in the same species in a GLP toxicity study. These analogues often have a substantially or completely different nucleotide sequence than the human ON, and hence, they are distinct molecular entities. One concern is that the intensity of class effects can vary substantially among ONs of different sequences, which can result in different manifestations of toxicity between the human ON and analogue that may be construed as evidence of EP but is actually a reflection of some non-pharmacology-based sequence-related difference. Companies with extensive experience in the safety assessment of antisense ONs have

learned over the years that certain ONs exhibit dramatically greater toxicity than other sequences for reasons unrelated to EP, and this anomalous toxicity may occur in approximately 10%–20% of the ONs tested (personal communication among Subcommittee members). The structure–activity relationship for this type of unexpected toxicity is not well understood, and hence, it would behoove sponsors to select active analogues that do not exhibit such properties before including them in a side-by-side comparison with the human ON in toxicity studies. However, because of the sense of urgency for most ON development programs, many sponsors elect to forgo preliminary toxicity screening of active analogues.

In addition, the fact that the analogue possesses a different sequence than the human ON poses a risk that the analogue could, by chance, elicit some type of mechanism-based (e.g., antisense) effect (i.e., an “off-target” inhibition of another gene product) that translates into toxicity. If unexpected toxicity occurred with an analogue, discerning whether the effect reflected EP or an off-target effect may be difficult. There are other possible differences in properties between the human ON and an analogue that are unrelated to EP but could yield differential toxicity that could be construed as evidence of EP. These include differences in pharmacokinetic (PK), biodistribution and tissue stability. Although the PK and tissue distribution profile is typically similar among ONs that are structurally related, there may be some influence of nucleotide sequence on ON disposition that could affect toxicologic potency.

In the event that the human molecule was not species cross-reactive and EP needed to be evaluated in at least 1 species, it may generally be more appropriate to conduct focused safety evaluations with analogues in a disease model in which the target may be over-expressed, with specific investigations of endpoints that would be expected to be affected by the EP, rather than subject the analogue to a general toxicity evaluation in parallel with the human ON. Studies in disease models might be challenging to accomplish and usually cannot always be performed under GLP-compliant conditions. However, the absence of a GLP setting should not be a deterrent from conducting such studies, as long as the execution and record-keeping practices for such studies are sound. However, even with more focused safety studies, the potential to encounter off-target toxicities with analogues exists and could yield results that are difficult to interpret.

The possible utility of analogues for later-stage investigations of reproductive toxicity and/or carcinogenicity was also discussed. For those human ONs that have activity in non-human primates, but not rodents, a rodent analogue may be used to assess EP-related reproductive toxicity, but such judgments should be based, at least in part, on the level of concern or uncertainty about whether inhibited expression of the targeted gene product would be expected to affect reproductive performance. Therefore, while the use of animal active analogues for general toxicity investigations should be approached with caution, it is recognized that such analogues may be the best option for addressing requirements for reproductive toxicity testing. In addition, such analogues may have a role in the carcinogenicity evaluation, considering that these studies are conducted only in rodents; however, such an undertaking should be carefully considered as per the criteria discussed above (i.e., regarding the level of concern about the consequences of target inactivation and other factors).

When evaluating the safety of an animal-active analogue, there are a number of tactical considerations about how to accomplish this. At the outset, it is important to understand the additional resources needed to manufacture and characterize a second test item. The most common paradigm has been to include 1 or more groups to be treated with the analogue in the GLP toxicity study, in parallel with the human sequence. For many programs, sponsors have elected to test only 1 dose level of the analogue, typically matching the high or middle dose level of the human sequence. However, the evaluation of a single dose level presents a risk that, should the group exhibit a unique or more pronounced toxicity that is regarded as a manifestation of EP, a regulatory issue may arise from not having characterized a no-adverse-effect level (NOAEL) for that effect. Hence, careful consideration needs to be given to the number of groups required for proper evaluation of an analogue. As mentioned, prescreening a range of doses may aid this decision process, although it may be more time-efficient to simply include multiple groups in a GLP toxicity study.

Questions were also addressed about whether the use of an active analogue as a test article in a GLP toxicity study should be subject to the same analytical stringency as the primary (human ON) test article. It was the Subcommittee's opinion that the prestudy analysis of the analogue need not be conducted under GLP or GMP conditions, as long as the material is well characterized by methodology similar to that employed for the primary test article (human ON) and the analysis results are well documented. However, as is expected for any study element that is not fully GLP compliant, use of non-GMP material in a GLP study should be addressed in the compliance section of the study report.

Similarly, regarding the dosimetry and/or exposure verification for analogues, some concessions should be allowed relative to full GLP compliance. It was acknowledged that the resource allocation is not trivial, especially for those programs involving multiple ONs with corresponding analogue combinations. For GLP-complaint determination of dosimetry, a sponsor would need to undertake method development and validation to enable analysis of the analogue concentration, homogeneity, and stability in dosing solutions under GLP-compliant conditions, in much the same manner as is done for the human sequence. Hence, most sponsors have elected to forego assessment of dosimetry for analogues. The rationale for this decision is based largely on the fact that the dosing solutions of the analogue are prepared and delivered in the same manner as the primary (human) test article. The absence of dosimetry verification for an animal-active analogue should be cited as an exception to GLP compliance in the study report.

Determination of exposure of the animals to the analogue in the context of a GLP toxicity study would require additional resource allocation in the form of bioanalytical method development and validation prior to study sample analysis. However, because most ONs are administered by intravenous or subcutaneous routes, and because absorption is complete with intravenous dosing and known to be extensive with subcutaneous dosing, exposure of the test system can be assumed to be somewhat similar for an analogue and the human sequence, thereby justifying reliance on the toxicokinetic data obtained with the primary test article (human sequence) to represent the likely exposure of the analogue. The primary caveat to this assumption is that, following systemic absorp-

tion, the *in vivo* stability of the analogue may differ substantially from that of the human sequence, which could translate into a different degree of tissue accumulation with repeated dosing that could dictate a different severity of toxicity. Such differences in toxicity between the analogue and human sequence may be inappropriately construed as evidence of EP. Therefore, it may be prudent to include additional animals in the analogue-treated groups for toxicokinetic (plasma and tissue) sample collection, to be stored frozen and possibly analyzed if an analogue exhibited a different degree of toxicity than the human sequence.

The use of inactive analogues as control articles. Inactive analogues have been used to permit the distinction of EP-related effects from non-pharmacologic effects. This approach is becoming increasingly prevalent for siRNA programs, particularly for those programs in which the siRNA is delivered via a specialized formulation and formulation-based toxicity is expected. This circumstance presents challenges in distinguishing between the effects of the formulation excipients, the non-specific ON class effects, and effects stemming from EP. In such cases, the inclusion of an inactive analogue can be very informative. However, the concerns expressed about the use of active analogues are relevant to the use of inactive analogues (i.e., that anomalous toxicity related to off-target mechanism-based activity or other sequence-dependent effects could manifest and could confound interpretation of the study data). In those cases in which the human ON is devoid of activity in the animal species, the human ON could serve as an inactive control ON and may be the best choice for such a control because it avoids the addition of another inactive sequence to the study design. However, it is important to ensure that the human ON is truly inactive in the animal species.

The role of formulations. Many ON development programs are moving forward with specialized delivery systems, which could impact the likelihood of encountering significant toxicity related to EP. On the one hand, formulations that afford targeted delivery to specific cell types could dramatically increase the potential for adverse manifestations of EP in those cell types. However, if such targeted delivery is directed against cancer cells or cells containing non-host pathogens, and if the pharmacologic objective is cellular destruction, adverse consequences of EP in those cell types may be moot. For several types of specialized formulations, it appears that the toxicity stemming from excipients or other properties of the formulation apart from the ON content may be much more pronounced than any EP effect of the ON, particularly if the formulated ON possesses little or no xenobiotic chemical modification. However, it is difficult to know *a priori* whether the formulation-based toxicity or the ON-related toxicity will be predominant. Hence, the increasing use of delivery formulations presents new considerations about the strategies and scope of EP assessment, and sponsors are encouraged to examine the circumstances of their program(s) and make appropriate proposals for regulatory interactions.

Aptamer ONs

Aptamers are a unique subclass of ONs that are designed to interact with a protein, as opposed to mRNA. The tertiary

structure of ONs is sufficiently complex to provide a rich array of conformations for interacting with the proteins, and a complex progressive selection process is applied to yield a molecule with very high affinity for the target protein, such that binding of the aptamer ON to the protein inhibits the function of the protein. Hence, the anti-human aptamer that is selected possesses a unique structure that is highly specific for the target protein. In this respect, aptamers behave like monoclonal antibodies, in that their cross-species activity may be limited if conservation of the protein sequence and structure across species is poor. Therefore, most aptamers exhibit activity in primate species but typically not in rodents or other non-primate species such as rabbits and dogs.

The leading developers in this space have often relied on pharmacology data from human or non-human primate models, and they have occasionally developed a rodent analogue aptamer to enable *in vivo* pharmacology studies when non-human primate models are not available or feasible. However, because aptamer discovery is a sequential selection process rather than a molecular design process, the analogue will always be substantially different in length, composition, and structure than the anti-human aptamer, and the only common properties may be the homologous target binding and basic nucleic acid structure. The analogue is a valuable tool for performing pharmacology studies of the target biology, but it may not completely mimic the clinical candidate.

Many of the aptamers that are currently undergoing clinical and nonclinical development are polyethylene glycol conjugates (PEGylated), which is commonly introduced to confer desirable pharmacokinetic properties. PEGylation can profoundly affect the activity of an aptamer, which dictates that the final stages of selection of an optimally active human aptamer must be done with PEGylated molecules. However, since the rodent analogue that is employed for target biology investigations is typically not PEGylated, there may be significant differences in the property of the analogue that preclude its valid use in toxicity studies. With PEGylated aptamers, the primary chemistry-related toxicity that is observed is vacuolation of various cells, reflecting uptake of the PEGylated molecule. Hence, the toxicity profile of a non-PEGylated rodent analogue aptamer cannot be compared directly to the PEGylated human aptamer.

For all of these reasons, aptamer companies have generally avoided the use of analogue aptamers in toxicity studies, and they have relied mainly on the testing done with the human aptamer in primate toxicity studies (i.e., 1-species evaluation of EP). As discussed for the other subclasses, judgments about the appropriate scope of EP assessment for aptamers should be made on a case-by-case basis.

Immunostimulatory ONs

Immunostimulatory (IS) ONs are much less species-specific than other subclasses and can exhibit exaggerated pharmacology across most species. The IS ONs interact with Toll-like receptors (TLRs) on immunocompetent cells and trigger intracellular events that translate into various cytokine-mediated responses. Common anatomic pathology findings stemming from the intended IS activity include injection site reactions, lymphoid hyperplasia, cellular infiltration in various organs, and related responses. These effects invariably dominate the

toxicity profile of IS ONs and are dose limiting. Thus, EP is quite evident from pharmacology and toxicity studies of IS ONs, and the main challenge is to determine which animal species is the most appropriate model for human responses.

In this regard, several recent publications have documented the differences between rodent and primate species with respect to the cellular distribution of TLRs, the downstream cytokine responses and other sequelae, and the structure-activity relationships for TLR activation. Discussion of the details of those species differences is beyond the scope of this summary document (this has been addressed by the Immunomodulatory Subcommittee of the OSWG). However, there is a growing body of evidence indicating that the severity and diversity of the anatomic pathology elicited by IS ONs in rodents may not be representative of human responses. Non-human primates appear to be a better model for human responses to IS ONs. Further discussions are needed to help determine whether the rat provides any additional value in the context of general toxicity assessment and first-in-human dose selection.

MicroRNA

Several new subclasses of ONs are emerging that have unique properties and will present new issues for assessment of EP. One of the most promising new subclasses is microRNA (miR). Various strategies to deliver anti-miRs or miR mimetics are being employed, but such programs are currently at an early stage. The mechanism for the modulation of gene regulation by miRs shares some features with the RNAi pathway, but with some important distinctions. MicroRNAs function by using the RNA-induced silencing complex to bind to short "seed regions" in mRNAs, which are typically present within the 3'-untranslated region of mRNAs for multiple interrelated genes. Therefore, unlike other approaches to blocking gene expression that are aimed at 1 target (e.g., antisense and RNAi), the introduction of a miR mimetic or anti-miR typically affects a constellation of largely interrelated gene products. The direct effect of an anti-miR is the derepression of expression (increase), but downstream genes may change in a positive or negative direction (quite commonly in both directions for different gene products). The aim of miR-based therapies is to produce some degree of overall modulation of a biological response by mimicking or inhibiting a disease-related miR. Another important distinction between miR-based therapy and siRNAs and ASOs is that the magnitude of modulation of any particular gene product is modest (typically no more than a 2-fold change), in contrast to the more dramatic inhibition intended for other ONs that affect gene expression. MicroRNAs function by fine tuning levels of expression. Although the experience is limited thus far, the conservation of miRs across species, combined with their mechanism of action, translates into robust cross-species activity, such that most miRs exhibit the desired changes in biomarkers in species commonly used for safety assessment of ONs (i.e., monkeys and rodents). Hence, animal-active analogues are generally not needed to assess EP for this class.

The extent to which the pattern of miR-induced modulation of gene product expression will be analogous across species is uncertain, although, theoretically, there should be considerable commonality. Because of the broader spectrum of mRNA

targets for an individual miR, it may be difficult to distinguish whether any specific change in gene expression reflects intended targeting versus off-targeting versus a compensatory response of the cell. Micro arrays and other means of characterizing changes in gene product expression can be used to obtain snapshots of the modulation profile, as such profiling is central to understanding the miR's pharmacologic response. From such information, one might also glean insights into whether certain ancillary effects may be anticipated based on the observed pattern of alterations in gene expression. However, it is questionable whether this type of information should serve as an impetus for special, dedicated toxicity investigations, apart from standard regulatory safety studies.

In summary, the current approach to identifying potential safety issues associated with the intended pharmacologic action of miRs needs to be considered, particularly because of the novelty of such molecules and the uncertainty about the array of gene targets affected and any downstream consequences of such effects. However, because the miRs currently under development have shown cross-species activity, and because they typically produce only a moderate degree of modulation in gene product expression, there should not be a heightened concern about the likelihood of encountering exaggerated pharmacology with these molecules, and there is no reason to expect that conventional toxicity studies will fail to reveal effects in the EP category that would be relevant to human safety.

Conclusions

The primary consensus recommendations for assessing EP in ON nonclinical safety programs are as follows.

Number of species for EP evaluation: The types of information to be considered in making a judgment regarding the value of 2 species for EP evaluation should include: the role of the target gene product; what is known about loss of its function (e.g., from knockout models); the potency and persistence of the ON-induced inactivation; the route of administration (and likelihood of extensive systemic exposure); the dosing frequency and duration; and the clinical indication (risk-benefit considerations). Different judgments may be made about the scope of the EP investigation for ONs that target a ubiquitous key regulatory protein versus a protein that is expressed only in specific cells (e.g., cancer cells). However, for ONs acting via familiar mechanisms and utilizing familiar chemical modifications, evaluation of EP in 1 species should generally suffice. Note that the recommendations regarding the number and choice of species for evaluation of EP should not be construed as recommendations about how to conduct a general toxicity evaluation of oligonucleotides.

Cross-species sequence homology or activity needed to obviate the use of an animal-active analogue: One-hundred-percent sequence homology between the human and animal target mRNA sequences is usually sufficient to expect pharmacological activity, although it would be desirable to also verify the activity. If there is moderate non-homology (e.g., 1 or 2 mismatches), an animal species can still be a valid model for assessment of EP (with the human ON), but investigations to substantiate pharmacologic activity should be conducted.

Use of animal-active analogues: Inclusion of active or inactive animal analogues for general toxicity investigations should be approached with caution. These analogues often have

substantially or completely different nucleotide sequences than the human ON and, hence, they are distinct molecular entities. As such, there is a risk that different manifestations of toxicity between the human ON and analogue could be construed as evidence of EP, but may actually be a reflection of a non-pharmacology-based sequence-related difference. When analogues are employed in general toxicity studies, a single dose level may suffice (equivalent to the high- or mid-dose level of the human ON), but, in the event that EP effects are observed at single dose level, additional investigations may be needed to characterize the NOAEL for exaggerated pharmacology. Prescreening may aid this decision process, although it may be more time-efficient to simply include multiple groups in a GLP toxicity study. Prestudy analysis of the analogue need not be conducted under GLP or GMP conditions, as long as the material is well characterized by methodology similar to that employed for the primary test article (the human ON), and providing that the absence of GLP compliance for the analytical testing is acknowledged in the study report. It is assumed that dosing solutions of the analogue are prepared and delivered in the same manner as the primary (human) test article and that the *in vivo* disposition of the analogue will be similar to that of the human sequence, which may obviate the need to verify dosimetry and pharmacokinetics of the analogue. While the usefulness of analogues in repeat-dose general toxicity studies is undermined to some extent by the uncertainties about interpretation of findings with a molecular entity possessing a different nucleotide sequence from that of the human ON, such analogues may play an important role in reproductive toxicity and carcinogenicity studies when the human ON is devoid of activity in lower (non-primate) species.

Considerations about ON subclass: Special considerations may apply to the assessment of EP for novel subclasses of ONs, and the paradigms established for antisense and other ONs directed against human gene expression may need to be reconsidered. For immunostimulatory ONs, EP is typically broadly expressed across species and can be readily evaluated in general toxicity studies. For aptamers, particularly those that are PEGylated, the use of rodent analogues lacking PEGylation to assess EP is of questionable validity. The approach for other novel subclasses should be developed on a case-by-case basis with regard to the mechanism of action, cross-species activity, nature of the molecular target, and other factors.

Author Disclosure Statement

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