Understanding the Biomarker Strategy of Antisense Oligonucleotide (ASO) Drugs from Non-Clinical and Clinical Studies

Nick White, PhD;
Clinical Pharmacology & Quantitative Pharmacology, Clinical Pharmacology and Safety Sciences, R&D, AstraZeneca, Cambridge, United Kingdom

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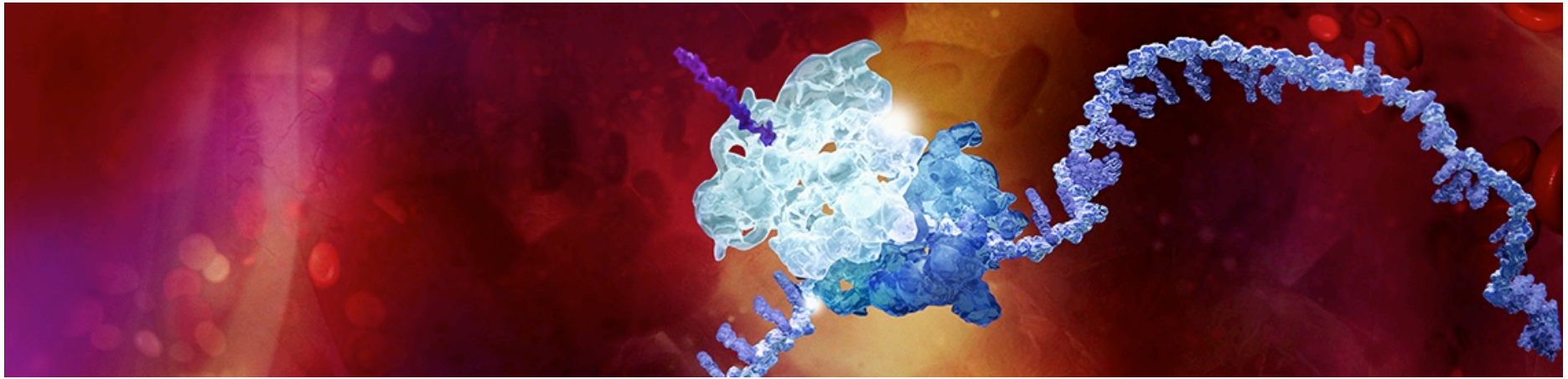
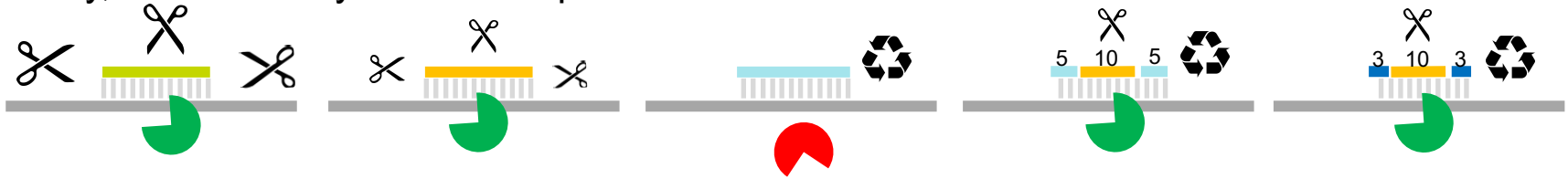


Antisense Oligonucleotides (ASOs)

- Oligonucleotides are unmodified, or chemically modified, single-stranded DNA molecules
- In general, they are relatively short (13–25 nucleotides) and hybridise to a unique sequence of an intracellular target gene
- Oligonucleotides are designed to specifically modulate the transfer of the genetic information to an expressed protein
- In 1978, Zamecnik and Stephenson first demonstrated that an oligonucleotide that is antisense (i.e., complementary) to a viral RNA could reduce protein translation and viral replication
- Oligonucleotides, in their native phosphodiester form, can be readily degraded via intracellular endonuclease & exonuclease activity, usually via 3’ to 5’ activity, leading to cytotoxic metabolites

Chemical Evolution of ASO Modifications

- Various modifications to ASO have been engineered to overcome nuclease activity, binding affinity, bioavailability & cellular uptake



Endogenous
RNA:DNA hybrid recognized and cleaved by RNAse H
+ Good affinity hybrid supporting RNAse H
- Extracellular DNA rapidly degraded and cleared. Limited uptake in vivo

Generation 1
Phosphorothioate (PS) DNA backbone
+ Nuclease resistance, plasma protein binding avoids renal clearance
- Decreased DNA:RNA affinity. Limited uptake in many cell types

Full 2'ribose modification
e.g. 2'O-Me, 2'MOE, 2'LNA, 2'cEt etc…
+ Full nuclease resistance, plasma protein and increased DNA:RNA affinity
- Does not support RNAse H mechanism. Limited uptake in many cell types

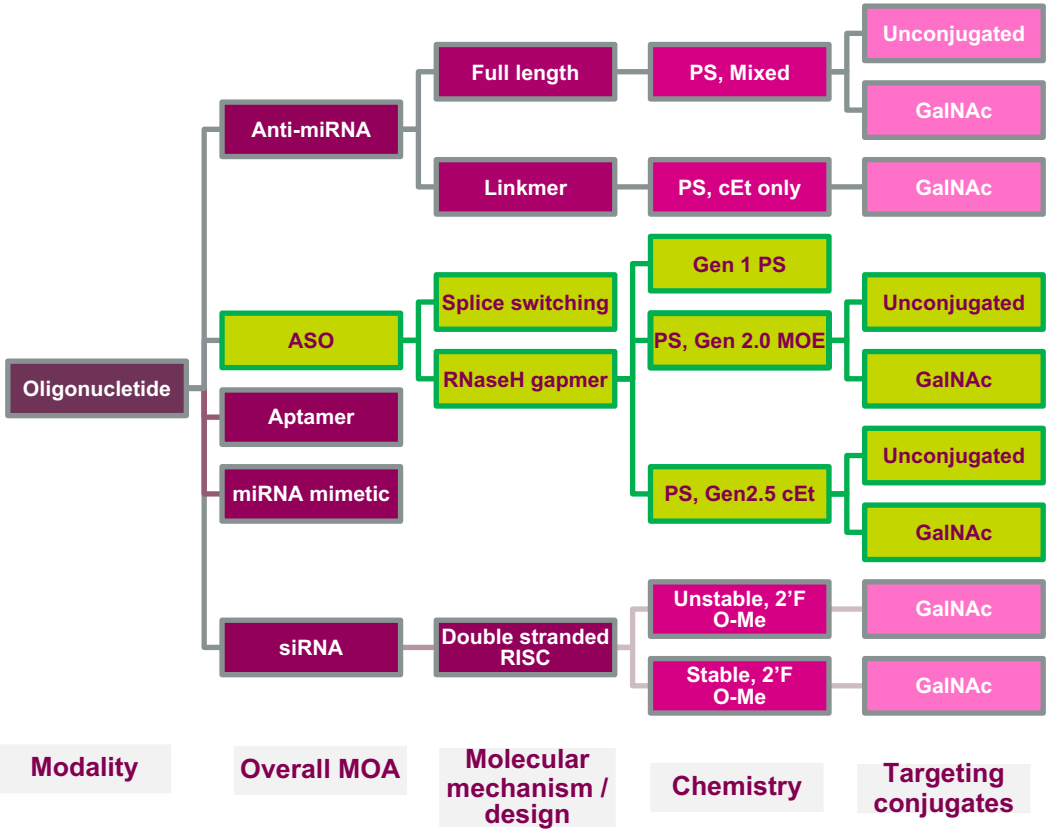
Generation 2
20-mer gapmer design with 2' MOE modified wings flanking DNA
+ Full exonuclease resistance, increased plasma protein, DNA:RNA affinity and support of RNAse H mechanism
- Limited productive uptake in many cells

Generation 2.5
14-16-mer gapmer design with 2' cEt (or LNA) flanking DNA
+ Shorter, full exonuclease resistance, increased plasma protein, DNA:RNA affinity and support of RNAse H mechanism
- Limited productive uptake in many cells. Higher off-target affinity

PS; Phosphorothioate
2'O-Me; 2'-*O*-Methyl
2'MOE; 2'-Methoxyethyl
2'LNA; 2'-Locked Nucleic Acid
2'cEt; constrained ethyl

Adapted from http://dx.doi.org/10.1038/nbt.3765

Oligonucleotide Therapies are a Diverse Family with Multiple Mechanisms-of-Action, Designs and Chemistry's



Antisense Oligonucleotides (ASOs); Main Classes

- On the basis of MoA two classes of antisense oligonucleotide have been described
  - RNase H-dependent oligonucleotides which induce the degradation of mRNA
  - Steric-blocker oligonucleotides, which physically prevent or inhibit the progression of splicing or the translational machinery

RNase H-dependent oligonucleotides

- The majority of the ASO drugs investigated in drug development pipelines function via an RNase H-dependent mechanism
- RNase H is a ubiquitous enzyme that hydrolyses the RNA strand of an RNA/DNA duplex
- RNase H-dependent oligonucleotides can inhibit protein expression when targeted to virtually any region of the mRNA

Steric-blocker oligonucleotides

- Unlike ASO that trigger target degradation, steric-blocking ASOs bind to target transcripts with high affinity
- This steric hindrance may prevent RNASe H-dependent degradation
- Blockade of splicing machinery & pre-mRNA allows manipulation of specific transcripts, redressing splice mutations

Pharmacokinetics (PK) & Pharmacodynamics (PD) of ASOs

- PK properties of ASOs are heavily dependent and largely defined by the chemical and structural architecture and phys-chem-properties of the drug
- In contrast the pharmacophore (i.e. PD properties) is defined by the ASO nucleotide sequence and governs the molecular features that determine target regulation
- Potentially, ASO assisted reduction of targeted RNA expression can be quite efficient, leading to significant down-regulation of target protein and mRNA expression
- Within the non-clinical species, the dose-effect on target gene, within the target organ is **key** to understanding the dose-response relationship following ASO administration and valuable for dose selection and building an exposure-response relationship

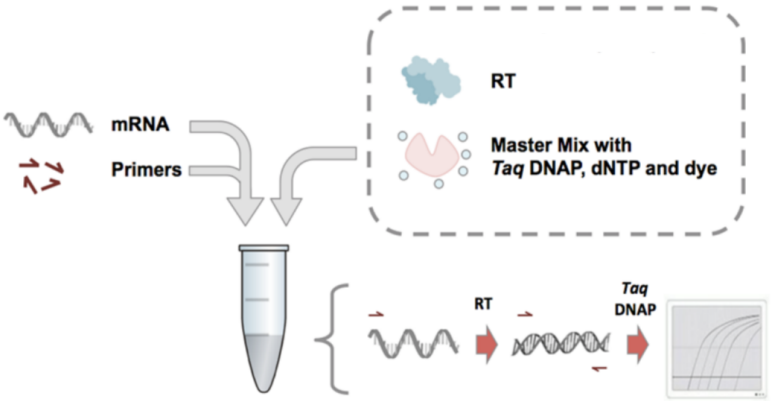
Biomarker Assessment of ASO Therapeutics; Context of Use Drives Analytical Platform and Qualification Level

- The anticipated change in target gene expression needs to be observed in target and secondary tissues
- The development stage at which the BM assessment is associated with
- What the BM data is being used for
- Which stakeholders are utilising these data
- Does the qualification fit with regulatory expectations

RT qPCR; the Most Prevalent Approach to Support mRNA End Points in Non-Clinical Studies

- Historically, the most commonly applied RT qPCR technique to support mRNA end-points where ΔΔCt methods, an approach that determines the change in the analyte RNA relative to reference/house-keeping RNA
- Such methodologies have their obvious limitations
- The favoured approach within AZ and our CRO vendors for quantification of on-target and off-target engagement of mRNA analytes is the application of a surrogate standard curve approach within the RT-qPCR method

RT qPCR; the Most Prevalent Approach to Support mRNA End Points in Non-Clinical Studies



- Extracted RNA from study sample or Oligo Std is starting material
- RNA, primers, RT and Master Mix are loaded to the reaction tube
- RT converts RNA to DNA
- Heat activation dissociates the Taq-DNAP, restoring the Taq polymerase activity
- The cycle is repeated and curve generated based on Log Copies

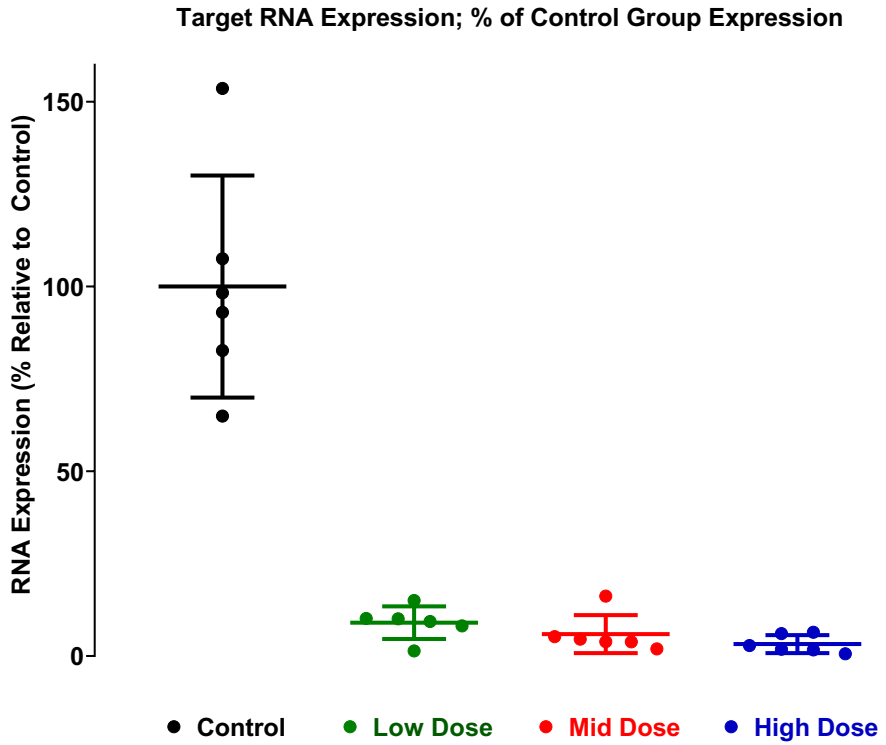
RT qPCR Methods Require Assessment of Key Parameters During Method Development and Qualification Activities

- **DEVELOPMENT**

- Design of primer probes & DNA/RNA oligo standard
- Specificity assessments
- Selectivity assessments
- Establish house-keeping gene levels is consistent and reproducible.
- Assess RNA linearity & input range

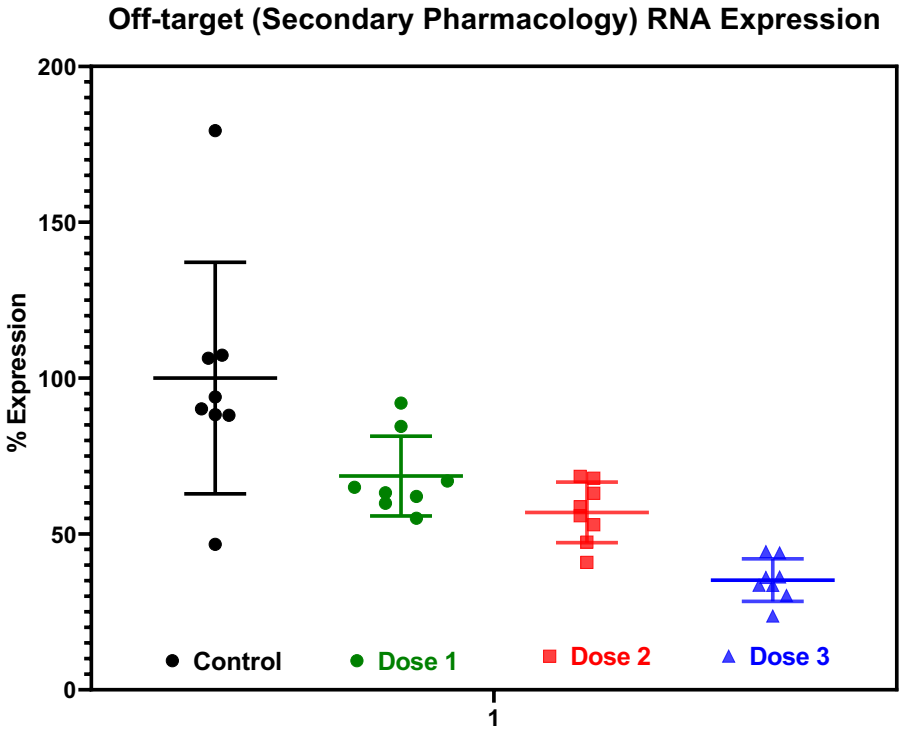
- **QUALIFICATION**
- No regulatory guidance to drive qualification design
- Traditional 4-6-20 BMV approach not appropriate
- **ASSESSMENTS INCLUDE**
- Calibration standard and matrix linearity, efficiency and specificity
- Limits of detection
- Lower limit of Quantification
- Intra- and inter-assay accuracy and precision
- RNA stability

RT-qPCR Allows the Degree of Target Gene Knock-down to be Estimated for an Administered Dose



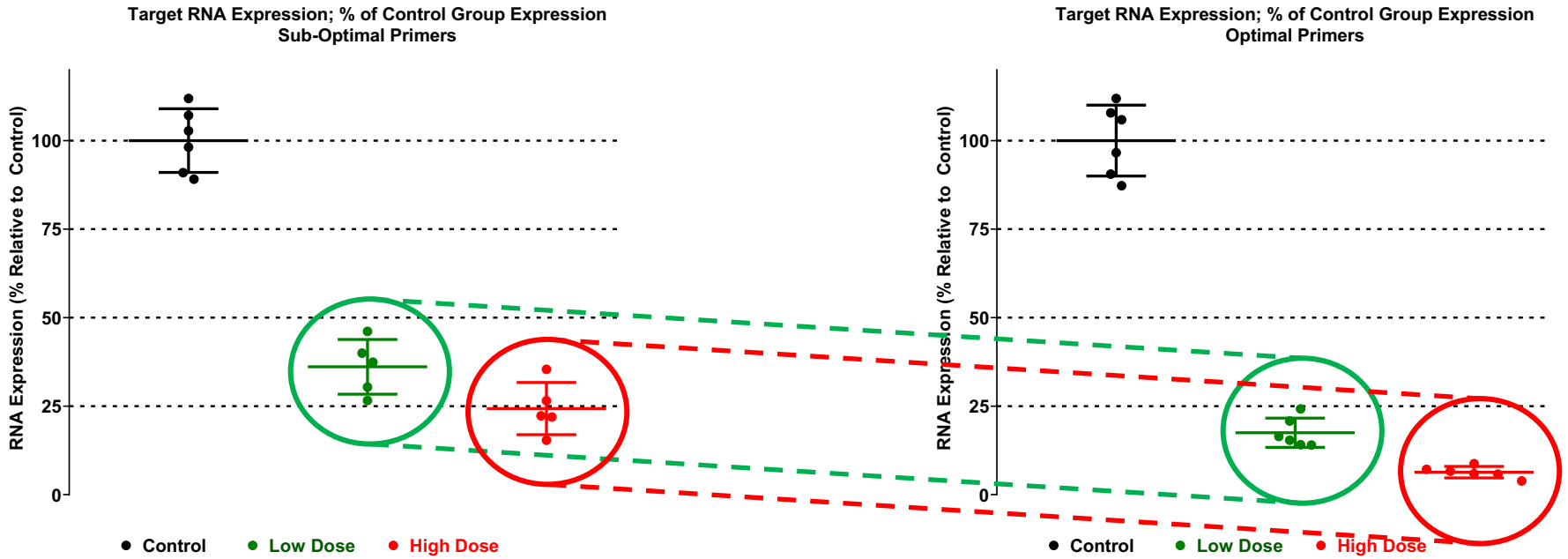
- Following repeat Toxicology and Dose Range Finding studies estimations of tissue RNA of the target gene can be made relative to the control/vehicle group
- PK-PD relationships can then be estimated to drive dose-selection
- Understanding BM in target organ and its context critical in data utilisation & interpretation

RT-qPCR Allows Assessment of Off-Target Pharmacology Relative to Dose; Critical in Safety Assessment



- ASO may have off-target or secondary pharmacology associated with the molecule
- RT qPCR PD data can provide a key piece of the puzzle to provide in-sight into the secondary pharmacology relative to dose

Primer-Probe Design & Oligo Standard Sequence are Key to Derive Meaningful Representative Data



- Understanding the gene target sequence is key when designing primer-probes and the Oligo Standard
- Protein encoding regions maybe missed leading to under or over estimation of knockdown

Understanding Target Gene Expression & ASO PD Effects in the Clinical Setting

- Understanding the effect of drug on genetic target expression is the ideal
- HOWEVER, obtaining target organ tissue to understand the PD may not be ethically feasible…
- THEREFORE, the bioanalytical scientist may have to approach an alternative biomarker avenue, undertaking a minimally invasive strategy and interrogating the option of proximal and/or distal biomarkers

Alternative Bioanalytical Approaches to Understand ASO PD in the Clinical Setting

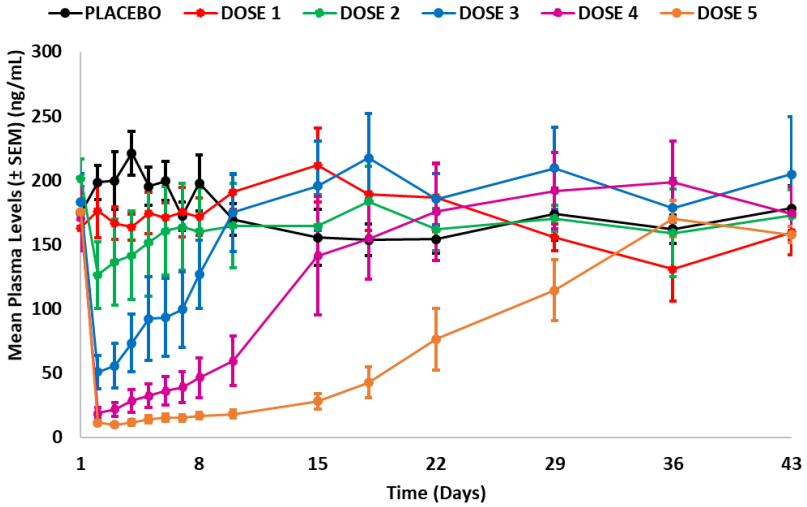
- **PROXIMAL**

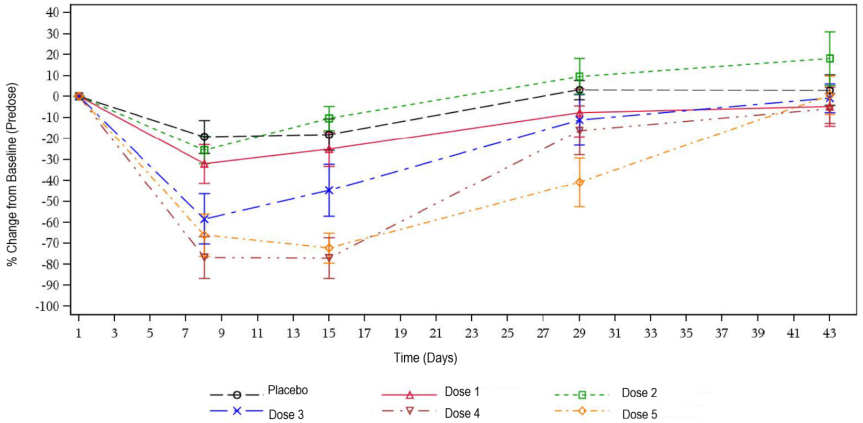
- CIRCULATING PROTEIN ENCODED BY TARGET GENE
- Downstream marker(s) that have potential to play a useful role in PD assessment
- Potential utility as it measures the circulating level of target ligand
- PD effects can be detected in **hours-to-days** post-administration
- Demonstrates Proof of Biology and Pharmacology

- **DISTAL**

- CLINICALLY VALIDATED END POINT OF PHARMACOLOGY
- 'Other' downstream marker(s) that has potential in PD assessment
- PD effects can be detected in **days-to-weeks** post-administration
- Demonstrates Proof of Pathway and Proof of Biology and biochemical coverage

Example Assessments of Proximal and Distal Marker Assessments





Proximal marker measurement allows the real time monitoring of systemically expressed protein

Distal marker measurement allows the real time monitoring of the Investigational Products (validated) pharmacology

Conclusions and Closing Remarks

- ASO drugs are specifically and selectivity designed to silence a target gene either via recruitment of RNase H or steric hindrance
- The dose-effect on target gene, within the target organ, is key to understanding the dose-response relationship following ASO administration and valuable for dose selection.
- RNA expression in target organ is the ideal biomarker for such assessments to understand the dose-response relationship
- RT qPCR is the bioanalytical tool of choice for such assessment in the non-clinical setting to understand target organ gene changes
- In the clinic this approach may not feasible, therefore other ‘accessible’ distal and proximal markers maybe employed to understand the pharmacodynamics and pharmacology of the drug

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Confidentiality Notice