N1C 2024 Annual Meeting

Individual Journeys, Shared Discoveries:
Partnering for Progress in Individualized Medicines

ASO design and safety

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ASO sequence has a profound effect on toxicity

Of the various classes of nucleic acid therapeutics, ASOs behave the *least* like a platform technology.

siRNAs have a more consistent structure and therefore pharmacokinetics

CRISPR in an LNP even more so…

Careful design and safety screening is essential for clinical success.

ASO Design and Safety

ASO sequence design guidelines and tools

Mechanisms of toxicity and in vitro assays that predict them

Chemical modification and formulation: impact on CNS safety

Designing ASOs

Exon Skipping ASO Design

For exon skipping, designing as few as 10 ASOs can be a good start!

1. A GC percentage of 40-60% appears optimal, avoid 3 or more consecutive Gs
2. ASOs of 18-22 nucleotides are generally optimal
3. For constitutive exons, targeting exonic regions works better than splice sites
4. Targeting splicing enhancer sites within an exon increases the chance of efficient exon skipping
5. For constitutive exons, target the first 30% of exon and/or use eSkip-Finder

   Chiba et al, *Nucleic Acids Res*, **49**, p. W193, 2021.
6. Targeting splice sites often works for cryptic exons, but careful of GC% and overlap with non target sequences

* Credit to Annemieke Aartsma-Rus
* These guidelines are based mainly on constitutively spliced exons

Consensus Guidelines for the Design and *In Vitro* Preclinical Efficacy Testing N-of-1 Exon Skipping Antisense Oligonucleotides

Annemieke Aartsma-Rus,[1–3] Alejandro Garanto,[1,4] Willeke van Roon-Mom,[1,2] Erin M. McConnell,[3] Victoria Suslovitch,[3,5] Winston X. Yan,[3] Jonathan K. Watts,[6] and Timothy W. Yu,[3,5,7] on behalf of the N=1 Collaborative

Nucleic Acid Ther. **33**, 17-25, 2023.

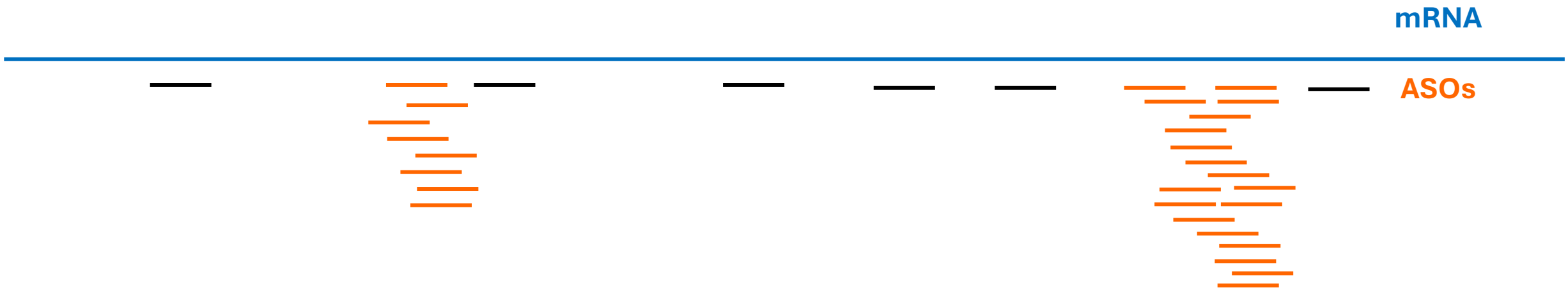
For gene silencing ASOs, there are many thousand possible sequences!

- Unlike for splice skipping, where design is focused around a single exon (typically ~100 bases), we have the whole pre-mRNA available as target space – can easily be over 100,000 bases!
- Introns provide both a challenge and an opportunity.

Sequence screening: Two-phase design

“sequence walk”

Sequence screening: Two-phase design



“microwalk” and
modification screen

Generating initial ASO sequences for a sequence walk

Factors that influence ASO efficacy

- Target mRNA accessibility
  - Self-structure
  - Protein binding
- ASO affinity for itself and for the target

Limited current public options for ASO design software

- PFRED
  - https://bio.tools/pfred *PLoS ONE* 2021, **16,** e0238753.
  - Does a pretty good job of specificity
  - Also designs siRNAs. Designs only for exons.
  - Limited attention to RNA self-structure.
- lncASO
  - https://iomics.ugent.be/lncaso/ **Now offline access only**
  - More focus on RNA structure
  - Less nuanced approach to specificity
  - Manual entry of target sequence – can enter introns but limited input length
  - Good at finding hits!

N1C is working on a data-integrated ASO design server

We are grateful for data coordination help from **La Jolla Labs** and others

- Searching within both introns and exons
- Focus on target RNA structure
- Nuanced understanding of specificity
  - Careful look at singly and doubly mismatched transcripts and gaps
  - Motifs previously seen to be toxic
- ML-based, data-informed algorithm (we will be seeking your data to continue to teach the algorithm)

If you have design server insights to share, please speak up!

What about allele selectivity?

Allele selective ASO design

- In our experience, making an ASO allele selective is normally feasible (“not the hard part”)
  - Sometimes adding a **second mismatch** helps improve discrimination between alleles
- The hard part… is that our target space is reduced from hundreds of kilobases to about 30 bases (i.e. the region that overlaps a SNP)
- If this region of RNA is not accessible in vivo, potency will be poor. Consider targeting a co-segregating SNP, remembering that introns are fair game.

Testing ASOs for safety

Factors that influence ASO safety

- ***Base-pairing mediated tox***: Full or partial complementarity to other expressed genes (including pre-mRNAs!)
  - A stretch of full complementarity is easy to search for using BLAST
  - Regions containing single or double mismatches are harder to predict
  - Most predicted off-target sites do not operate in practice
- ***Non-base-paring mediated tox:*** Toxic or immunogenic motifs
  - Protein binding
  - Immunostimulatory motifs

In vitro assays can predict many *in vivo* toxicities

Observed in vivo toxicity:	**Liver toxicity**
Mechanism:	**Protein mislocalization to the nucleolus**
Cell-based assay:	**Cell-based caspase assay**
Solution to minimize risk:	**Chemical modification of gap at position 2/3/4**

Shen *et al.*, *Nature Biotechnol.* 2019, **37**, 640.

In vitro assays can predict many *in vivo* toxicities

Observed in vivo toxicity:	**Immunostimulation, flu-like symptoms**
Mechanism:	**Innate immune recognition**
Cell-based assay:	**Bjab or PMBC cell assays**
Solution to minimize risk:	**Chemical modification and sequence selection**

PBMC: Burel et al, PMID 35976085
Bjab: Burel et al, Biorxiv, DOI 10.1101/2021.12.12.472280
Correlates with flu-like symptom AEs in clinical trials: Partridge et al, PMID 36631935

In vitro assays can predict many *in vivo* toxicities

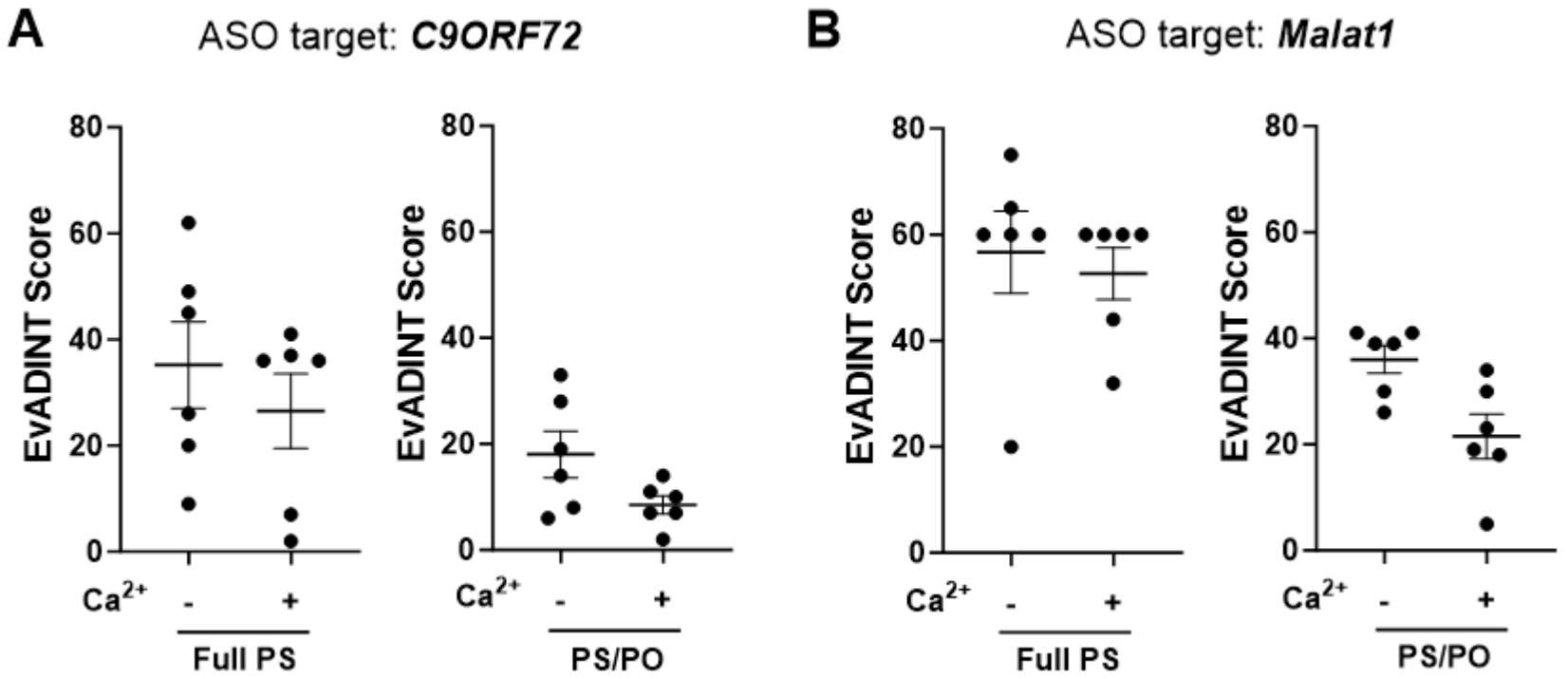
Observed in vivo toxicity:	**Acute motor phenotypes**
Mechanism:	**Possibly cell-surface protein binding?**
Cell-based assay:	**Calcium oscillation assay**
Solution to minimize risk:	**Reduce PS content, modify sequence (minimize 3'Gs)**

Hagedorn *et al.*, *Nucleic Acid Ther.* 2022, **32**, 151.

Minimizing acute neuromotor phenotypes in the CNS

…a bit of new data about *vehicle*

Polyanionic ASOs bind divalent cations from CSF. Calcium pre-saturation can improve motor phenotypes



Problems: Sometimes this affects solubility, complicates CMC, etc.

Moazami, Sarli et al, *Molecular Therapy*, in press

Can you just put calcium in your buffer?

Adding calcium to PBS caused the formation of micron-sized particles – which returned after sterile filtration and were present even when the solution was visibly clear

Formulation	Z-Average (d. nm) mean ± SD	PdI mean ± SD
PBS	n.a.	n.a.
PBS (3.6Ca)	12615 ± 2355	0.710 ± 0.257
PBS (3.6Mg)	n.a.	n.a.
PBS (8Ca)	11470 ± 594	0.793 ± 0.144
PBS (8Mg)	n.a.	n.a.

Moazami, Sarli et al, *Molecular Therapy*, in press

Can you just put calcium in your buffer?

Using HEPES instead of PBS allowed us to freely modulate divalent concentration with no particles

Formulation	Z-Average (d. nm) mean ± SD	PdI mean ± SD
PBS	n.a.	n.a.
PBS (3.6Ca)	12615 ± 2355	0.710 ± 0.257
PBS (3.6Mg)	n.a.	n.a.
PBS (8Ca)	11470 ± 594	0.793 ± 0.144
PBS (8Mg)	n.a.	n.a.
aCSF	n.a.	n.a.
aCSF (3.6Ca)	n.a.	n.a.
aCSF (3.6Mg)	n.a.	n.a.
aCSF (8Ca)	n.a.	n.a.
aCSF (8Mg)	n.a.	n.a.

Moazami, Sarli et al, *Molecular Therapy*, in press

PHYSIOLOGICAL SALT SOLUTIONS FOR BRAIN SURGERY

STUDIES OF LOCAL pH AND PIAL VESSEL REACTIONS TO BUFFERED AND UNBUFFERED ISOTONIC SOLUTIONS

K. A. C. ELLIOTT, PH.D., AND H. H. JASPER, M.D.*

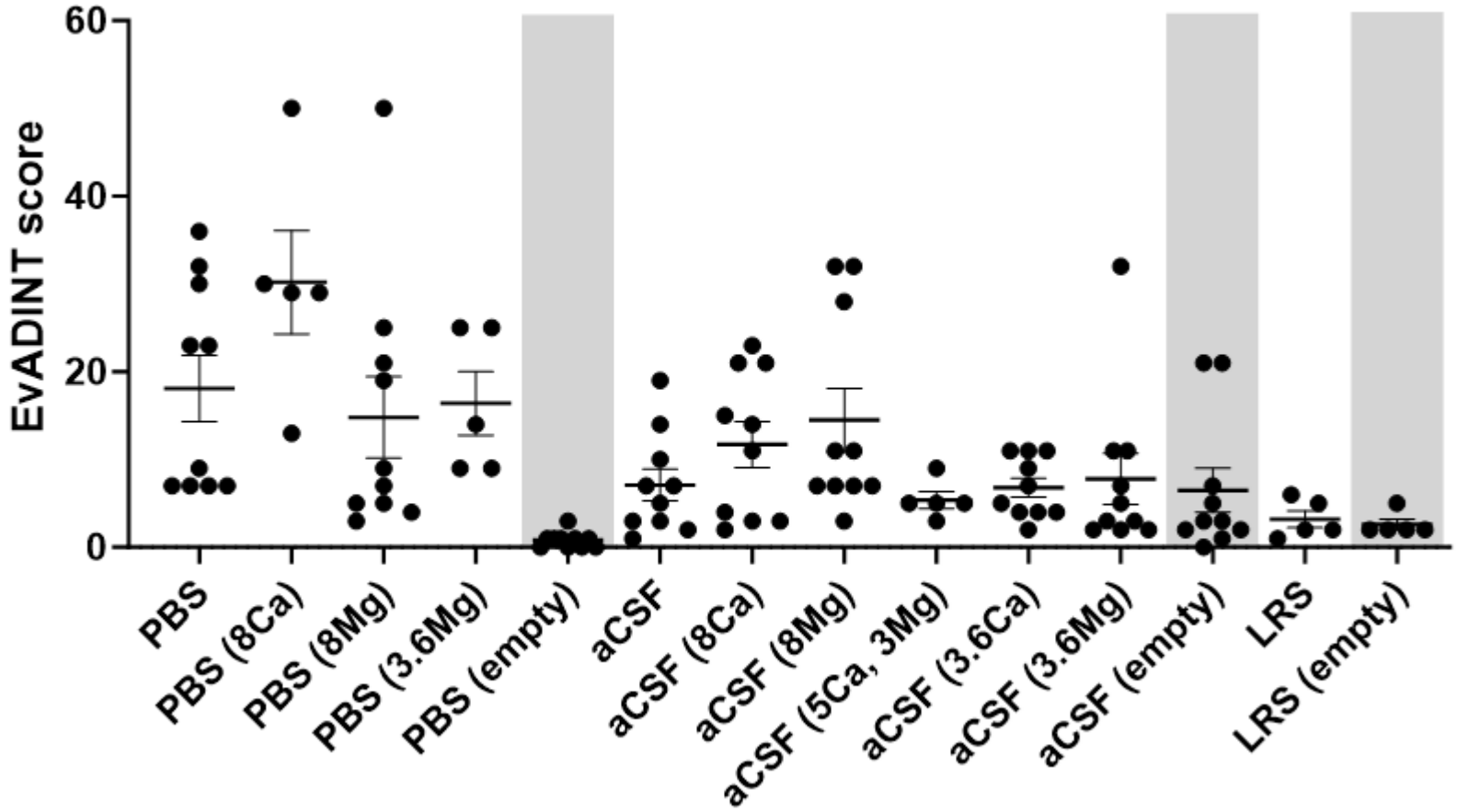
Department of Neurology and Neurosurgery, McGill University, and the Montreal Neurological Institute, Montreal, Canada

(Received for publication June 28, 1948)

Even USP-grade Elliotts B forms microparticles when heated or subjected to freeze thaw

Journal of Neurosurgery, **6,** p. 140, 1949

Surprising result: Phosphate-free buffers reduce motor phenotypes *even when calcium is not in the mix*



Mixed backbone ASO targeting *MALAT1*

Moazami, Sarli et al, *Molecular Therapy*, in press

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Thank You