An Integrated Service Platform Supporting ASO Drug Discovery: A Technology Showcase for the Design, Synthesis, *In Vitro* Screening and Functional Evaluation of ATXN2-targeting ASOs

WuXi Biology

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Abstract

Antisense oligonucleotides (ASOs) are a promising therapeutic modality that target RNA sequences to modulate gene expression. To better support the discovery of ASO agents, we have established an integrated ASO drug discovery platform, which encompasses sequence design, efficient oligonucleotide synthesis, as well as in vitro and in vivo efficacy and safety tests.

To demonstrate the capabilities of our platform, we performed a showcase to develop a gapmer ASO against a CNS target, ATXN2, which can be used to treat neurodegenerative disorders such as Amyotrophic lateral sclerosis (ALS). We used our in-house in silico design platform to create a series of ASOs targeting ATXN2 sequences, followed by in vitro screening across various cell lines. From a single round of design and screening, we identified ASOs that show knockdown efficacy comparable to existing clinical candidates, highlighting the robustness of our design platform. In vitro functional assays further confirmed that the selected ASOs effectively delayed the maturation of stress granules, a key pathological feature of ALS. RNA profiling data showed that the candidate ASOs had minimal off-target activity, thus confirming the high specificity of our designed ASOs. Our platform also offers assays to screen for ASO toxicity and immunogenicity. In vivo, we can evaluate the PK and PD of ASOs via different dosing routes such as intrathecal or intracerebroventricular routes, as well as detect early neurotoxicity via functional observational battery (FOB) assessment.

These results validate the capability of our end-to-end, integrated ASO drug discovery platform to develop potent and safe ASOs, thus offering a robust and efficient platform to advance ASO drug discovery.

Background

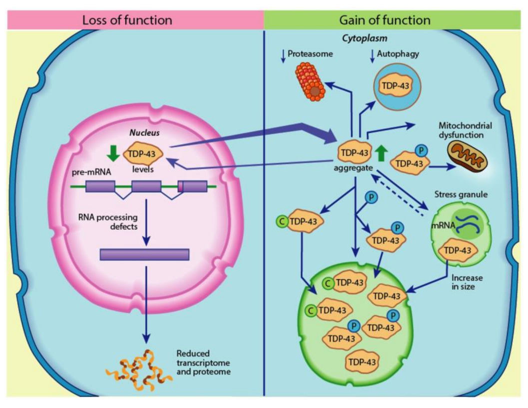


Image from J Neurol Neurosurg Psychiatry, 2021, 92(1): 86–95

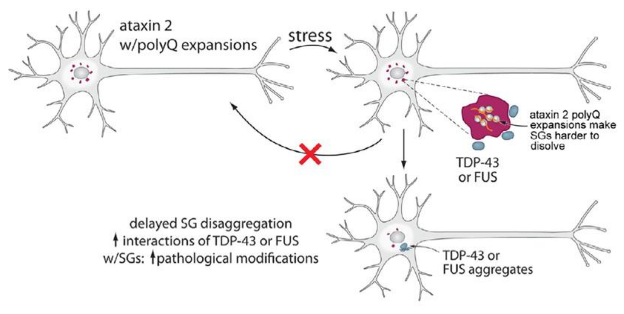


Image from Journal of cell biology, 2013, 201(3): 361-372.

- ✓ Many CNS diseases (e.g., ALS, FTLD, Alzheimer's disease) display pathogenic cytoplasmic TDP-43 aggregates (TDP-43 proteinopathies) that localize to stress granules
- ✓ ATXN2 KD disrupts stress granule formation and TDP-43 localization, leading to reduced TDP-43 aggregates

References:

[1] De Boer, Eva Maria Johanna, et al. "TDP-43 proteinopathies: a new wave of neurodegenerative diseases." *Journal of Neurology, Neurosurgery & Psychiatry* 92.1 (2021): 86-95.

[2] Li, Yun R., et al. "Stress granules as crucibles of ALS pathogenesis." *Journal of cell biology* 201.3 (2013): 361-372.

Methods and Results

1. Sequence design of *ATXN2*-targeting gapmer ASOs

Transcripts of target gene	Sequence information	Fragmentation	Homology comparison	Off-target filter	Efficiency & Patent evaluation
✓ Expression ✓ isoform	✓ SNP ✓ sequence context	✓ SNP avoidance ✓ GC ratio/distribution ✓ Position at transcripts	✓ Monkey ✓ Mouse/Rat	✓ Mature mRNA ✓ Pre-mRNA	✓ Sequence context ✓ Thermodynamic features ✓ Patent risk labeling

Figure 1-1. A general flowchart of ASO sequence design

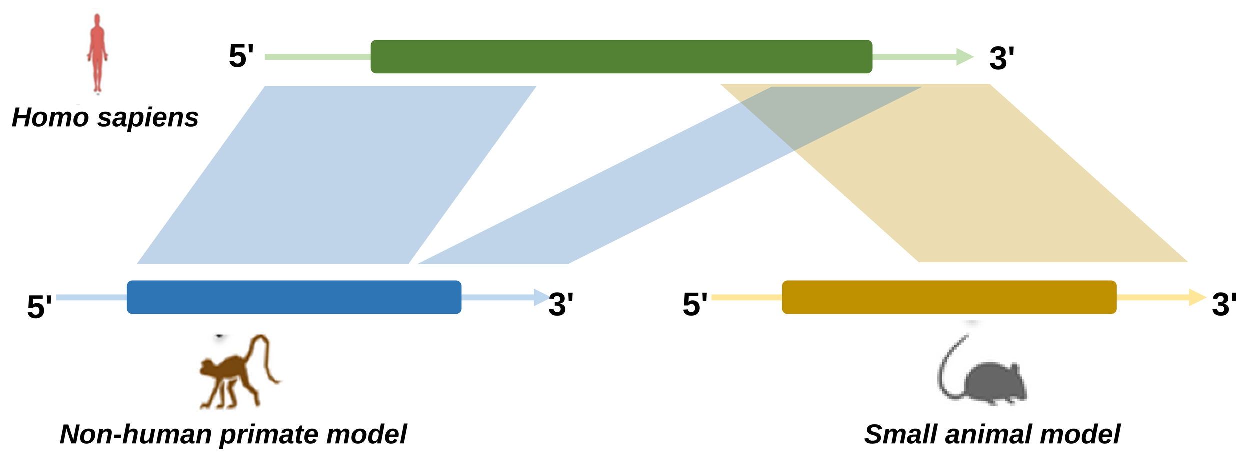


Figure 1-2. Cross-species comparison of candidate ASOs

Results-1

- ✓ A platform for ASO sequence design has been established.
- ✓ The ASO for further evaluations were selected based on multiple parameters, including off-targets, cross-species and efficacy.

2. Screening of focused ASO library for *ATXN2* knockdown

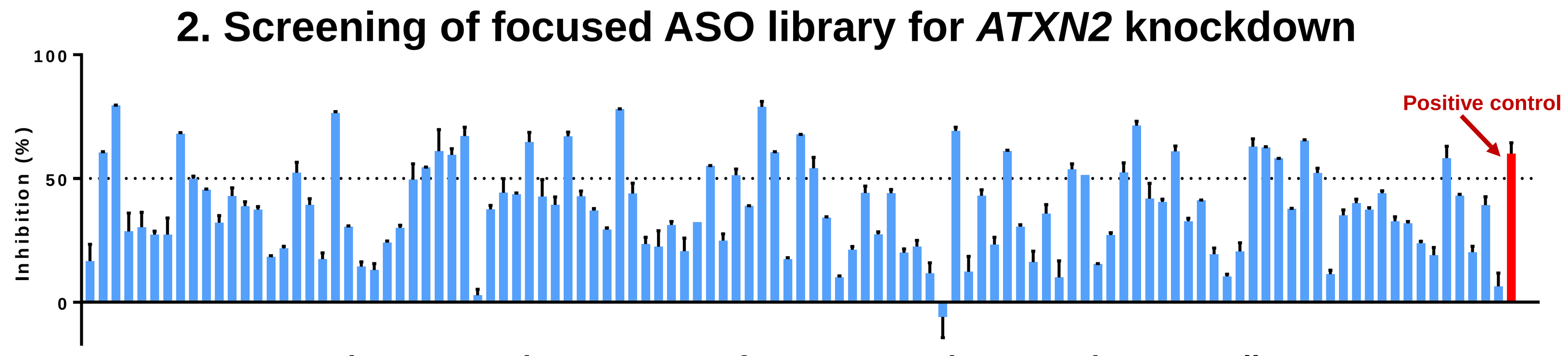


Figure 2-1. Primary screen of *ATXN2*-targeting ASOs in HeLa cells

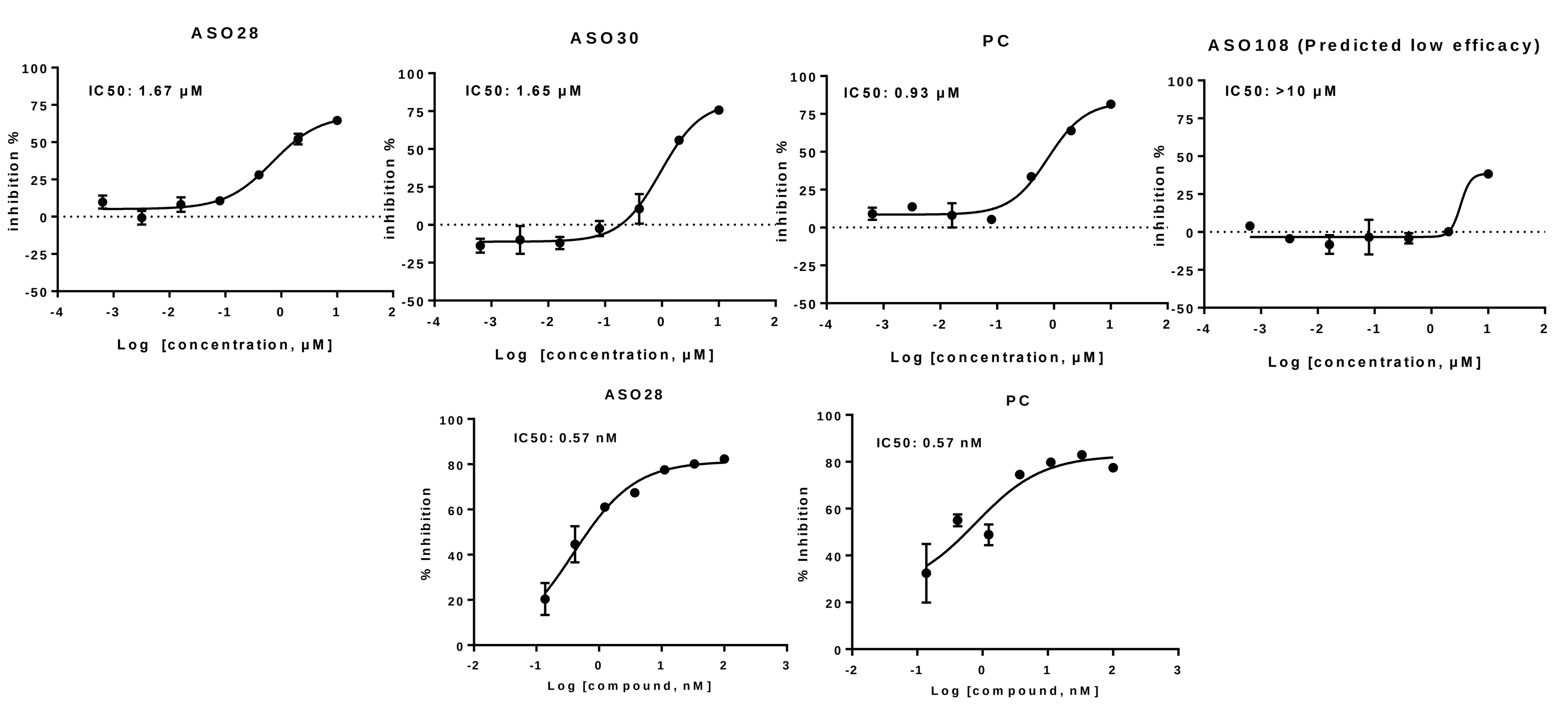


Figure 2-2. Dose response curves of top *ATXN2*-targeting ASOs in SH-SY5Y (Top) / neuro 2a cells (Bottom)

Results-2

- ✓ From just one round of design and screening, ~30% of candidates inhibit the target by >50% at low concentration (5 nM) by transfection in HeLa cells
- ✓ ~20% of candidates inhibit the target at similar or better levels than the positive control (a clinical-phase drug)
- ✓ Dose response tests show that our top ASO candidates have comparable IC50s to the positive control by electroporation in SH-SY5Y cells / transfection in neuro 2a cells
- ✓ Predicted sequence with low efficacy (ASO108) showed low knockdown efficacy (high IC50)
- ✓ The designed cross human and mouse ASO (ASO28) exhibits activity comparable to the positive control in both human and mouse cell lines.

3. Functional assay for effects of ATXN2-targeting ASOs on stress granule formation

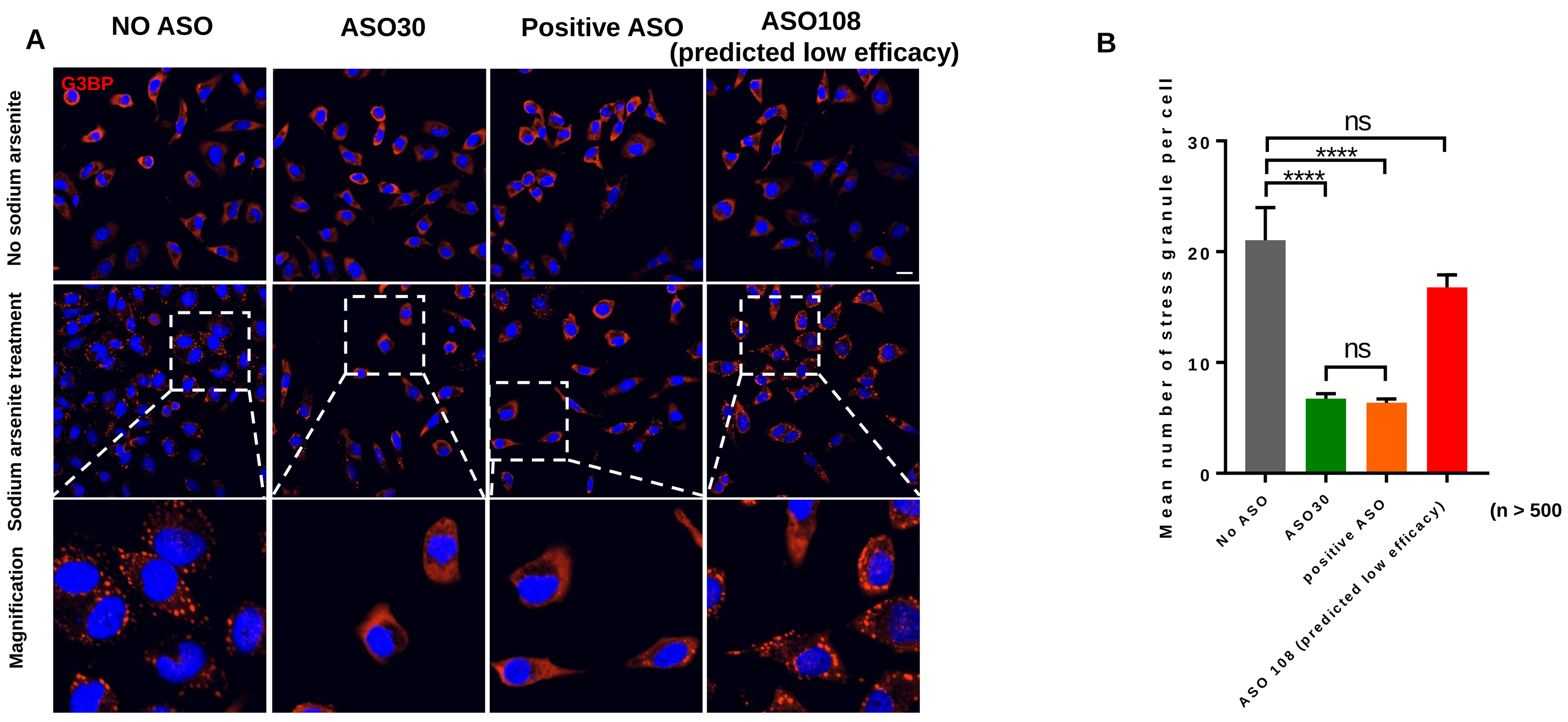


Figure 3. Selected ASOs effectively delayed the formation of stress granules

Results-3

- ✓ One of our top ASO candidates (ASO30) attenuated the formation of stress granules induced by sodium arsenite, comparable to the clinical-phase drug (Positive ASO)
- ✓ Predicted low-efficacy sequence (ASO108) did not significantly attenuate the formation of stress granules

4. Detection of off-target genes of ATXN2 ASOs by transcriptional profiling in SH-SY5Y cells

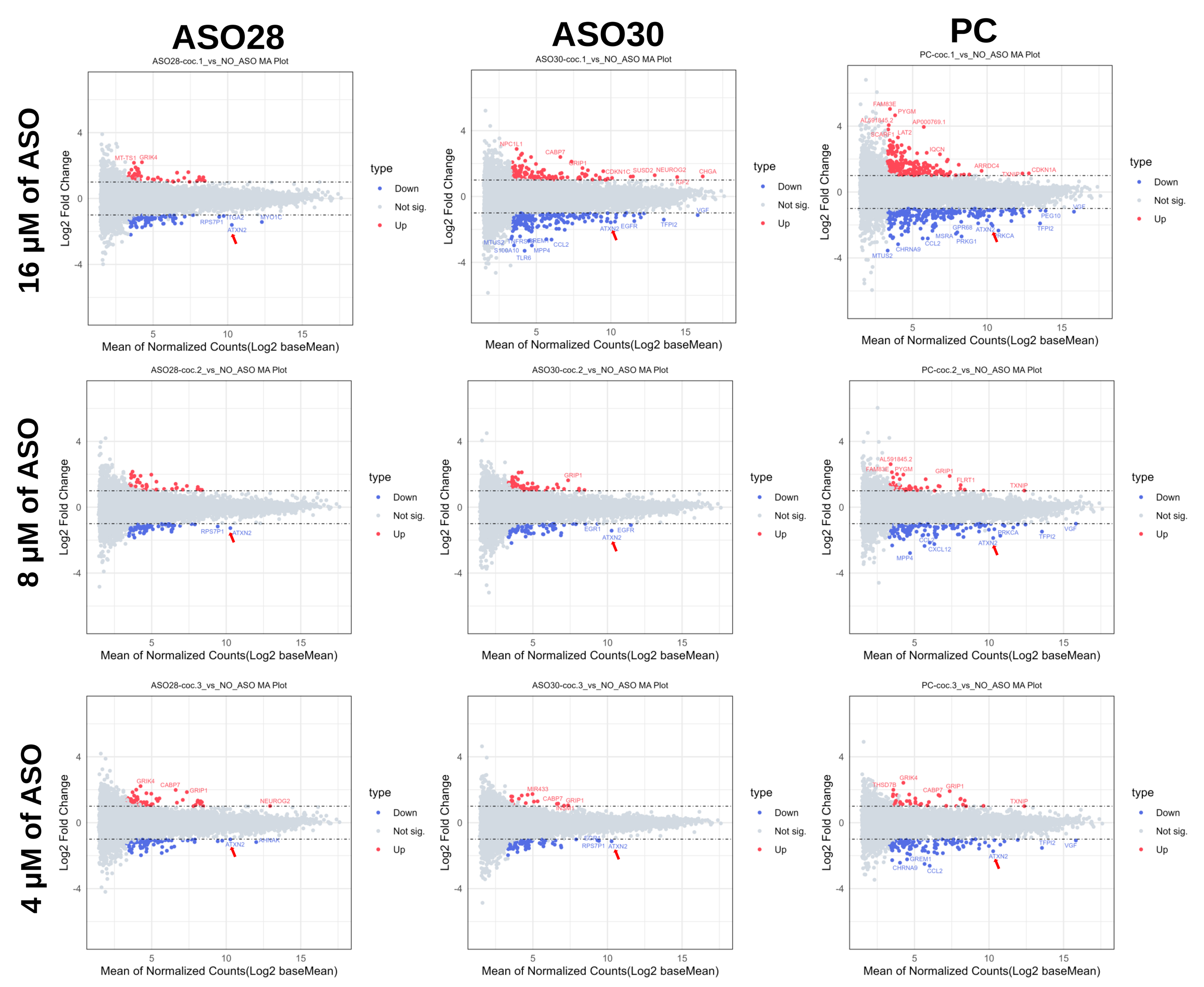


Table 1. Analyze potential off-target genes through DEG and dose-response

ASO ID	Potential off-target gene	Number of potential off-target gene
ASO28	MYO1C, EIF3CL, COL6A6, RPS7P1, ITGA2	5
ASO30	EGFR, ARHGAP26, POM121	3
PC	PLCB1, MYO9A, EGR1, etc.	52

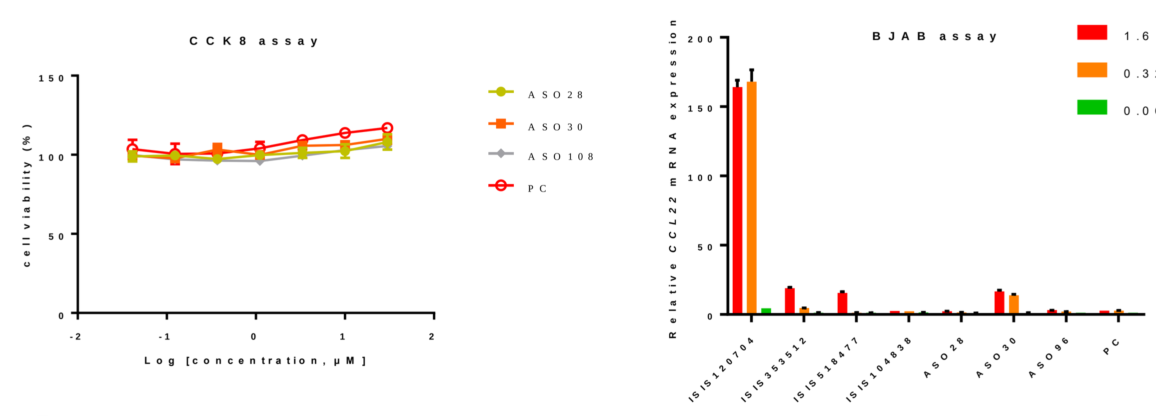
Table 2. Alignment of Potential off-target gene and ASOs

ASO ID	Potential off-target gene	Alignment (5'->3' from ASO strand)	Number of potential off-target gene
ASO28	MYO1C	||**:|||||||||||||||	1
ASO30	NA	NA	0
PC	PLCB1	||:*|*|||:||||*||*||	5
PC	MYO9A	:|||||:*|:||:||*||*|	
PC	EGR1	|*||||||||:|*|*|:*||	
PC	ZNF346	*||*|*|||*|||||*||||	
PC	MSRA	|*|**||*||||||||||*|	

Results-4

- ✓ Potential off-targets were detected by transcriptional profiling.
- ✓ Dose-dependent off-targets were detected against a background of differentially expressed genes (could be noise due to the sensitivity of transcriptional profiling, etc.)
- ✓ WuXi designed ASOs shows high potency and few/no potential off-targets.

5. Toxicity and immunogenicity assessment of candidate ASOs

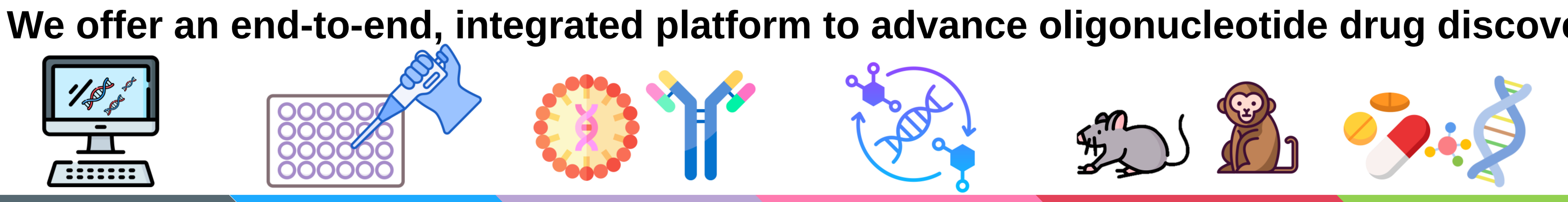


Immunogenicity Rank:
ISIS120704(high immunogenicity) > ISIS353512> ISIS518477> ISIS104838(safe)

Results-5

- ✓ The candidate ASOs showed no significant cytotoxicity as determined by CCK8 assay.
- ✓ The candidate ASOs showed no significant immunogenicity as determined by the BJAB assay.

6. We offer an end-to-end, integrated platform to advance oligonucleotide drug discovery



Sequence design of oligos	Hit to lead	Delivery tool	Lead optimization	In vivo PK/PD and safety	IND
✓ *In silico* sequence design ✓ siRNA, gapmer ASO, splice-switching ASO, upregulating saRNA/ASO, miRNA, etc. ✓ Cross-species activity prediction ✓ Off-target prediction ✓ Efficacy prediction ✓ Patent sequence search ✓ …	✓ High-throughput screening using cell lines (>1,500 cell bank), primary cells, iPSC-derived cells, reporter cells ✓ Cell viability, toxicity, and apoptosis ✓ Off-target analysis by RNA profiling ✓ Functional assays ✓ …	✓ Delivery tool discovery: ASMS, DEL, phage display, etc. ✓ GalNAc, antibody, peptide, lipid, aptamer conjugates ✓ LNP, exosomes vehicles ✓ Cell internalization, endosome release assays ✓ …	✓ Sequence optimization ✓ Modification optimization ✓ Delivery tool optimization ✓ Stability assays (S9, plasma, brain homogenate, etc.) ✓ Immunogenicity assays ✓ …	✓ Humanized mouse models (HDI, AAV, transgenic, etc.) ✓ Rat ✓ NHP ✓ PK/PD ✓ Acute tox study ✓ Disease /efficacy models ✓ …	✓ DMPK studies ✓ Dose-range finding studies in rodents and NHP (non-GLP) ✓ General toxicology studies in rodents and NHP (GLP) ✓ Genetic toxicology ✓ GMP synthesis ✓

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