

1 **Quantifying and Mitigating Motor Phenotypes Induced by Antisense Oligonucleotides in the Central**
2 **Nervous System**

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16

17 **ABSTRACT**

18 Antisense oligonucleotides (ASOs) are emerging as a promising class of therapeutics for neurological

19 diseases. When injected directly into the cerebrospinal fluid, ASOs distribute broadly across brain

20 regions and exert long-lasting therapeutic effects. However, many phosphorothioate (PS)-modified

21 gapmer ASOs show transient motor phenotypes when injected into the cerebrospinal fluid, ranging from

22 reduced motor activity to ataxia or acute seizure-like phenotypes. The effect of sugar and phosphate

23 modifications on these phenotypes has not previously been systematically studied. Using a behavioral

24 scoring assay customized to reflect the timing and nature of these effects, we show that both sugar and

25 phosphate modifications influence acute motor phenotypes. Among sugar analogues, PS-DNA induces

26 the strongest motor phenotype while 2'-substituted RNA modifications improve the tolerability of PS-

27 ASOs. This helps explain why gapmer ASOs have been more challenging to develop clinically relative to

28 steric blocker ASOs, which have a reduced tendency to induce these effects. Reducing the PS content of

29 gapmer ASOs, which contain a stretch of PS-DNA, improves their toxicity profile, but in some cases also

30 reduces their efficacy or duration of effect. Reducing PS content improved the acute tolerability of ASOs

31 in both mice and sheep. We show that this acute toxicity is not mediated by the major nucleic acid

32 sensing innate immune pathways. Formulating ASOs with calcium ions before injecting into the CNS
33 further improved their tolerability, but through a mechanism at least partially distinct from the
34 reduction of PS content. Overall, our work identifies and quantifies an understudied aspect of
35 oligonucleotide toxicology in the CNS, explores its mechanism, and presents platform-level medicinal
36 chemistry approaches that improve tolerability of this class of compounds.

37

38 INTRODUCTION

39 Antisense oligonucleotides (ASOs) are emerging as a promising class of therapeutics for neurological
40 diseases.¹⁻⁴ The groundbreaking ASO nusinersen was approved by the FDA in 2016 to treat spinal
41 muscular atrophy.⁵⁻¹² Nusinersen operates through a splice-switching mechanism and is fully modified
42 with 2'-O-methoxyethyl sugars and phosphorothioate linkages. Nusinersen showed an excellent safety
43 and efficacy profile in clinical trials. Another class of ASOs is designed to recruit RNase H to cleave target
44 RNA and thus silence gene expression. These so-called gapmer ASOs require a stretch (“gap”) of 8-10
45 non-sugar-modified DNA nucleotides in the middle to enable RNase H recruitment. In contrast to
46 nusinersen, early clinical evaluation of gapmer ASOs in the CNS faced dose-limiting toxicity resulting in
47 failed clinical trials. Nevertheless, after these false starts, a more advanced generation of gapmer ASOs
48 are in clinical development for neurological diseases including Huntington’s disease,¹³⁻¹⁵ Alzheimer’s
49 disease,¹⁶ Parkinson’s disease,¹⁷ and amyotrophic lateral sclerosis (ALS).^{4, 18-20} These compounds show
50 promising biomarker efficacy with reasonable toxicity profiles.

51 Looking at the chemical modification patterns used in early clinical trials vs RNase H-active compounds
52 currently in the clinic, a major difference is the number of PS backbone modifications. In early trials,
53 gapmer ASOs were fully PS modified (similar to nusinersen), while current variants of the compounds
54 use a mixed backbone where up to six linkages are substituted back to phosphodiester. In parallel in our
55 own work, during the development of gapmer ASOs for *C9ORF72*-driven ALS,²¹ we observed a range of
56 transient motor phenotypes most severe within the first 1-3 hours after intracerebroventricular
57 administration of the ASOs to mice. We were able to reduce these effects by reducing the backbone PS
58 content. We carried out the current study to explore the generality of this phenomenon, explore its
59 mechanism, and understand how it is affected by chemical modifications to both phosphates and
60 sugars.

61 Using a quantitative scoring assay for ASO-induced motor phenotypes, we now show that the acute
62 motor phenotypes produced by PS-modified gapmer ASOs in the brain can be minimized by using ASOs
63 containing a combination of phosphorothioate (PS) and unmodified phosphodiester (PO) linkages.
64 Interestingly, the ASO-induced motor phenotypes are profoundly affected by the sugar modifications
65 used: PS-DNA ASOs were the most toxic, while ASOs composed entirely of 2'-O-substituted RNA (2'-O-
66 methoxyethyl or 2'-O-methyl RNA) were less toxic.

67 We also now present experimental results that shed light on the mechanism underlying the observed
68 motor phenotypes. We show that they are not mediated by the major nucleic acid sensing innate
69 immune pathways, do not produce long-term toxicity and are observed in both small (mouse) and large
70 (sheep) brains. The toxicity profile of *both* fully PS and mixed-backbone (PO/PS) ASOs can be improved
71 by exposing ASOs to calcium-containing buffers before injection, indicating that PS-modified
72 oligonucleotides induce a local CSF disbalance in divalent ion composition. Finally, we show that mixed
73 (PO/PS) backbone ASOs with *in vivo* gene silencing efficacy comparable with full PS ASOs can be
74 engineered, defining the clear steps towards development of highly active and safe ASOs for other
75 neurodegenerative disorders.

76 Progress in chemical modification of oligonucleotides has been profoundly important in enabling clinical
77 success.²²⁻²⁴ Further improvements in modification and formulation of ASOs, as well as increased
78 mechanistic understanding of the factors defining efficacy and toxicity, is essential to expand the
79 therapeutic use of gapmer ASOs in the CNS.

80 **RESULTS**

81 **EvADINT scoring assay for acute behavioral toxicity**

82 After administration of ASOs into the CNS, we observed dose-dependent acute behavioral toxicity that
83 varied from lethargy, lack of responsiveness and ataxia to hyperactivity, seizures, and, in extreme cases,
84 death. This behavioral neurotoxicity was most striking in the first 1-3 hours after administration. Even
85 severely affected mice, unless they died, recovered fully by 24h and showed no further adverse effects.
86 To describe our studies of acute toxicity in a robust way, we needed a way to quantify this acute toxicity.
87 Various protocols have been used to quantitate behavioral toxicity under the umbrella of a “Functional
88 Observational Battery” (FOB), with variations for both acute and longitudinal neurotoxicity (reviewed in
89 ref²⁵. Regulatory documents such as the OECD guideline for neurotoxicity testing in rodents²⁶ do not

90 provide assay details that are well aligned with the transient toxicity seen after ASO administration to
91 mice.

92 We therefore developed a scoring assay optimized to quantify the transient motor phenotypes induced
93 after intracerebral oligonucleotide injection. We call this assay EvADINT (Evaluation of Acute Drug-
94 Induced NeuroToxicity). In this assay, we monitored mice at multiple time points over 24 hours after
95 injection, assigning a score for various parameters as described in Table 1. If a mouse died, it was given a
96 score of 75. Seizures, hyperactivity and other atypical motor behavior were scored depending on their
97 severity. The rest of the score was allotted based on how much time elapsed before the animal was able
98 to resume various aspects of normal mouse behavior. We weighted the observed phenotypes according
99 to their apparent severity. For example, sternal posture was weighted more heavily than normal
100 grooming since mice must be able to right themselves to carry out most other aspects of normal mouse
101 behavior, and because maintenance of sternal posture is simpler than eating, walking or grooming. For
102 similar intuitive reasons, seizures and death were weighted more heavily than the other factors such as
103 latency to resume normal mouse behavior(s). We varied the relative weightings of different factors in
104 our assay – death, seizures, and the various other behavioral observations – and observed that the
105 relative scores of different ASOs were very similar in all cases. Thus, the EvADINT scoring assay is robust
106 to variation in the precise weights assigned to each factor.

Behavioral element / observation	75				
	Severe	Moderate	Mild	Absent	
Death					
Seizure ^a	20	15	10	0	
Hyperactivity or other atypical motor behavior ^b	15	10	5	0	
Time required for:	0.5 h	1 h	2 h	4 h	≥24 h
Maintenance of sternal posture	0	4	8	12	20
Unstimulated movement	0	3	6	9	15
Movement without ataxia	0	2	4	6	10
Normal grooming/eating/nesting	0	1	2	3	5

107
108 **Table 1.** Breakdown of scoring for the EvADINT system for quantification of acute motor phenotypes. Examples of observed
109 phenotypes (movies and corresponding scores), and all actual mouse scores for all figures are given in the supporting
110 information. Personnel were blinded to ASO group during the scoring. ^aSeverity of seizures was ranked as follows: A *severe*
111 seizure had a duration of >30min and/or with constant or high intensity muscle contractions. A *moderate* seizure had a 10-30
112 min duration and moderate intensity muscle contractions or rapid and repetitive synchronous twitching, accompanied by
113 apparent short-term loss of consciousness. A *mild* seizure lasted <10min and featured low intensity muscle contractions or
114 short & infrequent bursts. ^bSeverity of hyperactivity or other atypical motor behavior was ranked as follows. Severe: >30min

115 duration, popcorning/jumping, constant. Moderate: 10-30min duration, slight hopping, other atypical motor behavior. Mild:
116 <10min duration, uncoordinated movements, twitching.

117

118 **Reduced phosphorothioate content and formulation with Ca²⁺ reduces acute toxicity**

119 In our parallel work on the development of ASOs that target the sense and antisense transcripts from
120 the ALS/FTD gene *C9ORF72*, we found that reducing the number of phosphorothioate-modified linkages
121 between pairs of 2'-*O*-methoxyethyl (MOE)-modified ribonucleotides improved tolerability without loss
122 of efficacy or duration of effect.²¹ A similar mixed backbone design is now in clinical development.¹⁵
123 Thus our lead backbone design, and the mixed backbone design that we focus on exclusively in this
124 study, contains a single PS linkage at each end of the ASO, followed by three PO linkages and then PS
125 linkages throughout the remaining (central) portion of the ASO (sequences and modification patterns
126 are shown in Table 2). With the EvADINT scoring system established, we set out to quantify this
127 observation and explore its generality and mechanism. We first established that our previous result was
128 reproducible under quantitative, blinded conditions. Thus, reducing the phosphorothioate content of a
129 *C9ORF72*-targeted ASO led to increased tolerability in the CNS (Figure 1A).

130 Phosphorothioate groups are more acidic than phosphates, and thus more anionic at physiological pH,
131 and a greater share of the negative charge is concentrated on the sulfur atom. We wondered whether
132 the more anionic character of phosphorothioate-modified ASOs was increasing their tendency to chelate
133 divalent cations from CSF. During ICV injections, to keep volumes small, the ASO concentration is high
134 (typically 1-4 mM). This is comparable to or higher than the concentration of Ca²⁺ in CSF, which is 1.3–
135 1.4 mM²⁷ – and moreover, each polyanionic ASO could potentially chelate multiple Ca²⁺ ions. We
136 wondered whether reducing the PS content of our ASOs was reducing toxicity simply by reducing their
137 tendency to chelate Ca²⁺ ions.

138 To explore whether the neurotoxicity we observed could be explained by Ca²⁺ chelation, we tested ASOs
139 that had been pre-saturated with Ca²⁺ before injection. We chose this pre-saturation approach because
140 we did not know exactly how much Ca²⁺ each ASO would chelate from the solution. We were concerned
141 that if we simply suspended our ASOs in buffer containing a physiological concentration of Ca²⁺, it might
142 be insufficient to compensate for the chelation abilities of ASOs at these high concentrations. And on
143 the other hand, we were concerned that injecting ASOs in buffer containing higher-than-physiological
144 concentrations of Ca²⁺ might lead to hypercalcemia-mediated toxicity. Thus, after HPLC purification of

145 ASOs, we transferred each ASO to a 3kDa-cutoff Amicon ultrafiltration cartridge, and washed with a
146 solution containing 20 mM Ca²⁺, twice with water, and once with PBS.²⁸ We then resuspended the ASO
147 in PBS and proceeded to injection. The intervening water washes are important to prevent the
148 irreversible precipitation of calcium phosphate resulting from excess Ca²⁺ in the presence of phosphate
149 buffer.

150 For this study, we chose a moderately high dose of 35 nmol ASO per mouse (equivalent to about
151 10mg/kg), somewhat higher than the dose typically required for effective gene silencing. Under these
152 conditions, for our *C9ORF72*-targeted ASO, we observed that pre-saturation with Ca²⁺ led to a robust
153 improvement in acute tolerability in the CNS (Figure 1A). Interestingly, the improvement in tolerability
154 occurred for both the full PS and the mixed backbone ASOs. Thus, the best-tolerated ASO was the
155 compound with reduced PS content and which had also been pre-saturated with Ca²⁺.

156 These *C9ORF72* ASOs targeted the human transcript; the acute motor phenotypes were observed
157 whether the ASOs had a target (as in the transgenic *C9ORF72* mouse models we used in the parallel
158 work on therapeutic development for *C9ORF72*)²¹ or not (as in the wild type mice used here).

159 To test whether these principles applied to other targeting and non-targeting sequences, we synthesized
160 ASOs targeting the noncoding RNA *Malat1* and the Huntingtin (*Htt*) mRNA (Sequences 6–9, Table 2). We
161 synthesized versions of these sequences in both full PS and mixed backbone formats, and in all cases
162 compared simple formulation in PBS with formulation in PBS after calcium pre-saturation. We injected
163 these compounds into mice (ICV) and observed motor phenotypes using the EvADINT assay (Figure 1B-
164 C). For the *Malat1*-targeting ASO (Figure 1B), we saw the same pattern as for the *C9ORF72*-targeted
165 ASO – namely, there was a substantial improvement in tolerability upon reducing the PS content, and
166 both the full PS and mixed backbone ASOs showed further improvement upon formulation with Ca²⁺.

167 For the *Htt*-targeted ASO, we saw higher toxicity and broader variability in tolerability across the groups
168 (Figure 1C). The improvement in tolerability of this mixed-backbone design was less clear than for the
169 other sequences. However, the improvement in tolerability from Ca²⁺ formulation was robust in the
170 context of both backbone variants. Thus, there may be a sequence-dependence to optimal backbone
171 design, and it is clear that reducing the PS content in this way does not reduce the need to select good
172 sequences.

173 Finally, we synthesized non-targeting control ASOs (Sequences 10 and 11, Table 2) and formulated them
174 in the same way as the first three sequences. We injected these ASOs into mice and scored neurotoxicity

175 using the EvADINT assay (Figure 1D). The patterns observed for this sequence are slightly different – in
 176 this case, calcium formulation had little or no effect on toxicity, while the reduction of PS content
 177 showed a dramatic improvement in toxicity.

178 Thus, each of the four sequences we tested showed a robust improvement in toxicity following a
 179 modest reduction in PS content, Ca²⁺ formulation, or both. The ASO-induced motor phenotypes were
 180 dose dependent across multiple sequences and design patterns (Supporting Figure S1). It is interesting
 181 that across all sequences, higher toxicity was often accompanied with higher inter-animal variability.
 182 That is, there appear to be important animal-to-animal variations in the actualization of this toxicity.

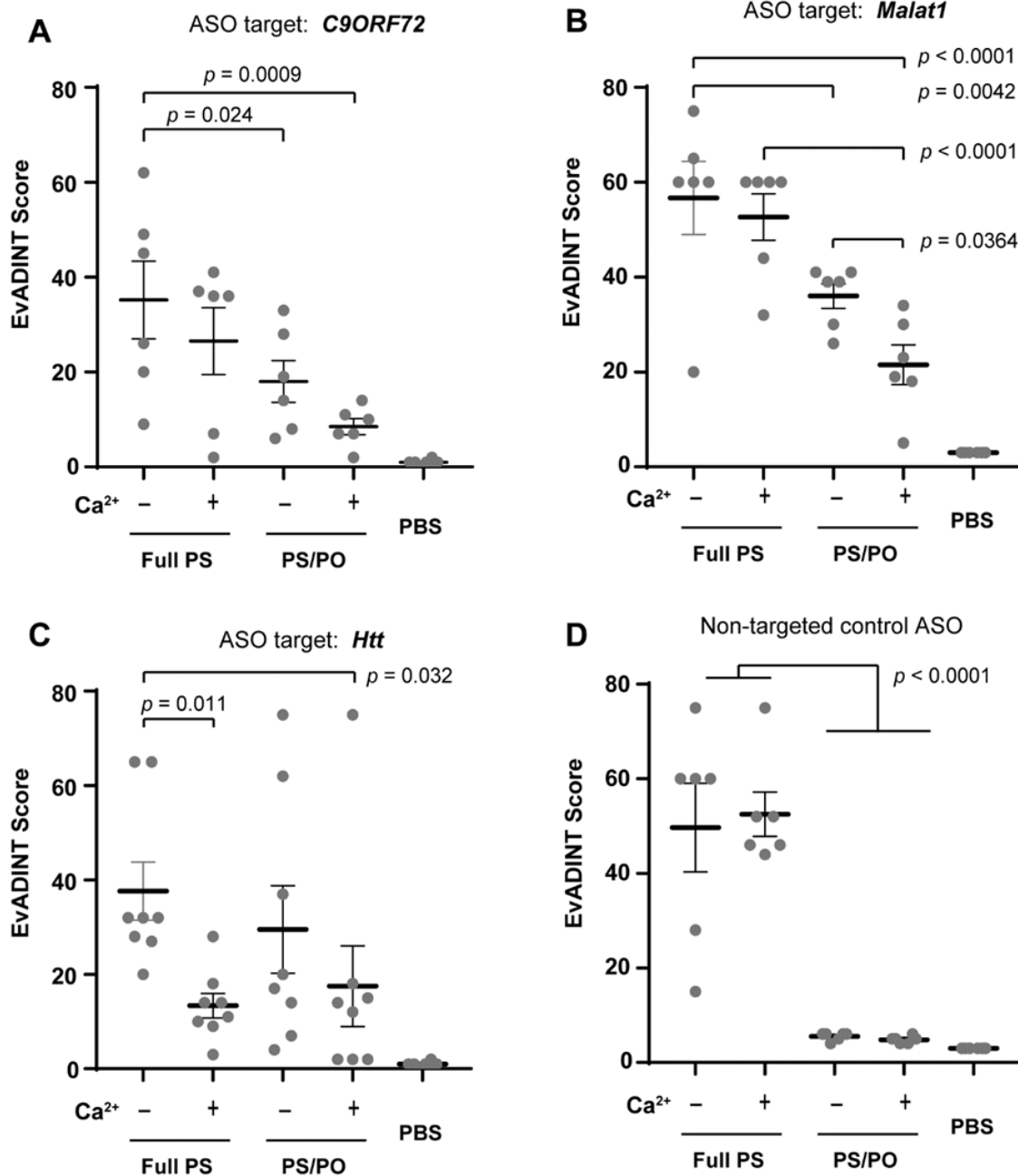
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184

Seq #	Name	Sequence and modification pattern	Mass calculated	Mass observed
1	C9ORF72-full PS	GsCsCsCsTsAsGsCsGsCsGsGsGsAsCsTsC	6560.6	6560.0
2	C9ORF72-PS/PO	GsC _o C _o C _o CsTsAsGsCsGsCsGsCsGsGsGsAsCsTsC	6465.6	6465.2
3	C9ORF72-DNA+PS	GsCsCsCsCsTsAsGsCsGsCsGsCsGsGsAsCsTsC	5820.8	5820.6
4	C9ORF72-MOE+PS	GsCsCsCsCsTsAsGsCsGsCsGsCsGsGsAsCsTsC	7153.3	7154.3
5	C9ORF72-OMe+PS	GsCsCsCsCsTsAsGsCsGsCsGsCsGsGsAsCsTsC	6332.0	6332.8
6	MALAT1-full PS	GsGsGsTsCsAsGsCsTsGsCsCsAsAsTsGsCsTsAsG	7232.1	7231.9
7	MALAT1-PS/PO	GsG _o G _o T _o CsAsGsCsTsGsCsCsAsAsTsG _o C _o T _o AsG	7136.3	7135.2
8	HTT-full PS	CsTsCsGsAsCsTsAsAsAsGsCsAsGsGsAsTsTsTsC	7217.1	7216.8
9	HTT-PS/PO	CsT _o C _o G _o AsCsTsAsAsAsGsCsAsGsGsAsT _o T _o TsC	7120.7	7120.0
10	NTC-full PS	CsCsTsAsTsAsGsGsAsCsTsAsTsCsCsAsGsGsAsA	7184.1	7184.0
11	NTC-PS/PO	CsC _o T _o A _o TsAsGsGsAsCsTsAsTsCsCsA _o G _o G _o AsA	7088.1	7087.1

185 **Table 2.** Sequences used in this study. Bold blue: MOE. Bold green: 2'-OMe. Black: DNA. Lower case
 186 letters s and o refer to phosphorothioate and phosphodiester linkages, respectively. NTC: non-target
 187 control ASO.

188



189

190 **Figure 1.** The tolerability of ASOs administered into the CNS is improved by modestly reducing the backbone PS
 191 content and by formulating with Ca²⁺ ions before injecting. Mice were injected ICV with 35 nmol of each ASO in 10
 192 μ L PBS (or with 10 μ L PBS as control) and behavior was scored by a blinded investigator over the following 24h
 193 using the EvADINT rubric. Sequences targeting (A) *C9ORF72*, (B) *Malat1*, or (C) *Htt*, or (D) a non-targeting control
 194 ASO, showed improvements in acute tolerability upon reducing the PS content, formulating with Ca²⁺ ions, or both.
 195 Each data point represents the EvADINT score from one mouse; n = 6–8; error bars represent SEM. P-values are
 196 calculated using one-way ANOVA within GraphPad Prism software and represent per-comparison error rates. Full
 197 sequences and modification patterns are given in Table 2.

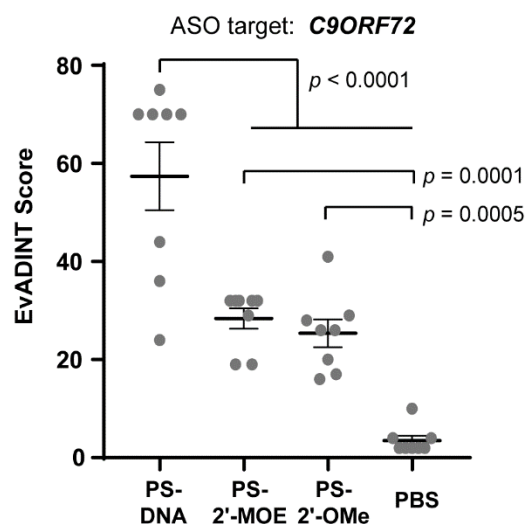
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199 Sugar 2'-modifications reduce acute motor phenotypes

200 Nusinersen, approved to treat spinal muscular atrophy,⁹⁻¹² contains a fully phosphorothioate backbone.
201 Yet it is well tolerated in the CNS⁸ and has received FDA approval.^{5, 6} This compound is modified at each
202 nucleotide with MOE; it is not a gapmer and does not require a stretch of DNA because it functions to
203 redirect splicing rather than recruiting RNase H. We therefore wondered whether fully-
204 phosphorothioate-modified ASOs containing different sugar modifications might show acute motor
205 phenotypes to a different extent.

206 To study this question, we synthesized fully-phosphorothioate ASOs modified *at every nucleotide* with
207 DNA, 2'-O-methyl RNA, or MOE, respectively (in contrast to the gapmer designs used in Figure 1). We
208 suspended these in PBS, injected them ICV at 35 nmol/mouse and scored acute motor phenotypes using
209 the EvADINT assay. The fully DNA oligonucleotide **3** was dramatically more toxic than the two
210 oligonucleotides containing 2'-modifications at every nucleotide (**4** and **5**; Figure 2). The two 2'-modified
211 versions showed a dramatic reduction in motor phenotypes. We also carried out the comparison of full
212 MOE with full DNA for a sequence targeting Malat1 and saw the same dramatic difference in toxicity
213 (data not shown: the Malat1 experiment was carried out before we had established the quantitative
214 EvADINT assay, but the clear difference we observed strongly suggests that the impact of sugar
215 modification on toxicity is not sequence specific.)

216



217

218 **Figure 2.** Fully PS ASOs containing 2'-modifications at each position are less toxic than those containing DNA at
219 each position. We injected 35 nmol of each ASO in PBS to the right lateral ventricle of mice, and recorded
220 behavioral outcomes according to the EvADINT rubric. Each data point represents the EvADINT score from one

221 mouse; n = 8; error bars represent SEM. P-values are calculated using one-way ANOVA within GraphPad Prism
222 software and represent per-comparison error rates. Full sequences and modification patterns are given in Table 2.

223

224 **PS-ASO-induced acute neurotoxicity is not mediated by the major nucleic acid sensing innate immune**
225 **pathways**

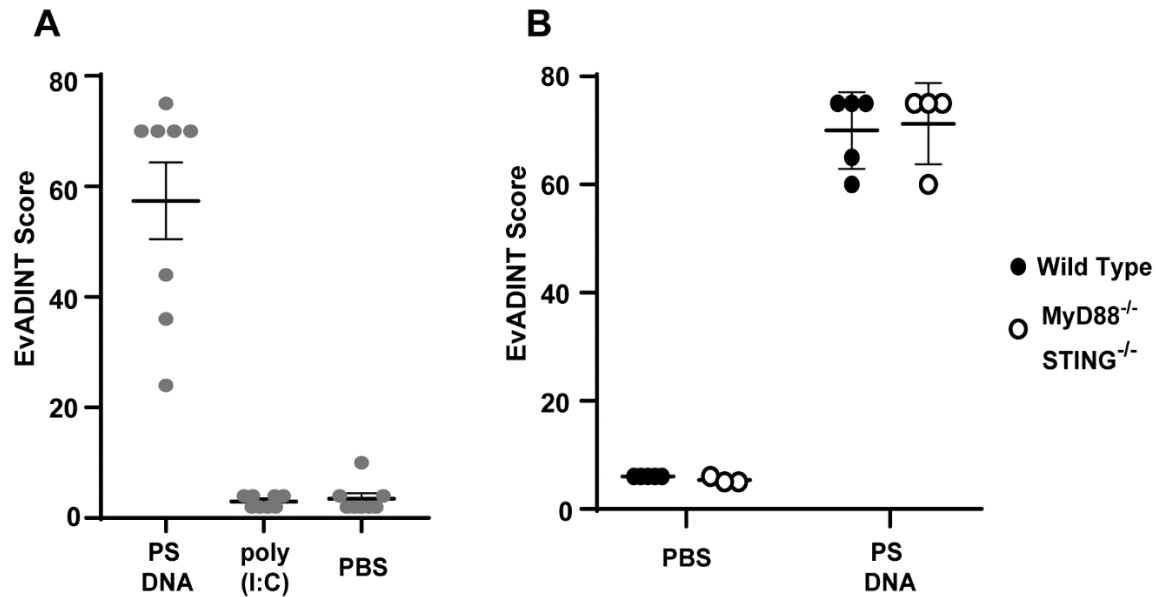
226 The acute toxicity we observe is most intense in the first hour after injection of mice. This timing
227 suggested to us that the acute toxicity is not mediated by the innate immune system, since innate
228 immune responses to nucleic acid stimuli typically do not peak until several hours after stimulation.^{29, 30}

229 To confirm in a more direct way whether innate immune responses could play a role, we directly
230 evaluated whether there was any contribution to this neurotoxicity from signaling through Toll-like
231 receptors (TLRs) 3, 7, or 9, or the CGAS-STING pathway. First, we evaluated mouse behavior after
232 injecting a 50 µg dose of poly(I:C), a compound and dose known to induce potent innate immune
233 stimulation through TLR3 or MDA-5.^{29,31} Mice treated with poly(I:C) showed no evidence of the acute
234 motor phenotypes seen with PS-modified ASOs, with EvADINT scores comparable to the buffer-only
235 control mice (Figure 3A).

236 We also evaluated the role of other endosomal receptors TLRs 7 and 9 as well as the cytosolic DNA
237 sensing cGAS-STING pathway by obtaining mice lacking both MyD88 and STING (Myd88^{-/-} STING^{-/-} double
238 knockout mouse). This strain lacks signaling components for both endosomal as well as cytosolic nucleic
239 acid sensing pathways. TLRs 7 and 9 signal through MyD88, whereas STING functions downstream of
240 cGAS following cytosolic DNA sensing. Injection of a PS-DNA ASO into these double knockout mice
241 showed an identical response relative to a background-matched control mouse, confirming that the
242 toxicity is not mediated by any of these nucleic acid sensors (Figure 3B).

243 Taken together, these experiments provide evidence that the acute toxicity which is the focus of this
244 work is not mediated by the major nucleic acid sensing innate immune pathways. Of course, this finding
245 does not preclude a role for other oligonucleotide-induced innate immune responses in the brain at
246 longer timepoints, as described by other authors.³²

247



248

249 **Figure 3.** The acute neurotoxicity we observe is not mediated by toll-like receptors 3, 7, or 9, by MDA-5, or by the
250 cGAS-STING pathway. (A) The highly immunogenic compound poly(I:C) produces none of the behavioral toxicity
251 seen for the PS-containing ASOs after ICV injection, showing that the toxicity is not signaling through TLR3 or MDA-
252 5. We injected 35 nmol of PS-DNA or 50 μ g of poly(I:C) in PBS to the right lateral ventricle of mice, and recorded
253 behavioral outcomes according to the EvADINT rubric. (B) Double knockout MyD88^{-/-} STING^{-/-} mice (hollow dots)
254 show an identical response to wild type mice (filled dots), showing that the acute toxicity is not mediated by TLR7,
255 TLR9 or the CGAS-STING pathway. In both panels, each data point represents the EvADINT score from one mouse;
256 error bars represent SEM. All PS-DNA samples were significantly different from all other samples in both panels
257 (One-way ANOVA, $p < 0.0001$). Full sequences and modification patterns are given in Table 2.

258

259

260 **The improved tolerability of mixed backbone ASOs applies to large brains**

261 We wondered whether the acute toxicity we observed was an artifact of the small brain size of mice.
262 This would make the concern significantly less relevant to researchers interested in therapeutic
263 development of ASOs. To test whether the phenomenon applied to larger brains, we injected two
264 sheep with fully phosphorothioate ASO (C9ORF72-full PS, Sequence 1), and four sheep with the mixed
265 backbone analogue of the same sequence (C9ORF72-PS/PO, Sequence 2).

266 Direct intrathecal injection, the route used for patients receiving ASO therapeutics, is not practical in
267 sheep because of difficulty accessing the intrathecal compartment and because CSF tends to be expelled
268 from the site where the dura is punctured, leading to poor uptake of ASO. Therefore, we used a
269 technique whereby a microcatheter was threaded up through the intrathecal space and the ASO was
270 delivered directly into the cisterna magna (see Methods). In all cases successful microcatheter
271 navigation was performed into cisterna magna. Both intracisternal contrast injection and cone beam
272 computed tomography confirmed the correct catheter position prior to ASO injection. Contrast material
273 opacification was seen in the cisterna magna, around the cerebellum and in the upper cervical spinal
274 canal. No complication was observed in relation to catheter navigation or contrast injection.

275 None of the four sheep that were given the mixed backbone ASO (C9ORF72-PS/PO, Sequence 2) showed
276 evidence of abnormal motor phenotypes. In contrast, both sheep that were given fully
277 phosphorothioate ASO (C9ORF72-full PS, Sequence 1) showed hindlimb weakness and gait instability
278 (wobbliness) within the first 24 hours. Thus, the acute toxicity of fully phosphorothioate ASOs is not
279 specific to mice but also applies to large brains. The ASOs were given in Lactated Ringer's solution, a
280 calcium-containing diluent readily available at USP-grade, which confirms that the toxicity improvement
281 mediated by reducing PS content is at least partly distinct from the question of Ca²⁺ chelation, in larger
282 brains (in this case, sheep) as in mice as described above.

283

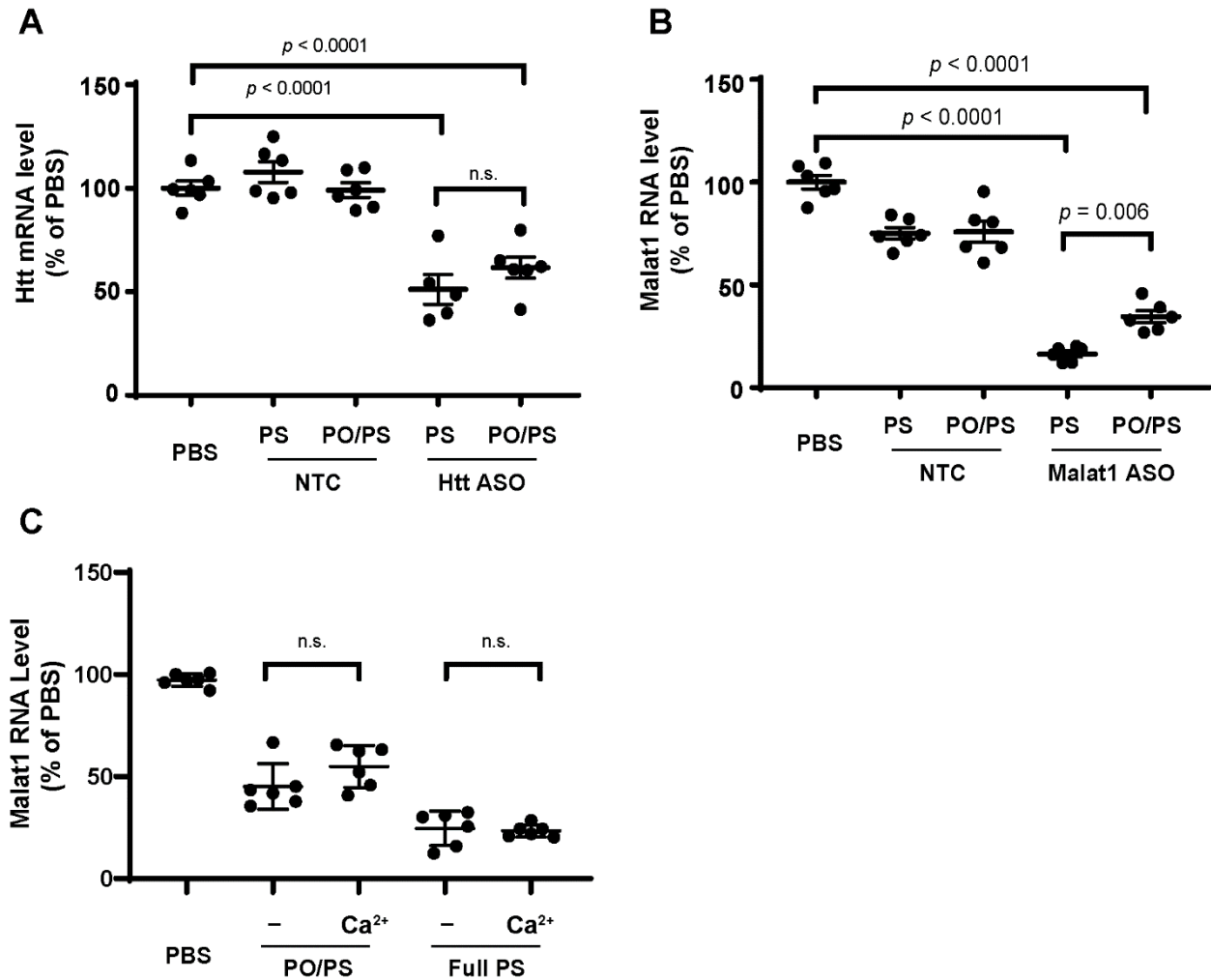
284 **Impact on efficacy of reducing phosphorothioate content and Ca²⁺ formulation**

285 We previously observed for C9ORF72-targeted ASOs that the mixed backbone strategy did not reduce
286 potency or efficacy as long as the phosphodiester linkages were between MOE nucleotides on both
287 sides. In contrast, when we modified the linkage between a MOE nucleotide and a deoxynucleotide, the
288 potency dropped dramatically.²¹ Thus, in this paper we have focused on a single mixed backbone

289 design, in which the three internal linkages in each MOE wing were PO, and all other positions were PS.
290 To study the effect on efficacy of this backbone design across these sequences, we compared the gene
291 silencing of ASOs against HTT and MALAT1 in their full PS and mixed backbone versions. To be able to
292 discriminate between compound efficacy, we chose a non-saturating dose for this element of our study:
293 we therefore injected 15 nmol of each ASO ICV, and harvested brains after 3 weeks.

294 We found that both HTT and MALAT1-targeted ASOs significantly reduced their target mRNA expression,
295 but there was a trend to reduced efficacy in mixed backbone format relative to the fully PS analogues
296 (Figure 4A-B). It is not clear whether this results from a reduction in nuclease stability or a reduction in
297 cellular uptake, since the PS linkage contributes to both factors. Nevertheless, this finding suggests that
298 there is merit in exploring alternate backbone architectures, including next-generation mixed backbone
299 designs,³³ that might allow improvements in acute toxicity while maintaining or improving potency and
300 efficacy.

301 Next, we tested whether calcium pre-saturation affected ASO efficacy. We prepared ASOs by dissolving
302 in PBS, either with or without a Ca²⁺ pre-saturation step, and injected them ICV into mice. In the context
303 of either a full PS or mixed PO/PS backbone, we observed that gene silencing efficacy was not affected
304 by the presence of the Ca²⁺ pre-saturation step (Figure 4C).



305

306 **Figure 4.** Effect of phosphorothioate reduction and calcium formulation on ASO efficacy. (A,B) Silencing of (A) *Htt*
307 and (B) *Malat1* RNA three weeks after a 15 nmol dose of either full PS of mixed backbone (PO/PS) ASOs. Each data
308 point represents one mouse; n=6 mice. (C) Formulation with calcium does not affect the gene silencing efficacy of
309 ASOs. ASOs were resuspended and delivered in PBS, with or without first saturating the ASOs with calcium (see
310 methods). In all panels, statistical significance was evaluated by one-way ANOVA followed by Tukey's multiple
311 comparisons test. Sequence numbers relate to the sequences and modification patterns given in Table 2.

312

313 DISCUSSION

314 Over the past few years, an increasing number of papers have described the use of gapmer ASOs in the
315 CNS. Many of these studies employ ASOs containing full-PS backbones^{4, 13, 34-36} or those for which the
316 modification pattern is not clearly disclosed.^{16, 18, 37, 38} Other recent papers do describe ASOs containing
317 a mixture of PS and PO linkages for use in the CNS.^{15, 17, 19, 20, 36, 39-42} However, to the best of our
318 knowledge, no comparative data on the neurotoxicology of these mixed-backbone ASOs relative to fully-
319 PS ASOs has been presented, nor has the relationship of sugar modification with acute motor
320 phenotypes been previously known or the underlying mechanism explored before now.

321 The mechanism for the acute neurotoxicity of PS-modified ASOs is likely multifactorial. We initially
322 wondered whether the more polyanionic nature of PS linkages was increasing chelation of divalent ions
323 by ASOs. Since divalent cations, and Ca²⁺ in particular, are key for synaptic signaling and
324 neurotransmission,^{43, 44} such depletion of divalent cations from CSF would be expected to produce acute
325 toxicity that would be expected to last until homeostasis of Ca²⁺ concentration in CSF was restored. Ca²⁺
326 chelation has been responsible for unexpected toxicities in other classes of drugs.⁴⁵ Supporting a role
327 for this mechanism, pre-saturating ASOs with Ca²⁺ before injection improved their tolerability. Other
328 groups have observed ASO-mediated Ca²⁺ chelation in cultured neurons.⁴⁶ However, mixed backbone
329 strategies improved toxicity even in the presence of such divalent ions, which suggests that Ca²⁺
330 chelation cannot fully explain the acute motor phenotypes induced by PS-modified gapmer ASOs.

331 Our experiments document that ASO-induced transient motor phenotypes are not a downstream
332 consequence of innate immune signaling through the major nucleic-acid-sensing immune pathways.
333 Treatment with poly(I:C) did not recapitulate the acute toxicity induced by treatment with PS-modified
334 ASOs, suggesting that the PS-ASO-induced motor phenotypes do not result from TLR3- or MDA-5-
335 mediated effects. And experiments in Myd88^{-/-} STING^{-/-} mice confirmed that PS-ASO-induced motor
336 phenotypes are not mediated by TLR7, TLR9, or cGAS/STING.

337 At least part of the acute neurotoxicity of PS-ASOs is likely to be mediated by protein binding, for
338 example, to cell surface receptors involved in neuronal signaling. PS-DNA shows extensive binding to a
339 variety of proteins,⁴⁷⁻⁵⁰ including cell-surface and trafficking proteins.⁵¹⁻⁵⁴ The origins of the high protein
340 binding of PS-oligos were recently explored with a structural study.⁵⁵ After systemic administration, an
341 earlier generation of mixed-backbone modification ASOs showed reduced binding to proteins such as
342 complement pathway members and clotting factors.⁵⁶⁻⁵⁹ An explanation of the acute motor phenotypes

343 related to protein binding is also consistent with the sugar modification data presented above, since PS-
344 MOE reduces nonspecific protein binding relative to PS-DNA,⁶⁰ for example, recent work showed that
345 PS-MOE bound various plasma proteins with 3 to 50-fold lower affinity relative to PS-DNA.⁶¹ In another
346 recent study, PS-2'OMe gapmers of two different sequences showed about 2.5-fold lower affinity
347 protein binding than PS-DNA of the same sequence.⁵⁵

348 The acute motor phenotypes discussed in this work do not appear to be related to other types of ASO-
349 induce toxicity – such as liver toxicity or immune stimulation. For example, in ongoing work in our
350 group, we have come across sequences that are well-tolerated in terms of acute motor phenotypes, but
351 still exhibit liver toxicity, and vice versa. The timing of these effects is also very different: with acute
352 motor phenotypes strongest in the first hour (perhaps driven by binding to cell-surface receptors as
353 discussed above), innate immune stimulation peaking at 1-2 days (driven by binding to toll-like receptors
354 and cytosolic nucleic acid sensors), and liver toxicity evident from 1 day to several days after dosing
355 (driven by factors including mislocalization of paraspeckle proteins to nucleoli⁶²).

356 This paper has focused on a single mixed-backbone design, and the impact of replacing three PS linkages
357 within each MOE wing with their corresponding (unmodified) PO linkages. It is striking that a relatively
358 small reduction in PS content makes such a dramatic difference in acute toxicity for multiple sequences.
359 Our work on *C9ORF72* showed that this design not only maintained but improved potency relative to the
360 full PS version.²¹ Other investigators have also disclosed that some ASOs show increased potency when
361 a subset of PS linkages are replaced by PO linkages.⁶³ Nevertheless, this design sometimes leads to a
362 modest loss in potency, as shown in Figure 4.

363 The well-tolerated nature of fully 2'-modified ASOs in the CNS (Figure 2) has allowed the rapid
364 development and FDA approval of nusinersen⁵⁻⁹ as well as the first personalized ASO drug, milasen.⁶⁴
365 However, recruitment of RNase H requires the presence either of DNA or a DNA analogue, typically as a
366 gapmer design.^{65,66} The fact that PS-DNA shows higher acute toxicity than the corresponding 2'-
367 modified nucleotides (Figure 2) suggests that a major area of focus for nucleic acid chemists should be
368 the development of sugar or phosphate-modified DNA analogues that elicit robust RNase H cleavage
369 while reducing the incidence of motor phenotypes when administered to the CNS. The development of
370 next-generation mixed-backbone ASOs is an active area of research in our group.^{33,67} In the meantime,
371 researchers can implement mixed backbone designs such as those described here, along with calcium
372 formulation, to improve the therapeutic index of gapmer ASOs for clinical use in the CNS.

373

374 **METHODS**

375 **Oligonucleotides**

376 All oligonucleotides were synthesized using ABI 394 or Akta OligoPilot synthesizers using standard
377 methods. Phosphoramidites were purchased from ChemGenes and diluted to 0.1 M in acetonitrile.
378 Sulfurization was accomplished using DDTT (0.1M, ChemGenes). Benzylthiotetrazole (0.25 M in
379 acetonitrile, TEDIA) was used as activator. All cytosine residues were 5-methylcytosine.

380 Oligonucleotides were deprotected by treatment with concentrated aqueous ammonia at 55°C for 16h
381 then concentrated and purified by ion-exchange HPLC (eluting with 30% acetonitrile in water containing
382 increasing gradients of NaClO₄) or ion-pairing reverse-phase HPLC (eluting with aqueous
383 triethylammonium acetate containing increasing gradients of acetonitrile). All oligonucleotides were
384 characterized by LCMS.

385 After HPLC purification, we carried out final desalting and buffer equilibration using ultrafiltration
386 (Amicon centrifugal filters, 3-kDa molecular weight cutoff, Millipore). For oligonucleotides administered
387 in PBS, we placed the oligonucleotide in the Amicon filter and washed with 2 changes of PBS. To
388 saturate calcium-binding sites within oligonucleotides, we placed the purified oligonucleotides in the
389 Amicon filter, washed them twice with a 20 mM solution of CaCl₂, twice with water, and once with PBS,
390 before resuspending the ASOs in PBS (Note, in follow up work after the completion of this study, we
391 found that a lower concentration of CaCl₂ was preferable to minimize compound loss during this wash
392 step, which is an issue for certain sequences). For the oligonucleotides used for sheep studies, we did
393 not carry out calcium saturation in this manner but rather resuspended in USP-grade Lactated Ringers
394 Solution (LRS, which contains 1.3 mM Ca²⁺).

395

396 **ICV administration of oligonucleotides in mice**

397 Mouse studies were carried out under UMass Medical School IACUC protocol A-2551. FVB/NCI mice (7-
398 13 weeks old) were anaesthetized by intraperitoneal injection of fentanyl/midazolam/dexmedetomidine
399 (0.1, 5, and 0.25 mg/kg, respectively, as a solution in sterile saline). Anaesthetized animals were
400 transferred to a Kopf small animal stereotaxic frame, ear bars placed and a hand-warmer was placed
401 underneath the animal to preserve core body-temperature. Ophthalmic lubricant was placed over each
402 eye and the fur covering the skull was removed. The scalp was aseptically prepared by thorough

403 alternate swabbing with betadine and 70% isopropanol (3x each) and allowed to dry before a medial
404 incision was made to expose the skull. The periosteum was dried with a sterile cotton swab and the
405 syringe containing oligonucleotide was moved to bregma. A point was marked on the skull 1 mm
406 dextralateral and 0.4 mm posterior from bregma and a 0.6–0.8-mm diameter hole drilled at this
407 location. The tip of the needle was advanced 2 mm ventrally through this hole into the lateral ventricle
408 and after a 2 minute wait, the oligonucleotide was injected over a period of 25 seconds (10 μ L total
409 injection volume). The needle was left in place for 3 minutes post-injection, then removed and the skin
410 closed with 5-0 vicryl suture. An intraperitoneal injection of flumazenil/atipamezole (0.5 mg/kg and 5
411 mg/kg respectively, in sterile saline) was used to reverse injected anesthetic agents. Buprenorphine was
412 also injected for analgesia (0.3 mg/kg, SC). Animals were removed from the stereotaxic frame and
413 allowed to recover in a warm cage, food and gel were provided, and the animals were observed
414 periodically over the next 24 hours according to the rubric laid out in Table 1.

415 For ICV injections in the Myd88^{-/-} STING^{-/-} mice (C57BL/6 background) and corresponding C57BL/6
416 controls, we used the protocol described above with slightly adjusted coordinates for the ICV injection
417 (1mm dextralateral, 2mm posterior, 3mm ventral.)

418

419 **STING/MyD88 double knockout mice**

420 Myd88^{-/-} mice on C57BL/6 background⁶⁸ were obtained from S. Akira (Osaka University, Osaka, Japan).
421 STING^{-/-} mice on C57BL/6 background⁶⁹ were originally from G. Barber (University of Miami, Florida) and
422 obtained from D. Stetson (University of Washington, Seattle). The two strains were intercrossed to
423 generate Myd88^{-/-} STING^{-/-} double knockouts. The mice were bred and maintained under pathogen-free
424 conditions in our animal facility. The Myd88 and STING deficiencies were confirmed by performing PCR
425 on DNA obtained after digesting a tail snip. For Myd88, specific primer pairs were used to distinguish the
426 WT or knockout allele in two separate reactions. Reaction 1 with primer sequences AGC CTC TAC ACC
427 CTT CTC TTC TCC ACA and AGA CAG GCT GAG TGC AAA CTT GTG CTG was used to detect the WT band at
428 1000 bp and reaction 2 with primer pairs AGC CTC TAC ACC CTT CTC TTC TCC ACA and ATC GCC TTC TAT
429 CGC CTT CTT GAC GAG were used to detect KO band at 1000 bp. For STING, reaction 1 with primer
430 sequences AGA ACG GAC AGC CAG TAA GTA TAC AG and CAA TGC TCT CAT AGC CTT CAC TAT C was
431 used to detect the WT band at 375 bp and reaction 2 with primer pairs AAC TTC CTG ACT AGG GGA GGA
432 GTA G and CAA TGC TCT CAT AGC CTT CAC TAT C was used to detect the KO band at 470 base pairs.

433

434 **ASO administration in sheep**

435 Sheep studies were carried out under UMass Medical School IACUC protocol A-2593. Jacob sheep were
436 fasted overnight in preparation for surgery. A 20G catheter was placed and secured in the jugular vein,
437 blood (5 mL) was drawn from the catheter for analysis, and the catheter was flushed with saline (0.9%
438 NaCl). We administered buprenorphine (0.01 mg/kg IM), acepromazine (0.05 mg/kg IM) and
439 glycopyrrolate (0.01 mg/kg IM) 30 min prior to induction of anesthesia. An intravenous cocktail of
440 ketamine (6 mg/kg) and diazepam (0.3 mg/kg) was administered to induce anesthesia, followed by
441 ketoprofen (2.2 mg/kg SQ) as analgesic and cefazolin (22 mg/kg IV) to minimize any risk of infection.
442 The animal was intubated, and a stomach tube was placed to prevent rumen gas pressure build up.
443 Anesthesia was maintained using vaporized isoflurane (1.5–3.5% in oxygen). Sheep were positioned in
444 lateral recumbency in an Allura Xper FD20 X-ray system (Philips Medical Systems, Best, Netherlands). A
445 19-gauge Tuohy needle was inserted in the lumbosacral (L7-S1) intrathecal space and then ~5 mL of CSF
446 was removed. Using the Touhy needle as entry point, a straight tip microcatheter (Excelsior SL-10;
447 Stryker Neurovascular) was inserted through the lumen of the needle to access the intrathecal space.
448 The microcatheter was navigated into the cisterna magna with an assistance of a 0.014" wire under
449 fluoroscopic guidance. The wire had a slight curve on the tip (Synchro Guidewire, Stryker Neurovascular)
450 to avoid any nerve or vascular structure damage. A microcatheter contrast injection (1 mL of
451 Omnipaque 240 mg/ml) was injected and the pattern of contrast material distribution was visualized
452 prior to the injection of ASO solution (2 mg/kg in ~3 mL of Lactated Ringers Solution; for comparison, the
453 mouse doses of 15-35 nmol/mouse equate to about 4-10 mg/kg). Cone beam computed tomography
454 (Allura Xper FD20 X-ray system) imaging was performed to confirm the final microcatheter position in
455 the cisterna magna in relation to the nerve and vascular structures. At the end of the procedure the
456 Touhy needle was withdrawn and the microcatheter was removed.

457

458 **EvADINT scoring system for acute neurotoxicity**

459 After ICV administration of ASOs to mice, a blinded investigator ranked the behavior of mice at multiple
460 timepoints using the rubric laid out in Table 1. If a mouse died within the first 24 hours, its score was
461 assigned to be 75; otherwise it was the sum of all other scores. Seizures, if observed, were scored based
462 on severity; hyperactive or spastic behavior was also scored based on severity and included twitching,
463 uncontrolled movement such as “popcorning” and other atypical motor phenotypes. Besides these
464 elements, the score was based on the time elapsed until mice resumed normal posture and behavior;

465 for example, if a mouse required more than 1 hour but less than 2 hours to be able to right itself
466 (resume and maintain sternal posture) it would be given a score of 8. Each mouse was individually
467 scored. Examples of scoring, with corresponding videos, are provided in Supporting Table S1. The
468 breakdown of scoring for each mouse is provided in Supporting Tables S2-S7.

469

470 **Evaluation of gene silencing in the CNS**

471 *For comparison of backbones on gene silencing efficacy:* Mice were euthanized at 3 weeks post-
472 treatment by cervical dislocation and the brain was immediately removed into ice-cold PBS. The brain
473 was placed in a brain matrix (Braintree scientific) and the most rostral 3 mm discarded. A 1-mm slice was
474 then taken and each side homogenized independently. The tissue was suspended in Affymetrix
475 homogenizing solution containing proteinase K and mechanically dissociated using a Qiagen Tissuelyser
476 with a 2mm tungsten carbide bead. The tubes were then incubated in a water bath at 65 °C until all
477 tubes appeared transparent. Tubes were centrifuged (16,000 xG, 15 minutes) and supernatant
478 transferred to a 96 well plate for storage at -80. *Htt*, *Malat1* and *Ppib* RNA levels were quantified using
479 the QuantiGene 2.0 assay kit (Affymetrix, QS0011) as previously described.⁷⁰

480 *For studying the effect calcium formulation on gene silencing efficacy:* Mice were euthanized at 2 weeks
481 post-injection via IP administration of 0.1mL of 390 mg/mL pentobarbital sodium and the brain and
482 spinal cord were immediately removed into ice-cold PBS. A 2mm section of the lumbar spinal cord was
483 cut and placed in an Eppendorf tube in -80°C. The brain was placed in a brain matrix and the most
484 rostral 3mm discarded. A 2-mm slice was taken, and the cortical section removed and placed in an
485 Eppendorf tube in -80°C. Tissue was homogenized in TRI-reagent using a Qiagen TissueLyser and 1 µg of
486 RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription kit (Life Technologies) per
487 the manufacturer's protocol. qRT-PCR was carried out using iTaq Supermix (Bio-Rad) on Bio-Rad CFX-96
488 real time machine using gene-specific primers: *Malat1* Primer1: 5' CTC CAA CAA CCA CTA CTC CAA 3';
489 Primer2: 5' GTA CTG TTC CAA TCT GCT GCT A 3'; probe: /56-FAM/TCA TAC TCC /ZEN/AGT CGC GTC ACA
490 ATG C/3IABkFQ/. For *Ppib* (internal control), Primer1: 5'CCG TAG TGC TTC AGC TTG A 3'; Primer2: 5' AGC
491 AAG TTC CAT CGT GTC ATC 3'; Probe: /56-FAM/TGC TCT TTC /ZEN/CTC CTG TGC CAT CTC /3IABkFQ/.

492

493

494 **SUPPORTING INFORMATION / DATA AVAILABILITY**

495 Supporting information is available in the online version of this file, and includes the following:

496 Supporting Figure S1 shows the dose responsiveness of acute motor phenotypes. Supporting Table S1
497 and associated movies show examples of mouse phenotypes and corresponding assigned scores. Tables
498 S2-S10 provide a breakdown of the EvADINT scoring for each mouse and each treatment.

499

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508

509 **AUTHOR CONTRIBUTIONS**

510 MPM and PMK synthesized and purified oligonucleotides; MPM, JMR-B, FW and AW carried out mouse
511 experiments; FW, JMR-B and JKW developed the EvADINT scoring assay; MPM, JMR-B, M.Marosfoi, RMK
512 and HG-E carried out the sheep study; M.Motwani and KAF developed the knockout mice, JKW
513 supervised the study and wrote the manuscript; all authors edited the manuscript.

514

515 **COMPETING INTERESTS**

516 JKW is Scientific Advisory Board member of PepGen and ad hoc consultant for BridgeBio and Flagship
517 Pioneering. RHB is co-founder and Scientific Advisory Board member of ApicBio.

518

519 **REFERENCES**

- 520 1. Seguin, R., Antisense Oligonucleotides for Treatment of Neurological Diseases. In
521 *Oligonucleotide-Based Drugs and Therapeutics: Preclinical and Clinical Considerations for*
522 *Development*. (eds. N. Ferrari & R. Seguin) 389-409 (Wiley, Hoboken; 2018).
- 523 2. Evers, M.M., Toonen, L.J. & van Roon-Mom, W.M. (2015). Antisense oligonucleotides in therapy
524 for neurodegenerative disorders. *Adv Drug Deliv Rev* 87, 90-103.
- 525 3. Wahlestedt, C. (1994). Antisense oligonucleotide strategies in neuropharmacology. *Trends*
526 *Pharmacol. Sci.* 15, 42-46.
- 527 4. Smith, R.A., Miller, T.M., Yamanaka, K., Monia, B.P., Condon, T.P., Hung, G., Lobsiger, C.S., Ward,
528 C.M., McAlonis-Downes, M., Wei, H. et al. (2006). Antisense oligonucleotide therapy for
529 neurodegenerative disease. *J. Clin. Invest.* 116, 2290-2296.
- 530 5. Aartsma-Rus, A. (2017). FDA Approval of Nusinersen for Spinal Muscular Atrophy Makes 2016
531 the Year of Splice Modulating Oligonucleotides. *Nucleic Acid Ther* 27, 67-69.
- 532 6. Corey, D.R. (2017). Nusinersen, an antisense oligonucleotide drug for spinal muscular atrophy.
533 *Nat. Neurosci.* 20, 497-499.
- 534 7. Hua, Y., Sahashi, K., Hung, G., Rigo, F., Passini, M.A., Bennett, C.F. & Krainer, A.R. (2010).
535 Antisense correction of SMN2 splicing in the CNS rescues necrosis in a type III SMA mouse
536 model. *Genes Dev.* 24, 1634-1644.
- 537 8. Passini, M.A., Bu, J., Richards, A.M., Kinnecom, C., Sardi, S.P., Stanek, L.M., Hua, Y., Rigo, F.,
538 Matson, J., Hung, G. et al. (2011). Antisense Oligonucleotides Delivered to the Mouse CNS
539 Ameliorate Symptoms of Severe Spinal Muscular Atrophy. *Sci. Trans. Med.* 3, 72ra18-72ra18.
- 540 9. Rigo, F., Hua, Y., Krainer, A.R. & Bennett, C.F. (2012). Antisense-based therapy for the treatment
541 of spinal muscular atrophy. *J. Cell Biol.* 199, 21-25.
- 542 10. Singh, N.N., Howell, M.D., Androphy, E.J. & Singh, R.N. (2017). How the discovery of ISS-N1 led
543 to the first medical therapy for spinal muscular atrophy. *Gene Ther.* 24, 520-526.
- 544 11. Finkel, R.S., Mercuri, E., Darras, B.T., Connolly, A.M., Kuntz, N.L., Kirschner, J., Chiriboga, C.A.,
545 Saito, K., Servais, L., Tizzano, E. et al. (2017). Nusinersen versus Sham Control in Infantile-Onset
546 Spinal Muscular Atrophy. *N. Engl. J. Med.* 377, 1723-1732.
- 547 12. Mercuri, E., Darras, B.T., Chiriboga, C.A., Day, J.W., Campbell, C., Connolly, A.M., Iannaccone,
548 S.T., Kirschner, J., Kuntz, N.L., Saito, K. et al. (2018). Nusinersen versus Sham Control in Later-
549 Onset Spinal Muscular Atrophy. *N. Engl. J. Med.* 378, 625-635.
- 550 13. Kordasiewicz, H.B., Stanek, L.M., Wancewicz, E.V., Mazur, C., McAlonis, M.M., Pytel, K.A.,
551 Artates, J.W., Weiss, A., Cheng, S.H., Shihabuddin, L.S. et al. (2012). Sustained therapeutic
552 reversal of Huntington's disease by transient repression of huntingtin synthesis. *Neuron* 74,
553 1031-1044.
- 554 14. Tabrizi, S., Leavitt, B., Kordasiewicz, H., Czech, C., Swayze, E., Norris, D.A., Baumann, T., Gerlach,
555 I., Schobel, S., Smith, A. et al. (2018). Effects of IONIS-HTTRx in Patients with Early Huntington's
556 Disease, Results of the First HTT-Lowering Drug Trial (CT.002). *Neurology* 90.
- 557 15. Tabrizi, S.J., Leavitt, B.R., Landwehrmeyer, G.B., Wild, E.J., Saft, C., Barker, R.A., Blair, N.F.,
558 Craufurd, D., Priller, J., Rickards, H. et al. (2019). Targeting Huntingtin Expression in Patients with
559 Huntington's Disease. *N. Engl. J. Med.* 380, 2307-2316.
- 560 16. DeVos, S.L., Miller, R.L., Schoch, K.M., Holmes, B.B., Kebodeaux, C.S., Wegener, A.J., Chen, G.,
561 Shen, T., Tran, H., Nichols, B. et al. (2017). Tau reduction prevents neuronal loss and reverses
562 pathological tau deposition and seeding in mice with tauopathy. *Sci. Trans. Med.* 9, eaag0481.
- 563 17. Zhao, H.T., John, N., Delic, V., Ikeda-Lee, K., Kim, A., Weihofen, A., Swayze, E.E., Kordasiewicz,
564 H.B., West, A.B. & Volpicelli-Daley, L.A. (2017). LRRK2 Antisense Oligonucleotides Ameliorate α -
565 Synuclein Inclusion Formation in a Parkinson's Disease Mouse Model. *Mol. Ther. Nucl. Acids* 8,
566 508-519.

- 567 18. Jiang, J., Zhu, Q., Gendron, Tania F., Saberi, S., McAlonis-Downes, M., Seelman, A., Stauffer,
568 Jennifer E., Jafar-nejad, P., Drenner, K., Schulte, D. et al. (2016). Gain of Toxicity from ALS/FTD-
569 Linked Repeat Expansions in *C9ORF72* Is Alleviated by Antisense Oligonucleotides Targeting
570 GGGGCC-Containing RNAs. *Neuron* *90*, 535-550.
- 571 19. Becker, L.A., Huang, B., Bieri, G., Ma, R., Knowles, D.A., Jafar-Nejad, P., Messing, J., Kim, H.J.,
572 Soriano, A., Auburger, G. et al. (2017). Therapeutic reduction of ataxin-2 extends lifespan and
573 reduces pathology in TDP-43 mice. *Nature* *544*, 367.
- 574 20. McCampbell, A., Cole, T., Wegener, A.J., Tomassy, G.S., Setnicka, A., Farley, B.J., Schoch, K.M.,
575 Hoye, M.L., Shabsovich, M., Sun, L. et al. (2018). Antisense oligonucleotides extend survival and
576 reverse decrement in muscle response in ALS models. *J. Clin. Invest.* *128*, 3558-3567.
- 577 21. Tran, H., Moazami, M.P., Yang, H., McKenna-Yasek, D., Douthwright, C., Pinto, C., Metterville, J.,
578 Shin, M., Sanil, N., Dooley, C. et al. (2021). Potent mixed backbone antisense oligonucleotide
579 safely suppresses expression of mutant *c9orf72* transcripts and polydipeptides: First in human
580 pilot study. *Research Square* (preprint) DOI: [10.21203/rs.3.rs-211236/v1](https://doi.org/10.21203/rs.3.rs-211236/v1).
- 581 22. Khvorova, A. & Watts, J.K. (2017). The chemical evolution of oligonucleotide therapies of clinical
582 utility. *Nature Biotechnology* *35*, 238-248.
- 583 23. Watts, J.K., The medicinal chemistry of antisense oligonucleotides. In *Oligonucleotide-Based*
584 *Drugs and Therapeutics*. (eds. N. Ferrari & R. Seguin) 39-90 (Wiley, Hoboken; 2018).
- 585 24. Wan, W.B. & Seth, P.P. (2016). The medicinal chemistry of therapeutic oligonucleotides. *J. Med.*
586 *Chem.* *59*, 9645-9667.
- 587 25. Moser, V.C. (2011). Functional Assays for Neurotoxicity Testing. *Toxicol. Pathol.* *39*, 36-45.
- 588 26. OECD GUIDELINE FOR THE TESTING OF CHEMICALS (1997). Test No. 424: Neurotoxicity Study in
589 Rodents DOI [10.1787/20745788](https://doi.org/10.1787/20745788).
- 590 27. Murphy, V.A., Smith, Q.R. & Rapoport, S.I. (1986). Homeostasis of brain and cerebrospinal fluid
591 calcium concentrations during chronic hypo- and hypercalcemia. *J. Neurochem.* *47*, 1735-1741.
- 592 28. In more recent unpublished work, we have identified that 5 mM calcium is a better
593 concentration for this saturation protocol, since for some sequences 20 mM calcium leads to
594 reduced solubility.
- 595 29. Town, T., Jeng, D., Alexopoulou, L., Tan, J. & Flavell, R.A. (2006). Microglia Recognize Double-
596 Stranded RNA via TLR3. *The Journal of Immunology* *176*, 3804-3812.
- 597 30. Jiang, M., Zhang, S., Yang, Z., Lin, H., Zhu, J., Liu, L., Wang, W., Liu, S., Liu, W., Ma, Y. et al. (2018).
598 Self-Recognition of an Inducible Host lncRNA by RIG-I Feedback Restricts Innate Immune
599 Response. *Cell* *173*, 906-919.e913.
- 600 31. Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A.,
601 Kawai, T., Ishii, K.J. et al. (2006). Differential roles of MDA5 and RIG-I helicases in the recognition
602 of RNA viruses. *Nature* *441*, 101-105.
- 603 32. Toonen, L.J.A., Casaca-Carreira, J., Pellise-Tintore, M., Mei, H., Temel, Y., Jahanshahi, A. & van
604 Roon-Mom, W.M.C. (2018). Intracerebroventricular Administration of a 2'-O-Methyl
605 Phosphorothioate Antisense Oligonucleotide Results in Activation of the Innate Immune System
606 in Mouse Brain. *Nucleic Acid Ther* *28*, 63-73.
- 607 33. Debacker, A.J., Sharma, V.K., Meda Krishnamurthy, P., O'Reilly, D., Greenhill, R. & Watts, J.K.
608 (2019). Next-Generation Peptide Nucleic Acid Chimeras Exhibit High Affinity and Potent Gene
609 Silencing. *Biochemistry* *58*, 582-589.
- 610 34. Ostergaard, M.E., Southwell, A.L., Kordasiewicz, H., Watt, A.T., Skotte, N.H., Doty, C.N., Vaid, K.,
611 Villanueva, E.B., Swayze, E.E., Bennett, C.F. et al. (2013). Rational design of antisense
612 oligonucleotides targeting single nucleotide polymorphisms for potent and allele selective
613 suppression of mutant Huntingtin in the CNS. *Nucleic Acids Res* *41*, 9634-9650.

- 614 35. DeVos, S.L., Goncharoff, D.K., Chen, G., Kebodeaux, C.S., Yamada, K., Stewart, F.R., Schuler, D.R.,
615 Maloney, S.E., Wozniak, D.F., Rigo, F. et al. (2013). Antisense Reduction of Tau in Adult Mice
616 Protects against Seizures. *J. Neurosci.* *33*, 12887-12897.
- 617 36. Meng, L., Ward, A.J., Chun, S., Bennett, C.F., Beaudet, A.L. & Rigo, F. (2014). Towards a therapy
618 for Angelman syndrome by targeting a long non-coding RNA. *Nature* *518*, 409.
- 619 37. Friedrich, J., Kordasiewicz, H.B., O'Callaghan, B., Handler, H.P., Wagener, C., Duvick, L., Swayze,
620 E.E., Rainwater, O., Hofstra, B., Benneyworth, M. et al. (2018). Antisense oligonucleotide-
621 mediated ataxin-1 reduction prolongs survival in SCA1 mice and reveals disease-associated
622 transcriptome profiles. *JCI insight* *3*, e123193.
- 623 38. Luo, X., Fitzsimmons, B., Mohan, A., Zhang, L., Terrando, N., Kordasiewicz, H. & Ji, R.-R. (2018).
624 Intrathecal administration of antisense oligonucleotide against p38 α but not p38 β MAP kinase
625 isoform reduces neuropathic and postoperative pain and TLR4-induced pain in male mice. *Brain*.
626 *Behav. Immun.* *72*, 34-44.
- 627 39. Sztainberg, Y., Chen, H.M., Swann, J.W., Hao, S., Tang, B., Wu, Z., Tang, J., Wan, Y.W., Liu, Z.,
628 Rigo, F. & Zoghbi, H.Y. (2015). Reversal of phenotypes in MECP2 duplication mice using genetic
629 rescue or antisense oligonucleotides. *Nature* *528*, 123-126.
- 630 40. Moore, L.R., Rajpal, G., Dillingham, I.T., Qutob, M., Blumenstein, K.G., Gattis, D., Hung, G.,
631 Kordasiewicz, H.B., Paulson, H.L. & McLoughlin, H.S. (2017). Evaluation of Antisense
632 Oligonucleotides Targeting ATXN3 in SCA3 Mouse Models. *Mol. Ther. Nucl. Acids* *7*, 200-210.
- 633 41. Mohan, A., Fitzsimmons, B., Zhao, H.T., Jiang, Y., Mazur, C., Swayze, E.E. & Kordasiewicz, H.B.
634 (2017). Antisense oligonucleotides selectively suppress target RNA in nociceptive neurons of the
635 pain system and can ameliorate mechanical pain. *Pain* *159*, 139-149.
- 636 42. Ling, K.K., Jackson, M., Alkam, D., Liu, D., Allaire, N., Sun, C., Kiaei, M., McCampbell, A. & Rigo, F.
637 (2018). Antisense-mediated reduction of EphA4 in the adult CNS does not improve the function
638 of mice with amyotrophic lateral sclerosis. *Neurobiol. Dis.* *114*, 174-183.
- 639 43. Berridge, M.J. (1998). Neuronal Calcium Signaling. *Neuron* *21*, 13-26.
- 640 44. Brini, M., Cali, T., Ottolini, D. & Carafoli, E. (2014). Neuronal calcium signaling: function and
641 dysfunction. *Cell. Mol. Life Sci.* *71*, 2787-2814.
- 642 45. Bosakowski, T. & Levin, A.A. (1987). Comparative acute toxicity of chlorocitrate and fluorocitrate
643 in dogs. *Toxicology and Applied Pharmacology* *89*, 97-104.
- 644 46. Olson, R.E., Cacace, A.M., Hagedorn, P., Hog, A.M., Jensen, M.L., Nielsen, N.F., Li, D., Brown, J.M.
645 & Mercer, S.E. (2016). Tau antisense oligomers and uses thereof. Patent application WO
646 2016/126995 A1.
- 647 47. Brown, D.A., Kang, S.H., Gryaznov, S.M., DeDionisio, L., Heidenreich, O., Sullivan, S., Xu, X. &
648 Nerenberg, M.I. (1994). Effect of phosphorothioate modification of oligodeoxynucleotides on
649 specific protein binding. *Journal of Biological Chemistry* *269*, 26801-26805.
- 650 48. Lebedeva, I. & Stein, C.A. (2001). Antisense oligonucleotides: promise and reality. *Annual Review*
651 *of Pharmacology and Toxicology* *41*, 403-419.
- 652 49. Levin, A.A., Yu, R.Z. & Geary, R.S., Basic Principles of the Pharmacokinetics of Antisense
653 Oligonucleotide Drugs. In *Antisense Drug Technology: Principles, Strategies, and Applications*,
654 Edn. 2nd. (ed. S.T. Crooke) 183-215 (CRC Press, Boca Raton; 2008).
- 655 50. Geary, R.S. (2009). Antisense oligonucleotide pharmacokinetics and metabolism. *Expert Opin*
656 *Drug Met* *5*, 381-391.
- 657 51. Weidner, D.A., Valdez, B.C., Henning, D., Greenberg, S. & Busch, H. (1995). Phosphorothioate
658 oligonucleotides bind in a non sequence-specific manner to the nucleolar protein C23/nucleolin.
659 *FEBS Letters* *366*, 146-150.
- 660 52. Stein, C.A., Wu, S., Voskresenskiy, A.M., Zhou, J.F., Shin, J., Miller, P., Souleimanian, N. &
661 Benimetskaya, L. (2009). G3139, an anti-Bcl-2 antisense oligomer that binds heparin-binding

- 662 growth factors and collagen I, alters in vitro endothelial cell growth and tubular morphogenesis.
663 Clin. Cancer Res. 15, 2797-2807.
- 664 53. Miller, C.M., Donner, A.J., Blank, E.E., Egger, A.W., Kellar, B.M., Østergaard, M.E., Seth, P.P. &
665 Harris, E.N. (2016). Stabilin-1 and Stabilin-2 are specific receptors for the cellular internalization
666 of phosphorothioate-modified antisense oligonucleotides (ASOs) in the liver. Nucleic Acids
667 Research 44, 2782-2794.
- 668 54. Wang, S., Allen, N., Vickers, T.A., Revenko, A.S., Sun, H., Liang, X.H. & Crooke, S.T. (2018).
669 Cellular uptake mediated by epidermal growth factor receptor facilitates the intracellular
670 activity of phosphorothioate-modified antisense oligonucleotides. Nucleic Acids Res 46, 3579-
671 3594.
- 672 55. Hyjek-Skladanowska, M., Vickers, T.A., Napiorkowska, A., Anderson, B.A., Tanowitz, M., Crooke,
673 S.T., Liang, X.H., Seth, P.P. & Nowotny, M. (2020). Origins of the Increased Affinity of
674 Phosphorothioate-Modified Therapeutic Nucleic Acids for Proteins. J. Am. Chem. Soc. 142, 7456-
675 7468.
- 676 56. Agrawal, S., Rustagi, P.K. & Shaw, D.R. (1995). Novel enzymatic and immunological responses to
677 oligonucleotides. Toxicology Letters 82-83, 431-434.
- 678 57. Zhang, R., Iyer, R.P., Yu, D., Tan, W., Zhang, X., Lu, Z., Zhao, H. & Agrawal, S. (1996).
679 Pharmacokinetics and tissue disposition of a chimeric oligodeoxynucleoside phosphorothioate in
680 rats after intravenous administration. J. Pharmacol. Exp. Ther. 278, 971-979.
- 681 58. Zhao, Q., Tamsamani, J., Iadarola, P.L., Jiang, Z. & Agrawal, S. (1996). Effect of different
682 chemically modified oligodeoxynucleotides on immune stimulation. Biochem. Pharmacol. 51,
683 173-182.
- 684 59. Agrawal, S., Jiang, Z., Zhao, Q., Shaw, D., Sun, D. & Saxinger, C. (1997). Mixed-Backbone
685 Oligonucleotides Containing Phosphorothioate and Methylphosphonate Linkages as Second
686 Generation Antisense Oligonucleotide. Nucleosides Nucleotides 16, 927-936.
- 687 60. Henry, S.P., Kim, T.-W., Kramer-Stickland, K., Zanardi, T.A., Fey, R.A. & Levin, A.A., Toxicologic
688 Properties of 2'-O-Methoxyethyl Chimeric Antisense Inhibitors in Animals and Man. In *Antisense
689 Drug Technology: Principles, Strategies and Applications*. (ed. S.T. Crooke) 327-363 (CRC Press,
690 Boca Raton; 2007).
- 691 61. Gaus, H.J., Gupta, R., Chappell, A.E., Østergaard, M.E., Swayze, E.E. & Seth, P.P. (2019).
692 Characterization of the interactions of chemically-modified therapeutic nucleic acids with
693 plasma proteins using a fluorescence polarization assay. Nucleic Acids Res 47, 1110-1122.
- 694 62. Shen, W., De Hoyos, C.L., Migawa, M.T., Vickers, T.A., Sun, H., Low, A., Bell, T.A., 3rd, Rahdar, M.,
695 Mukhopadhyay, S., Hart, C.E. et al. (2019). Chemical modification of PS-ASO therapeutics
696 reduces cellular protein-binding and improves the therapeutic index. Nat. Biotechnol. 37, 640-
697 650.
- 698 63. Ferrari, N. & Seguin, R. (2012). Oligonucleotide inhibitors with chimeric backbone and 2-amino-
699 2'-deoxyadenosine. PCT application PCT/CA2012/000169.
- 700 64. Kim, J., Hu, C., Moufawad El Achkar, C., Black, L.E., Douville, J., Larson, A., Pendergast, M.K.,
701 Goldkind, S.F., Lee, E.A., Kuniholm, A. et al. (2019). Patient-Customized Oligonucleotide Therapy
702 for a Rare Genetic Disease. N. Engl. J. Med. 381, 1644-1652.
- 703 65. Damha, M.J., Wilds, C.J., Noronha, A., Brukner, I., Borkow, G., Arion, D. & Parniak, M.A. (1998).
704 Hybrids of RNA and arabinonucleic acids (ANA and 2'-F-ANA) are substrates of Ribonuclease H. J.
705 Am. Chem. Soc. 120, 12976-12977.
- 706 66. Verbeure, B., Lescrinier, E., Wang, J. & Herdewijn, P. (2001). RNase H mediated cleavage of RNA
707 by cyclohexene nucleic acid (CeNA). Nucleic Acids Research 29, 4941-4947.

- 708 67. Sharma, V.K., Singh, S.K., Krishnamurthy, P.M., Alterman, J.F., Haraszti, R.A., Khvorova, A.,
709 Prasad, A.K. & Watts, J.K. (2017). Synthesis and biological properties of triazole-linked locked
710 nucleic acid. *Chem. Commun.* 53, 8906-8909.
- 711 68. Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K. &
712 Akira, S. (1998). Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-
713 mediated function. *Immunity* 9, 143-150.
- 714 69. Ishikawa, H. & Barber, G.N. (2008). STING is an endoplasmic reticulum adaptor that facilitates
715 innate immune signalling. *Nature* 455, 674-678.
- 716 70. Coles, A.H., Osborn, M.F., Alterman, J.F., Turanov, A.A., Godinho, B.M., Kennington, L., Chase, K.,
717 Aronin, N. & Khvorova, A. (2016). A High-Throughput Method for Direct Detection of
718 Therapeutic Oligonucleotide-Induced Gene Silencing In Vivo. *Nucleic Acid Ther* 26, 86-92.

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