



# The Combination of Methyl-Phosphoramidate Inter-Nucleotide Linkages and 2'-O-Methyl in Selected Positions in the Antisense Oligonucleotide Enhances the Performance of RNaseH1 Active PS-ASOs

Lingdi Zhang, Xue-hai Liang, Cheryl Li De Hoyos, Michael Migawa, Joshua G. Nichols, Graeme Freestone, Jun Tian, Punit P. Seth, and Stanley T. Crooke

Antisense oligonucleotides (ASOs) that mediate RNA target degradation by RNase H1 are used as drugs to treat various diseases. Previously we found that introduction of a single 2'-O-methyl (2'-OMe) modification in position 2 of the central deoxynucleotide region of a gapmer phosphorothioate (PS) ASO, in which several residues at the termini are 2'-methoxyethyl, 2' constrained ethyl, or locked nucleic acid, dramatically reduced cytotoxicity with only modest effects on potency. More recently, we demonstrated that replacement of the PS linkage at position 2 or 3 in the gap with a methyl-phosphoramidate (MsPA) linkage also significantly reduced toxicity without meaningful loss of potency and increased the elimination half-life of the ASOs. In this study, we evaluated the effects of the combination of MsPA linkages and 2'-OMe nucleotides on PS ASO performance. We found that two MsPA modifications at the 5' end of the gap or in the 3'-wing of a Gap 2'-OMe PS ASO substantially increased the activity of ASOs with OMe at position 2 of the gap without altering the safety profile. Such effects were observed with multiple sequences in cells and animals. Thus, the MsPA modification improves the RNase H1 cleavage rate of PS ASOs with a 2'-OMe in the gap, significantly reduces binding of proteins involved in cytotoxicity, and prolongs elimination half-lives.

**Keywords:** methyl-phosphoramidate, 2'-O-methyl, RNase H1, antisense oligonucleotide

## Introduction

**R**NASE H1-DEPENDENT GAPMER phosphorothioate (PS) Antisense oligonucleotides (ASOs) induce degradation of targeted RNAs and are used to treat various diseases. Compared with Generation 2 (G2.0) 5-10-5 gapmer PS ASOs, which contain five 2'-methoxyethyl (2'-MOE) modified nucleotides at each end (ie, the wings) and a 10-nucleotide deoxynucleotide gap and PS linkages, G2.5 gapmer ASOs are typically 16-mers with three 2' constrained ethyl (cEt) nucleotides in the wings and a 10-nucleotide deoxynucleotide gap and PS linkages. ASOs containing locked nucleic acid (LNA) nucleotides in the wings are also

considered to be G2.5 ASOs, but clinical use has been limited by substantial toxicities [1–3]. Compared with G2.0 ASOs, the G2.5 ASOs have substantially higher affinity for target sequences in RNAs and, therefore, have higher potencies.

However, some G2.0 ASOs and large fraction of G2.5 ASOs bind with high affinity to many proteins and specifically interact with RNase H1 and paracellular proteins to form a toxic ribonucleoprotein (RNP) complex that mislocalizes to the nucleolus where it inhibits preribosomal RNA transcription and processing and ultimately causes apoptosis [4,5]. Recently, we found that introduction of a 2'-O-methyl (2'-OMe) nucleotide at gap position 2 (referred to here as the gap2 OMe modification) of gapmers with 2'-MOE, cEt, or

Department of Core Antisense Research, Ionis Pharmaceuticals, Inc., Carlsbad, California, USA.

© Lingdi Zhang et al., 2022; Published by Mary Ann Liebert, Inc. This Open Access article is distributed under the terms of the Creative Commons Attribution Noncommercial License [CC-BY-NC] (<http://creativecommons.org/licenses/by-nc/4.0/>) which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and the source are cited.

LNA wings and PS backbones significantly altered protein binding and reduced the formation of toxic RNP complexes, improving the therapeutic indices of many toxic sequences [5]. However, for some ASOs, the introduction of the gap2 OMe caused a modest reduction in RNase H1-mediated degradation of the targeted RNA.

The PS linkages in the ASOs used clinically to protect the deoxynucleotide residues in the gap from degradation by nucleases. PS-rich oligonucleotides bind proteins in a sequence and chemical modification-dependent manner [4,6,7]. PS modifications appear to be a major determinant of non-specific protein binding [6–9], although 2' sugar modifications also contribute, with more hydrophobic modifications tending to bind more proteins more tightly [7,9]. Nonspecific protein binding that results in RNP complex formation can cause pro-inflammatory effects [10] or other RNP complexes can be formed that cause cytotoxicity [3].

ASOs with mesyl phosphoramidate (MsPA) linkages have enhanced the potency for targeting microRNA miR-21 compared with a PS-ASO [11–13]. The MsPA linkage is negatively charged but does not have a negatively charged sulfur atom, which is the key pharmacophore for protein binding. We found that replacement of all PS linkages with MsPAs in a gapmer PS-ASO reduced activity; however, replacing three to five PS linkages near the 5'-terminus with MsPAs improved potency, reduced cytotoxicity, and resulted in a longer duration of pharmacodynamic effect in a mouse model [14]. Introduction of MsPA linkages in the gap region also tended to reduce protein binding and cytotoxicity, and lead to increased activity in cells upon transfection compared with the parent with PS linkages [14]. These observations prompted us to examine if the combination of the gap2 OMe modification with the MsPA backbone would further improve ASO performance.

In this study, we evaluated the effects of the combination of Gap2 OMe modification with one to five MsPA linkages at different positions in the context of a G2.5 gapmer ASO. We found that although the gap2 OMe modification results in reduced cytotoxicity as expected, which was more significant than the MsPA backbone modifications alone, PS ASOs with MsPA backbone modifications had better potency than the gap2 OMe-modified ASOs. Introduction of two MsPA linkages at certain positions near the 5' end of the gap and in the 3' wing increased potency and maintained and reduced cytotoxicity of multiple PS ASO sequences tested both *in vitro* and *in vivo*. The increased potency is likely due to the enhancement of cleavage by RNase H1 by mesyl phosphoramidate moieties. Together, these results suggest that a combination of two MsPA linkages and gap2 OMe improves the therapeutic index of gapmer PS ASOs.

## Materials and Methods

### ASOs and antibodies

ASOs were synthesized as described previously [14]. The ASOs used in this study are listed in Supplementary Table S1. RNase H1 antibody (15606-I-AP) was purchased from ProteinTech and Ku70 antibody (ab83501) was from Abcam.

### Cell culture and ASO treatment

Cell lines, including HeLa, NIH3T3, Hepa1–6, and MHT, were grown at 37°C, 7.5% CO<sub>2</sub> in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. For electropora-

tion with ASOs, 20,000 cells per well were mixed with different concentrations of ASOs (5 μM, serial dilution 1:3 with 7 doses, and no ASO control) and were electroporated at 160V for MHT cells, and 140V for NIH3T3 cells. The cells were transferred to 96-well plates and grew for 24 h for RT-PCR. Primary hepatocytes MHT were prepared from mouse liver as described previously [5]. For high-throughput electroporation of ASOs for caspase 3/7 Glo assay, cells were mixed with ASOs for a final ASO concentration of 20 μM in a final volume of 100 μL and added to a BTX high-throughput electroporation plate. The cells were then electroporated 140V for Hepa 1–6 cells, using the ECM 830 high-throughput electroporation system.

### Animal studies

Animal experiments were conducted according to American Association for the Accreditation of Laboratory Animal Care guidelines and were approved by the institution's Animal Welfare Committee. Male BALB/c mice aged 6–8 weeks were obtained from Charles River Laboratories. If not otherwise specified, three animals were used per treatment. ASOs or saline were administered subcutaneously on study day 1. On study days specified, animals were anesthetized using 2%–4% isoflurane, and blood was collected by cardiac puncture or tail bleed. Blood samples were processed to plasma and evaluated for ALT and AST using a Beckman Coulter AU480 Bioanalyzer.

### RNA preparation and qRT-PCR assay

Mouse liver punches were homogenized using a Bio-Gen PRO200 Homogenizer (PRO Scientific). Total RNAs from mouse liver or cell culture were isolated using RNeasy 96 Kits (Qiagen) according to protocols supplied by the manufacturers. TaqMan One-step qRT-PCR was performed using AgPath-ID One-Step RT-PCR Reagents (Thermo Fisher Scientific). Reverse transcription was performed at 48°C for 10 min, the reactions were then denatured at 95°C for 10 min, and 40 cycles of PCRs were conducted at 95°C for 15 s and 60°C for 20 s within each cycle, using an Applied Biosystems StepOnePlus Real-Time PCR system. Expression levels of target RNA were normalized to total RNA quantified using Quant-iT RiboGreen RNA Reagent (Thermo Fisher Scientific).

### Caspase 3/7 assays

For quantitative high-throughput analysis of caspase activation, ASOs were introduced to Hepa1–6 cells through electroporation and grown at 37°C overnight. Caspase-Glo 3/7 Reagent (Promega) was added directly to the cells in 96-well or 384-well plates at a volume equal to the sample volume. Luminescence was recorded after 30 min. Background readings were determined from wells containing culture medium without cells and were subtracted from the control or assay readings. Relative caspase activity (%) was calculated as:  $100\% \times \text{luminescence reading of a treated sample} / \text{luminescence reading of a mock-treated control}$ .

### Affinity selection and protein analyses

Affinity selection using biotinylated ASO 902459 was performed as described previously [8]. Proteins were eluted

using ASO 558807 or ASOs with two MsPA modifications in Supplementary Fig. S1B. In Fig. 3C, the affinity selection using biotinylated ASO 902459 with OMe RNA LZ37 as bait, and proteins were eluted by using the ASO/LZ37 RNA duplex. Eluted proteins were separated on 4%–12% SDS-PAGE gel, and proteins were detected by immunoblotting [15].

#### RNase H1 cleavage assay

MBP-tagged RNase H1 protein (5 nM), purified as previously described [9], was incubated with 300 nM preannealed duplex of ASO and FITC-RNA (FITC-GC-558807) for 0–10 min in buffer described previously [9]. The cleaved products were analyzed by 20% urea PAGE, visualized with STORM 860 WO Phosphor Screen, and quantified using ImageJ.

#### NanoBRET assay

The detailed method was previously described [6,9]. For competitive binding assays, the 3–10–3 Alexa-linked cEt PS-ASO (936533) was added at 10 nM and the unconjugated competing ASO added at the indicated concentrations in 50  $\mu$ L water.  $10^6$  RLU/well of purified fusion protein PC4 [16] was then added in 50  $\mu$ L 2 $\times$  binding buffer for a final volume of 100  $\mu$ L. After incubation at room temperature for 15 min, substrate addition and BRET readings were carried out. The reaction was in 1 $\times$  binding buffer: 100 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 mM EDTA, and 0.1% NP40.

## Results

### MsPA backbone improves potency, whereas gap2 OMe reduces toxicity in cytotoxic PS ASOs

To compare the effects of the gap2 OMe modification and the MsPA backbone modification, the modifications were tested in the context of an ASO 558807, which targets *CXCL12* pre and mRNA and which was previously shown to induce severe cytotoxicity [5] (Fig. 1A). Consistent with previous results [17], introducing two MsPA linkages at different positions of the gap caused modestly increased potency compared with the parent PS ASO when ASOs were electroporated into NIH3T3 cells (Fig. 1A). The MsPA linkages also reduced the cytotoxicity as evidenced by reduced caspase activity in Hepa1–6 cells (Fig. 1A), where caspase activity is robust [5,18].

To further compare the performance of these different modifications, PS ASOs were subcutaneously administered to mice, and *CXCL12* mRNA levels in mouse liver were analyzed using qRT-PCR. The gap2 OMe modifications reduced the ASO potency (Fig. 1A, B), consistent with pre-

vious observations [5]. Although incorporation of MsPA linkages caused modest reduction in ASO potency in mice as compared with the parental ASO, the mesyl modified ASOs displayed slightly higher potency than the gap2 OMe-modified ASO indicated by ED<sub>50</sub>.

We speculate that the increased potency of the ASOs with the MsPA backbone modification results from increased RNA cleavage, as ASOs with the MsPA backbone modification enhanced cleavage in an assay with purified RNase H1 and an FITC-conjugated complementary RNA substrate (Supplementary Fig. S1A). The backbone modifications of OMe (ASO 926053) and MsPA linkages at position 2 and position 3 (ASO 1375426, 1375427, and 1375428) in the gap prevented the RNase H1 cleavage activity across from these positions, resulting the loss of bigger cleaved fragment C [14].

The reduction in potency of the PS ASOs with MsPA linkages relative to the parent is likely due to reduced PS ASO uptake into liver cells, since MsPA linkages reduced total protein binding, especially when placed in the gap (Supplementary Fig. S1B) and protein binding is essential for productive uptake and subcellular distribution [10]. Furthermore, MsPA linkages in the gap region reduced binding to a model protein with a single PS ASO binding site, PC4 [16,19] (Supplementary Fig. S1C) using a Nano-BRET assay. When MsPA linkages were placed in the 5' wing region, a greater loss in protein binding was found than when the MsPA linkages were placed in the 3' wing (Supplementary Fig. S1B), consistent with our previous findings that the 5' half of a gapmer is the preferred binding site for many proteins [7,20].

Unlike the parent PS ASO (ASO 558807), treatment of mice with the gap2 OMe-modified PS ASO (936053) did not cause a dramatic increase in ALT and AST levels (Fig. 1A). Even at the highest dose used (150 mg/kg), the gap2 OMe-modified ASO did not cause ALT/AST elevation. Incorporation of MsPA linkages (ASO 1375426, 1375427, and 1375428) also decreased ALT and AST levels relative to the parental ASO, although not to the extent that the gap2 OMe modification did.

The ASO with MsPA linkages at gap positions 2 and 3 (ASO 1375427) induced less ALT and AST than the ASOs with MsPA linkages at gap positions 1 and 2 (ASO 1375428) or 3 and 4 (ASO 1375426). A similar trend was also observed when levels of mRNAs encoding *P21*, *RNase H1*, and *Tnfrsf10b*, other markers of the cytotoxicity pathway [5], were evaluated (Fig. 1A). Together, these results suggest that the two MsPA linkages at 5' end of the gap reduce cytotoxicity but to a lesser extent than does incorporation of a single gap2 OMe modification; however, the gap2 OMe may cause some loss of potency.

**FIG. 1.** MsPA backbone improves activity, whereas gap2 OMe reduces toxicity. **(A)** Different sequences and chemical modifications of *CXCL12* PS-ASOs indicate MsPA modifications improved potency than gap 2 OMe ASO *in vitro* and *in vivo*, also reduced toxicity at certain levels. Blue letters indicate cEt, black PS DNA, subscript pink  $\mu$ =MsPA, red indicates 2'-OMe. For *in vitro* studies, activity was done in NIH3T3 cells and toxicity was done in Hepa1–6 cells by electroporation. For *in vivo* studies, BALB/c mice ( $N=3$  animals per group) were subcutaneously administered a single dose of parental, gap2 OMe, and MsPA PS-ASOs at indicated doses for 72 h. *CXCL12* mRNA by 50% (ED<sub>50</sub>), Levels of ALT, AST, *Cdkn1a* mRNA, *Tnfrsf10b* mRNA, and *RNase H1* in the liver. **(B)** Percentage of *CXCL12* mRNA in livers of mice treated subcutaneously with indicated ASO relative to saline control.  $n=3$ . PS, phosphorothioate; ASO, antisense oligonucleotide; 2'-OMe, 2'-O-methyl; MsPA, mesyl-phosphoramidate.

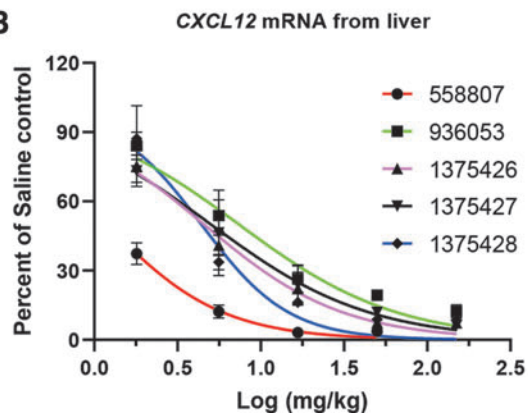
**A**

	<i>In vitro</i>			<i>In vivo</i>					
	Caspase (% Mock)	IC50 (nM)	Dose (mg/kg)	ALT	AST	ED50 (mg/kg)	P21 (% Mock)	Tnfrsf10b (% Mock)	H1 (% Mock)
558807 (Parent) GCATGTTCTCACATTA	848	170	1.8	31	52	1.182	48	96	80
			5.6	25	96		87	122	101
			16.7	770	907		1148	870	152
			50	7666	11092		11488	11757	446
936053 (2'-OMe) GCATGTTCTCACATTA	Ref(5)	Ref(5)	1.8	29	59	7.393	98	151	101
			5.6	29	132		142	121	104
			16.7	27	60		137	132	98
			50	23	49		132	148	114
			150	29	74		169	191	91
1375426 (MsPA Double P3,4) GCATGTTCTCACATTA	292	100	1.8	27	82	4.56	113	124	81
			5.6	24	82		114	150	159
			16.7	20	102		106	115	107
			50	55	112		250	213	106
			150	4325	4514		6388	4869	294
1375427 (MsPA Double P2,3) GCATGTTCTCACATTA	193	87	1.8	45	94	5.023	79	116	93
			5.6	24	47		230	178	123
			16.7	28	81		179	127	107
			50	29	66		180	280	155
			150	1519	1529		2171	971	197
1375428 (MsPA Double P1,2) GCATGTTCTCACATTA	550	99	1.8	28	70	4.404	142	149	162
			5.6	25	41		111	104	88
			16.7	35	112		45	118	117
			50	473	637		788	586	236
			150	3945	4376		12412	12528	535

Footnote: blue: cEt; Red: 2'Ome; μ, MsPA linkage; others are PS linkages

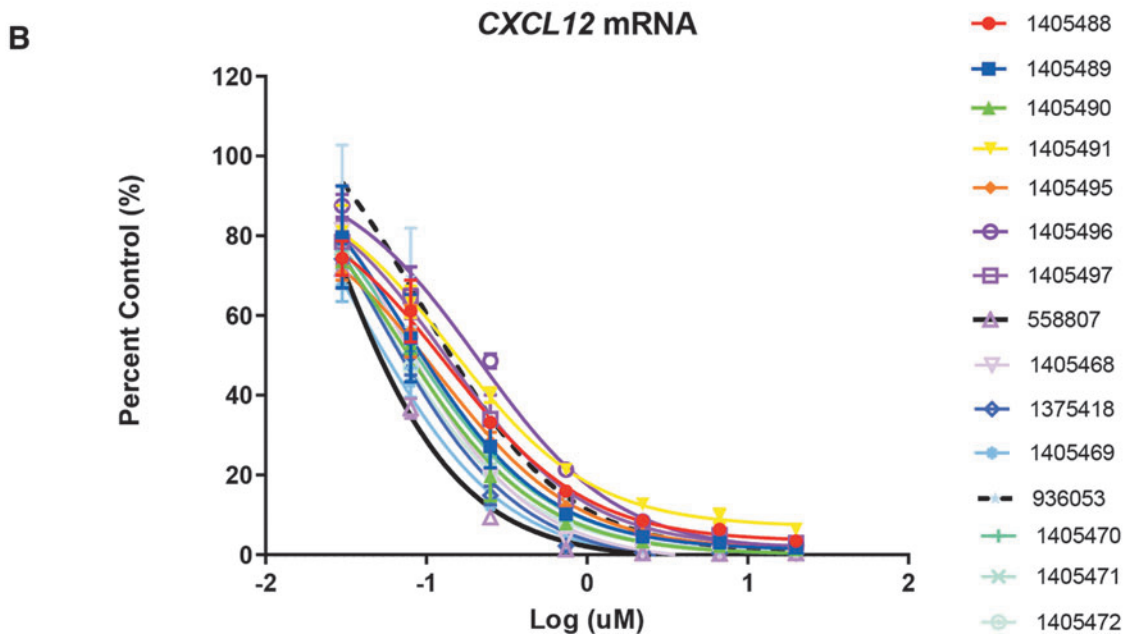


**B**



**A**

Ionis #	Sequence	Comment	Caspase (% Mock)	IC <sub>50</sub> (nM)
558807	GCATGTTCTCACATTA	Parent	2230	54
936053	GCATGTTCTCACATTA	2'-OMe	102	144
1405489	GCATG <sub>μ</sub> TTCTCACATTA	2-OMe, mu	182	94
1405496	GCATG <sub>μ</sub> TTCTCACATTA	2-OMe, mu	148	197
1405490	GCATG <sub>μ</sub> TTCTCACATTA	2-OMe, mu	107	78
1405495	GCATG <sub>μ</sub> TTCTCACATTA	2-OMe, mu	74	91
1405488	GCATG <sub>μ</sub> TTCTCACATTA	2-OMe, mu	103	110
1405497	GCATGTTCTCA <sub>μ</sub> CATTA	2-OMe, mu	95	126
1405491	GCATGTTCTC <sub>μ</sub> A <sub>μ</sub> CATTA	2-OMe, mu	95	130
1405468	G <sub>μ</sub> C <sub>μ</sub> A <sub>μ</sub> TGTTCTCACATTA	mu	1480	88
1405470	G <sub>μ</sub> C <sub>μ</sub> A <sub>μ</sub> TGTTCTCACATTA	2-OMe, mu	133	86
1375418	GCATGTTCTCACAT <sub>μ</sub> T <sub>μ</sub> A	mu	1983	74
1405471	GCATGTTCTCACAT <sub>μ</sub> T <sub>μ</sub> A	2-OMe, mu	73	78
1405469	G <sub>μ</sub> C <sub>μ</sub> A <sub>μ</sub> TGTTCTCACAT <sub>μ</sub> T <sub>μ</sub> A	mu	1980	58
1405472	G <sub>μ</sub> C <sub>μ</sub> A <sub>μ</sub> TGTTCTCACAT <sub>μ</sub> T <sub>μ</sub> A	2-OMe, mu	148	73



**FIG. 2.** The combination of MsPA linkages with the gap2 OMe modification reduces toxicity and enhances potency. (A) Summary of caspase activity (red indicates high; green indicates low) and potency as indicated by IC<sub>50</sub> values (green indicates high potency; red indicates low). Blue letters indicate cEt, black PS DNA, subscript pink  $\mu$  MsPA, red 2'-OMe. Red arrow indicates parental ASO and blue arrow the ASO modified with gap2 OMe but not MsPA. Green dots indicate the ASOs with the lowest toxicity and highest activity.  $n=4$ . (B) Dose-response curves for targeting CXCL12 mRNA in NIH3T3 cells by electroporation in Fig. 2A,  $n=3$ .

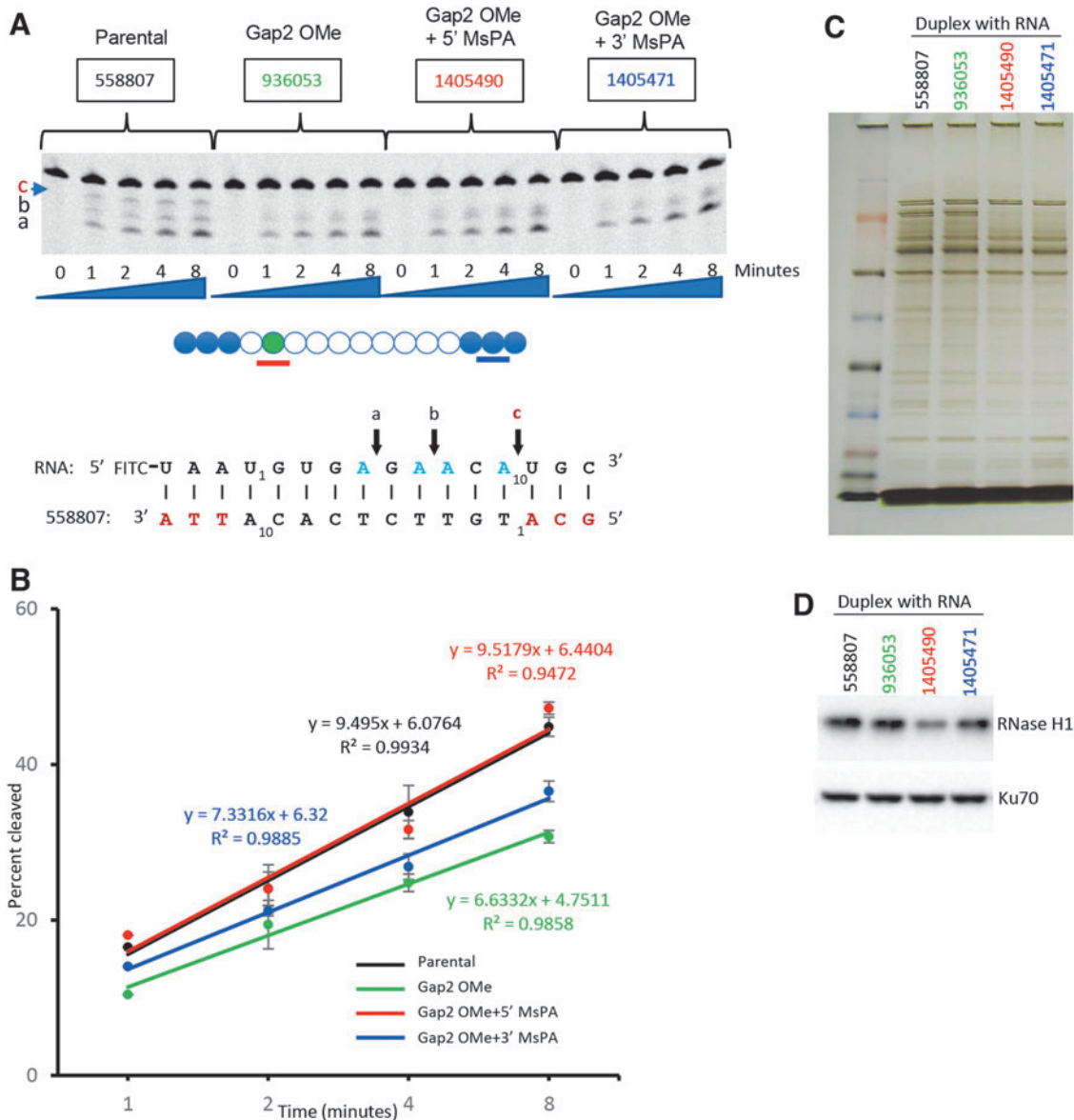


*The combination of MsPA linkages with the gap2 OMe modification reduces cytotoxicity and enhances potency relative to the gap2 OMe PS ASO*

Next, PS ASOs were synthesized with both gap2 OMe and MsPA linkages (Fig. 2A). The MsPA linkages were incorporated in either 5' or 3' wings or at different positions in the gap. PS ASOs were delivered by electroporation into Hepa1-6 cells, and caspase activity was measured to evaluate the cytotoxicities of the PS ASOs (Fig. 2A). As expected, the PS ASO with a single gap2 OMe modification (ASO 936053) displayed dramatically reduced caspase activity compared with that caused by the parental PS ASO (ASO 558807), which caused an increase of ~22-fold in caspase activity

relative to mock treatment. PS ASOs containing the gap2 OMe modification and MsPA linkages, all caused minimal increase in caspase activity compared with the mock treatment. In contrast, the PS ASOs with the MsPA linkages without the gap2 OMe modification induced increased levels of caspase activity that were significant, but less than that the parent PS ASO caused (Fig. 2A).

Interestingly, the combination of MsPA linkage modifications at certain positions of the PS ASOs with the gap2 OMe was advantageous. The gap2 OMe modification (ASO 936053) caused a reduction of 2.5-fold in potency in NIH3T3 cells relative to the parent (Fig. 2A, B), but when two MsPA linkages were introduced at positions 2 and 3 of the gap (ASO 1405490) or in the 3' wing (ASO 1405471) in combination



**FIG. 3.** MsPA linkage modifications enhance RNase H1 cleavage and off-rate. (A) Analysis of RNase H1 cleavage of FITC-labeled RNA,  $n = 3$ . In the carton, the *circles* indicate residues with the *blue circles* denoting the wings and the *open circles* the gap; the *green circle* indicates the gap2 OMe. The lines show the positions of MsPA linkages. (B) Percentage cleaved product as a function of time in RNase H1 cleavage assay,  $n = 3$ . (C) Gel analysis of affinity selection with parent and ASOs modified with gap2 OMe or with gap2 OMe and MsPA. (D) Western blot showed the ASOs with modifications of MsPA and gap2 OMe bind less RNase H1 proteins, Ku70 as control.

with the gap2 OMe, potency was comparable with that of the parent (ASO 558807). Importantly, the caspase activity induced by the PS ASOs with both MsPA linkages and gap2 OMe were comparable with that of the ASO with the single gap2 OMe modification. The effects of the MsPA backbone linkages on ASO potency were depended on the position in which they were placed. For example, little activity improvement was observed when two MsPA linkages were placed at gap positions 7 and 8 in combination with the gap2 OMe modification (ASO 1405491).

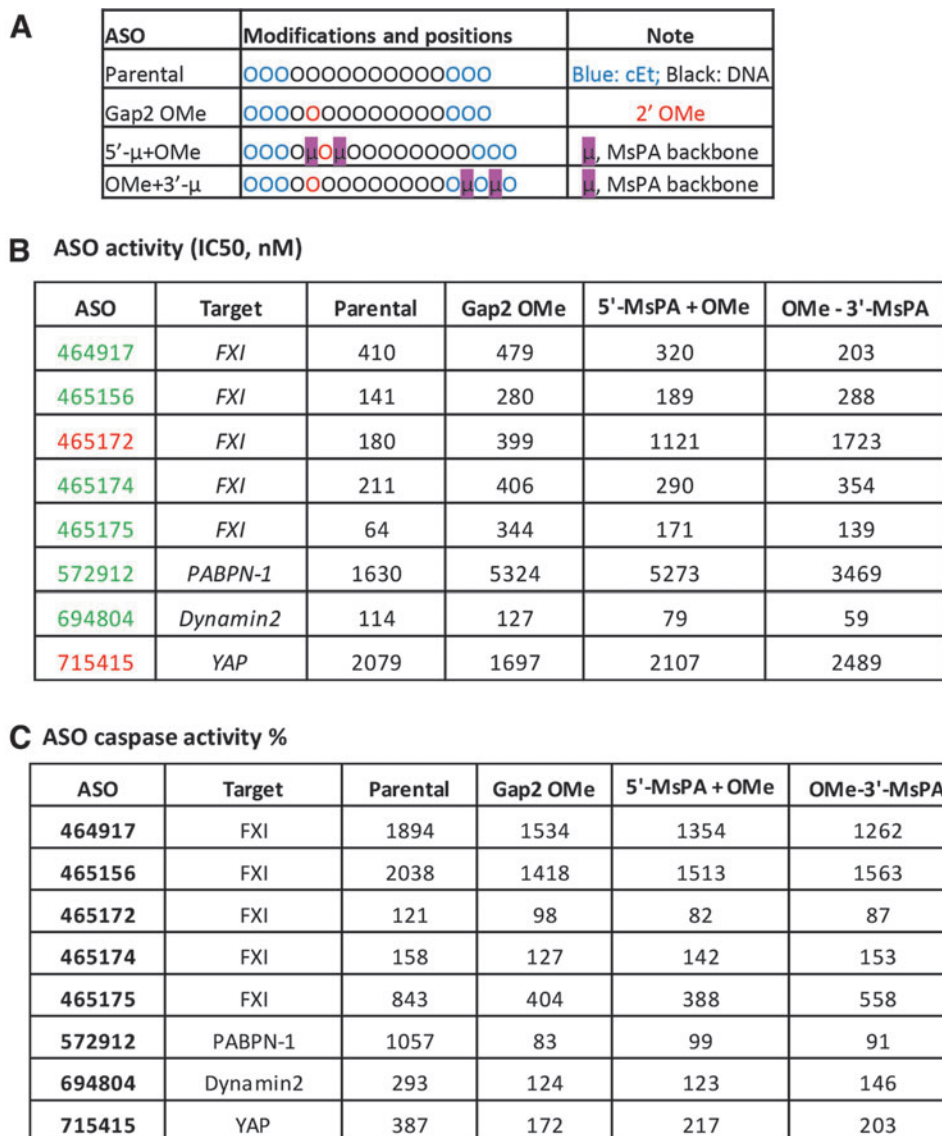
#### MsPA linkage modifications increase RNase H1 cleavage rate

The increase in potency of gap2 OMe-modified ASOs by introduction of two MsPA linkage modifications suggests that the later modifications may enhance RNase H1 cleavage of target RNAs. To evaluate this possibility, an RNase H1 cleavage assay was performed using purified human RNase H1 and an FITC-conjugated complementary RNA substrate (Supplementary Table S1). The incorporation of a gap2 OMe (ASO 936053) altered the cleavage

pattern of the RNA substrate relative to that observed with the parent ASO (Fig. 3A), as reported previously [5].

The cleavage patterns of the ASOs with both gap2 OMe and MsPA linkages (ASOs 1405490 and ASO 1405471) were similar to that of the ASO with only the gap2 OMe modification (Fig. 3A). The gap2 OMe modification caused slower cleavage as measured by the percentage of cleaved products over time (Fig. 3B). The ASOs with gap2 OMe and two MsPA linkages modifications induced cleavage at rates comparable with or slightly less than the parental ASO (Fig. 3B). Thus, the incorporation of MsPA linkages improved the cleavage rate of the reaction mediated by the gap2 OMe-modified ASO, consistent with the increased activity in cells (Fig. 2).

Since the cleavage rate was determined under multiple turnover conditions, the increased rate may reflect an increase in the off-rate of RNase H1 from the duplex. We thus examined RNase H1 binding to ASO/RNA duplex using affinity selection and western analysis. The ASOs with MsPA linkages and a gap2 OMe (ASOs 1405490 and 1405471) have reduced global protein binding compared with the parent ASO (Fig. 3C). In particular, the duplexes between modified



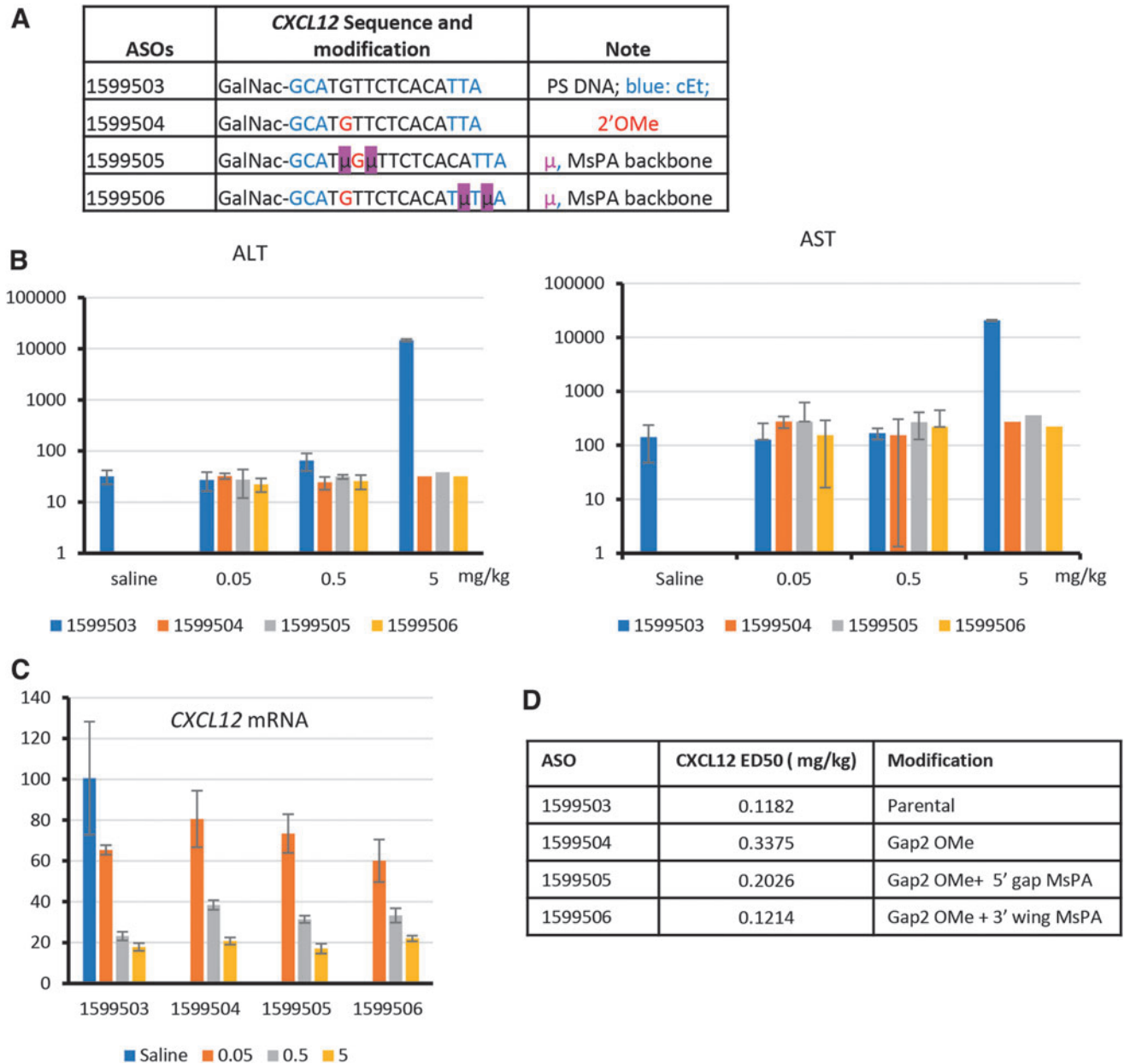
**FIG. 4.** The combination of gap2 OMe and MsPA linkages enhances results in high potency without toxicity. (A) The cartoon indicated the four kinds of 3-10-3 ASOs for each group tested in cells. (B) The IC<sub>50</sub>s of ASO activities for different groups that target different genes. ASO activities targeting *FXI* were done in primary hepatocyte MHT cells and targeting *PABPN1*, *Dynamin2*, and *YAP* were done in mouse NIH3T3 cell line,  $n=3$ . (C) The toxicities of the different ASOs groups were detected by caspase activity in Hepa 1-6 cells,  $n=4$ .

PS ASOs and complementary RNA bound less RNase H1 than the duplex containing the parent PS ASO, especially when MsPA was placed in the gap (Fig. 3D), suggesting that the increased cleavage rate may be due to a higher off-rate, leading to a faster turnover of RNase H1.

*Effects of gap2 OMe and MsPA linkage modifications were observed in cytotoxic PS ASOs of different sequences*

To determine the effects of MsPA linkages on the ASO with a gap2 OMe modification were unique to the ASO

targeting *CXCL12* mRNA, similar designs were applied to ASOs targeting several different mRNAs (Fig. 4A, B). PS ASO potency was determined by electroporation into a cell type that expresses that targeted mRNA. The results showed that the PS ASOs with two MsPA linkages in either the gap or the 3' wing and the gap2 OMe modification had higher potency than the ASO with only the gap2 OMe for six of the nine ASOs tested (Figs. 2, 4B and Supplementary Fig. S2). Caspase activity induced by these ASOs was analyzed in Hepa1-6 cells, and the introduction of MsPA backbone did not substantially alter the cytotoxicity of the gap2 OMe ASOs (Fig. 4C). These results indicate that in most cases, the



**FIG. 5.** The combinations of double MsPA linkage modifications with gap2 OMe on ASOs enhance activity and maintain safety in mice. **(A)** The GalNac-ASOs with different modifications targeting *CXCL12* are listed. **(B)** ALT and AST indicate the combination of MsPA linkages and gap2 OMe modification reduce the toxicity similar to gap 2' OMe,  $n=3$ . **(C)** The dose-response of *CXCL12* mRNA levels by different modifications,  $n=3$ . **(D)** The combination of gap2 OMe and MsPA linkages improved the activity compared with gap2 OMe ASO,  $n=3$ .



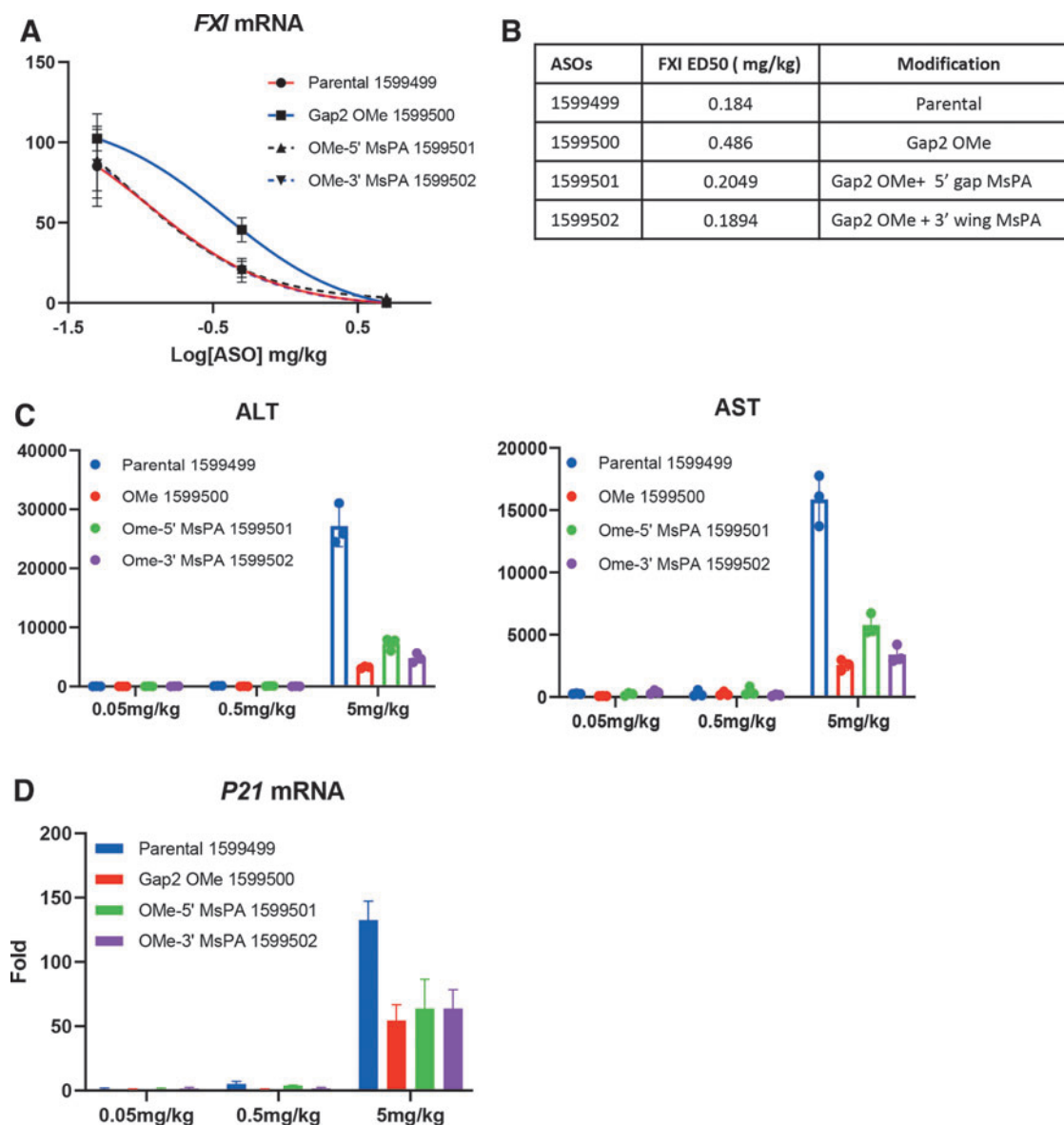
combination of MsPA linkages with the gap2 OMe modification results in higher potency and comparable low cytotoxicity as the PS ASOs with the gap2 OMe only.

*MsPA linkage modifications increase the activities of gap2 OMe ASOs in mice*

Next, we evaluated the effects of the combined gap2 OMe and MsPA modifications on ASO potency in mice. *N*-Acetylgalactosamine (GalNac)-conjugated ASOs targeted to *CXCL12* used in these experiments were administrated by subcutaneous injection (Fig. 5A). After 72 h, mice were sacrificed, and blood chemistry was analyzed (Fig. 5B).

Consistent with the *in vitro* observations, the MsPA linkages did not substantially alter the cytotoxicity profile of the gap2 OMe ASO as measured by analyses of ALT and AST levels (Fig. 5B) or by *P21* mRNA levels (Supplementary Fig. S3).

However, the incorporation of MsPA linkages in the context of the gap2 OMe (ASOs 1599505 and 1599506) resulted in increased potency relative to the gap2 OMe ASO 1599504. In fact, the PS ASOs with the combination of both MsPA and gap2 OMe had potencies almost equivalent to that of the parental ASO 1599503 (Fig. 5C, D). PS ASOs targeting *FXI* were also tested in mice, confirming two MsPA linkages in either the gap or 3' wing in combination with the



**FIG. 6.** Analyses of ASOs targeting *FXI* for activity and safety in mice. **(A)** The dose–response of *FXI* mRNA levels by different modifications,  $n = 3$ . **(B)** The combination of gap2 OMe and MsPA linkages improved the activity compared with gap2 OMe ASO,  $n = 3$ . **(C)** ALT and AST indicate the combination of MsPA and 2' OMe modifications have the similar ability to gap 2' OMe to reduce the toxicity compared with parental ASO (1599499),  $n = 3$ . **(D)** The double MsPA linkage and gap2 OMe modifications maintain the safety shown the similar levels of *P21* mRNA as gap2 OMe ASO, compared with parental ASO (1599499).

gap2 OMe resulted in potency that was greater than the PS ASO with the gap2 OMe modification and equal to the parent PS ASO (1599499) with a cytotoxicity profile nearly equivalent to that of the gap2 OMe PS ASO (Fig. 6).

## Discussion

Previously we showed that the gap OMe modification dramatically reduces the cytotoxicity of gapmer PS-ASOs [5]. Furthermore, the MsPA modifications at certain positions of gapmer PS-ASOs can reduce both immune response and cytotoxicity and improve the duration of action of PS ASOs by enhancing the nuclease stability [14]. In this study, we evaluated the combination of the MsPA linkage and gap2 OMe on potency and therapeutic index. Although the gap2 OMe modification reduces the toxicity, it also impairs potency. Our results showed the two MsPA modifications either in the gap or in the 3' wing enhanced activities of the gap2 OMe-modified ASOs likely due to increased RNase H1 cleavage rate.

As shown by analysis of caspase activity in Hepa1–6 cells and liver function markers in mice, the combination of the MsPA linkages and the gap2 OMe modification considerably reduced toxicity of the PS-ASOs tested. This effect was not limited to a single ASO sequence or targeted mRNA. In conclusion, we demonstrated that the combination of MsPA and gap2 OMe improves potency while maintaining safety of gapmer PS-ASOs. These findings will facilitate the discovery and development of better ASO drugs.

## Acknowledgments

We thank all members of Crooke group for stimulating discussions, and Luyi Zhao for technical assistance in collecting blood for mice experiment.

## Data Availability

No data related to this study were deposited to databases. Research data are available upon request.

## Author Disclosure Statement

All authors are employees of Ionis Pharmaceuticals.

## Funding Information

Internal funding from Ionis Pharmaceuticals. Funding for open access charge: Ionis Pharmaceuticals internal funding.

## Supplementary Material

Supplementary Table S1  
Supplementary Figure S1  
Supplementary Figure S2  
Supplementary Figure S3

## References

- Burdick AD, S Sciabola, SR Mantena, BD Hollingshead, R Stanton, JA Warneke, M Zeng, E Martsen, A Medvedev, *et al.* (2014). Sequence motifs associated with hepatotoxicity of locked nucleic acid—modified antisense oligonucleotides. *Nucleic Acids Res* 42:4882–4891.
- Kakiuchi-Kiyota S, LO Whiteley, AM Ryan, and N Mathialagan. (2016). Development of a method for profiling protein interactions with LNA-modified antisense oligonucleotides using protein microarrays. *Nucleic Acid Ther* 26:93–101.
- Shen W, CL De Hoyos, H Sun, TA Vickers, XH Liang, and ST Crooke. (2018). Acute hepatotoxicity of 2' fluoro-modified 5-10-5 gapmer phosphorothioate oligonucleotides in mice correlates with intracellular protein binding and the loss of DBHS proteins. *Nucleic Acids Res* 46:2204–2217.
- Shen W, XH Liang, H Sun, and ST Crooke. (2015). 2'-Fluoro-modified phosphorothioate oligonucleotide can cause rapid degradation of P54nrb and PSF. *Nucleic Acids Res* 43:4569–4578.
- Shen W, CL De Hoyos, MT Migawa, TA Vickers, H Sun, A Low, TA Bell, 3rd, M Rahdar, S Mukhopadhyay, *et al.* (2019). Chemical modification of PS-ASO therapeutics reduces cellular protein-binding and improves the therapeutic index. *Nat Biotechnol* 37:640–650.
- Vickers TA and Crooke ST. (2016). Development of a quantitative BRET affinity assay for nucleic acid-protein interactions. *PLoS One* 11:e0161930.
- Liang XH, W Shen, H Sun, GA Kinberger, TP Prakash, JG Nichols and ST Crooke. (2016). Hsp90 protein interacts with phosphorothioate oligonucleotides containing hydrophobic 2'-modifications and enhances antisense activity. *Nucleic Acids Res* 44:3892–3907.
- Liang XH, W Shen, H Sun, TP Prakash and ST Crooke. (2014). TCP1 complex proteins interact with phosphorothioate oligonucleotides and can co-localize in oligonucleotide-induced nuclear bodies in mammalian cells. *Nucleic Acids Res* 42:7819–7832.
- Zhang L, TA Vickers, H Sun, XH Liang and ST Crooke. (2021). Binding of phosphorothioate oligonucleotides with RNase H1 can cause conformational changes in the protein and alter the interactions of RNase H1 with other proteins. *Nucleic Acids Res* 49:2721–2739.
- Crooke ST, XH Liang, RM Crooke, BF Baker and RS Geary. (2021). Antisense drug discovery and development technology considered in a pharmacological context. *Biochem Pharmacol* 189:114196.
- Miroshnichenko SK, OA Patutina, EA Burakova, BP Chelobanov, AA Fokina, VV Vlassov, S Altman, MA Zenkova and DA Stetsenko. (2019). Methyl phosphoramidate antisense oligonucleotides as an alternative to phosphorothioates with improved biochemical and biological properties. *Proc Natl Acad Sci U S A* 116:1229–1234.
- Patutina OA, SK Gaponova Miroshnichenko, AV Sen'kova, IA Savin, DV Gladkikh, EA Burakova, AA Fokina, MA Maslov, EV Shmendel, *et al.* (2020). Methyl phosphoramidate backbone modified antisense oligonucleotides targeting miR-21 with enhanced in vivo therapeutic potency. *Proc Natl Acad Sci U S A* 117:32370–32379.
- Hammond SM, OV Sergeeva, PA Melnikov, L Goli, J Stoodley, TS Zatsepin, DA Stetsenko, and MJA Wood. (2021). Methyl phosphoramidate oligonucleotides as potential splice-switching agents: impact of backbone structure on activity and intracellular localization. *Nucleic Acid Ther* 31:190–200.
- Anderson BA, GC Freestone, A Low, CL De-Hoyos, WJD Iii, ME Østergaard, MT Migawa, M Fazio, WB Wan, *et al.* (2021). Towards next generation antisense oligonucleotides: methylphosphoramidate modification improves

- therapeutic index and duration of effect of gapmer anti-sense oligonucleotides. *Nucleic Acids Res* 49:9026–9041.
15. Vickers TA, M Rahdar, TP Prakash and ST Crooke. (2019). Kinetic and subcellular analysis of PS-ASO/protein interactions with P54nrb and RNase H1. *Nucleic Acids Res* 47: 10865–10880.
  16. Hyjek-Skladanowska M, TA Vickers, A Napiorkowska, BA Anderson, M Tanowitz, ST Crooke, XH Liang, PP Seth and M Nowotny. (2020). Origins of the increased affinity of phosphorothioate-modified therapeutic nucleic acids for proteins. *J Am Chem Soc* 142:7456–7468.
  17. Szaflarski W, M Leśniczak-Staszak, M Sowiński, S Ojha, A Aulas, D Dave, S Malla, P Anderson, P Ivanov and SM Lyons. (2021). Early rRNA processing is a stress-dependent regulatory event whose inhibition maintains nucleolar integrity. *Nucleic Acids Res* 50:1033–1051.
  18. Migawa MT, W Shen, WB Wan, G Vasquez, ME Oestergaard, A Low, CL De Hoyos, R Gupta, S Murray, *et al.* (2019). Site-specific replacement of phosphorothioate with alkyl phosphonate linkages enhances the therapeutic profile of gapmer ASOs by modulating interactions with cellular proteins. *Nucleic Acids Res* 47:5465–5479.
  19. Vickers TA, MT Migawa, PP Seth and ST Crooke. (2020). Interaction of ASOs with PC4 is highly influenced by the cellular environment and ASO chemistry. *J Am Chem Soc* 142:9661–9674.
  20. Liang XH, H Sun, W Shen and ST Crooke. (2015). Identification and characterization of intracellular proteins that bind oligonucleotides with phosphorothioate linkages. *Nucleic Acids Res* 43:2927–2945.

Address correspondence to:

*Lingdi Zhang, PhD*  
*Department of Core Antisense Research*  
*Ionis Pharmaceuticals, Inc.*  
*2855 Gazelle Court*  
*Carlsbad, CA 92010*  
*USA*

*E-mail: lingdizhang68@gmail.com*

*Stanley T. Crooke, PhD*  
*Department of Core Antisense Research*  
*Ionis Pharmaceuticals, Inc.*  
*2855 Gazelle Court*  
*Carlsbad, CA 92010*  
*USA*

*E-mail: scrooke@ionisph.com*

Received for publication February 12, 2022; accepted after revision March 30, 2022; Published Online July 20, 2022.