Localization and Quantification of Oligonucleotide Therapeutic Drugs: Immunoassays Specific for Nucleic Acid Modifications Independent of Sequence

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ABSTRACT

Oligonucleotide therapeutics (ONTs) have increased in development since their inception in 1978. ONTs, such as antisense oligonucleotides (ASO) and small interfering RNA (siRNA), are short fragments of nucleic acid complementary to a specific mRNA and upon administration can modulate protein synthesis in patients. ONTs have been applied across diverse disease areas including oncology, neurology and infectious disease prevention. However, a significant number of hurdles remain to bring this class of drugs to more widespread use. One such limitation, improving the "drug-likeness" of ONTs, is facilitated by chemical modifications to the sugar-phosphate backbone and/or nitrogenous bases. Modifications are designed to increase ONT drug stability, uptake, and efficacy, but may increase the likelihood of toxicity. First generation chemical modifications include phosphorothioate (PS) modification of the phosphate portion of the backbone, whereas second generation modifications include adding substituent groups to the 2' position of the ribose portion of the backbone, specifically 2'-O-methyl (OMe), 2'-O-methoxy-ethyl (MOE), and 2'-fluoro modifications of RNA. Third generation modifications include peptide nucleic acids (PNA), locked nucleic acids (LNA), morpholino phosphoroamidate (PMO) modifications, and others.

We have developed, optimized, and validated a library of monoclonal antibody reagents that detect chemical modifications independent of nucleic acid composition, structure, strandedness, configuration, or platform. These "universal detection reagents" can be used to localize and quantify ONTs in lysates and biofluids by various analytical assays. While LC-MS/MS and ligand binding assays (LBA) are the mainstay of analytical assays for ONTs, immunoassays based on the antibodies described here represent an orthogonal approach to solving problems associated with demonstrating the safety and efficacy of this class of drug.

Here we report the ability to localize and quantify ONTs using panels of monoclonal antibodies specific for PS, 2'-MOE or 2'-OMe modifications. We demonstrate the performance of these antibodies by various *in vitro* assays that show the utility of these specialty reagents to facilitate the collection of analytical data to support ADMET studies useful to ensure ONT approval by regulatory agencies.

INTRODUCTION

ONT drugs, such as ASOs and siRNAs, are short fragments of nucleic acids complementary to a specific mRNA that can suppress or amplify the expression of a gene, for instance by inhibiting or promoting transcription and translation of the corresponding protein. ONTs and mRNA vaccines have been researched for decades and now the rate of ONT drug approval is increasing resulting in burgeoning pipelines for new drug development. Problems with getting ONT drugs to market remain: (1) poor stability and high sensitivity to endo and exonucleases in cells and serum, (2) poor uptake into target cells or tissues, (3) off-target effects due to partial complementation to an unintended target, and (4) immunogenicity or immuno-stimulation. However, emerging challenges include targeting extrahepatic tissue via a systemic administration of drugs and the lack of analytical tools to demonstrate absorption, distribution, metabolism, excretion, and toxicity (ADMET). Analytical tools are the primary limiting factor in the collection of analytical data needed to satisfy regulatory agencies who require such data to ensure efficacy and safety.

While custom-made antibodies have been developed for specific ONTs, the resultant antibody likely recognizes a plethora of epitopes such as the nucleic acid sequence itself, conformation, modification, and many other possible epitopes. As such, these antibodies are typically generated and used "in-house" and may not have utility for other ONTs or across drug platforms and may not be available for use except by their originators, or as gifts and may have use limitations.

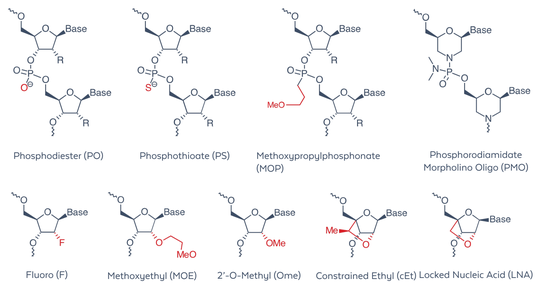

Figure 1. Common chemical modifications used to enhance the 'drug likeness" of ONTs.

To overcome these obstacles, we have targeted highly specific epitopes within ONTs consisting solely of the chemical modifications themselves (see Figure 1). We have designed, developed, optimized and validated panels of antibodies specific for each of the most common chemical modifications, PS, 2'-MOE or 2'-OMe used in many drug designs.

Here we report the ability to localize and quantify ONTs using these monoclonal antibodies as analytical tools. By validating these antibodies using a combination of chemically defined ONT drugs as either positive or negative controls, we have determined the ability to detect these chemical modifications (see Figure 2) independent of nucleic acid composition, structure (e.g., ASO, siRNA, or mRNA), strandedness (e.g., ss or ds), configuration (e.g., RNA:RNA, DNA:DNA, or RNA:DNA), or platform (e.g., gapmer).

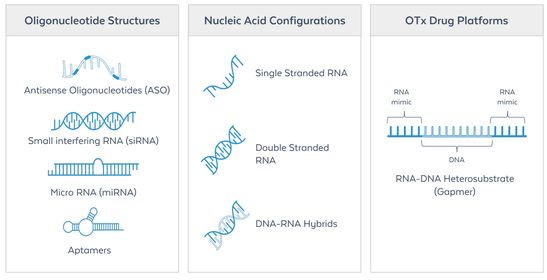

Figure 2. Developed monoclonal antibody reagents as analytical tools detect chemical modifications independent of nucleic acid composition, structure, strandedness, configuration, or platform.

As there is a need to expand the analytical toolbox for the collection of analytical data to support the development and regulatory approval of this class of drug, we opine that immunoassays should be considered as an alternative or orthogonal method to support existing analytical assays to assess ADMET.

INTRACELLULAR LOCALIZATION (IF)

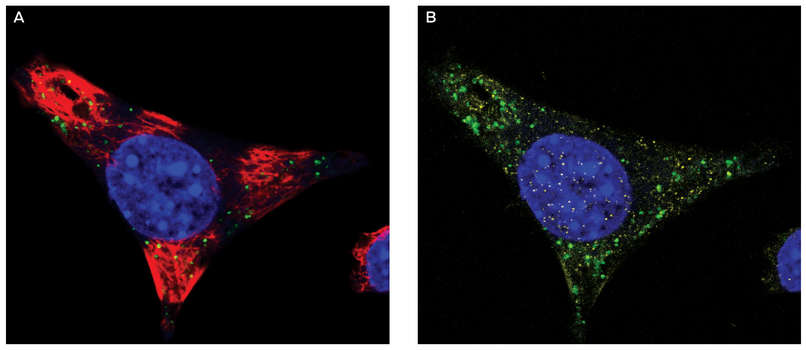

Figure 3. Intracellular localization of ASO using Anti-PS monoclonal antibody clone PS05. Mouse glioma cells (GL261) derived from C57 black mice were cultured and treated with ASO drug. After fixation with paraformaldehyde, cells were stained with DAPI (blue) and anti-PS antibody clone PS05 (green) either with (A) or without (B) staining using anti-alpha tubulin clone DM1A (red). The anti-PS antibody was used at a 1:2000 dilution. Punctate cytoplasmic staining is consistent with endosomal storage of ASO within the cell, as expected for this ONT drug. Vehicle only treated cells showed no staining (not shown).

CO-LOCALIZATION (IF)

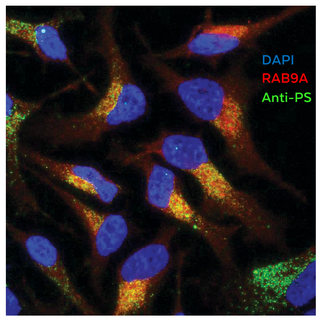

Figure 4. Co-localization of ONT with endosomal marker. HeLa cells were cultured, fixed with paraformaldehyde and reacted with anti-PS antibody clone PS03 (red) at a 1:1000 dilution. Nuclei were stained with DAPI (blue) and endosomes were identified using a RAB9A marker (green). Co-localization is seen for ONT and endosomes (yellow). Data generated by Inês Fial, Nucleic Acid Accelerator (NATA), Harwell, UK.

QUANTIFICATION OF ONT DRUG (E)

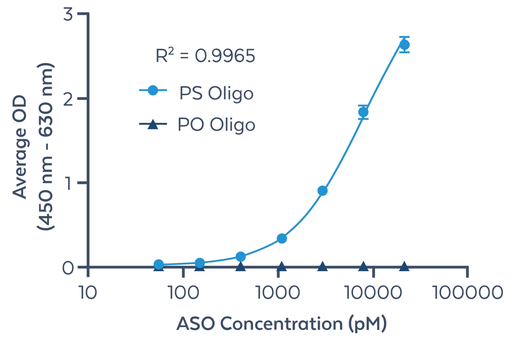

Figure 5. Quantification of fully PS modified ASO by ELISA using anti-PS monoclonal antibody (clone PS04). Non-modified PO oligonucleotide of the same sequence was used as a negative control (PO Oligo). The curve was generated using PS-modified ASO diluted in buffer from 55 pM to 21 nM in concentrations and subsequently detected using anti-PS monoclonal antibody. A standard curve was plotted as the average OD result versus the log of ASO concentration in pM using a 4PL best fit formula. The lower limit of quantitation (LLOQ) as defined as the lowest standard detected is 55 pM. The limit of detection (LOD) was determined to be <11pM based on standard definitions of the term. The upper limit of quantitation (ULOQ) as shown in 21 nM. Further optimization and sensitivity enhancement will likely increase the LLOQ by one log unit.

DRUG RANKING (E)

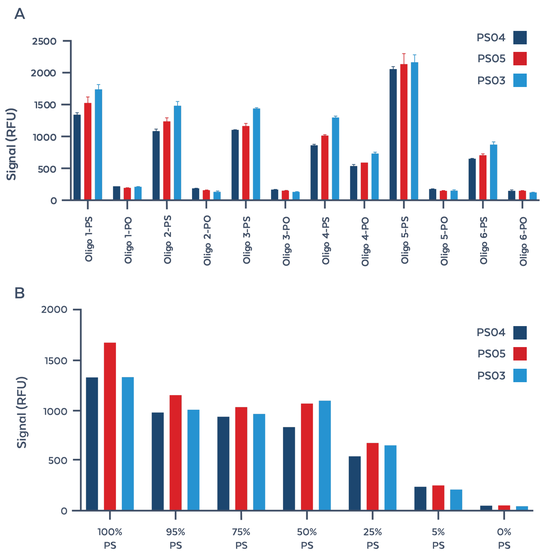

Figure 6. ELISA of selected Anti-PS monoclonal antibodies against candidate OTx drugs. Panel A shows sequence/chemistry reactivity of anti-PS antibody clone PS04, clone PS05, and clone PS03 against a panel of six (6) candidate OTx drugs with different configurations, e.g., ASO, DNA, gapmer. Reactivity is shown for selected clones against PS modified drug and unmodified PO negative control. Drug ranking can be useful to correlate immunodetection by anti-PS antibodies with the biological activity of the drug. Panel B shows the reactivity of each of these antibodies, respectively, against PS linkages in a PS modified OTx drug when the degree of PS modification is lowered from 100% to 95%, 75%, 50%, 25% and 5%, respectively. While the signal strength trends downward with the degree of modification, all antibodies tested in this series reacted against an OTx drug with as few as only 5% modified bases, e.g., 1 modified base out of a total of 20 bases, relative to baseline (0% data not shown). Anti-PS antibodies were used at a 1:10,000 dilution followed by fluorescein conjugated anti-Mouse IgG secondary antibody.

ANTIBODY PANELS TO PS, MOE, OMe

Differential Specificity of Antibodies Specific for Chemical Modifications

Table 1. List of clones by designation, indicating isotype and showing reactivity by ELISA against a panel of positive and negative control ASOs and an FDA approved gapmer, Inotersen. Clones are listed for reactivity against PS (Panel A), 2'-MOE (Panel B) and 2'-OMe (Panel C)

A

Clone ID	Specificity	Isotype	ELISA Reactivity					Affinity Binding		
			Fully Modified ASO	33% Modified ASO	3% Modified ASO	Unmodified ASO	Gapmer	Affinity Constant (K_D) nM	Association Rate (K_a) $M^{-1}s^{-1}$	Dissociation Rate (K_d) S^{-1}
PS01	Phosphorothioate (PS)	IgG_1 kappa	+/-	-	-	-	-	ND	ND	ND
PS02		IgG_{2b} kappa	+	-	-	-	-	ND	ND	ND
PS03		IgG_{2b} kappa	+++	++	-	-	+++	0.31	6.63×10^4	2.06×10^{-5}
PS04		IgG_{2b} kappa	+++	++	-	-	+++	1.50	2.03×10^5	3.05×10^{-4}
PS05		IgG_{2b} kappa	+++	++	-	-	+++	1.15	1.62×10^5	1.86×10^{-4}
PS06		IgG_1 kappa	++	ND	ND	-	+/-	ND	ND	ND
PS07		IgG_1 kappa	++	+	-	-	ND	20.4	1.10×10^6	2.24×10^{-2}
PS08		IgG_{2b} kappa	++	+	-	-	-	ND	ND	ND
PS09		IgG_{2b} kappa	+++	-	-	-	+++	<0.001	5.63×10^4	$<1.0 \times 10^{-7}$

B

Clone ID	Specificity	Isotype	ELISA Reactivity					Affinity Binding		
			Fully Modified ASO	33% Modified ASO	3% Modified ASO	Unmodified ASO	5-10-5 Gapmer (inotersen)	Affinity Constant (K_D) nM	Association Rate (K_a) $M^{-1}s^{-1}$	Dissociation Rate (K_d) S^{-1}
MOE1	2'-O-Methoxyethyl (2'MOE)	IgG_1 kappa	ND	ND	ND	ND	+++	1.51	1.33×10^5	2.01×10^4
MOE3		IgG_1 kappa	ND	ND	ND	ND	++	<0.001	2.09×10^5	$< 1.0 \times 10^{-7}$
MOE4		IgG_1 kappa	ND	ND	ND	ND	++	0.003	1.17×10^5	3.23×10^{-7}
MOE9		IgG_1 kappa	ND	ND	ND	ND	+++	2.37	1.04×10^5	2.48×10^{-4}
MOEC		Y_1K-Y_1K-$Y_{20}K$ cocktail	ND	ND	ND	ND	+	0.003	8.22×10^4	2.53×10^{-7}

C

Clone ID	Specificity	Isotype	ELISA Reactivity					Affinity Binding		
			Fully Modified ASO	33% Modified ASO	3% Modified ASO	Unmodified ASO	5-10-5 Gapmer (inotersen)	Affinity Constant (K_D) nM	Association Rate (K_a) $M^{-1}s^{-1}$	Dissociation Rate (K_d) S^{-1}
OMe1	2'-O-Methyl (2'OMe)	IgG_1 kappa	++	ND	ND	-	-	ND	ND	ND
OMe2		IgG_{2b} kappa	+++	ND	ND	-	-	ND	ND	ND
OMe3		IgG_3 kappa	+	ND	ND	-	-	ND	ND	ND
OMe4		IgG_1 kappa	+++	ND	ND	-	-	ND	ND	ND
OMe5		IgG_{2b} kappa	+++	ND	ND	-	-	ND	ND	ND

Key: (-) negative; (+/-) weak; (+) positive; (++) strong; (+++) very strong; (ND) not determined. Affinity binding determined using Sartorius Octet R4 with SA Biosensor and biotinylated gapmer. Gapmer contains PS and MOE but not OMe modifications. Radar chart shows aggregate nucleotide base specificity of anti-2'-MOE clones by relative ELISA titer. ASO used has the same nucleic acid sequence, however, panel A contains PS modifications only and panel C contains OMe modifications only.

BIODISTRIBUTION (IF)

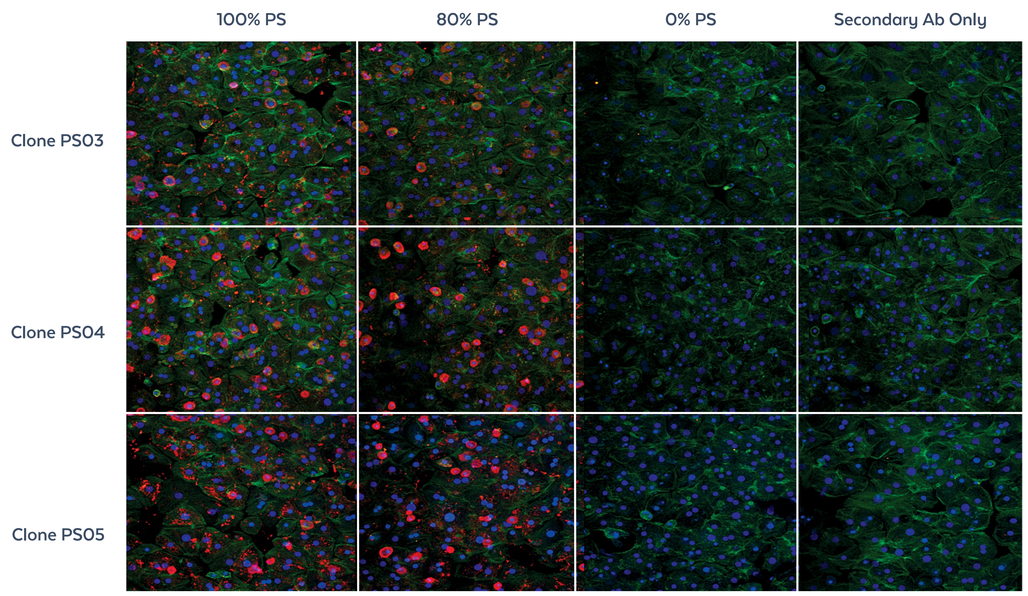

Figure 7. Biodistribution of ONT drug using selected Anti-PS monoclonal antibodies. Human hepatocytes were treated with ASO by free uptake and then stained by immunofluorescence microscopy with DAPI (blue), phalloidin (green), and anti-PS antibody (red). Anti-PS antibodies were used at a dilution range of 1:200 to 1:1000. The top panel of images represents staining using clone PS03, the middle panel clone PS04, and the bottom panel clone PS05, respectively. Tissue was treated either with 100% modified ASO, 80% modified ASO, unmodified ASO, or with secondary antibody only as a negative control. Panels for 100% and 80% modified drug show clear staining of cells containing the ASO. The larger cells stained red are believed to be apoptotic hepatocytes.

CONCLUSIONS

A limiting factor in the development of oligonucleotide drugs is the availability of analytical tools needed to perform assays required by regulatory agencies to assess efficacy and safety of these drugs. The defined specialty reagents presented here, as universal detection reagents, facilitate conventional immunoassays for data collection. These tools offer considerable time and cost savings and may advance the rate at which these drugs are approved for use. Either as a freestanding method or an orthogonal approach, immunoassays like ELISA, IHC, and IF allow for the ranking of drug performance, quantification of ONTs, deposition of drug within tissue, and the localization of drug within cells. As ONT drugs are considered small molecule drugs and therefore require immunogenicity studies, these antibodies may also serve as positive control antibodies in both preclinical and clinical assays designed to assess immune response. We opine that highly specific antibody-based immunoassays capable of detecting as little as a single modified internucleotide linkage in 20 represents a significant advance and should be thoroughly explored for their utility.

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