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Structural Determinants for the Interactions of Chemically Modified Nucleic Acids with the Stabilin-2 Clearance Receptor

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Supporting Information

ABSTRACT: The Stabilin receptors are systemic clearance receptors for some classes of chemically modified nucleic therapeutics. In this study, the recombinant human secreted ecto-domain of the small isoform of Stabilin-2 (s190) was purified from cell culture and evaluated for direct binding with a multitude of antisense oligonucleotides (ASOs) using a fluorescence polarization-based assay. The tested ASOs varied in their backbone composition, modification of the ribose 2′ position, overall length of the oligo, and sequence of the nucleotide bases. A fully phosphorothioate (PS) ASO with a 5−10−5 pattern of flanking 2′-*O*-methoxyethyl modifications was then used to test the effects of pH and salt concentration on receptor binding. These tests concluded that the PS backbone was the primary determinant for ASO binding and that decreasing pH and increasing salt generally increased the rate of ligand dissociation and fit within the biological parameters expected of a constitutive recycling receptor. These results will be useful in the rational design of therapeutic oligonucleotides for enhancing their affinity or avoidance of the Stabilin receptors.

Antisense oligonucleotides (ASOs) are short (14−25) chemically modified nucleic acids that have made rapid progress for the treatment of congenital and acquired metabolic diseases.[1] The effectiveness of an ASO relies on several parameters, including biological stability, adherence to cell-surface proteins, internalization within the cells, and escape from endosomes and specificity to the target RNA.[2,3] To increase their stability in biological fluids, they are often designed with a phosphorothioate linkage in which the free nonbridging oxygen atom of the phosphodiester backbone is replaced with a sulfur atom, rendering the polymer resistant to nucleases.[4] The PS backbone also enhances the avidity of ASO for plasma and cell-surface proteins that promote distribution to tissues and cellular accumulation.[5] Gen 2 ASOs typically have the gapmer design in which a central region of DNA nucleotides is flanked by 2′-modified nucleotide analogues that further enhance nuclease stability and RNA binding affinity.[6] Commonly used 2′-modified analogues used in gapmers include 2′-methoxyethyl RNA (MOE), constrained ethyl BNA (cEt), and locked nucleic acid (LNA)[7] (Figure 1).

Our collaborative group discovered that the Stabilin class of receptors, of which there are two members, is responsible for the systemic clearance of phosphorothioate antisense oligonucleotides (PS-ASOs).[8] Both human Stabilin-1 and Stabilin-2 are ∼315 kDa type 1 receptors with a single transmembrane domain and a short cytoplasmic tail.[9] Stabilin-1 is more widely expressed within endothelial cells and alternatively activated macrophages.[10] Stabilin-2 is expressed at a high level in the liver, spleen, bone marrow, and lymph node sinusoidal endothelium and at a lower level in specific tissues within the muscle, brain, and kidney.[11−13] Both receptors share the same domain organization in which the extracellular portion consists of seven Fasciclin-1 domains separated by four clusters consisting of four to six EGF/EGF-like domains, and an X-Link domain that binds hyaluronan in Stabilin-2 but is dysfunctional in Stabilin-1.[14] Both receptors bind with ligands such as heparin,[15] PS-ASOs,[8] phosphatidylserine,[16,17] and oxidized low-density lipoprotein.[18] Each protein can also internalize their own unique ligands such as SPARC[19] and placental lactogen[20] for Stabilin-1 and hyaluronan[21] and chondroitin sulfates A, C, and D for Stabilin-2.[22] Sodium dodecyl sulfate−polyacrylamide gel electrophoresis analysis of the receptor demonstrates that Stabilin-1 is expressed as two high-molecular weight proteins (1:1 ratio) that migrate as a tight doublet in contrast to Stabilin-2, which is expressed as 315 and 190 kDa isoforms in an approximately 1:1 ratio in native tissues.[23] For the experiments outlined in this report, we utilized the ecto-domain of the recombinant 190 kDa isoform (s190) of Stabilin-2 as it has a high level of expression and/or secretion in cell lines and may be purified to near 100% purity

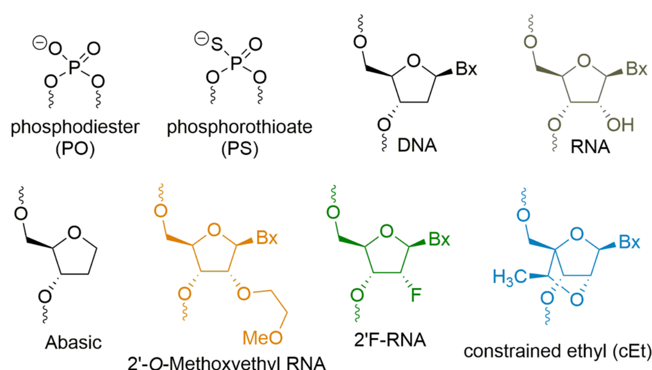

Figure 1. Structures of chemical modifications used in this study.

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using affinity chromatography. Both isoforms have the same activity against PS-ASOs.[8]

Previously, we used the recombinant 190 kDa isoform expressed in cell lines and the s190 purified protein to assess PS-ASO binding and internalization. From both enzyme-linked immunosorbent assay (ELISA)-like assays and internalization data with [^{125}I]PS-ASO (5−10−5 oligo), we determined that the binding affinity was ∼140 nM.[8] Competition assays were utilized to determine the effect of chemical modifications and oligonucleotide composition on Stab2 binding. The competition assays did not accurately inform the direct binding of the competitors or their lower affinity for the receptor. The objective in this report was to assess direct binding of a variety of ASOs to determine which chemistries (Figure 1) provide the weaker and stronger interaction between the nucleic acid and s190 using a sensitive fluorescence polarization (FP) assay.[24,25]

A series of experiments were performed using different variants of an ASO targeting phosphatase and tensin homologue (PTEN) mRNA to determine their affinity for s190. The interaction between the protein receptor and PTEN ASO was then challenged by pH and salt dependence.

Table 1 (Figure 2A) provides a summary of results for the initial binding experiments with the PTEN ASOs. It was found that the receptor has a significantly higher affinity for single-stranded ASO (ASO 1) than for double-stranded molecules in which the same ASO was bound with a RNA complement (ASO 8). The phosphorothioate linkage is highly preferred for binding (ASO 1) in contrast to the phosphodiester oligo (ASO 5), and the affinity rapidly drops as the oligo length is reduced to 15 bases (ASO 6) and 10 bases (ASO 7). The five flanking bases with 2′ modifications did not affect the overall affinity, indicating that the PS modification on the oligo backbone is the primary contributor for Stabilin-2 binding (compare ASO 5 with ASO 1, 9, and 10).

Table 1. Binding of PTEN ASOs to s190 (Stab2)[a]

ASO	Sequence	ASO Design	Kd (nM)
1	X-CTGCTAGCCTCTGGATTTGA	5-10-5 MOE PS	12.6 (18.8)
2	X-CTGCTAGCCTCTGGATTTGA	5-10-5 MOE MBB	35.5
3	X-CTGCTAGCCTCTGGATTTGA	uniform DNA PS	11.4
4	X-CTGCTAGCCTCTGGATTTGA	uniform MOE PS	7.0
5	X-CTGCTAGCCTCTGGATTTGA	5-10-5 MOE PO	>1,000
6	X-CTGCTAGCCTCTGGA	15-mer PS	83.9
7	X-CTGCTAGCCT	10mer PS	>1,000
8	X-CTGCTAGCCTCTGGATTTGA/ TCAAATCCAGAGGCTAGCAG	5-10-5 MOE PS / RNA complement	>1,000
9	X-CTGCTAGCCTCTGGATTTGA	5-10-5 cEt PS	16.1
10	X-CTGCTAGCCTCTGGATTTGA	5-10-5 2′-F PS	10.6

[a]Orange letters indicate MOE, gray letters RNA, black letters DNA, green letters 2′F RNA, and blue letters cEt modifications. All oligonucleotides are PS-modified, except for underlined letters, which are natural phosphodiesters (PO). Oligos with a mixture of PS and PO linkages are mixed backbone (MBB); X = Alexa 647 Fluor. The number in parentheses is a value from duplicate measurements.

The same experiments were repeated using a "gapmer" designed set of oligos (Figure 2B and Table 2). The control for this group is PTEN ASO 1. All of these PS-based oligos (ASO 11−15) bound with affinities within 1 order of magnitude of each other (15−73 nM). The T_{20} (ASO 17) and A_{20} (ASO 18) oligos were also compared against each other, which resulted in A_{20} having a 20-fold weaker affinity for the receptor. The explanation for this observation is the assumption that because of the helical self-stacking of the purine bases, A_{20} is more rigid and, therefore, has a lower binding affinity. Rigidity may also be the reason for which the double-stranded PTEN ASO has an affinity lower than that of the single-stranded ASO in Table 1. The tight binding with the A-basic ASO (ASO 16) confirms that the PS modification is the binding motif for Stabilin-2, which is in agreement with the PTEN ASOs in which the PO version of the PTEN ASO does not bind with s190.

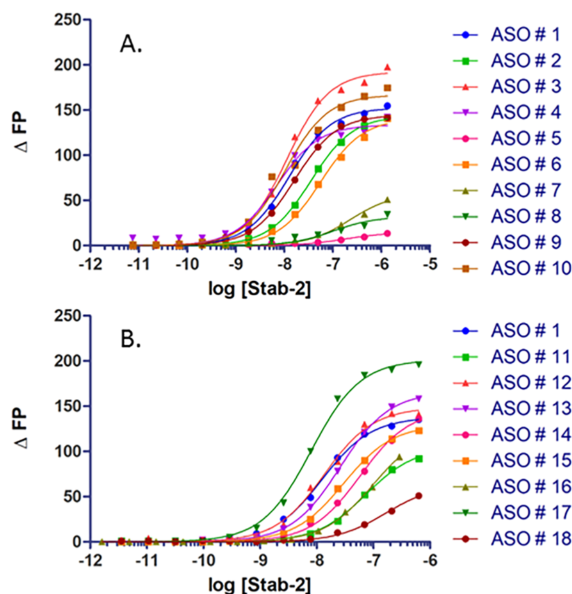

Figure 2. Direct ASO binding to s190 (Stab2). (A) PTEN ASOs with various chemistries. (B) ASOs with differing sequences and designs.

Table 2. Effect of Sequence and Design on Binding to s190 (Stab2)[a]

ASO	Sequence	ASO Design	Kd (nM)
1	X-CTGCTAGCCTCTGGATTTGA	PTEN 5-10-5 MOE PS	12.4
11	X-GCTCCTTCCACTGATCCTGC	PTB1B 5-10-5 MOE PS	73.4
12	X- TGCATCCCCCAGGCCACCAT	ICAM1 DNA PS	9.0
13	X-TCCCATTTCAGGAGACCTGG	CRP 3-14-3 MOE PS	26.2
14	X-GCTGATTAGAGAGAGGTCCC	TNF-α 5-10-5 MOE PS	58.6
15	X-CCTTCCCTGAAGGTTCCTCC	Control 5-10-5 MOE PS	29.8
16	X-NNNNNNNNNNNNNNNNNNNN	A-basic	96.5
17	X-TTTTTTTTTTTTTTTTTTTT	T_{20} DNA PS	7.9
18	X-AAAAAAAAAAAAAAAAAAAA	A_{20} DNA PS	165

[a]Orange letters indicate MOE and black letters DNA. N indicates a PS-modified abasic oligonucleotide, and X = Alexa 647 Fluor.

The effect of pH on ASO binding is important to examine as once the receptor is internalized in early endosomes, the pH decreases during endosomal maturation. Using a mixture of mono- and divalent 10 mM sodium phosphate buffers containing 150 mM NaCl, the fluorescence-based assay was repeated under four pH conditions (Figure 3A). As the pH decreased from 7.4 to 5.0, the affinity decreased. For most protein−ASO interactions that have been observed thus far, the opposite trend or no change in binding affinity is the typical result. In other proteins and receptors, the higher affinity at lower pH may be the result of a more positive charge that is attractive to the polyanionic PS ASO.[26] However, this is clearly not the case with this receptor. Most, if not all, professional endocytic receptors release their cargo under low-pH (<5.5) conditions,[27] and this may be the reason for the results observed for Stabilin-2.

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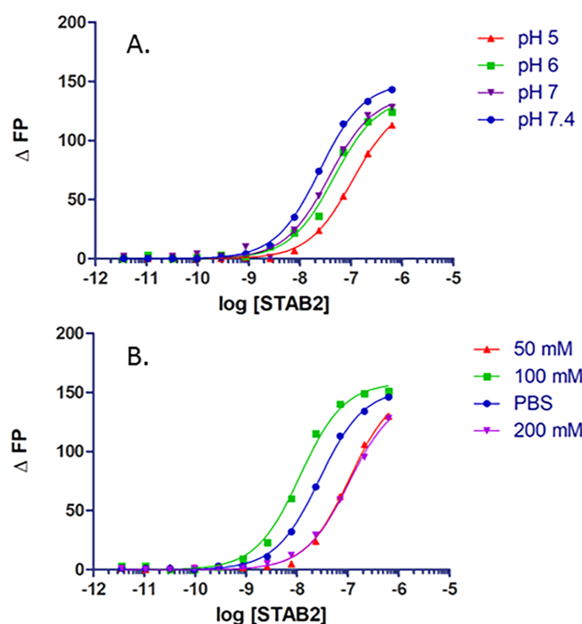

Figure 3. ASO 1 binding to s190 under various (A) pH and (B) salt conditions. The phosphate-buffered saline concentration is 150 mM.

Previous experiments with an ELISA type assay revealed that PS ASO–protein binding is dependent on ionic bonding.[8] We repeated this assay with the FP method and found that, as before, the affinity of the ASO for the receptor decreases as the ionic strength increases (Figure 3B). It is somewhat surprising that binding affinity is weakest at the lowest salt concentration and may be a result of an artifact from the experimental method or that low concentrations of salt perturb protein structure enough to decrease the level of ASO binding. Any perturbation to salt concentration may alter the physical and chemical environment of the binding site(s). There are no structural data for this receptor, and the s190 used in these experiments contains 1359 amino acids, including 104 cysteine residues; thus, predicting overall and specific subdomain structures is not possible at this time (Figure S1).

This fluorescence-based assay confirmed the results from our previous report describing the high affinity of the PS-based ASO for the Stabilin receptors.[8] This is the first report in which direct binding affinities have been observed with a multitude of different PS and non-PS ASOs that could not have been attained otherwise. With this information, it is clear that the length of the PS backbone and the single-stranded nature of the nucleic acid are the primary determinants for binding to the Stabilin receptors. In addition, the nucleotide sequence does not substantially affect the affinity for the receptor.

The sequence-independent tissue accumulation properties of PS ASOs in the liver have been used advantageously for the clinical development of ASO therapeutics. Our binding data show that PS ASOs can bind the Stabilin receptors, and presumably other cell-surface proteins, in a PS-dependent but sequence-independent manner and provide a rationale for the predictable liver accumulation properties of single-stranded PS ASOs in animals. Our data also emphasize the importance of interactions with cell-surface proteins for the promotion of cellular internalization of nucleic acid-based therapeutics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b00126.

> Methodology for the purification of s190 and fluorescence polarization assay and the amino acid sequence and domain organization of s190 (Figure S1) (PDF)

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Notes

The authors declare no competing financial interest.

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Supporting Information

Structural determinants for the interactions of chemically modified nucleic acids with the Stabilin-2 clearance receptor

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Purification of the soluble 190-HARE ecto-domain (s190)

A stable cell line expressing the s190 ecto-domain was previously developed and described in Harris et al[1]. Cells were cultured in 4-chamber Celldisc flasks (Greiner-bio One) at 37°C, 5% CO_2. The s190 protein is secreted from the cells in growth medium containing DMEM supplemented with 8% fetal bovine serum and 50 μg/mL hygromycin B. A batch of 500 mL of condition medium was incubated with 1 mL of packed mAb30 resin. Monoclonal antibody 30 (mAb30) is a monoclonal antibody against rat HARE (175-kDa) isoform that also reacts against the human receptor[2]. The antibody was conjugated with cyanogen-bromide activated sepharose (#C9142, SigmaAldrich) according to the manufacturer's instructions. Both resin and conditioned medium were rotated slowly overnight at 4°C and the resin was separated from the medium using a single gravity flow column (#9704352, BioRad, Hercules, CA, USA). Excess protein and media were washed from the resin using 10 bed volumes of saline (500 mM NaCl, 20 mM sodium phosphate monobasic, pH 7.2). s190 protein was eluted from the resin using four sequential bed volumes of 100 mM glycine, pH 3 which dripped into a 15 mL conical containing 4 bed volumes of 1 M unbuffered Tris buffer, pH 11. The resin was immediately rinsed with saline (150 mM NaCl, pH 7.2) and stored at 4°C for re-use. The eluted protein was concentrated and buffered exchanged with 1X PBS (150 mM NaCl, 20 mM sodium phosphate monobasic) using Vivaspin Turbo 4 concentrators (#VS04T41, MWCO = 100,000, Sartorius) down to a volume of 0.3-0.5 mL and quantified by the Bicinchoninic (BCA) assay (BioRad, Hercules, CA, USA).

Fluorescence polarization assay

Fluorescence polarization experiments were performed using ALEXA647-labeled ASOs synthesized at Integrated DNA Technologies (Coralville, IA, USA). Measurements were performed in 1X phosphate buffered saline (PBS), except for the experiments to determine salt and pH dependence of binding. For those evaluations a 10 mM phosphate buffer with a sodium chloride concentration of 50 to 200 mM and a pH of 5, 6, or 7 was utilized. The assay was set up in 96-well costar plates (black flat-bottomed non-binding) purchased from Corning, NY, USA. Binding was evaluated by adding ALEXA647-labeled ASOs to yield 2 nM concentration to each well containing 100 μL of Stabilin-2 protein from sub nM to low μM concentration. Readings were taken using the Tecan (Baldwin Park, CA, USA) InfiniteM1000 Pro instrument (λ_{ex} =635 nm, λ_{em} =675 nm). Using polarized excitation and emission filters, the instrument measures fluorescence perpendicular to the excitation plane (the 'P-channel') and fluorescence that is parallel to the excitation plane (the 'S-channel'), and then it calculates FP in millipolarization units (mP) as follows: $mP = [(S - P * G) / (S + P * G)] * 1000$. The 'G-factor' is measured by the instrument as a correction for any bias toward the P channel [3]. Polarization values of each ALEXA647-labeled ASO in 1X PBS at 2 nM concentration were subtracted from each measurement. K_d values were calculated with GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA) using non-linear regression for curve fit assuming one binding site.

Fig. S1: Amino acid sequence of the s190 ectodomain of Stabilin-2 that was purified and used in the fluorescence polarization assay. Color coding is indicated below.

TKLALFESLPNLLMRLEQMPDYPIFRGYIIQYNLANAIEAADAYTVFAPNNNAIENYIREKKVLSLEEDV
LRYHVVLEEKLLKNDLHNGMHRETMLGFSYFLSFFLHNDQLYVNEAPINYTNVATDKGVIHGLGKVLEIQ
KNRCDNNATTIIRGRCRTCSSELTCPFGTKSLGNEKRRCIYTSYFMGRRTLFIGCQPKCVRTVITRECCA
GFFGPQCQPCPGNAQNVCFGNGICLDGVNGTGVCECGEGFSGTACETCTEGKYGIHCDQACSCVHGRCNQ
GPLGDGSCDCDVGWRGVHCDNATTEDNCNGTCHTSANCLTNSDGTASCKCAAGFQGNGTICTAINACEIS
NGGCSAKADCKRTTPGRRVCTCKAGYTGDGIVCLEINPCLENHGGCDKNAECTQTGPNQAACNCLPAYTG
DGKVCTLINVCLTKNGGCSEFAICNHTGQVERTCTCKPNYIGDGFTCRGSIYQELPKNPKTSQYFFQLQE
HFVKDLVGPGPFTVFAPLSAAFDEEARVKDWDKYGLMPQVLRYHVVACHQLLLENLKLISNATSLQGEPI
VISVSQSTVYINNKAKIISSDIISTNGIVHIIDKLLSPKNLLITPKDNSGRILQNLTTLATNNGYIKFSN
LIQDSGLLSVITDPIHTPVTLFWPTDQALHALPAEQQDFLFNQDNKDKLKEYLKFHVIRDAKVLAVDLPT
STAWKTLQGSELSVKCGAGRDIGDLFLNGQTCRIVQRELLFDLGVAYGIDCLLIDPTLGGRCDTFTTFDA
SGECGSCVNTPSCPRWSKPKGVKQKCLYNLPFKRNLEGCRERCSLVIQIPRCCKGYFGRDCQACPGGPDA
PCNNRGVCLDQYSATGECKCNTGFNGTACEMCWPGRFGPDCLPCGCSDHGQCDDGITGSGQCLCETGWTG
PSCDTQAVLPAVCTPPCSAHATCKENNTCECNLDYEGDGITCTVVDFCKQDNGGCAKVARCSQKGTKVSC
SCQKGYKGDGHSCTEIDPCADGLNGGCHEHATCKMTGPGKHKCECKSHYVGDGLNCEPEQLPIDRCLQDN
GQCHADAKCVDLHFQDTTV*GVFHLRSPLGQYKLTFDKAREACANEAATMATYNQLSYAQKAKYHLCSAGW*
*LETGRVAYPTAFASQNCGSGVVGIVDYGPRPNKSEMWDVFCY*RMKDVNCTCKVGYVGDGFSCSGNLLQVL
MSFPSLTNFLTEVLAYSNSSARGRAFLEHLTDLSIRGTLFVPQNSGLGENETLSGRDIEHHLANVSMFFY
NDLVNGTTLQTRLGSKLLITASQDPLQPTETRFVDGRAILQWDIFASNGIIHVISRPLKAPPAPVKGELG
TELGSEGKPIPNPLLGLDSTRTGHHHHHH

Remnant of the signal sequence from vector plasmid
S190 HARE ectodomain
EGF/EGF-like domains
Fasciclin-1 domains
Link domain
Remnant of the original vector MCS sequence
V5 epitope tag
6xHis tag

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