Peptide Nucleic Acids Are Potent Modulators of Endogenous Pre-mRNA Splicing of the Murine Interleukin-5 Receptor-α Chain

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ABSTRACT: Antisense oligonucleotides (ASOs) that bind target pre-mRNA with high affinity have been shown to alter splicing patterns and offer promise as therapeutics. Previous studies have shown that ASOs fully modified with 2′-*O*-methoxyethyl (2′-*O*-MOE) sugar residues redirect constitutive and alternative splicing of the murine interleukin-5 receptor-α (IL-5Rα) chain pre-mRNA in cells, resulting in inhibition of the membrane-bound isoform and enhanced expression of the soluble isoform. Here, we show that antisense peptide nucleic acids (PNAs) alter splicing of the IL-5Rα pre-mRNA in a fashion similar to their 2′-*O*-MOE-modified counterparts of the same sequence. Moreover, using PNA as the splicing modulator, the length of the antisense oligomer could be shortened from 20 to 15 nucleobase units to obtain a comparable effect. Treatment of cells with antisense PNA resulted in dose-dependent, specific downregulation of IL-5Rα membrane isoform mRNA expression and enhanced levels of the soluble IL-5Rα isoform transcript, with an EC50 equivalent to that observed in parallel with the corresponding 2′-*O*-MOE ASO. The pronounced activity of antisense PNAs in modulating IL-5Rα mRNA splicing observed in our study identifies these compounds as a promising new class of lower molecular weight splicing modulators.

The mechanism of action of antisense oligonucleotides in controlling gene expression can be broadly divided into two categories: RNase H-mediated cleavage of the target mRNA after hybridization of the antisense DNA-like strand, and RNase H-independent mechanisms of action (*1*). The latter category includes inhibition or modulation of splicing (*2*−*4*), translation arrest (*5*), and disruption of necessary RNA structure (*6*). The RNase H-dependent mode of action is the most common one and is exploited by all of the antisense drug candidates which are currently in clinical evaluation. However, this mechanism imposes very stringent structural requirements for the antisense drug constructs in terms of conformational, backbone charge, steric, and configurational aspects (*7*). Thus, the presence of 2′-deoxy sugars for maintaining DNA-like conformation ($C_{2'}$-endo or $O_{4'}$-endo) or 2′-arabino and not ribo configurations (*8*, *9*) for the pentofuranose sugar moiety of the nucleotide residue is required for RNase H activation. The above structural requirements hamper the use of numerous other classes of high-affinity modifications such as 2′-ribo modifications (RNA mimetics) (*8*) and backbone modifications such as PNA,[1] MMI (*10*), and morpholino (*11*) functionalities in RNase H-dependent antisense pathways. However, these high-affinity modifications can be successfully employed in oligomers where the mode of action depends on simple binding to the mRNA target and does not require RNase H activity for the gene-silencing effect.

Among these high-affinity modifications, peptide nucleic acids (PNAs) are nucleic acid analogues that contain an uncharged pseudo-peptide backbone with *N*-(2-aminoethyl)-glycine units to which the nucleobases are attached via methylene carbonyl linkers (*12*−*15*) (Figure 1). PNA is capable of sequence-specific recognition of DNA and RNA obeying the Watson−Crick hydrogen-bonding scheme, and the hybrid complexes exhibit extraordinary thermal stability and unique ionic strength effects.

PNA has been used for many therapeutic and genetic applications (*16*), including monitoring telomere length (*17*), screening for genetic mutations (*18*, *19*), affinity capture of nucleic acids (*20*), and antisense-mediated target reduction (*21*). Besides binding target mRNA with high affinity, PNAs are highly resistant to nuclease and protease degradation (*21*) and display mismatch sequence discrimination (*14*), thus making them interesting third generation antisense molecules with potential therapeutic application. PNA oligomers have demonstrated in vitro transcriptional and translational block of many genes (*22*). Furthermore, PNA oligomers have been

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[1] Abbreviations: ASOs, antisense oligonucleotides; Boc, *tert*-butyloxycarbonyl; Cbz, benzyloxycarbonyl; DIEA, *N*,*N*-diisopropylamine; DVB, divinylbenzene; ESI-MS, electrospray ionization mass spectrometry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; IL-5Rα, interleukin-5 receptor-α chain; 2′-*O*-MOE, 2′-*O*-methoxyethyl; Lys, L-lysine, MBHA, *p*-methylbenzhydrylamine; MMI, methyl−methylene imino; PNA, peptide−nucleic acid; RPA, RNase protection assay; RP-HPLC, reversed-phase high-performance liquid chromatography.

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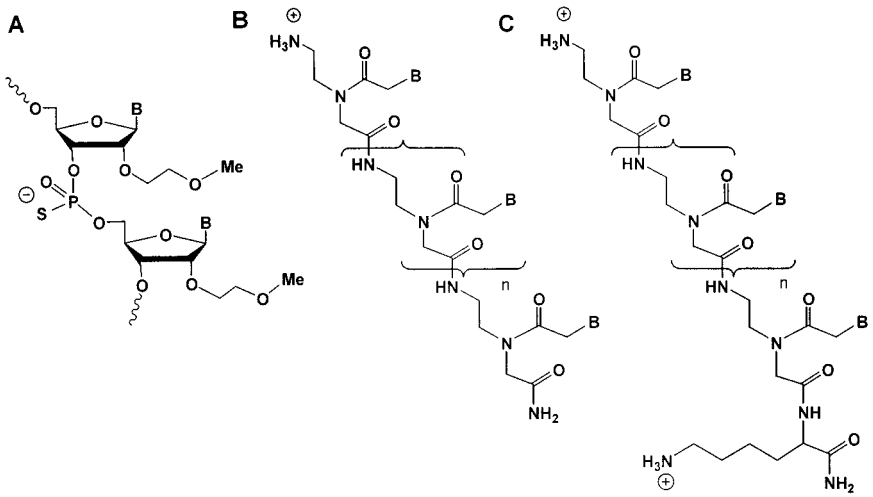

FIGURE 1: Structure of 2′-*O*-MOE phosphorothioate oligonucleotides (A) and PNA oligomers without (B) and with (C) a terminal L-lysine residue.

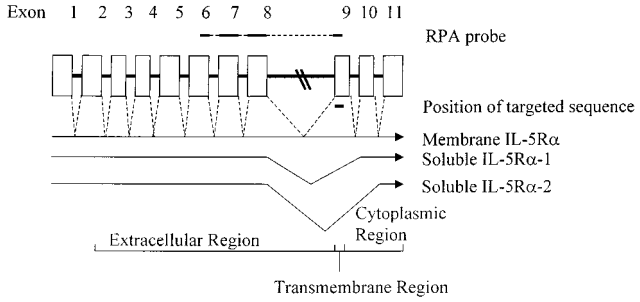

FIGURE 2: Schematic representation of the alternative splicing of the murine IL-5Rα chain gene and positions of the RPA probe and targeted sequence on the transcript. Arrows indicate normal generation of mRNA isoform species. Dashed lines indicate the presence of constitutive splicing to remove introns. Although not pictured, removal of introns occurs similarly from soluble receptor transcripts.

shown to induce triplex-mediated mutagenesis of a chromosomal gene in mouse cells (*23*).

Cytokines play a major role in the control of immune responses, contributing to immune cell activation and differentiation, trafficking, and the regulation of programmed cell death (*24*−*28*). These activities are achieved through complex signal transduction processes mediated by binding to specific receptors on the cell surface. Many cytokine receptors have evolved to finely regulate these signals by utilizing multiple subunits and alternative splicing strategies to enumerate soluble isoforms of one of these subunits capable of binding cytokines in the extracellular space (*29*, *30*). The function of these soluble cytokine receptor subunits can be either to neutralize the activity of the cytokine or to foster productive signaling by increasing the cytokine's half-life (*30*). The murine interleukin-5 receptor (IL-5R) is composed of α and β subunits which combine to form the membrane-bound, signaling-competent heterodimer (*31*−*34*). However, the α chain pre-mRNA is also alternatively spliced, resulting in soluble isoforms lacking the transmembrane coding region which do not complex with the β chain (*33*) (Figure 2). These soluble receptor proteins can bind IL-5, but this interaction does not appear to lead to IL-5 signaling events (*35*). In contrast to the mouse system, alternative splicing is required to generate the membrane-bound IL-5Rα chain in human (*36*). Thus, therapeutic strategies that modulate the expression of the IL-5Rα chain isoforms may be of utility in inflammatory diseases such as eosinophilic syndromes where IL-5 has been strongly implicated in the resulting pathology.

The previous work of Kole and colleagues has shown that 2′-*O*-methyl-modified ASOs can induce correction of aberrantly spliced mutant human β-globin constructs stably expressed in mammalian cells (*37*, *38*), suggesting their potential usefulness for treating β-thalassemia. Hodges and Crooke demonstrated that 2′-*O*-methyl ASOs specific for the branch point were more effective inhibitors of splicing of adenovirus transcript containing the β-globin branch point and 3′ splice site than 2′-deoxyphosphorothioate ASOs (*3*). Taylor et al. demonstrated the effectiveness of 2′-*O*-MOE-modified ASOs for altering pre-mRNA splicing of the *bcl-x* transcript (*39*). Recently, we have observed that 2′-*O*-MOE-modified ASOs modulate alternative splicing of the murine IL-5Rα pre-mRNA such that the transmembrane-encoding exon 9 is skipped, resulting in a decrease in membrane-bound isoform expression coupled with an increase in mRNA levels of the soluble isoform (*40*). To determine whether a PNA oligomer could also function as a selective modulator of alternative mRNA splicing, an antisense sequence shown to produce exclusion of exon 9 from the IL-5Rα processed mRNA was synthesized as both a 2′-*O*-MOE and PNA oligomer and compared in vitro in cells. The patterns of IL-5Rα mRNA expression were then examined using an RNase protection assay with probes that distinguish the membrane and soluble forms of the receptor mRNA, thus allowing a direct comparison of activity.

EXPERIMENTAL PROCEDURES

Cell Culture. The B lymphoma cell line, BCL_1, was purchased from the ATCC (Rockville, MD). BCL_1 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 10 mM Hepes, pH 7.2, 50 μM 2-ME, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco, Grand Island, NY).

Antisense Oligonucleotide and PNA Synthesis. The 2′-*O*-MOE antisense oligonucleotides and antisense PNA oligomers synthesized for this study are shown in Table 1. 2′-*O*-

Table 1: Sequence and Backbone Modification of the Oligomers Synthesized

oligo	sequence	backbone	MW (g/mol) calcd	MW (g/mol) found
1	GCCATTCTACCAAGGACTTC	2′-*O*-MOE P═S	7922.1	7925.1
2	TCT ACC AAG GAC TTC	2′-*O*-MOE P═S	5918.3	5920.0
3	H-TCT ACC AAG GAC TTC-Lys-NH_2	PNA	4148.7	4150.0
4	H-TCT ACC AAG GAC TTC-NH_2	PNA	4020.5	4021.6
5	H-TCA ACC TAG AAC TTC-Lys-NH_2	PNA	4132.7	4133.9

Methoxyethylribose-modified phosphorothioate oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems, model 380B), as described previously (*40*). Oligonucleotides were analyzed by capillary gel electrophoresis and judged to be at least 90% full-length material. PNA oligomers were synthesized in a 10 μmol scale on a 433A peptide synthesizer (Applied Biosystems, model 433A) using commercially available Boc/Cbz-protected monomers (Applied Biosystems) and synthesis protocols based on previously published procedures (*41*−*43*). The coupling efficiency was monitored by qualitative Kaisertest (*44*). After cleavage and deprotection the PNA oligomers were purified by RP-HPLC. After chromatographic purification, the oligomers were analyzed by ESI-MS, lyophilized, and stored at −20 °C.

Preloading of the Solid Support. A polystyrene resin (2% DVB) bearing a MBHA linker was swollen in DCM for 6 h and preloaded with either the PNA monomers or Boc-Lys-(2-Cl-Z)-OH using the combination of HATU and DIEA for monomer activation. The resin substitution was determined by quantitative Kaisertest (*45*).

Cell Transfection. BCL_1 cells (1×10^7 cells in PBS) were transfected with oligonucleotides by electroporation, at 200 V, 1000 μF, using a BTX Electro Cell Manipulator 600 (Genetronics, San Diego, CA). RNA was harvested 24 h following electroporation and purified using the RNeasy method (Qiagen, Santa Clarita, CA).

Riboprobe Design and RNase Protection Assay. RNase protection experiments were conducted using Riboquant kits according to the manufacturer's instructions (Pharmingen, San Diego, CA). A custom riboprobe was designed to protect the mRNA sequence corresponding to the distal half of exon 6, all of exons 7 and 8, and the proximal half of exon 9 and purchased from Pharmingen. Ten micrograms of total RNA was analyzed in each experiment. Signals were quantitated using a Molecular Dynamics PhosphorImager.

RESULTS

PNA Synthesis. PNA synthesis was carried out on MBHA polystyrene resins preloaded with the first monomer residue by using the Boc strategy and employing HATU/DIEA for monomer activation. The C-terminal lysines for **3** and **5** were introduced by using a resin preloaded with BOC-Lys(2-Cl-Z)-OH. Synthesis on a 10 μmol scale yielded after RP-HPLC purification more than 20 mg of PNA oligomer with a purity of >95%, which were further analyzed by ESI-MS (Table 1).

Effect of Antisense PNA Oligomers of the Same Sequence as a Uniformly 2′-Methoxyethyl-Modified Phosphorothioate Antisense Oligonucleotide That Redirects Splicing of the Murine IL-5Rα Subunit. Previous experiments have shown that high-affinity, uniformly 2′-*O*-MOE-modified antisense oligonucleotides (20mers) targeted to exon sequences just downstream of the 3′ splice site of intron 8 in the murine IL-5Rα chain induce exclusion of exon 9, the exon that encodes the transmembrane domain (*40*). The interaction of these ASOs with the IL-5Rα pre-mRNA causes the selective exclusion of exon 9 from the processed mRNA, resulting in inhibition of expression of the membrane-bound isoform of the receptor and simultaneous superinduction of the soluble isoform species. These oligonucleotides do not accommodate RNase H-mediated cleavage (*46*) and appear to produce these changes in isoform expression by redirecting the splicing machinery to the next downstream splice acceptor site in that the total amount of mRNA measurable in RNase protection experiments evaluating this phenomenon is conserved.

Because a uniform 2′-*O*-MOE modification is known to confer high-affinity hybridization properties to oligonucleotides (*47*), we wished to evaluate whether another high-affinity modification, PNA, would also induce antisense-mediated splicing activity in cells. Therefore, two PNA 15mers, one with (**3**) and one without (**4**) a C-terminal Lys residue, corresponding to residues 1−15 from the 3′-end of our most potent uniform 2′-*O*-MOE 20mer antisense oligonucleotide (**1**) targeted against exon 9 of IL-5Rα, were studied. The Lys residue bearing a protonated δ-amino group at physiological pH was introduced to determine the effect of an additional positive charge in the PNA backbone on the biological activity of the oligomer in this particular setup. Their potency to modulate splicing was investigated in comparison to the corresponding uniform 2′-*O*-MOE 15mer (**2**) as well as the parent 2′-*O*-MOE oligonucleotide (**1**) in IL-5Rα-expressing murine BCL_1 B cell lymphoma cells.

As shown in Figure 3, electroporation of **1** into BCL_1 cells resulted in a dose-dependent reduction of the membrane isoform of the IL-5Rα coupled with a concomitant increase in expression of the soluble isoform (lanes 2−5). Reducing the length by five nucleotides (**2**) slightly compromised the activity, as expected for a compound with lower affinity for the target (lanes 6−9 and Figure 4). Importantly, however, the PNA 15mer (**3**) produced at least an equivalent inhibition of the membrane isoform of IL-5Rα compared to the 2′-*O*-MOE oligonucleotide (**2**) of the same length and sequence (lanes 11 and 12 compared to lanes 6 and 7 and Figure 4A). Furthermore, this length-reduced PNA exhibited essentially the same antisense activity as the parent 20mer oligonucleotide (compare lanes 11 and 12 with lanes 2 and 3). The effects of the uncharged PNA (**4**) were similar to those of the Lys-modified oligomer (**3**); however, they appear to be slightly less pronounced (lanes 18−21 and Figure 4). A three-base mismatch control PNA (**5**) had little effect on IL-5Rα expression of either isoform (lanes 14−17 and Figure 4), indicating that the activities of the antisense PNAs are based

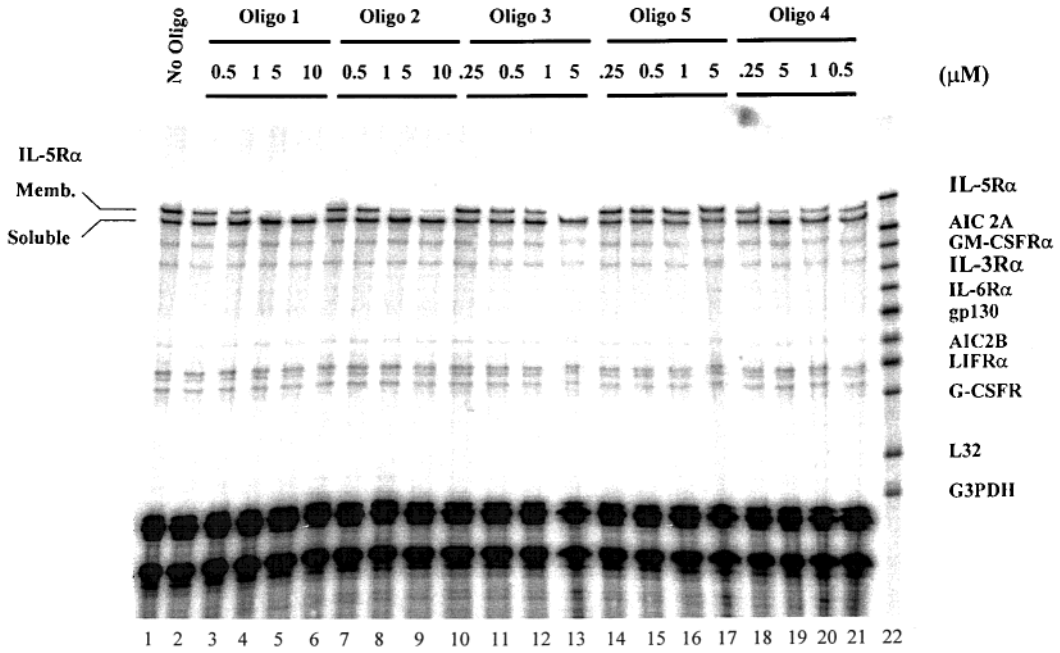

FIGURE 3: Antisense PNA 15mers redirect splicing of the mouse IL-5Rα pre-mRNA in BCL_1 cells. RPA analysis of membrane and soluble IL-5Rα mRNA species (arrows) following electroporation transfection of BCL_1 cells with uniformly modified 2′-*O*-MOE antisense oligonucleotides or antisense PNAs targeting the exon 9 sequence near the 3′ intron 8 splice site. PNA 15mers with (**3**) and without (**4**) coupling to Lys are shown versus a three-base mismatch PNA sequence (**5**) and 2′-*O*-MOE 20mer (**1**) and 15mer (**2**). RNA from cells that were electroporated in the absence of oligonucleotide is shown in lane 1. Undigested probe is shown in lane 22. The experiment shown is representative of two similar trials.

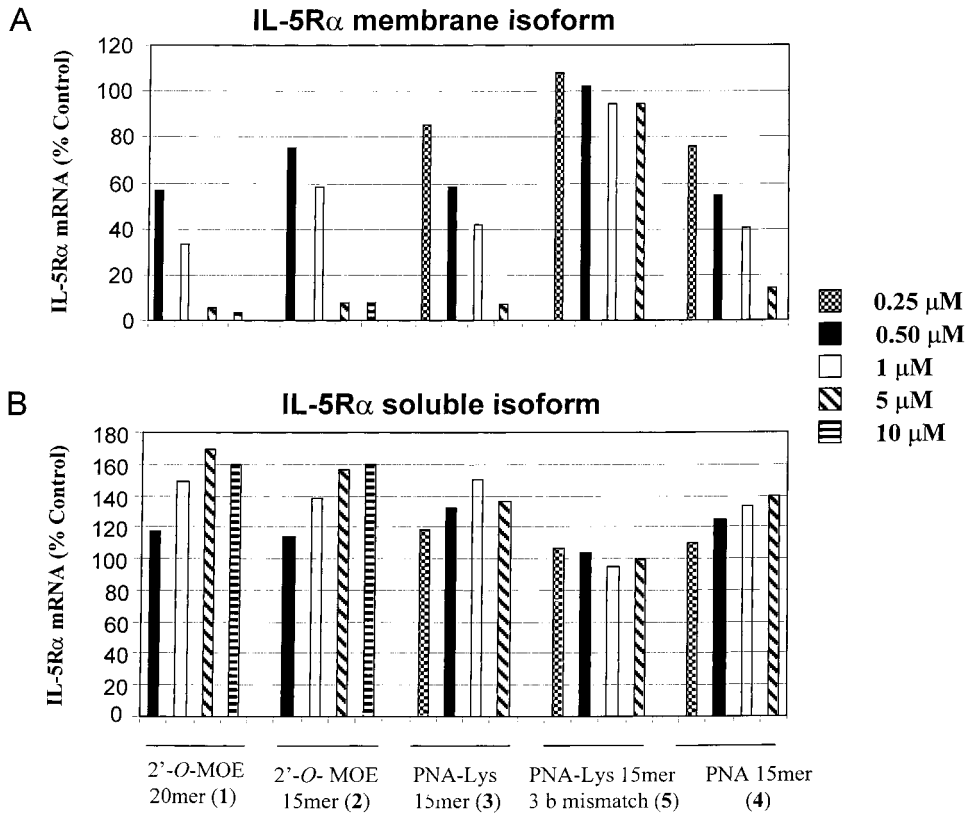

FIGURE 4: Comparison of antisense PNA oligomers versus 2′-*O*-MOE antisense oligonucleotides in altering splicing of mouse IL-5Rα. Quantitation of RPA phosphorimage analysis of effects of antisense PNA oligomers and 2′-*O*-MOE-modified antisense oligonucleotides depicted in Figure 3 on membrane (upper panel; A) and soluble (lower panel; B) IL-5Rα mRNA levels in BCL_1 cells. The data shown are derived from analysis of a RPA gel normalized to L32 levels and are representative of two similar experiments.

on hybridization to the target sequence of the pre-mRNA and are consistent with an antisense mechanism of action.

Quantification of the data shown in Figure 3 normalized to the L32 housekeeping gene is shown in Figure 4. From this representation, it is apparent that the antisense PNA **3** also induces increased expression of the soluble form of IL-5Rα in BCL_1 cells, with activity similar to that of its 2′-*O*-MOE counterpart, particularly at the low doses that can be

directly compared (Figure 4B). Thus, the antisense PNA 15mer (**3**) is an effective splicing modulator of the murine IL-5Rα pre-mRNA in cells, and its activity compares favorably to that of a 20mer 2′-*O*-MOE antisense oligonucleotide (**2**).

DISCUSSION

An underlying tenet of antisense drug discovery predicts that increased hybridization affinity of anti-gene compounds for target mRNA will result in improved pharmacological activity. PNA oligomers exhibit significantly greater hybridization affinity than the first generation DNA phosphorothioates and even second generation 2′-*O*-MOE-modified ASOs (*14*, *48*). It has been unequivocally demonstrated that PNA can specifically inhibit both translation initiation and elongation by forming high-affinity duplexes with target RNA (*49*). In this report, a PNA oligomer outperformed a uniformly modified 2′-*O*-methyl oligonucleotide and a *N*3′→*P*5′ phosphoramidate (*50*) oligomer in inhibiting the translation initiation of mutated H-ras mRNA in vitro in a rabbit reticulocyte extract. In the same system, translation elongation of the polypeptide chain was also inhibited by PNA antisense oligomers. However, so far, there is no documented evidence of pharmacology in PNA oligomers in controlling splicing alteration and a direct comparison of PNA to other high-affinity 2′-*O*-alkyl modifications. It has also been claimed that the PNA oligomers exhibit higher on-rates of hybridization to RNA and DNA, and it has been shown that the PNA−RNA hybrids and PNA−DNA hybrids exhibit higher T_m's than the corresponding DNA−DNA hybrids (*51*).

In the present study, we compared the relative activities of PNA oligomers of 15 nucleobase units to both 15mer and 20mer ASOs uniformly modified with 2′-*O*-MOE. Previous work has established that 2′-*O*-MOE uniformly modified ASOs can selectively redirect pre-mRNA splicing activity in cells (*39*, *40*). Using PNA oligomers and 2′-*O*-MOE-modified ASOs targeted to the identical sequence within exon 9 of the mIL-5Rα chain, our results indicate that an antisense PNA 15mer can function as an effective splicing modulator, with activity similar to that of its 2′-*O*-MOE antisense 20mer counterpart, as measured by either inhibition of membrane IL-5Rα isoform expression or by induction of soluble isoform levels in BCL_1 cells. As one of the goals of this study was to demonstrate the effectiveness of PNA as a reduced-length oligomer within the exact same target sequence, we did not compare the activity of the 20mer PNA to the parent 20mer 2′-*O*-MOE-modified ASO.

The altered physicochemical and structural properties of PNA are responsible for its observed superior antisense properties. The neutral pseudopeptide backbone of PNA may be responsible for the increased on-rate of hybridization due to the overall charge reduction. The inherent rigidity of PNA, which is due to the presence of the amide linkages in the backbone, reduces the entropy of the system and leads to an increased preorganization for duplex formation and the observed high affinity to complementary RNA. Furthermore, the pseudopeptide backbone, with its nonnucleosidic nature, is not a substrate for endo- and exonucleases and also provides stability against peptidases and proteases, making PNA completely stable against enzymatic degradation (*51*).

In contrast to negatively charged antisense oligonucleotides, the neutral backbone of PNA also offers an enhanced potential to improve cellular uptake. This could be improved even further by conjugating it to basic peptides and other cationic molecules, which are predicted to have favorable interactions with negatively charged cell membranes. In fact, Kole et al. very recently found that the presence of cationic Lys residues significantly enhanced free cellular uptake of PNA oligomers (*52*). Furthermore, the introduction of positive charges has a favorable effect on the solubility of the PNA oligomers in aqueous solutions. Our present data, however, do not show a clear difference in activity between the PNA 15mer with (**3**) and without (**4**) a C-terminal lysine. This may be due to the use of electroporation involved in these studies to engender transfection of BCL_1 cells and the fact that we linked only one lysine residue to PNA.

The IL-5Rα antisense oligomers hybridize to a purine-rich region near the 3′ ss of intron 8. Different mechanisms of oligonucleotide-induced redirection of splicing have been implicated in cell-based systems (*39*, *40*). For example, ASOs may trigger recognition of a cryptic ss near a normally dominant ss, instead of inducing skipping of an exon (*37*). It will be important to analyze PNA activity in different splicing systems to evaluate potential cell-type-specific effects, as well as possible differences in interactions with splicing factors regulating unique splicing activities. Further work will be required to better understand the general application of PNA oligomers in splicing modulation.

IL-5 has been shown to govern eosinophil release from the bone marrow and is critical for the development of tissue eosinophilia following inflammatory stimuli (*53*). Neutralizing IL-5 monoclonal antibodies and both IL-5 and IL-5Rα antisense approaches using 2′-*O*-MOE chemistry have shown activity in animal models of disease (*54*−*56*). With regard to an antisense approach, these studies have to date only evaluated 2′-*O*-MOE-modified "gapmer" RNase H-dependent oligonucleotides. Thus, PNA antisense oligomer targeting of the IL-5Rα chain is an attractive alternative which may provide in vivo pharmacological activity.

The results presented here suggest that PNA antisense oligomers of only 15 nucleobase units can display modulatory activity in experimental splicing systems in vitro similar to that observed using 2′-*O*-MOE uniformly modified 20mers. This result clearly establishes the role of PNA oligomers in controlling gene expression by modulating splicing. Furthermore, the observation that reducing the length by 25% does not compromise the efficacy of the corresponding antisense oligomer is important for validating the prediction that length reduction is possible with appropriate novel chemistries. This not only will have favorable impact on the cost of the synthesis but also could lead to improved pharmacological properties of the antisense drugs by enhancing their cellular permeation and oral absorption properties.

In vivo analysis, however, will be required to address whether the biophysical characteristics of PNAs provide distribution advantages and translate to improved efficacy in immune system-mediated disease models compared to advanced chemistries such as 2′-*O*-MOEs. Both an in vitro report and a recently published in vivo study in brain have shown increased antisense potency with PNA sequences compared to 2′-deoxyphosphorothioates (*21*, *57*). Recently, PNA has been conjugated to cell permeation peptides and lipophilic molecules to improve its uptake properties and

evaluated in vitro (*58*−*62*). These conjugates have demonstrated delivery of PNA oligomers without electroporation in some cases. Obviously, the next logical extension is the evaluation of PNA as conjugates of permeation peptides, oligomeric cationic groups, and other cell permeation enhancers. In vivo evaluation of antisense PNA oligomers and conjugates of these uptake enhancers will provide the ultimate answers in choosing PNAs as next generation therapeutic agents. This might also open the exciting possibility of using novel chemical modifications in antisense drugs, which can work through a non-RNase H mode of action.

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