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Splice switching efficiency and specificity for oligonucleotides with locked nucleic acid monomers

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Abstract

The use of antisense oligonucleotides to modulate splicing patterns has gained increasing attention as a therapeutic platform and, hence, the mechanisms of splice switching oligonucleotides are of interest. Cells expressing luciferase pre-mRNA interrupted by an aberrantly spliced β -globin intron, HeLa pLuc705, were used to monitor splice switching activity of modified oligonucleotides by detection of expression of functional luciferase. It was observed that phosphorothioate 2'-O-methyl RNA oligonucleotides containing locked nucleic acid monomers provide outstanding splice switching activity. However, similar oligonucleotides with several mismatches do not impede splice switching activity which indicates risk for off-target effects. The splice switching activity is abolished when mismatches are introduced at several positions with locked nucleic acid monomers suggesting that it is the locked nucleic acid monomers that give rise to low mismatch discrimination to target pre-mRNA. The results highlight the importance of rational sequence design to allow for high efficiency with simultaneous high mismatch discrimination for splice switching oligonucleotides and suggest that splice switching activity is tunable by utilizing locked nucleic acid monomers.

Introduction

Aberrant mRNA that code for defective protein isoforms is a crucial factor for several genetic diseases by failure to remove introns or by inaccurate recognition of exon-intron boundaries during pre-mRNA splicing [1]. Manipulation of pre-mRNA splicing is one approach to find novel therapies for inherited diseases such as cystic fibrosis [2], β -thalassemia [3] and Duchenne's muscular dystrophy [4]. An application that has gained increasing attention since its initial discovery in 1993 is the use of oligonucleotides (ONs) that bind in an antisense manner to pre-mRNA and modulate splicing patterns by blocking spliceosomal pre-mRNA processing, so called splice switching ONs (SSOs) [5]. Preferably, an SSO should be stable in serum, hybridize efficiently with target pre-mRNA, be non-toxic and not recruit RNase H [6, 7].

RNase H competent phosphorothioate DNA (PS DNA) ONs are classified as the 1st generation of antisense ONs (asONs) and they were introduced to inhibit protein expression at mRNA level [8]. The 2nd generation of asONs is characterized by different 2'-modifications, such as 2'-O-methyl RNA (2'-OMe RNA), that increase nuclease resistance and ameliorate base-pairing affinity. The 2nd generation asONs are RNase H incompetent [9-11] which makes them suitable as SSOs [12]. The 3rd generation of asONs are further developed with modified riboses and/or phosphate linkages to exhibit improved affinity to the target RNA, low toxicity, high serum stability and improved pharmacokinetics [6]. From a pharmaceutical point of view, one of the most promising modified asON, defined by content of 2'-C,4'-C-oxy-methylene-linked bicyclic ribonucleotide monomers, is locked nucleic acid (LNA) [13, 14].

Incorporation of LNA monomers to an ON confer increased affinity for complementary RNA [15] and for an LNA/DNA ON with LNA monomers evenly distributed over the sequence (mixmer) the increase in melting temperature per substitution is greater compared to an ON with clustered LNA substitutions (gapmer) [16]. This phenomena is termed 'structural saturation' [17] and it has been observed that LNA pyrimidines enhance duplex stability more than purines, especially when the substituted LNA pyrimidines are neighboring purines [17, 18]. Incorporation of evenly distributed LNA monomers in SSOs should therefore be advantageous by effective competition with splicing factors for access to the target pre-mRNAs [19]. Such chimeric PS LNA/DNA mixmers do not recruit RNase H and they have shown great potency to switch pre-mRNA splicing *in vivo* [20].

In this study a previously described SSO [21-24] was synthesized as phosphorothioate mixmer consisting of 2'-OMe RNA with addition of one third LNA monomers, evenly distributed in the sequence but mainly at pyrimidine positions. Corresponding SSOs with various introduced mismatches were utilized to assess mismatch discrimination. In

addition, we investigated the importance of introduced LNA monomers for splice switching efficiency utilizing mixmers where every second position was an LNA monomer. Other modified ONs, such as PS DNA, peptide nucleic acid (PNA), DNA spiegelmers (L-DNA) and PS 2'-OMe RNA without LNA monomers were also included in the study for comparisons. Splice switching activity was tested with a cell based splicing reporter system [24].

Encouraged by previous data where 2nd and 3rd generation asONs have successfully been used in splice switching experiments [7] [20], the main scope with this study was to design SSOs that display high activity at low concentrations and investigate the impact of mismatches to target pre-mRNA, which so far have not been thoroughly examined for SSOs with LNA monomers in the sequence.

Experimental

Synthesis of oligonucleotides and peptide-PNA conjugate

PS 2'-OMe RNA and PS DNA ONs were synthesized on an ÄKTA™ oligopilot™ 10 plus and purified on ÄKTAexplorer™ 100. Details are found in supplementary data. PS LNA/2'-OMe RNA mixmers were obtained from RiboTask (Odense, Denmark) and L-DNA was obtained from Noxxon Pharma (Berlin, Germany).

The cell-penetrating peptide, M918 [22], and PNA were synthesized on an Applied Biosystems™ model 433A (Applied Biosystems, Warrington, PA) stepwise synthesizer by *t*-Boc chemistry using a 4-methylbenzhydrylamine-polystyrene resin (Iris Biotech, Marktredwitz, Germany) to generate an amidated C-terminus. Amino acids (Neosystem, Strasbourg, France) were coupled as hydroxybenzotriazole esters while PNA monomers (Applied Biosystems, Warrington, PA) were coupled with 2-(7-aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate. After cleavage of peptide and PNA from the resin using hydrogen fluoride, synthesis products were purified using a Supelco Discovery™ HS C18-10 reversed-phase HPLC column (Sigma-Aldrich, Stockholm, Sweden) and analyzed using a Perkin Elmer proTOF™ 2000 MALDI O-TOF mass spectrometer (Perkin Elmer, Upplands Väsby, Sweden). Conjugation of peptides with 3-nitro-2-pyridinesulfonyl (Npys) cysteine to cysteine containing PNA via a disulfide bridge was performed overnight in 20% acetonitrile/water containing 0.1% trifluoroacetic acid (TFA) and purified by reversed-phase HPLC.

Cell culture

HeLa pLuc 705 cells were grown in Dulbecco's modified Eagle's medium with glutamax (DMEM) supplemented with 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were grown at 37 °C in a 5% CO₂ atmosphere. All media and chemicals were purchased from Invitrogen, Stockholm, Sweden.

Splice switching assay

To facilitate evaluation of SSOs, Kole and co-workers have constructed a splicing reporter system [24] with a plasmid carrying a luciferase-coding sequence that is interrupted by an insertion of intron 2 from β -globin pre-mRNA, carrying an aberrant splice site that activates a cryptic splice site. Unless this aberrant splice site is masked by an SSO, the pre-mRNA of luciferase will give rise to expression of non-functional luciferase (Figure 1). By using HeLa pLuc 705 cells which are stably transfected with this plasmid, it is possible to evaluate various SSOs by measuring the induced luciferase activity and thereby generate a positive read-out [23].

HeLa pLuc 705 cells (120,000) were evenly seeded 24 h before experiments in 24-well plates (Sigma-Aldrich, Stockholm, Sweden). Cells were washed with phosphate buffered saline (Invitrogen, Stockholm, Sweden) and treated for 16 h in 200 μ l transfection mixture. The mixture consisted of 100 μ l DMEM (supplemented with 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate and 10% fetal bovine serum) and 100 μ l DMEM, ON and LipofectamineTM 2000 (2.5 μ l/ μ g ON). Thereafter, the cells were washed twice with phosphate buffered saline and lysed using 100 μ l cell culture lysis buffer (Promega, Falkenberg, Sweden) for 15 min at room temperature.

PNA was conjugated to the cell-penetrating peptide, M918 [22], via a disulfide bond and transfection was performed for 1 h with 5 or 10 μ M conjugate in 300 μ l DMEM, followed by replacement of the transfection solution to 400 μ l full growth media. Cells were grown overnight and thereafter, washed with phosphate buffered saline and lysed.

Luciferase activity in 20 μ l cell lysate was measured in relative luminescence units (RLU) on a FlexstationTM II (Molecular Devices, Sunnyvale, CA) after addition of 80 μ l Promega luciferase substrate (Promega, Falkenberg, Sweden) and normalized to protein content determined by Lowry's protein assay [25] (Bio-Rad Laboratories, Sundbyberg, Sweden). Luciferase activities are presented either as RLU, RLU/mg protein or as fold increase in RLU over untreated cells. Experiments are performed at least twice in triplicate.

Cell viability

Cell viability was determined from mitochondrial activity using wst-1 assay which is based on the cleavage of the tetrazolium salt, wst-1, by mitochondrial dehydrogenases in viable cells [26]. HeLa pLuc 705 cells were seeded onto a 96-well plate (Sigma-Aldrich, Stockholm, Sweden), at 20 000 cells/well, 24 h before the experiment. Cells were then exposed to 250 nM ON in the same way as for the splice switching assay and the viability was measured after 16 h. Cells were then exposed to wst-1 according to the manufacturer's protocol (Roche, Bromma, Sweden). Absorbance (450 over 690 nm) was measured on a Digiscan absorbance plate reader (Labvision, Värmdö, Sweden). Untreated cells were defined as 100% viable.

Analysis of luciferase pre-mRNA

Cells were grown and treated with ON in the same manner as described for the splice switching assay but lysed with TRIzolTM (Invitrogen, Stockholm, Sweden) and total cellular RNA was extracted according to the manufacturer's protocol. RNA from two wells were pooled and treated with Avian Myeloblastosis Virus reverse transcriptase (Fermentas, Helsingborg, Sweden) according to the manufacturer's protocol. PCR was performed with Taq DNA polymerase (In vitro, Stockholm, Sweden), dNTP (Fermentas,

Helsingborg, Sweden), forward primer (5'- TTG ATA TGT GGA TTT CGA GTC GTC) and reverse primer (5'- TGT CAA TCA GAG TGC TTT TGG CG). Cycles of PCR proceeded at 94°C for 30 s, 55°C for 30 s 72°C for 30 s for totally 30 cycles. The PCR products were separated on a 1,7 % agarose gel with ethidium bromide at 100 V for 45 min and identified by comparison with bands from 1 kb DNA ladder (Invitrogen, Stockholm, Sweden). Electrophoresis bands on the agarose gel were visualized by UV in a Molecular Imager Gel Doc system (Bio-Rad Laboratories, Sundbyberg, Sweden) and bands from PCR products correspond to the aberrant (268 nucleotides) and the correct (142 nucleotides) mRNA respectively. Proportion of band intensity was calculated densitometrically with ImageJ software (<http://rsb.info.nih.gov/ij/>)

Statistical analyses

All results are means $\pm$ standard deviation for at least two independent experiments performed in triplicate. Statistics were calculated using ANOVA, with Bonferroni's multiple comparison tests for post hoc analyses ($***P < 0,001$, $**P < 0.01$; $*P < 0.05$).

Results

Design of splice switching oligonucleotides

To assess the potency of SSOs with LNA monomers we designed a PS LNA/2'-OMe RNA mixmer with 33% LNA monomers (LNA1), positioned to achieve structural saturation [17], for targeting the aberrant β -globin splice site with LNA monomers (Table 1). In resemblance with previous splice switching experiments utilizing PNA [22, 23], we used a control sequence for LNA1(LNAinv1) holding four mismatches to target pre-mRNA (Table 1). The interval between LNA residues in LNA1 (position 1, 4, 7, 11, 15 and 18) was not changed for LNAinv1 (Table 1). Splice switching sequences consisting of only PS 2'-OMe RNA monomers and only PNA monomers were included in the study together with its corresponding control ONs holding four mismatches (Table 1). Unmodified PNA is uncharged and therefore not suitable for transfection with cationic lipids, Lipofectamine 2000 [27]. Consequently, disulfide conjugates of PNA and the cell-penetrating peptide M918 [22] were used instead (Table 1).

In addition, we designed two analogs to LNA1 with 50% LNA monomers where every second position is an LNA monomer. One analog is a PS LNA/2'-OMe RNA mixmer (LNA2-2OMe) designed to investigate splice switching efficiency when increasing the proportion of LNA monomers from 33% to 50% (Table 1). The other analog is a PS LNA/DNA mixmer (LNA2-DNA) designed to investigate whether the splice switching efficiency for PS LNA/DNA mixmers differs from PS LNA/2'-OMe RNA mixmers (Table 1). A phosphodiester DNA enantiomer [28], known as spiegelmer or L-DNA, and an RNase H recruiting PS DNA without any mismatches were also included in the study to investigate whether these modified ONs can induce splice switching (Table 1).

Splice switching activity

Splice switching activity increases when introducing LNA monomers to a PS 2'-OMe RNA SSO, demonstrated by elevated levels of correctly spliced luciferase in cells treated with PS LNA/2'-OMe RNA mixmer compared to cells treated with PS 2'-OMe RNA SSO (Table 1, Figure 2). This result is in accordance with the well characterized high binding affinity for LNA residues [6]. Concomitantly, the SSOs with 50% LNA monomers (LNA2-2OMe and LNA2-DNA) are more effective in the splice switching assay than LNA1 which hold 33% LNA monomers (Table 1, Figure 2). The high splice switching activity for SSOs with LNA monomers is appealing since efficiency at low concentrations is an appreciable characteristic for therapeutic purposes [29]. The PS LNA/2'-OMe RNA mixmers with 33% LNA monomers actually induce higher luciferase activity at 50 nM than the corresponding PS 2'-OMe RNA sequence induces at 100 nM (Figure 3).

Furthermore, there is no significant difference for phosphorothioate SSOs consisting of 50% LNA monomers complemented with 2'-OMe RNA or DNA (Figure 2). Both enantiomeric L-DNA and RNase H recruiting PS DNA show very low splice switching activity (Figure 2).

The PS LNA/2'-OMe RNA control sequence (LNAinv1) with four mismatches including one LNA mismatch, generates almost similar effect as the correct sequence, LNA1 (Table 1, Figure 2) and, interestingly, this behavior is persistent at all concentrations examined (Figure 3). Conversely, for PS 2'-OMe RNA the control sequence with four mismatches induces significantly lower splice switching activity than the correct sequence (Figure 2, Figure 3).

Sequences that reveal importance of LNA residues

To investigate the influence of LNA monomers for mismatch discrimination within splice switching, four additional PS LNA/2'-OMe RNA control sequences were designed. One scrambled sequence (LNAscr) and three sequences with inverted stretches causing six or eight mismatches to target luciferase pre-mRNA (LNAinv2, LNAinv3 and LNAinv4) (Table 1). The inverted stretches are chosen to introduce mismatches for either mainly LNA monomers (LNAinv3) or for mainly 2'-OMe RNA monomers (LNAinv2 and LNAinv4). In resemblance with LNA1, the control sequences consist of 33% LNA monomers and the internal distribution of LNA monomers in the sequences is kept intact, hence, position 1, 4, 7, 11, 15 and 18 are LNA monomers (Table 1).

Introduction of several mismatches for LNA monomers have considerable effect for discrimination of target pre-mRNA (Figure 4). Sequences with the same number of mismatches to target pre-mRNA but different number of mismatches for LNA monomers (LNAinv2 and LNAinv3) display significantly different splicing pattern (Table 1, Figure 4). It is obvious that splice switching activity is abolished when mismatches are introduced for mainly LNA monomers (LNAinv3) while surprisingly high activity occurs when treating cells with LNAinv2 which have mismatches at mainly 2'-OMe RNA monomers. The SSO with eight mismatches in total but only one LNA mismatch (LNAinv4) induces more than double luciferase response as compared to LNAinv3 that hold only six mismatches, of which three are LNA mismatches.

The splice switching activity for PS LNA/2'-OMe RNA mixmers with 33% LNA monomers was confirmed in RT-PCR experiments with RNA extracted from cells after 100 nM treatments (figure 4). To further unmask splice switching actions mediated by PS LNA/2'-OMe RNA mixmers, we compared cells treated with LNA1 and LNAinv1 (Table 1) to cells treated with corresponding PNA sequences conjugated to the cell-penetrating peptide M918 [22]. In our experimental setup, cells had to be treated with 5 μ M peptide-

PNA conjugate to mediate similar level of luciferase activity as cells treated with 100 nM LNA1 and Lipofectamine 2000 (Figure 5). No distinction in luciferase activity, compared to untreated cells, was observed for cells treated with 5 μ M of the conjugate holding four mismatches (data not shown). To assure that the control conjugate with four mismatches (M918-S-S-PNA_{inv}) was appropriately conveyed into cells we wanted to observe luciferase activity deviating from untreated cells. To observe such deviation 10 μ M control conjugate had to be applied to cells (Figure 5) indicating that PNA_{inv} has very low affinity to target pre-mRNA.

Oligonucleotide toxicity

SSOs constructed as PS LNA/DNA mixmers have been reported to not exhibit toxic effects *in vivo* [13, 20] and none of the ON transfections in this study had notable effect on the protein levels in cell lysate (data not shown). However, hepatotoxicity has been reported for asONs with LNA content *in vivo* [30] and SSOs with high proportion LNA might exhibit toxic side effects on cells. Therefore, wst-1 assays were carried out to determine whether PS LNA/2'-OMe RNA mixmers with 50% LNA monomers or PS 2'-OMe RNA ONs influence cellular proliferation. Data obtained from the wst-1 assay at highest employed concentration, 250 nM, show no toxicity (data not shown) and corroborates with the observed protein levels in cell lysates and previous report [7].

Discussion

Therapeutics for diseases derived from aberrant splicing can potentially be developed with SSOs as active substance. In our experiments, introduction of LNA monomers enhance splice switching efficiency for PS 2'-OMe RNA SSOs (Figure 2 and Figure 3) and increased proportion of LNA monomers confer increased splice switching activity (Figure 2). High activity at low concentrations is beneficial when developing SSOs for therapeutic applications since it implies that low doses are needed [20, 29], enabling a wider therapeutic dosing window. This opportunity for SSOs holding LNA monomers is not unambiguously an advantage since our data reveal that off-target effects for such SSOs is a substantial risk that is linked to number of LNA monomers.

It has been reported that 2'-OMe RNA bind target RNA better than DNA [31] so choice of monomers to complement with LNA in mixmers for splice switching is likely to affect the efficiency for SSOs. However, it seems that the high affinity that LNA monomers display to target RNA overrules the differences in affinity from remaining monomers in an SSO since virtually no difference in efficiency between PS LNA/2'-OMe RNA and PS LNA/DNA mixmers was observed (Figure 2).

Efficiency at low concentrations for SSOs, steric block asONs and RNase H recruiting asONs with LNA monomers has been demonstrated before [13, 20, 32, 33]. Nevertheless, the effect of different mismatches for such asONs has not been widely examined within splice switching assays [20, 34]. The initial control sequence in this study, LNAinv1, has five out of totally six LNA monomers that remain unchanged as compared to the correct sequence, LNA1, and it seems that the high affinity to target pre-mRNA displayed by the unchanged monomers outrange the effect of mismatches (Table 1, Figure 2 and Figure 3). Furthermore, two SSOs, LNAinv2 and LNAinv3 (Table 1) holding six mismatches but different number of mismatches at LNA monomers, induce significantly different splice switching activity (Figure 4). This reveals the important role that LNA monomers play for potential off-target effects within splice switching. Conversely, SSOs based on Phosphorodiamidate Morpholino Oligomers (PMO) or PNA display attractive high mismatch discrimination ([35-37] and Figure 5). These types of modified ONs are therefore conceivable alternatives to LNA mixmers. However, PMO and unmodified PNA are uncharged which affect solubility and restrain cellular uptake.

Introduction of LNA monomers in an ON stabilizes a perfectly matched duplex more than a mismatched duplex [38]. Potential toxicity due to low mismatch discrimination could be compensated by rational design of short SSOs or introduction of L-DNA or abasic nucleotides [39] as spacers to avoid non-unique regions at the target pre-mRNA. In analogy with the high efficiency but low mismatch discrimination for LNA mixmers

observed in this splice switching assay, similar effects have been observed in mRNA antisense experiments with mixmers holding LNA monomers [30, 40]. These data and our results accentuate the importance of LNA monomers for effective binding and the importance of rational sequence design to avoid off-target effects and related toxicity, even though no toxicity was observed in this study. Tuning the proportion of LNA monomers in SSOs is needed to optimize the trade-off between splice switching efficiency and risk for off-target effects.

In summary, our data suggest that splice switching activity increases when introducing LNA monomers to PS 2'-OMe RNA ONs and activity is further increased with higher proportion of LNA monomers. However, the striking information is that the LNA monomers in such mixmers give rise to low mismatch discrimination to target pre-mRNA and this fact has to be considered when utilizing the potent LNA monomers in mixmers for splice switching.

Supplementary Data

Supplementary material regarding synthesis and purification of ONs is available online.

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Figure legends

Figure 1: Reporter system for splice switching based on a plasmid carrying a luciferase-coding sequence with insertion of intron 2 from β -globin pre-mRNA containing an aberrant splice site that activates a cryptic splice site. Unless the aberrant splice site is masked by a splice switching oligonucleotide non-functional luciferase is expressed.

Figure 2: Luciferase activity after treatment with 50 nM SSO. LNA1 (33% LNA) is more efficient than 2OMe (no LNA). LNA2-2OMe and LNA2-DNA (50% LNA) are more efficient than LNA1. Control sequence, 2OMeinv (no LNA) with four mismatches, induce lower luciferase activity than the correct sequence, 2OMe. LNAinv1 induce splice correction despite four mismatches in the sequence.

Figure 3: Luciferase activity after treatment with SSO at various concentrations. The sequences with inverted stretches have 4 mismatches where LNAinv1 have one LNA mismatch. LNAinv1 prove to have similar effect as the correct sequence. LNA1 (33% LNA) has superior efficiency at 50 and 100 nM as compared to 2OMe (no LNA).

Figure 4: (a) Luciferase activity after treatment with 50 nM LNA1 (33% LNA) and corresponding control ONs. LNAinv1 has four mismatches including one LNA mismatch (4:1), LNAinv2 (6:1), LNAinv3 (6:3), LNAinv4 (8:1) and LNAscr is a randomly scrambled sequence. **(b)** RT-PCR analysis after treating cells with 100 nM splice switching oligonucleotide. PCR fragments derived from luciferase mRNA with and without splice switching are 142 and 268 base pairs respectively. Estimation of the proportion correctly spliced mRNA is shown below each lane and derives from densitometric analysis of one representative RT-PCR experiment.

Figure 5: PNA conveyed into cells with the cell penetrating peptide M918 at 5 μ M concentration achieve splice switching activity in the same range as 100nM LNA1 (33% LNA) conveyed with Lipofectamine 2000. The PNA with an inverted stretch, PNAinv, has four mismatches, analogous to LNAinv1, and display desirable specificity while LNAinv1 display similar effect as LNA1. The peptide conjugated to PNAinv had to be applied at 10 μ M to be able to monitor luciferase activity different from untreated.

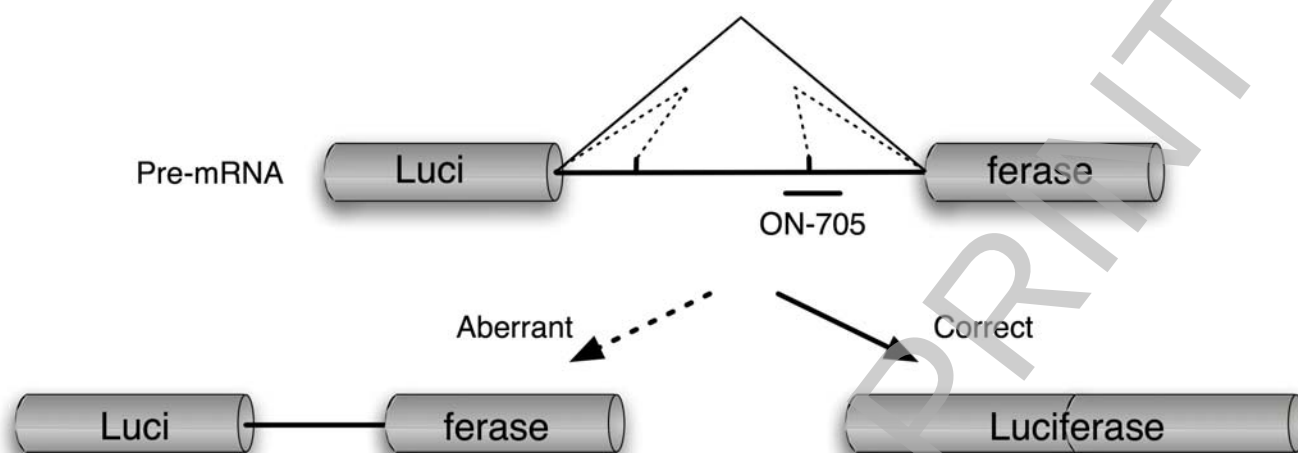


Figure 1

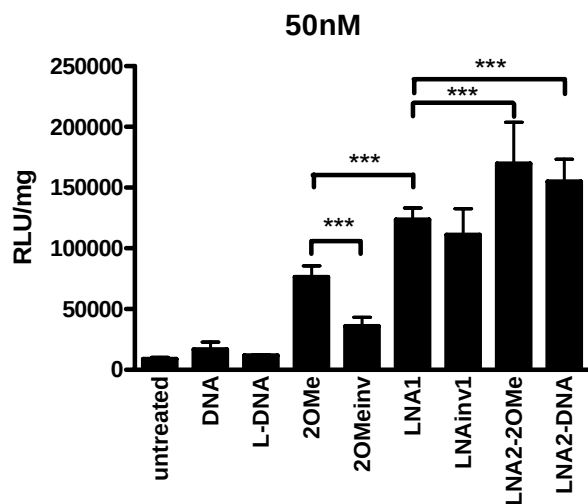


Figure 2

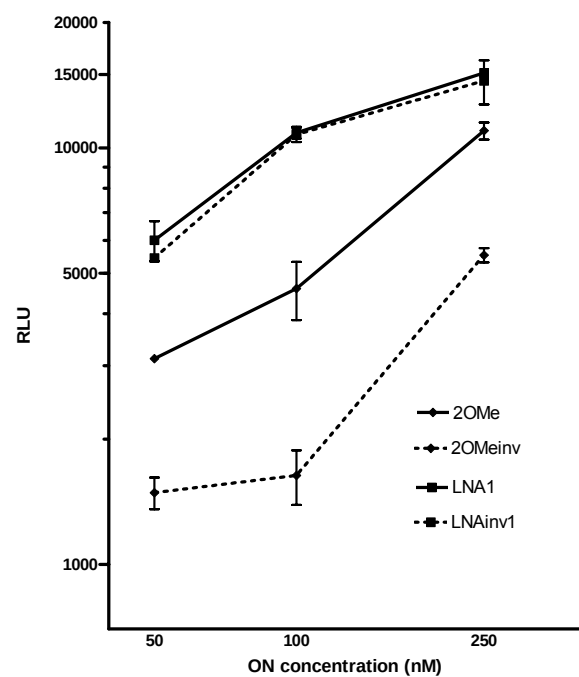


Figure 3

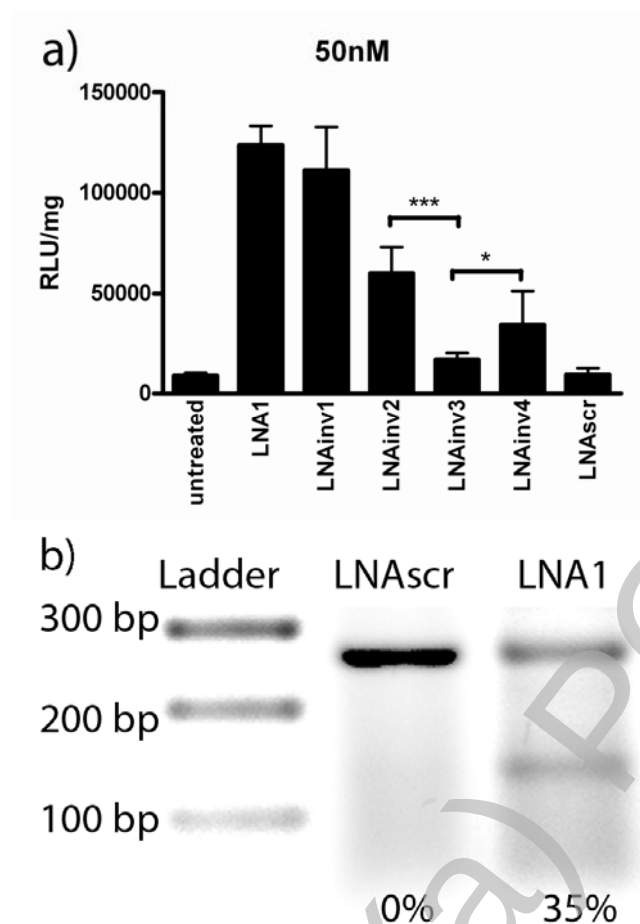


Figure 4:

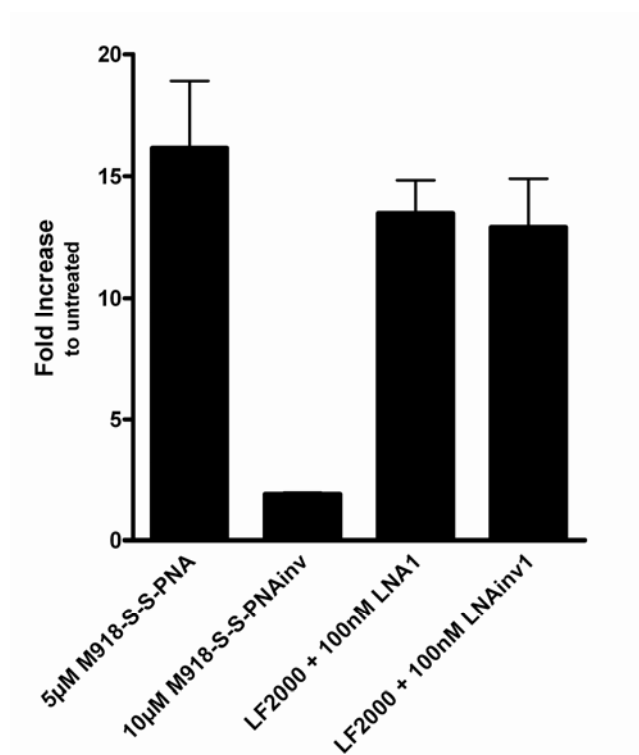


Figure 5

Table 1: Oligonucleotide and peptide sequences. Control ONs have internal inverted stretches which give rise to mismatches to target pre-mRNA. Both peptide and PNA have amidated C-terminal.

Name	Sequence	Mismatches	LNA-mismatches
2OMe	5'- CCU CUU ACC UCA GUU ACA	0	0
2OMeinv	5'- CCU CUU ACA <u>CUC</u> GUU ACA	4	0
LNA2-2OMe	5'- CcU cUt AcC tCa GtU aCa	0	0
LNA2-DNA	5'- CcU cUt AcC tCa GtU aCa	0	0
DNA	5'- CCT CTT ACC TCA GTT ACA	0	0
L-DNA	5'- CCT CTT ACC TCA GTT ACA	0	0
LNA1	5'- cCU cUU aCC UcA GUt ACa	0	0
LNAinv1	5'- cCU cUU aCA <u>CtC</u> GUt ACa	4	1
LNAinv2	5'- cCU cUU aGA <u>CtC</u> CUt ACa	6	1
LNAinv3	5'- cCU <u>aCU cCA UtC</u> GUt ACa	6	3
LNAinv4	5'- cCU cUU aGA <u>CtC</u> CUt <u>CAa</u>	8	1
LNAscr	5'-tCA gAU tCC AtC ACc UUc	14	6
PNA	C KK CCT CTT ACC TCA GTT ACA KK-NH₂	0	0
PNAinv	C KK CCT CTT ACA <u>CTC</u> GTT ACA KK-NH₂	4	0
M918	C(Npys)MVT VLF RRL RIR RAS GPP RVR V-NH₂		

LNA ONs are PS LNA/2'-OMe RNA mixmers where the LNA monomers are indicated as small letters. PS 2'-OMe RNA positions are capitals. For DNA, capitals indicate PS DNA positions and for L-DNA capitals indicate L-DNA phosphorodiester positions (spiegelmer). Inverted stretches are underlined and bold letters represent amino acids. Observe for LNAinv3, the stretch of nine inverted monomers give rise to only six mismatches.