PNA-mediated modulation and redirection of Her-2 pre-mRNA splicing: Specific skipping of *erbB-2* exon 19 coding for the ATP catalytic domain

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Abstract. The Her-2 receptor coded for by the proto-oncogenic *erbB-2* gene is a clinically validated target for treatment of a significant genetic subclass of breast cancers, and Her-2 is also overexpressed or mutated in a range of other cancers. In an approach to exploit antisense mediated splicing interference as a means of manipulating *erbB-2* expression in a therapeutically relevant fashion, we have studied the effect on mRNA splicing of a series of peptide nucleic acid (PNA) oligomers targeting specific intron-exon junctions in the *erbB-2* pre-mRNA. In particular, we are interested in identifying PNA oligomers that specifically induce skipping of exon 19 as this exon is coding for the ATP catalytic domain of Her-2, and if expressed such truncated version of the Her-2 protein should be functionally inactive in a dominant negative fashion. Therefore, antisense compounds having efficient *erbB-2* exon 19 skipping activity could be very interesting in terms of drug discovery. In the present study we identified PNA oligomers having such activity in SK-BR-3 and HeLa cancer cells in culture.

Introduction

The proto-oncogene *erbB-2* is a member of the *EGFR/erbB* family that encodes the 185,000 Da Her-2 receptor tyrosine kinase. The human *erbB-2* gene, located on chromosome 17, is amplified and/or overexpressed in breast, cervix, colon, endometrial, lung, and pancreatic cancers (1). In particular, erbB-2 is overexpressed up to 100 times in 25-30% of all breast cancer cases and it is well established that over-expression correlates with large tumor size, higher malignancy, and poor patient prognosis. Activation of the ligandless receptor occurs through heterodimerization with the other erbB receptors, for which Her-2 is the preferred co-receptor, resulting in activation of a complex signalling cascade with endpoints of cell growth regulation, division and survival (2). Additionally, activation of the highly overexpressed Her-2 receptor may occur by spontaneous formation of homodimers, which also have a high level of constitutive kinase activity (1). The Her-2 dimers are long-lived: they are recycled from endosomes to the cell membrane, in contrast to the EGFR1 dimers, which are degraded in the lysosomes (3). It was found that *erbB-2* over-expression is associated with resistance to anti-estrogen therapy and confers increased chemoresistance to cancer cells (4). In addition to overexpression and gene amplification, neu (rat homologues of human Her-2) constitutive activation can occur by point-mutation Val>Glu at amino acid 664 in the transmembrane domain (5). These facts together with a strong correlation between Her-2 over-expression and the aggressiveness of the cancer phenotype, i.e. spreading to the lymph nodes, high grade, lack of steroid hormone receptors, lower overall survival (2), makes the development of anti-Her-2 therapies of particular interest.

Over the past decade a wide variety of drugs targeting Her-2 function have been discovered. The strategies of anti-Her-2 drug design includes immunological approaches, development of kinase inhibitors, as well as gene therapy (2). The most promising anti-Her-2 therapy to date is based on the recombinant humanized antibody Trastuzumab (Herceptin®) targeted against the extracellular domain (ECD) of Her-2. While treatment with Herceptin improves overall survival in early stages of Her-2 overexpressing breast cancer (6), more than 5% of the patients receiving Herceptin monotherapy develop cardiotoxicity (7,8), and the cardio-toxicity rate is increased when Herceptin is used in combination with chemo-therapy (9). Furthermore, not all cancers exhibiting Her-2 overexpression are responsive to Herceptin therapy (10). While this treatment is effective for breast cancers, patients with non-small cell lung cancers (NSCLC) for instance generally do not respond to this therapy (11). Moreover, the ECD of the Her-2 protein can be shed from the cell surface by proteolytic cleavage and/or via alternatively initiated translation (12) and this may neutralize the therapeutic anti-bodies before reaching the tumor cells (13). In addition, an N-truncated membrane-associated p95Her-2 fragment possesses kinase activity *in vitro* (14). The pattern of expression of this isoform is different from that of the full-length Her-2 protein: the shorter isoform is especially

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prevalent in nodal tissues and in primary tumors from patients with node-positive breast cancers (15), delineating a group of patients with shorter overall survival (16). Since p95Her-2 expressing tumors are resistant to Herceptin (6) and less sensitive to hormonal and chemotherapy (16), alternative Her-2 targeting approaches are warranted.

Another strategy is aimed at targeting the Her-2 intracellular domain to interfere with kinase activity. The discovery of small-molecule tyrosine kinase inhibitors (TKIs) targeted selectively to the ATP-binding site of Her-2 was complicated by cross reactivity with a range of other kinases (1,17). However, this kind of ATP competitive inhibitors (reversible and irreversible) have been discovered for both EGFR1 and Her-2, and their efficiency are currently being evaluated in clinical trials [e.g. CI-1033, irreversible pan-erbB inhibitor, and lapatinib/Tykerb®, inhibitor of both erbB-1 and erbB-2) (18-20) or already approved for treatment of NSCLC patients (gefitinib/Iressa® and erlotinib/Tarceva® (21)]. The latter TKIs were found to be well tolerated and to exhibit high response rates as a single agent in phase I and II. However, in combination with standard chemotherapy the addition of these TKIs did not improve survival compared to standard chemotherapy alone (22). The objective tumor response to gefitinib was in the range of 9-19% (22), but it is interesting to note that the treatment by gefitinib gave higher response in a small group of patients, who had accumulated mutations in EGFR exons 18-24 encoding the receptor kinase domain (21,23,24). While the strategies described above are aimed at inhibiting the function of already expressed protein, inhibition and/or modulation of the expression of the protein at the mRNA (translational/splicing) level offers potential advantages in terms of efficient blocking of Her-2 synthesis.

Several studies have reported antisense targeting towards the translation start codon or the 3'-untranslated region of the Her-2 mRNA (25). For instance, phosphorothioate antisense oligonucleotides targeted to the translation initiation site of Her-2 mRNA (15-mer targeted to the initiation codon) was shown to suppress the expression of Her-2 oncoprotein (26,27) as well as to inhibit cell proliferation, and activate apoptotic processes.

Many cancer-related proteins are synthesized as a result of alternative splicing (28). The splicing of such proteins may be modified by antisense oligonucleotides (29,30) thereby shifting the balance between splice isoforms which may result, for example, in augmentation of the sensitivity to chemotherapeutic treatment (31,32). There are several examples of disease-related proteins, for which splicing of pre-mRNA can be shifted by antisense oligonucleotides (tau, c-myc, IL-5r, bcl-x, dystrophin, ß-globin) (25,33-35) to achieve a therapeutically beneficial outcome. Only antisense agents that do not activate RNaseH are efficient in splicing interference, and to have therapeutic potential they must possess high biostability, high target affinity and specificity, and good *in vivo* bioavailability (34).

PNA is a non-charged DNA mimic that hybridizes to complementary DNA or RNA sequence with high affinity and sequence specificity (36). PNA possesses many desirable features as a splicing modulator: it is stable in serum, the PNA/RNA complex is RnaseH resistant, and it interferes effectively with splicing *in vivo* (37). Furthermore, recent progress in improving cellular uptake and bioavailability of PNA through backbone modification and chemical (peptide) conjugation (38-42) is facilitating cellular as well as *in vivo* studies (37).

In the present study we explored the possible PNA oligomers that could redirect and/or inhibit the splicing of Her-2 pre-mRNA in two cancer cell lines (HeLa and SK-BR-3), in order to identify lead targets for new (PNA derived) RNA interference anticancer drugs.

Materials and methods

PNA synthesis and modifications. PNA synthesis, HPLC purification and MALDI-TOF mass spectrometry were performed as reported previously (43). The PNAs were lyophilized and stored at 4˚C until used. Stock solutions were made in water and stored at -20˚C. N-(9-aminoaciridinyl)-6-aminohexanoic acid (Acr) was linked to PNA on solid support at the N-terminal of the PNA through the eg1 (8-amino-3,6-dioxaoctanoyl) linker (Acr-PNA). Fluorescein (Fl)-labeled PNA oligomers were prepared using a previously described 'Fl-lysine' monomer (44).

Thermal denaturation. Measurements were performed on a Cary 300 Bio UV-visible spectrophotometer (Varian, Cary, NC, USA) connected to a temperature controller. Thermal melting profiles were obtained in 10 mM Na-phosphate (pH 7.0) containing 0.1 mM EDTA and 100 mM NaCl using heating-cooling cycles in the range of 5-95˚C at a rate of 0.5˚C/min. The melting temperature (Tm) was determined as the maximum of the first derivative of the heating curve. Cuvettes of 1.0 cm path length and 1.0 ml volume were used for all experiments.

Cell culture. The study was performed on two cell lines: cervical carcinoma HeLa cells and breast cancer SK-BR-3 cells, obtained from American Type Culture Collection (Manassas, VA). HeLa cells were cultivated in RPMI-1640 medium and SK-BR-3 cells were cultivated on McCOY's 5A modified medium (Sigma) both supplemented with 1% GlutaMAX (Gibco), heat-inactivated 10% fetal bovine serum (FBS; Sigma), and penicillin/streptomycin (50 IU/ml).

Cell transfection. HeLa and SK-BR-3 cells were plated on a 6-well plate 16-20 h before the transfection. Cells were washed by PBS and transfected by Acr-PNA in complex with Lipofectamine (LFA, Life Technologies) for 4 h in Opti-MEM (Life Technologies) medium at the final volume of 1 ml. An equal volume of the cultivation medium was added and the cells were cultivated further for a total of 24 or 72 h. The Acr-PNA/LFA complex was prepared as follows. The 0.5 mM stock solution of Acr-PNA was diluted in sterile water and the solution (100 μl) was incubated for 30 min at room temperature, mixed with 100 μl LFA in Opti-MEM and incubated for 15 min. The cells were washed in PBS once, then 800 μl Opti-MEM was added to the cells. Finally, 200 μl Acr-PNA/LFA complex was added to the cells. PNAs were used in designated concentrations and LFA was used at the final concentration of 6 μg/ml. In the control experiments with the non-PNA treated cells only LFA was added.

RT-PCR and sequencing. Twenty-four and 72 h after transfection, total RNAs of HeLa and SK-BR-3 cells were isolated using RNeasy kit (Qiagen). RNA of 360 ng/reaction (HeLa) and 30 ng/reaction (SK-BR-3) were used as templates in reverse transcription PCR reaction (10 μl), according the manufacture protocol (One Step RT-PCR kit, Qiagen). Amplification of ß-actin mRNA fragment was used as the amplification control. Primers were designed using Primer3 software (frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi): the sequences and targets of the primers are depicted in Table II. RT-PCR was programmed as follows: 55˚C for 35 min, 1 cycle; 95˚C for 15 min, 1 cycle; 94˚C for 1 min; 55˚C for 1 min; 72˚C for 0.5 min, 27 cycles. The PCR products were separated on 2% agarose gel and stained with ethidium bromide. The gel image was acquired, using the ImageMaster (Pharmacia Biotech).

For sequencing analysis, the bands of interest were cut out from the gel and eluted with QIAquick Gel Extraction kit (Qiagen). The isolated product of PCR was sequenced directly (MWGAG Biotech) with reverse primer from the primer set L (Table II), or the isolated fragment was TA cloned in pCRII-TOPO plasmid according to the manufacture protocol (Invitrogen). The sequencing was carried out with one of the M13 reverse and M13 forward (-20) primers by ABI PRISM BigDye Terminator v3.1 Cycle Sequensing kit (Applied Biosystems) and analysed on ABI 3100 Sequencer (Applied Biosystems).

Western blotting. HeLa cells were lysed in lysis buffer: 1% SDS, 10 mM Tris pH 7.2, with addition of protease inhibitor cocktail (Roche Diagnostics); then boiled for 10 min, and the protein concentration was measured using the BCA Protein Assay (Pierce). Forty micrograms of the total protein was separated on 7.5% polyacrylamide gel and transferred to a PVDF membrane. The non-specific binding was blocked by incubation the membrane in PBS with 2% of non-fat dried milk overnight. Rabbit polyclonal antibodies Neu (C-18) (Santa Cruz Biotechnology, Santa Cruz, CA) were used for the detection of Her-2 protein. Mouse monoclonal anti-ß-actin antibodies clone AC-15 (Sigma) was used for the detection of ß-actin. Rabbit anti-mouse-HRP and swine anti-rabbit-HRP conjugated antibodies (Dako) were used as the secondary antibodies. The signal was detected by chemiluminescence assay (ECL-Plus, Amersham Biosciences UK Limited, England). Prestained Protein Ladder (Fermentas) was used as a marker.

Confocal microscopy. HeLa and SK-BR-3 cells were plated into 4-well Lab-Tek chambered coverglass (Nunc) the day before transfection. The culture medium was discarded, cells were washed in PBS once, and Acr-Fl-PNA2542/LFA complex was added with a final PNA concentration 200 nM. In the co-localisation experiment, cells were co-transfected with 20 μg/ml tranferrin [Tetramethylrhodamine conjugate (T2872), Molecular Probes]. Six and 24 h after the transfection started, live (non-fixed) cells were analysed using MultyProbe 2001 Laser Scanning Confocal System equipped with the argon laser and the red laser diode; excitation wavelengths 488 and 638 nm, correspondingly (Radiance 2000, BioRad). The system was connected to a Nikon Eclipse TE200 microscope (oil immersion 60X1.4 NA objective Nikon, Tokyo, Japan). Lasersharp 200 software package (BioRad) was used for the image acquisition and processing.

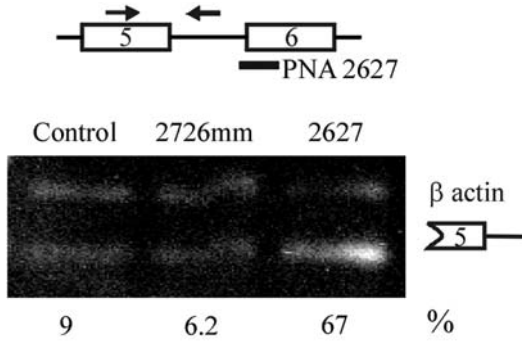

Figure 1. The effect of Acr-PNA on Her-2 mRNA splicing. HeLa cells were transfected with 1 μM of Acr-PNA 2627 and 4-mismatch Acr-PNA 2726mm, targeted to 5'ss of exon 6. Total RNA was isolated 24 h after transfection and subjected to RT-PCR with primer set E (arrows, Table II) located upstream of the PNA target site to avoid the possible inhibition of reverse transcriptase (51). Accumulation of the unspliced RNA appeared upon treatment with full-matched Acr-PNA 2627 but not with mismatched Acr-PNA 2726mm and not with LFA treatment alone (control). ß-actin fragment was amplified as an internal control with primer set K (Table II). The values under the gel image indicate the relative amount of unspliced RNA. In this and subsequent figures the numbered boxes are exons, lines are introns, arrows are primer positions.

Results

Splicing inhibition by PNA antisense. Among the possible strategies for cellular delivery of PNA we chose to use a simple modification by 9-aminoacridine (Acr-PNA) that allows lipofectamine mediated delivery (45). Consequently, a set of acridine modified 15-mer PNAs targeted to intron-exon junction sites of Her-2 pre-mRNA were synthesized (Table I) and their antisense activity on mRNA splicing was studied by RT-PCR. HeLa cells were transfected with Acr-PNA/LFA complex for 4 h in Opti-MEM medium (45) and incubated further for 24 h, after addition of an equal volume of growth medium (10% serum). Twenty-four hours after the transfection, total RNA was extracted and used as template for the semi-quantitative RT-PCR assay. Several of the PNAs, (2623, 2624, 2625, 2627 and 2538) that were targeted to 5' splice sites of different exons (Table I), showed splicing inhibitory activity at 3.2 μM as detected by the appearance of a PCR product containing an exon-intron splice junction, whereas mismatch controls showed no such effect. For example, as illustrated in Fig. 1, the full-matched Acr-PNA 2627 sequence specifically inhibits splicing at concentration 1 μM, and results in accumulation of the unspliced fragment, whereas the corresponding 4-bases mismatch Acr-PNA 2726mm showed no change in relative abundance of the unspliced form.

Of the PNAs targeted to 3' splice sites (ss) of the exons (Table I), only two, Acr-PNA 2424 (exon 17) and Acr-PNA 2696smm (exon 19), exhibited splicing inhibitory activity, as illustrated in Fig. 2. The remaining PNAs (Table I) appeared ineffective in terms of splicing inhibition: 2626 (5'ss exon 5), 2527 (3'ss exon 8) and 2540 (3'ss exon 18, data not shown).

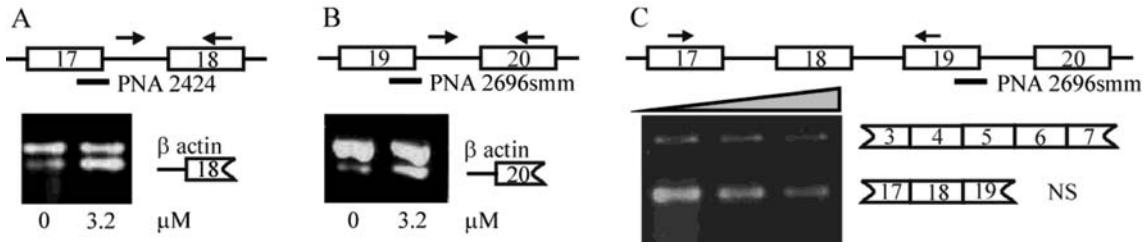

Figure 2. Splicing inhibition of Her-2 pre-mRNA induced in HeLa cells by Acr-PNAs targeted to the 3'ss of two exons. (A) Treatment with Acr-PNA 2424 targeted to the 3'ss of exon 17 results in the accumulation of the unspliced RNA. (B) Treatment with Acr-PNA 2696smm targeted to the 3'ss of exon 19 results in the accumulation of unspliced RNA. At the same time, the amount of normal spliced (NS) mRNA (exons 17-19) was reduced in a concentration dependent way (C). ß-actin (panels A and B) or Her-2 upstream mRNA fragment (exons 3-7, panel C) were amplified as internal controls.

Table I. Number, sequences and target position of the PNAs at the Her-2 mRNA (NM_004448).

PNA no. (Acr-PNA)	Target position		PNA sequence	PNA effect
5'ss targeted PNA				
2623	Exon 2	5'ss	Acr-eg1-GCCGGTGCACACTGG-Lys-NH2	x
2725mm	Exon 2	5'ss	Acr-eg1-GCC**C**GTG**G**ACA**TC**GG-Lys-NH2	-
2624	Exon 3	5'ss	Acr-eg1-TCCTGGATATCCTGG-Lys-NH2	x
2724mm	Exon 3	5'ss	Acr-eg1-TC**T**TGG**TA**ATCC**C**GG-Lys-NH2	-
2625	Exon 4	5'ss	Acr-eg1-TTTCAAGATCTCTGT-Lys-NH2	x
2626	Exon 5	5'ss	Acr-eg1-TGGCCTGAGGACAGA-Lys-NH2	-
2627	Exon 6	5'ss	Acr-eg1-AGTGCGCGTCACTGT-Lys-NH2	x
2726mm	Exon 6	5'ss	Acr-eg1-AGT**T**CGC**TG**CAC**G**GT-Lys-NH2	-
2538	Exon 9	5'ss	Acr-eg1-ACCATAGCACACTGA-Lys-NH2	x
2701	Exon 19	5'ss	Acr-eg1-CAGATGCCCTGGGCA-Lys-NH2	+
2707	Exon 19	5'ss	Acr-eg1-ATCCAGATGCCCTGG-Lys-NH2	+
2794	Exon 19	5'ss	Acr-eg1-TCCAGATGCCCTGGG-Lys-NH2	+
2727mm	Exon 19	5'ss	Acr-eg1-TC**G**AGA**GT**CCCT**C**GG-Lys-NH2	-
2541smm	Exon 19	5'ss	Acr-eg1-TCCAGATGCC**T**TGGG-Lys-NH2	+
2834smm	Exon 19	5'ss	Acr-eg1-TCCAGATGCC**A**TGGG-Lys-NH2	+
2835smm	Exon 19	5'ss	Acr-eg1-TCCAGATGCC**G**TGGG-Lys-NH2	+
3'ss targeted PNA				
2527	Exon 8	3'ss	Acr-eg1-GTGGGTACCTCGGGC-Lys-NH2	-
2424	Exon 17	3'ss	Acr-eg1-TCACCTCCGTTTCCT-Lys-NH2	x
2540	Exon 18	3'ss	Acr-eg1-CTGACCTTGTAGACT-Lys-NH2	-
2780	Exon 19	3'ss	Acr-eg1-GGCTTACGTCTAAGA-Lys-NH2	+
2781mm	Exon 19	3'ss	Acr-eg1-GG**G**TT**CA**GTCTAACA-Lys-NH2	-
2696smm	Exon 19	3'ss	Acr-eg1-GGGCTT-CGTCTAAGA-NH2	x
Fl-2542	Exon 8	3'ss	Acr-eg1-Fl-GTGGGTACCTCGGGC-Lys-NH2	-

Mismatch (mm) and single mismatch (smm) PNAs listed, where mismatched nucleotides are indicated in bold letters. ss, splicing site; Acr, 9-aminoacridine; eg1, 8-amino-3,6-dioxaoctanoic acid; Fl, fluorescein; (x), splicing inhibition; (+), splicing redirection; (-), no effect on splicing; parts of PNAs targeting introns are underlined.

Splicing redirection by PNA antisense. The Her-2 kinase domain is encoded by exons 18-24 and is highly conserved within the ErbB family. The DNA sequence that encodes the Her-2 kinase domain harbors several somatic mutations (missense, in-frame deletions and duplication/insertions) in gastric, lung, breast and colorectal cancers. The majority of these mutations are located in exons 19 and 20 (46-49) and may constitutively activate the kinase domain (50). For example, frequently mutated sites in exon 19 (amino acids 737-769) are L755S (transition mutation of T-C at nt 2264), and D769H (2305G>C). The latter mutation is located in the middle of an α-helix of the Her-2 kinase domain, and is

Table II. The sequences, target positions and product length of primers used in the study.

Primer set	Primer name	Sequence	Product size	PNAs studied
Her-2 primers				
A	Ex1	5'-CTCCTCCTCGCCCTCTTG-3'	342 bp	2623, 2725mm
	Int1	5'-CTTTGCGGGAAAACTTTCTG-3'		
B	Ex2	5'-GGGAAACCTGGAACTCACCT-3'	386 bp	2624, 2724mm
	Int2	5'-CTCACCAGCCCTTTCTTCTG-3'		
C	Ex3	5'-CCCAGCTCTTTGAGGACAAC-3'	490 bp	2625
	Int3	5'-ACTGCAGCGTGACTGAAATG-3'		
D	Ex4	5'-AACCAGCTGGCTCTCACACT-3'	130 bp	2626
	Int4	5'-GCAGGCACTGGGTTGTAAGT-3'		
E	Ex5	5'-TGGGGAGAGAGTTCTGAGGA-3'	188 bp	2627, 2726mm
	Int5	5'-AGACCCTCATCAGCATCACC-3'		
F	Ex8	5'-GGTGTGAGAAGTGCAGCAAG-3'	171 bp	2538
	Int8	5'-GGGCCAGGGGTAGAGAGTAG-3'		
G	Int17	5'-GTGAGGCGGGGTGAAGTC-3'	171 bp	2424
	Ex18	5'-GCACCTTCACCTTCCTCAGC-3'		
H	Ex17	5'-GATCCGGAAGTACACGATGC-3'	393 bp,	2794, 2727mm, 2541smm,
	Ex20	5'-TCCCGGACATGGTCTAAGAG-3'	294 bp[a]	2834smm, 2835smm
I	Int19	5'-GCTGTGGTTTGTGATGGTTG-3'	213 bp	2780, 2781mm
	Ex20	5'-TCCCGGACATGGTCTAAGAG-3'		2696smm
Her-2 internal control				
J	Ex3	5'-CCCAGCTCTTTGAGGACAAC-3'	479 bp	2696smm, 2794, 2780, 2727mm, 2781mm,
	Ex7	5'-ACAGATGCCACTGTGGTTGA-3'		2541smm, 2834smm, 2835smm, 2540
Primers used in semi-nested PCR and sequencing				
L	Int18	5'-CTACAAGGTCAGGGCCAGGT-3'	1200 bp	
	Ex20	5'-TCCCGGACATGGTCTAAGAG-3'		
M	Int18	5'-CTACAAGGTCAGGGCCAGGT-3'	331 bp	
	Ex19	5'-GGGATGTGTTTTCCCTCAAC-3'		
Actin primers				
K	ActF	5'-CCCTGGAGAAGAGCTACGAG-3'	257 bp	2424, 2540, 2541, 2627,
	ActR	5'-CAGGGCAGTGATCTCCTTCT-3'		2726mm, 2623, 2725mm, 2624, 2724mm, 2625, 2626, 2527, 2538

[a]Length of abberant exon 19 skipped fragment.

important for ATP binding (50). These mutations are speculated to contribute to the development of human cancers (46,50) and therefore they may be potential targets for antisense therapy.

Therefore, we investigated whether skipping exon 19 could be achieved, using splicing interference by targeting the 5'-exon-intron junction with Acr-PNA 2794. The data presented in Fig. 3A show that transfection of HeLa cells with this PNA (but not a mismatch control Acr-PNA 2727mm) results in a reduction in the amount of normally spliced mRNA and a concomitant appearance of a shorter fragment (294 bp, primer set H); the length of which corresponds to the exon 19 skipped fragment of mRNA. To confirm this interpretation, we isolated this fragment from the gel and determined the

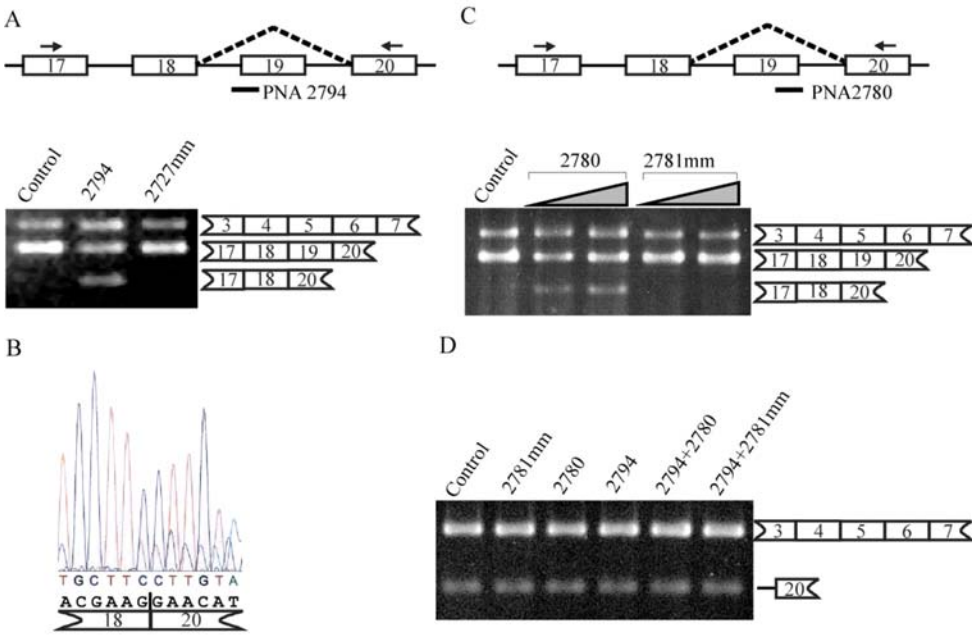

Figure 3. Redirection of Her-2 pre-mRNA splicing by antisense PNA in HeLa cells. RT-PCR analysis of Her-2 mRNA. (A) The full-matched Acr-PNA 2794 (4 μM) targeted to the exon 19 5'ss site induces the synthesis of aberrant mRNA without exon 19 (dotted line on the scheme). At the same time, the amount of the normally spliced mRNA form is reduced. Treatment with LFA alone or Acr-PNA 2727mm that contains four mismatches have no effect on splicing. (B) Sequence analysis of the aberrant fragment confirms the exclusion of exon 19. (C) Targeting of 3'ss of exon 19 with Acr-PNA 2780 also induces exon skipping in a concentration-dependent manner (1.6 and 3.2 μM), whereas its mismatch control Acr-PNA 2781mm does not have such an effect. (D) Targeting of 5'ss and 3'ss sites of exon 19 with 4 μM PNAs 2794 and 2780 alone or in combination do not change the level of unspliced Her-2 RNA. The position of primer set I is shown on Fig. 2B. Note that primers were positioned downstream of the PNA target site; thus PNAs do not interfere with the formation of the PCR product (51). The amplification of upstream Her-2 mRNA fragment from exon 3 to exon 7 is an internal control.

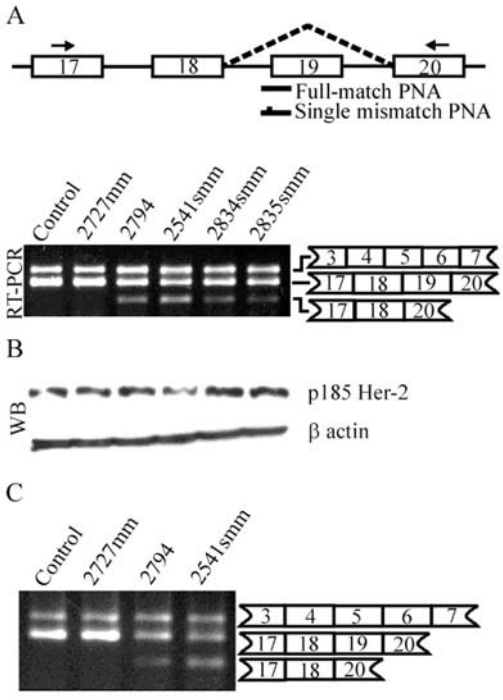

Figure 4. The effect of substituting a single base in the PNA sequence on Her-2 pre-mRNA splicing. The PNAs (4 μM) targeted to the 5'ss of exon 19 induce skipping of the exon during splicing (dashed line on the scheme indicates aberrant splicing). HeLa cells treated with LFA only (control) or with 4 bases mismatch Acr-PNA 2727mm were used as a control. Seventy-two hours after transfection RT-PCR (A) and Western blotting (WB) (B) were performed. (C) The single mismatch Acr-PNA 2541smm is a stronger splicing modulator than its full match analog Acr-PNA 2794 in HeLa cells. Amplification of the upstream fragment of Her-2 mRNA (exons 3-7) used as an internal control.

Table III. The PNAs and DNA sequences and the melting temperature (Tm) of the complexes of DNA 2794 with each PNA.

PNA no.	Sequence	Tm (°C)
2794	TCC AGA TGC CCT GGG	81
2541smm	TCC AGA TGC C**T**T GGG	72
2834smm	TCC AGA TGC C**A**T GGG	74
2835smm	TCC AGA TGC C**G**T GGG	77
DNA		
2794	CCC AGG GCA TCT GGA	

The positions of the mismatch nucleotides are bold and underlined. smm, single mismatch PNA.

sequence both by direct sequencing of the isolated PCR fragment and by cloning. The result of both methods confirms that the shorter fragment is indeed the exon 19 skipped form of the mRNA (Fig. 3B). The splicing redirection by Acr-PNA 2794 was also observed in the SK-BR-3 cell line (Fig. 5A). Shifting the PNA target two bases upstream (Acr-PNA 2701) or one base downstream (Acr-PNA 2707) from the position of Acr-PNA 2794 decreases the PNA splicing redirection activity to less then 60% (data not shown).

Similarly, targeting of the 3' exon-intron junction by Acr-PNA 2780 also resulted in a (smaller) reduction of the amount of normal spliced mRNA and accumulation of the exon 19

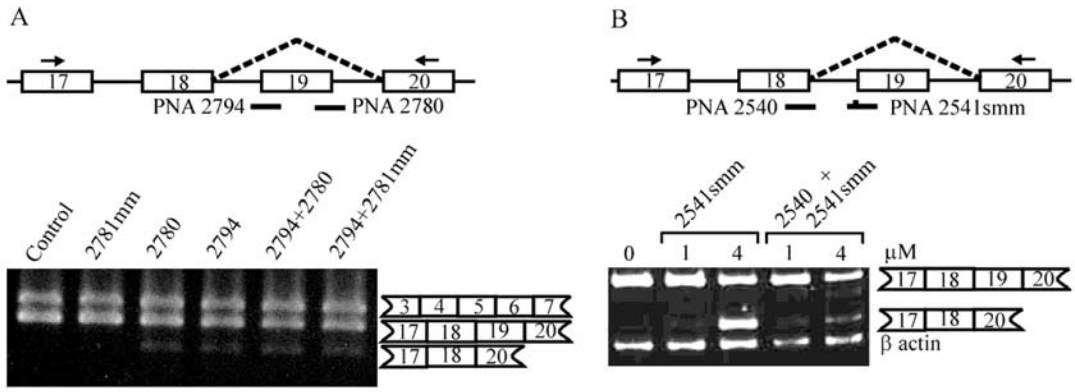

Figure 5. Co-targeting of 5' and 3' sites of exon 19 with both Acr-PNA 2794 (4 μM) and Acr-PNA 2780 (4 μM) in SK-BR-3 cells do not modify the exon skipping effect induced by Acr-PNA 2794 alone (A). Whereas upstream co-targeting of both splice sites of intron 18 with Acr-PNA 2541smm and Acr-PNA 2540 reduces the skipping effect induced by Acr-PNA 2541smm alone (B). Her-2 upstream mRNA (exons 3-7, panel A) or ß-actin (B) fragments were amplified as internal controls.

skipped fragment in a concentration dependent manner (Fig. 3C). This effect is sequence specific, since mismatch control Acr-PNA 2781mm does not affect the splicing (Fig. 3C). However, neither for Acr-PNA 2780 nor Acr-PNA 2794 accumulation of the unspliced form was detected in HeLa (Fig. 3D) or in SK-BR-3 cells (data not shown).

Effect of substitution of one nucleotide in PNA sequence. To evaluate the specificity of PNA 2794 in more detail, the antisense activity of PNAs with a single mismatch were compared to that of the fully matched PNA. Towards this aim, three new Acr-PNAs were synthesized where one of the cytosines in the Acr-PNA 2794, corresponding to the last intron 18 guanine was substituted with thymine, adenine or guanine, resulting in single mismatched PNAs: 2541 (C>T), 2834 (C>A) and 2835 (C>G) (Table I). We anticipated a reduction in activity of the single mismatch PNAs (PNA smm) reflecting a reduction in binding affinity to the target as indicated by Tm measurements (Table III). However, most surprisingly, the results showed that the single mismatched Acr-PNA 2541smm is the most effective among the four PNAs in terms of exon 19 skipping activity both in HeLa (Fig. 4) and in SK-BR-3 cells (data not shown). In light of this surprising result, we verified the intron 18 - exon 19 junction sequence in both HeLa and SK-BR-3 cell lines by semi-nested PCR (Table II), followed by sequencing, and the obtained sequence corresponds to the GenBank accession no. NT_010755 (gene 'ERBB2', nt: 1568689..1609211).

To further evaluate the activity of parent and single mismatch PNAs targeted to the 5' splice site of exon 19, Her-2 protein level was measured by Western blot analysis using the HeLa cell protein extracts collected 72 h after transfection. The results indicate a slight inhibition of Her-2 protein expression by Acr-PNA 2541smm, whereas the parent, mismatched and two other single mismatch PNAs have no detectable effect on Her-2 protein level (Fig. 4B). Moreover, no shorter protein isoform(s) could be detected. However, it is noteworthy that the splicing redirection effect remains 72 h after transfection (Fig. 4A).

In light of the above surprising results, we also tested a one-base deletion Acr-PNA 2696smm targeted to the 3'ss of exon 19. This PNA inhibits the splicing, which was confirmed by a reduction in the amount of normal spliced mRNA (Fig. 2C). In contrast to its full matched analog Acr-PNA 2780, the single base deletion Acr-PNA 2696smm does not redirect the splicing machinery, but resulted in accumulation of the unspliced fragment (Fig. 2B).

Co-targeting. Since the targeting of 5' exon 19 junction induced splicing redirection we next investigated whether co-targeting of adjacent splice junctions would modify this effect. Co-targeting of the 5'ss and the 3'ss of exon 19 did not differ from the exon skipping effect induced by Acr-PNA 2794 alone (Fig. 5A). On the contrary, co-targeting of the 5' exon 19 junction with the 3' exon 18 junction virtually abolished exon 19 skipping by Acr-PNA 2541smm (Fig. 5B). However, a reduction of normally spliced mRNA was observed (Fig. 5B, lane 5).

Cellular delivery. Although cationic lipid-assisted delivery of Acr-PNAs results in measurable effects on Her-2 mRNA splicing; micromolar concentrations of PNA are required which are significantly higher than warranted for drug discovery. This low cellular potency could be a consequence of low splicing modulation activity inherent to PNA molecules or could be due to a suboptimal delivery strategy. Thus, to study the cellular uptake and intracellular distribution of the anti-Her-2 PNAs, we used a fluorescein labeled PNA, Acr-Fl-PNA 2542 (Table I) for confocal fluorescence microscopy analysis. The results (Fig. 6) show that the Acr-Fl-PNA is readily taken up by the majority of both HeLa and SK-BR-3 cells. Green punctate fluorescence in the cytoplasm as well as on the cell membrane was observed (Fig. 6, left panel); however, only very weak if any signal was detected in the nuclei of the HeLa or in the SK-BR-3 cells. The transfection was carried out using of 200 nM and 1 μM concentrations in both cell lines, and the uptake was found to be proportional to the PNA concentration (data not shown).

To examine whether the uptake of Acr-Fl-PNA was a result of endocytosis, cells were co-transfected with the classical endocytotic marker transferrin (Fig. 6, central panel). The superimposed images of both cell types clearly show significant but not complete overlap (yellow) between the Acr-Fl-PNA green spots and endocytotic red vesicles defined

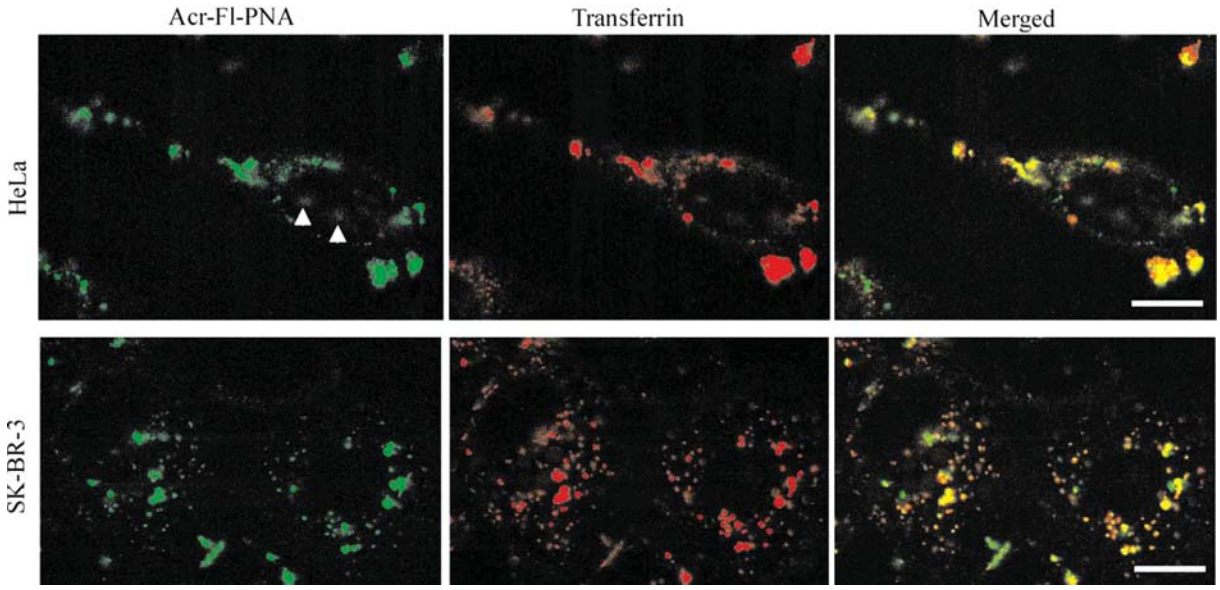

Figure 6. Confocal laser scanning microscopy images of unfixed HeLa and SK-BR-3 cells. Cells were transfected with 200 nM of Acr-Fl-PNA2542/LFA complex and transferrin 20 μg at 37˚C. Six hours (HeLa) and 24 h (SK-BR-3) after transfection living (non-fixed) cells were washed and viewed by confocal microscopy. Left panel: green fluorescence images of cells showing the internalization of Acr-Fl-PNA. Central panel: red fluorescence images of the same cells stained with transferrin. Right panel: superimposed images of green and red fluorescent images, indicating the co-localisation of Acr-Fl-PNA with transferrin (yellow spots). The weak signal in the HeLa cell nuclei (arrows) is a bleed-through of the upper level signals. Bar, 10 μm.

by transferrin (Fig. 6, right panel). Thus, most of the Acr-Fl-PNA share the endosomal localization with transferrin and are therefore not available for RNA interference in the nucleus (or in the cytoplasm).

Discussion

Many studies have demonstrated that intron-exon junction sites are very sensitive to interference by antisense PNA (45,52). In line with this general contention we have now identified several antisense PNA oligomers targeted to intron-exon junctions of Her-2 pre-mRNA that specifically interfere with correct splicing of this mRNA. In particular, we found that PNA oligomers targeted to the 5' intron-exon junction of exon 19 induces skipping of exon 19, which encodes the ATP catalytic domain of Her-2 and also harbors many Her-2 cancer related mutations.

The precise molecular mechanism for exon skipping induced by these PNAs is not known, but it is likely simple steric hindrance by PNA bound to RNA that will obstruct proper recognition of the 5' splice site by the spliceosome. Because the splicing machinery is flexible (32), obstruction of the 5' site could redirect the spliceosome to the next available splice site, namely, the 5' splice site of exon 20.
Recently it was shown that exon skipping can be induced not only by targeting the splice site, but also by targeting an exonic splicing enhancer (ESE) motif (35). These splicing regulatory sequences are facilitating the exon recognition by the spliceosome through the binding of serine-arginine rich proteins (SR proteins), and blocking these ESEs by antisense molecule may provoke exon skipping (53). Employing ESE predictive software (RESCUE-ESE, 54), we found that PNA 2794 partially overlaps exon 19 predicted ESE (data not shown). Thus, the effective splicing redirection by this antisense PNA may be a consequences of both blocking the splice acceptor site and interfering with the ESE motif. The fact that not all PNAs that target intron-exon junction sites of Her-2 pre-mRNA result in the appearance of the exon skipping form or affect the splicing, suggests that not all splicing sites that were targeted are equally accessible for the antisense PNAs.

Exon 19 consists of 99 nt and encodes 33 amino acids in-frame fragment (http://www.ensembl.org), which corresponds to a part of an α-helix (amino acids 761-775) of the Her-2 kinase domain (46). Although our RT-PCR results strongly indicate that the exon 19 missing mRNA is present, our data do not permit to conclude whether it is translated into protein. We could not detect the shorter isoform by Western blotting either due to incomplete separation between the two isoforms (the 33 amino acid truncated protein is only lighter by approximately 5 kDa), or due to insufficient amount of protein. It was previously shown that truncated proteins from antisense-induced alternatively spliced mRNA could be translated (55,56), therefore we do not exclude the possibility that such truncated Her-2 protein is indeed synthesized upon PNA treatment. Most interestingly from a therapeutic point of view, such truncated Her-2 protein should function in a dominant-negative manner because the kinase domain is partially missing, while the dimerization domain is intact.

The exact position of the PNA at the intron-exon junction is clearly important. Acr-PNA 2794 covering 5 nucleotides at the 3' junction of intron 18 and 10 nucleotides in 5' junction of exon 19 (data not shown) appears to be the most sensitive site in terms of splicing modulation, since shifting one nucleotide down- (Acr-PNA 2707) and two nucleotides upstream (Acr-PNA 2701) of the targeted position reduced the effect of exon skipping.

It is surprising that in two cases single mismatch PNA oligomers (Acr-PNA 2541smm and Acr-PNA 2696smm) are more potent splicing interference agents than their fully matched counterparts (PNAs 2794 and 2780). However, there

are numerous reports that describe gene silencing effects obtained by imperfectly matched oligonucleotides, e.g. by miRNAs. Natural (57,58) or designed (60) miRNAs regulate translation most effectively being only partially complementary to the targeted mRNA (59-61). For LNA antisense oligonucleotides it has also been reported that LNAs having one or two mismatches were equally efficient compared to the fully complementary molecule (62). For the action of siRNA, the identity of the substituted nucleotide is important. It was shown that a G:U wobble base pare is well tolerated for siRNA-mediated gene silencing, whereas the introduction of other nucleotides at a given position may dramatically affect the silencing effect (63). Our results show for the first time (to our knowledge) that a specific single mismatch antisense PNA is more effective in terms of splicing modulation than the perfectly matched PNA. We have not systematically studied how the position of the mismatch affect the splicing. However, the obtained data demonstrate that the identity of the altered base is important for the efficacy. We found in one case that a G-T pair at the endogenous RNA target site yields higher efficacy (Acr-PNA 2541smm) than the two other mismatches, G-A and G-G (this also is true for the native Watson-Crick G-C pair), and this cannot be explained on the basis of higher duplex stabilities. In the other case, a single base deletion in the PNA (Acr-PNA 2696smm) yielded higher potency for splicing interference. Further studies are necessary to understand the details and molecular mechanism(s) of these effects.

Taken together, the reported data show that the splicing of Her-2 pre-mRNA can be affected (inhibited/redirected) to varying degrees by PNA antisense targeted to intron-exon junction sites. The redirection of splicing induced by the full matched PNA depends on the target position and may be improved by introduction of mismatch nucleotides in the PNA sequence, whereas the identity of a mismatched base is of importance. These results could be of potential value in developing antisense-based anti-cancer therapy.

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