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Design and Application of a Peptide Nucleic Acid Sequence Targeting the p75 Neurotrophin Receptor

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Abstract—Novel antisense peptide nucleic acid (PNA) constructs targeting p75NTR as a potential therapeutic strategy for amyotrophic lateral sclerosis (ALS) were designed, synthesised and evaluated against phosphorothioate oligonucleotide sequences (PS-ODN). An 11-mer antisense PNA directed at the initiation codon dose-dependently inhibited p75NTR expression and death signalling by nerve growth factor in Schwann cell cultures. Inhibition of p75NTR production was not detected in cultures treated with the non-sense PNA or antisense PNA directed at the 3′-terminus sequence. The 19-mer PS-ODN sequences also failed to confer any activity against p75NTR but, unlike the PNA sequences, were toxic in vitro at comparable doses.

Expression of the death-signalling 75 kilodalton neurotrophin receptor (p75NTR) is associated with both neurodevelopment[1] and neurodegenerative disorders.[2,3] One disease implicating a role of p75NTR and its apoptotic effects is motor neuron disease (MND). Amyotrophic lateral sclerosis (ALS), which accounts for approximately 85% of MND incidence, is a progressive and fatal paralytic disorder characterized by degeneration of cortical, brainstem and spinal cord motor neurons.[4] The abnormal re-expression of p75NTR in mature degenerating spinal motor neurons of humans and transgenic mice expressing ALS pathology suggests an active role of this receptor.[5] Recent evidence suggests that p75NTR may be implicated in promoting caspase-dependent death in spinal motor neurons of familial ALS-expressing mice.[6] These two events prompted us to evaluate an antisense strategy comparing oligonucleotide (ODN) and peptide nucleic acid (PNA)[7] sequences directed against p75NTR as a potential therapy. Here we demonstrate that a simple 11-mer antisense PNA targeting the initiation codon inhibits p75NTR protein expression in a dose-dependent manner in rat Schwann cell cultures. Furthermore, this antisense PNA conferred significant protection against nerve growth factor (NGF)-induced p75NTR-mediated Schwann cell death. This study suggests that antisense therapies harnessing PNA chemistry may provide a useful paradigm for inhibiting death-signalling pathways in neurodegenerative diseases such as MND.

Two classes of antisense[8] agents were employed in this study for comparison, phosphorothioate oligonucleotide (PS-ODN) and peptide nucleic acid (PNA) molecules (Fig. 1).[9] Long 19-mer PS-ODN sequences targeting the 5′-terminus of p75NTR and used previously to rescue axotomised sensory and motor neurons of neonatal rats[10] were evaluated in vitro for inhibitory effects in Schwann cell[11] cultures (Table 1).

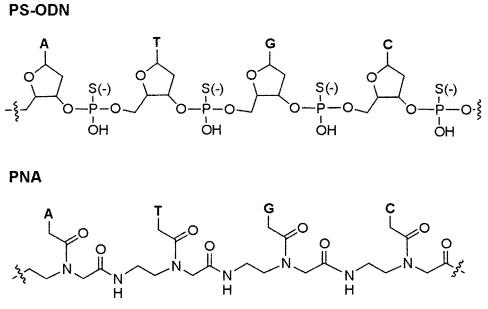

Figure 1. Schematic representation of a general phosphorothioate oligonucleotide (PS-ODN) and peptide nucleic acid (PNA).

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The structure of the PNA (Fig. 1) confers useful properties that make it an ideal agent for use in antisense therapies. These include a resistance to nucleases and proteases, enhanced target hybridisation independent of salt concentration and crucial to any therapeutic paradigm, a capacity to cross the blood–brain barrier[12] after systemic administration. Short PNA sequences (10, 11-mer) with comparable T_m values to lengthy PS-ODNs (19-mer) were designed (Table 1) against the 3′-terminus (nt 1359-1368) and initiator codon (nt 106-116) of the rat p75NTR gene (coding region length = 1388 nucleotides) and tested for efficacy in vitro. Sequences were designed to minimize intramolecular hairpins or intermolecular dimers. Because of the small size associated with the PNA sequences, a BLAST nucleotide search for short nearly exact matches against human and murine databases using the National Centre for Biotechnology Information (NCBI) is necessary to confirm the 'uniqueness' of each sequence to avoid non-specific binding that may lead to complications.

The PNA sequences were synthesized using an Expedite 8909 nucleic acid synthesizer adapted for PNA protocols. The nucleotide protecting groups (Bhoc) are removed at the end of the synthesis with TFA in the presence of the scavenger *m*-cresol. This protocol affords the PNA$(TFA)_n$ salt ($n = 10$, 11) which has ramifications for the PNAs solubility in tonic buffers.[13] Purification by preparative HPLC yields highly pure materials as adjudged by mass spectrometry[14] (Fig. 2). Clearly evident for AS-PNA_{106} are major peaks corresponding to multiply-charged PNA molecules at m/z 1469.5 ($[M+2H]^{2+}$), 980.2 ($[M+3H]^{3+}$), 735.6 ($[M+4H]^{4+}$) and 588.7 ($[M+5H]^{5+}$). Minor neighboring peaks can for the most part be attributed to multiple adducts of Na^+ or K^+ common with this technique. Interestingly, the ESIMS obtained are cone voltage dependant. At a cone voltage of 50 V (Fig. 2b), the spectrum is simplified. Peaks corresponding to values m/z 1469.5 ($[M+2H]^{2+}$), 980.2 ($[M+3H]^{3+}$) completely dominate the spectrum with no evidence of higher charged (lower m/z) species seen, consistent with the results obtained for NS-PNA. A similar result is obtained for FluAS-PNA_{106} in which a cone voltage of 30 V produces a spectrum dominated by higher charged species e.g., $[M+6]^{6+}$. This phenomenon can be regarded as a variation of 'charge-stripping'.[15] In this case, high cone voltages strip or remove the weakly bound ammonium protons from the PNA salt.

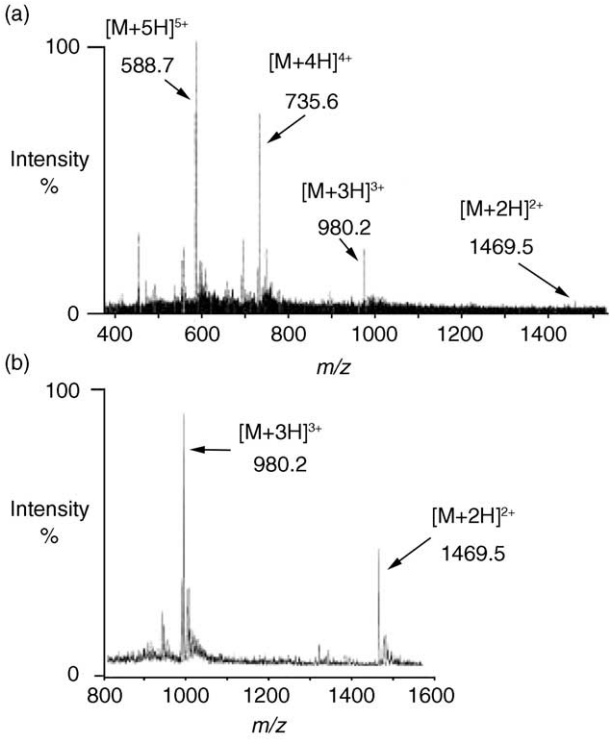

Figure 2. Positive electrospray ionisation mass spectra (ESIMS) of **N**-CAT TGC ACG CC-**C** recorded on a Micromass Platform II MS at cone voltages of (a) 35 V and (b) 50 V. Solutions of the PNA for analysis were prepared in 3% (v/v) formic acid in $MeCN/H_2O$ (1/1).

AS-PNA_{106} targeting the translational start codon inhibited p75NTR protein levels in a dose-dependent manner at 1–100 μM concentrations (Fig. 3a). Inhibition

Table 1. Phosphorothioate oligonucleotide and peptide nucleic acid sequences

Type	Sense type[a]	Length	Target[b]	Base sequence[c]	Molecular weight (Da)
PS-ODN	AS	19-mer	nt 110–128	**5′**-ACC TGC CCT CCT CAT TGC A-**3′**	9050.1
	NS	19-mer	—	**5′**-CTC CCA CTC GTC ATT CGA C-**3′**	9050.1
PNA[d]	AS	10-mer	nt 1359–1368	**N**-ACT CGC TGC A-**C**	2686.0
	AS	11-mer	nt 106–116	**N**-CAT TGC ACG CC-**C**	2937.4
	NS	11-mer	—	**N**-CAC CAG CCT TG-**C**	2937.4
	FluAS[e]	11-mer	nt 106–116	**FluOO**-CAT TGC AGC CC-**C**	3588.5

[a]AS = antisense, NS = nonsense, FluAS = Fluorescent-labelled antisense sequence.
[b]Based on *R. norvegicus* sequence, Genbank accession number NM_012610.
[c]Optimal PNA-DNA duplex T_m without intra-PNA hairpin and inter-PNA dimer calculated with Primer Premier 5.0 (Premier Biosoft). nt = nucleotide number. PS-ODNs and PNAs were solubilised in mouse-tonicity phosphate-buffered saline (MT-PBS), pH 7.4.
[d]PNAs were synthesised using an automated Expedite 8909 nucleic acid synthesizer (Applied Biosystems) adapted for PNA synthesis. Synthesis was performed on a 2 μmol scale with PS-PEG-XAL resin using Fmoc/Bhoc protected monomers. PNA was cleaved using trifluoroacetic acid with 20% (v/v) *m*-cresol in polytetrafluoroethylene spin columns (Millipore). PNA pellets were precipitated, washed with ice-cold ether and air-dried prior to purification. Preparative HPLC was performed on PNA pellets resolubilised in 1/1 distilled water/acetonitrile using preparative C_{18} columns. Purified fractions were collected, freeze dried and sequences analysed via electrospray ionization mass spectrometry (ESIMS).
[e]Fluorescent-labelled PNAs were synthesized manually with the addition of two 2-aminoethylglycine spacers and 5-carboxyfluorescein succinimidyl ester (Molecular Probes) to the 5′ end of the PNA. 5(6)-carboxyfluorescein, succinimidyl ester (Molecular Probes) dissolved in dimethylformamide in the presence of 0.07 M diisopropyl ethylamine was manually injected through the column for 30 min post-PNA synthesis. Purification and analysis were performed as forementioned, however samples were kept in the dark.

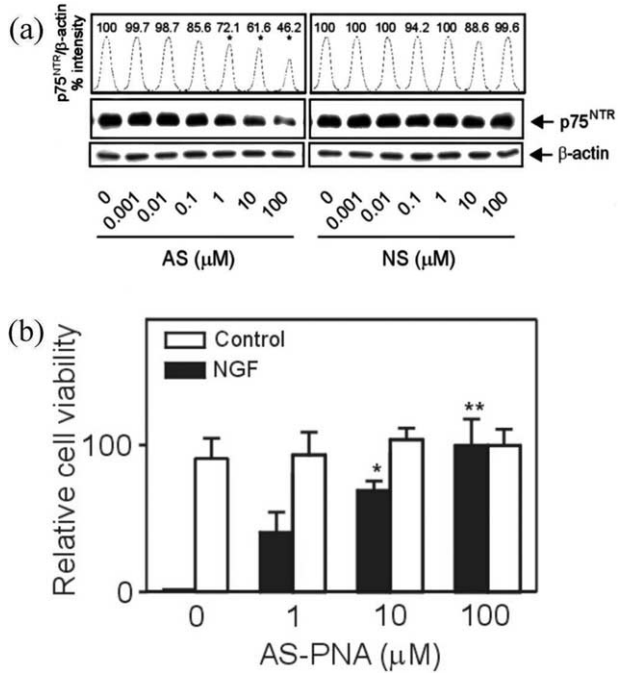

Figure 3. Antisense PNA targeting the 5′-terminus (AS-PNA$_{106}$) downregulates full-length p75^{NTR} protein and NGF-induced signalling in vitro. (a) Immunoblot analysis of Schwann cell cultures treated with AS or NS-PNA$_{106}$ and quantification of p75NTR immunoblots normalised to β-actin after 48 h. (b) NGF-induced p75NTR-mediated Schwann cell death as inhibited by AS-PNA$_{106}$ ($n=4$, mean ± SD, $^{*}p<0.05$, $^{**}p<0.01$).

of p75NTR production was not detected in cultures treated with either NS-PNA or AS-PNA$_{1359}$ directed at the 3′-terminus sequence but unlike the AS- or NS-PS-ODN, neither PNA sequence was toxic to cells at comparable doses. Although efficacious at low doses on cultured sensory neurons,[10] the higher concentrations of PS-ODN deployed in this study suggest that there is non-specific and sequence-independent toxicity, consistent with previous reports for this type of molecule.[9] As further evidence of PNA specificity, AS-PNA$_{106}$ did not alter β-actin levels at any dose tested (Fig. 3a). Significant reductions in p75NTR protein levels were detected using AS-PNA$_{106}$ concentrations between 1–100 μM after 48 h. In the best result, a 54% reduction of p75NTR protein, relative to control cultures, was achieved with 100 μM AS-PNA$_{106}$ (Fig. 3a).

To assess whether AS-PNA$_{106}$ could directly inhibit ligand-dependent p75NTR-mediated cell death, Schwann cells were exposed to β-NGF (10 ngmL^{-1}).[16] In the absence of AS-PNA$_{106}$, cell viability was significantly reduced (Fig. 3b). However, in the presence of 10 and 100 μM AS-PNA$_{106}$, 92 and 100% viability was detected, respectively. We can conclude from this result that the p75NTR gene switching by AS-PNA$_{106}$ as illustrated by Western analysis was also sufficient to significantly block NGF-induced p75NTR killing in vitro. As a final test, the uptake and intracellular distribution of AS-PNA$_{106}$ was determined using fluorescein tagging (Fig. 4). Uptake and accumulation of FluAS-PNA$_{106}$ in the cytosol rather than endosomal and lysosomal compartments of cultured Schwann cells was seen at 48 h

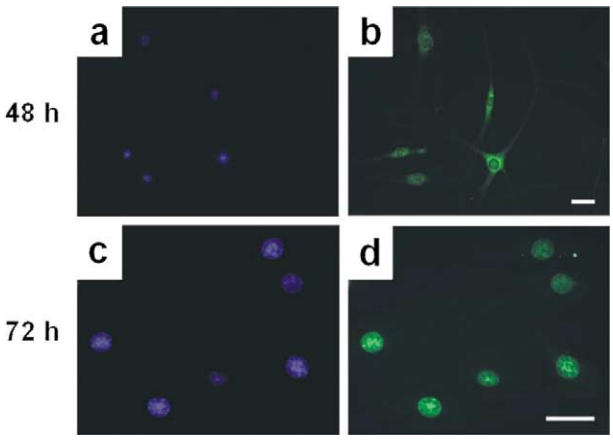

Figure 4. Cultured Schwann cells treated with fluAS-PNA$_{106}$ conjugates (100 μM) for 48 h (a,b) or 72 h (c,d) display cytoplasmic and nuclear localisation respectively; scale bar, 10 μm. 4,6-Diamidino-2-phenylindole (DAPI) dihydrochloride has been used as a nuclear specific control stain.

incubation (Fig. 4b). Prolonged exposure (72 h) resulted in complete nuclear localisation (Fig. 4d), supporting a direct antigene effect of PNA in addition to antisense inhibition of mRNA, based on its increased affinity for DNA targets.

Specific PNA sequences were found to be superior to PS-ODN in this antisense paradigm. The data described in this paper demonstrates that antisense PNA-mediated interference with p75NTR expression in culture is indeed sufficient to modify cellular responses to exogenous neurotrophin ligands. The data presented here supports recent reports that antisense inhibition is more effective when targeting upstream elements such as the 5′-untranslated region or initiation codon of the luciferase[9] or huntingtin genes[17] respectively. Truncation of p75NTR, as created by AS-PNA$_{1359}$ may continue to induce ALS pathology. We are at present investigating AS-PNA$_{106}$ as a potential therapeutic in a mouse model (SOD1^{G93A}) of ALS.

Acknowledgements

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References and Notes

1. (a) Barrett, G. L.; Bartlett, P. F. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 6501. (b) Hempstead, B. L. *Curr. Opin. Neurobiol.* **2002**, *12*, 260.
2. (a) Death signalling pathways mediated by p75NTR may underlie an array of adult pathological conditions associated with the nervous system, including axotomy, epilepsy and

stroke. For further information see: Frade, J. M.; Rodriguez-Tebar, A.; Barde, Y. A. *Nature* **1996**, *383*, 166. (b) Dechant, G.; Barde, Y. A. *Nat. Neurosci.* **2002**, *5*, 1131.
3. (a) p75NTR has also been implicated in neurons or glia Alzheimer's disease: Mufson, E. J.; Kordower, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 569. (b) Multiple sclerosis: Dowling, P.; Ming, X.; Raval, S.; Husar, W.; Casaccia-Bonnefil, P.; Chao, M.; Cook, S.; Blumberg, B. *Neurology* **1999**, *53*, 1676.
4. Rowland, L. P.; Schneider, M. D. *New Engl. J. Med.* **2001**, *344*, 1688.
5. Lowry, K. S.; Murray, S. S.; McLean, C. A.; Talman, P.; Mathers, S.; Lopes, E. C.; Cheema, S. S. *Amyotroph. Lateral Scler. Other Motor Neuron Disord.* **2001**, *2*, 127.
6. Li, M.; Ona, V. O.; Guégan, C.; Chen, M.; Jackson-Lewis, V.; Andrews, J.; Olszewski, A. J.; Steig, P. E.; Lee, J.-P.; Przedborski, S.; Friedlander, R. M. *Science* **2000**, *288*, 335.
7. (a) Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254*, 1497. (b) Eriksson, M.; Nielsen, P. E. *Nature Struct. Biol.* **1996**, *3*, 410.
8. (a) Taylor, M. F.; Wiederholt, K.; Sverdrup, F. *Drug Discovery Today* **1999**, *4*, 562. (b) Baker, B. F.; Monia, B. P. *Biochimica Biophysica Acta* **1999**, *1489*, 3. (c) Ramazeilles, C.; Mishra, R. K.; Moreau, S.; Pascolo, E.; Emanuelle, J.-J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 7859.
9. Braasch, D. A.; Corey, D. R. *Biochemistry* **2002**, *41*, 4503.
10. Cheema, S. S.; Barrett, G. L.; Barlett, P. F. *J. Neurosci. Res.* **1996**, *46*, 239.
11. These peripheral glia were chosen since they express high levels of p75NTR but not TrkA co-receptors.
12. Tyler, B. M.; Jansen, K.; McCormick, D. J.; Douglas, C. L.; Boules, M.; Stewart, J. A.; Zhao, L.; Lacy, B.; Cusack, B.; Fauq, A.; Richelson, E. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 7053.
13. In related work, **N**-TCC GTA GGA TGA-**C** as its TFA salt precipitates out of MT-PBS, pH 7.4. Solubility is excellent in 0.9% w/v NaCl solution. This comparative insolubility is most likely the result of a counterion exchange reaction between the trifluouroacetate and phosphate (excess) that decreases the solubility of the PNA in aqueous solution.
14. ESIMS results. AS-PNA (11mer): Cone Voltage 50 V; m/z 1469.5 (45%, $[M+2]^{2+}$), 980.0 (100%, $[M+3]^{3+}$), 735.1 (10%, $[M+4]^{4+}$). NS-PNA (11-mer): Cone Voltage 35 V; 980.2 (25%, $[M+3]^{3+}$), 735.6 (65%, $[M+4]^{4+}$), 588.7 (100%, $[M+5]^{5+}$). FluAS-PNA (11-mer): Cone Voltage 30V; 898.6 (15%, $[M+4]^{4+}$), 718.8 (60%, $[M+5]^{5+}$), 599.1 (100%, $[M+6]^{6+}$). AS-PNA (10-mer): Cone Voltage 35V; 896.3 (10%, $[M+3]^{3+}$), 672.7 (35%, $[M+4]^{4+}$).
15. (a) Schroder, D.; Engeser, M.; Schwarz, H.; Harvey, J. N. *ChemPhysChem.* **2002**, *3*, 584. (b) Aubry, C.; Polce, M. J.; Holmes, J. L.; Mayer, P. M.; Radom, L. *J. Am. Chem. Soc.* **1997**, *119*, 9039.
16. Cells were treated with PS-ODNs or PNAs diluted in Dulbecco's modified Eagle's medium (DMEM), pH 7.4, supplemented with 10% (v/v) FCS, 1% (v/v) PS, 10 mgmL^{-1} BPE and 2 μM forskolin. Aliquots of culture medium from wells were centrifuged at 5000×g for 5 min and supernatant fractions assayed for lactate dehydrogenase activity (Roche) as a marker of cytotoxicity. For cell survival assays, cells were treated with PNA for 48 h then 10 ng/mL NGF-2.5S (β-NGF, PeproTech) in deprived media containing DMEM with 50 μg/mL BPE only for 24 h. Viability was assessed by addition of 0.5 mg/mL MTT to wells, incubation for 3 h, solubilisation of cells in DMSO and absorbance measurements at 530 nm.
17. Boado, R. J.; Kazantsev, A.; Apostol, B. L.; Thompson, L. M.; Pardridge, W. M. *J. Pharmacol. Exp. Ther.* **2000**, *295*, 239.