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Efficacy of peptide nucleic acid and selected conjugates against specific cellular pathologies of amyotrophic lateral sclerosis

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ABSTRACT

Cellular studies have been undertaken on a nonamer peptide nucleic acid (PNA) sequence, which binds to mRNA encoding superoxide dismutase 1, and a series of peptide nucleic acids conjugated to synthetic lipophilic vitamin analogs including a recently prepared menadione (vitamin K) analog. Reduction of both mutant superoxide dismutase 1 inclusion formation and endoplasmic reticulum stress, two of the key cellular pathological hallmarks in amyotrophic lateral sclerosis, by two of the prepared PNA oligomers is reported for the first time.

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder which affects the upper and lower motor neurons of the brain, brain stem and spinal cord.[1] It causes progressive muscle weakness, paralysis and death within 3–5 years of diagnosis and there is currently no effective treatment. Around 10% of ALS cases are familial (fALS), caused by genetic mutations. However, the majority of ALS (90%) is sporadic, with no previous family history.[2] Mutations in the superoxide dismutase 1 (SOD1) gene account for approximately 20% of familial ALS cases and the most common mutation in North America is A4V (alanine to valine).[3,4] SOD1 is a major cytosolic protein which catalyzes the reduction of harmful, free superoxide radicals into molecular oxygen and hydrogen peroxide.

The etiology of ALS remains unclear but the formation of intracellular ubiquitin-positive inclusions containing misfolded proteins is a characteristic pathological hallmark.[5] Misfolded SOD1 inclusions are present in both sporadic human[6] and familial ALS patients, as well as transgenic SOD1^{G93A} mice,[7] the most widely used animal disease model in preclinical studies. Stress in the endoplasmic reticulum (ER) is also recognized to be an important pathway to motor neuron death in ALS. ER stress is triggered when misfolded proteins accumulate within the ER. This triggers the unfolded protein response (UPR), a signaling pathway that aims to relieve the stress and thus restore homeostasis. However if ER stress is prolonged, apoptosis is triggered. The transition of UPR from cell survival to cell death is mediated by CCAAT-enhancer binding protein homologous protein (CHOP),[8] a transcription factor which translocates to the nucleus when activated. We and others previously demonstrated that ER stress is present in sporadic ALS patient tissues[9] as well as in cells expressing mutant SOD1 and transgenic SOD1^{G93A} mice.[10–14] Novel pharmacological agents that prevent the formation of misfolded mutant SOD1 inclusions and inhibit the activation of CHOP in cells expressing mutant SOD1, and hence the pro-apoptotic phase of UPR, would therefore have therapeutic application in ALS.

Antisense agents such as peptide nucleic acids (PNA) are short single stranded nucleic acid analogs designed to specifically bind to complementary messenger ribonucleic acid (mRNA) targets through Watson and Crick hydrogen bonding. As a result, these antisense agents can silence a particular gene of interest. PNA is a third generation oligonucleotide, which replaces the traditional phosphate backbone of RNA/DNA with a peptide backbone made up of repeating *N*-(2-aminoethyl)glycine units and the sugar moiety replaced with a methylene carbonyl linker where the nucleic bases are attached (Fig. 1).

The modified backbone of PNA gives rise to resistance to enzymatic degradation and leads to higher affinity binding, rates of

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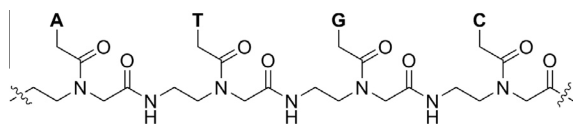

Figure 1. General structure of peptide nucleic acid (PNA) containing the four nucleobases of adenine (A), thymine (T), guanine (G) and cytosine (C).

association and subsequently an increase in duplex stability as there are no repulsive electrostatic interactions.[15] Additionally, the neutrality of the backbone significantly reduces the chance of undesired nonspecific interactions such as binding to cellular proteins.[16] PNA exhibits low toxicity in cells and is stable of a wide pH range, particularly toward the acidic end of the scale where DNA can be denatured.

The major limitation of PNA as an efficient antisense drug is its low phospholipid solubility due to the hydrophilic nature of the molecule, PNA does not readily cross cell membranes. Hydrophobic vitamins such as vitamin K and vitamin E are hypothesized as good candidates for conjugation to PNA. The main function of vitamin K is post-translational modification during the biosynthesis of vitamin K dependent proteins.[17] The synthetic form of this vitamin, vitamin K3 (menadione) has been found to readily cross the blood brain barrier as it is a small lipophilic molecule.[18] Despite its larger size, highly lipophilic vitamin E (tocopherol) has also been found to cross the blood brain barrier.[19] While the main task of this vitamin is as an antioxidant, vitamin E has also been shown to alleviate oxidative stress by promoting normal cell function.[20]

We have previously described the synthesis and conjugation of tocopherol (vitamin E) analogs to PNA for the investigation of effects on hybridization.[21] In this further study, we present the synthesis of a menadione (vitamin K) analog, its conjugation to the same nine nucleobase PNA oligomer and the subsequent hybridization results. In addition, this novel vitamin K conjugated oligomer, along with the previously synthesized three vitamin E derived conjugated PNA oligomers and the unconjugated PNA oligomer, were studied using cellular assays to determine their effects on the formation of mutant SOD1 inclusions and induction of on ER stress.

2. Results and discussion

2.1. Synthesis and thermodynamic studies of the menadione-PNA conjugate

The menadione analog was synthesized by the adaptation of the methods of Abell et al.[22] Commercially available menadione was directly brominated using molecular bromine in the presence of sodium acetate and glacial acetic acid to yield the brominated adduct **1**,[23] followed by reduction of the dione using potassium hydroxide and in situ methylation with dimethyl sulfate to give **2** in 45% yield over the two steps (Scheme 1). Alkylation of **2** using ethyl bromoacetate was achieved via a copper transmetalation reaction through a diaryl cuprate intermediate from treatment with *n*-butyllithium and copper bromide dimethyl sulfide. Increasing the number of equivalents of ethyl bromoacetate from 1.1 to 1.5 and extending the reaction time from 5 to 18 h resulted in an optimized yield of 55%, a considerable improvement over the previously reported 34% yield for this step.[22] Reduction of the ethyl ester **3** using lithium aluminum hydride proceeded in high yield to give the corresponding alcohol **4** which was succinylated using the anhydride to afford **5**. Ceric ammonium nitrate (CAN) was utilized to oxidatively deprotect the methoxy groups and restore the quinone functionality that is characteristic of the vitamin K family affording **6** in 84% yield.

Automated PNA synthesis was performed on PAL resin using standard protocols[24] and two PNA sequences were prepared as previously described: H-GCACGACTT-NH_2 (**PNA1**) and the complementary sequence H-AAGTCGTGC-NH_2 (**PNA2**).[21] Coupling of the menadione analog **6** to the growing oligomer of **PNA1** was then undertaken using the same automated synthetic protocols. Purification by reverse-phase high performance liquid chromatography (RP-HPLC) gave the desired menadione-PNA conjugated oligomer of **6-PNA1** (Fig. 2). Characterization was undertaken using MALDI-TOF mass spectrometry where m/z calculated for $C_{119}H_{150}N_{55}O_{33}$ requires $[M+H]^+$ 2734.069 and m/z of $[M+H]^+$ 2734.375 was found.

In order to determine the suitability of the conjugated menadione-PNA oligomer **6-PNA1**, the thermodynamics of hybridization to both the complementary PNA (**PNA2**) and DNA oligomers was undertaken to ensure conjugation did not impair hybridization (Tables 1 and 2). Two complementary methods were chosen for thermodynamic analysis of the duplexes, UV monitored melting curves (UVM)[25,26] and isothermal titration calorimetry (ITC).[27] Data for the control duplexes of **PNA1**/**PNA2** and **PNA1**/DNA was previously recorded[21] and is provided here to facilitate the comparison of the data obtained with the conjugated duplexes. The change in the thermodynamic differences between the two methods agree well with each other.

In the case for both conjugated duplexes, conjugation of the menadione analog does not appear to significantly affect the stability of the duplexes as reflected in the recorded thermal melting temperature (T_m). As expected, lower thermal stability and thus affinity was observed for the mixed duplexes of PNA and DNA oligomers.[28,29] However, the duplex binding affinity, which is related to the enthalpy change ($\Delta H°$) is reduced in both of the conjugated duplexes to the same extent (approximately 73 kJ mol^{-1} on average). As a result of this, the free energy ($\Delta G°$) is also reduced leading to less favorable duplex formation at 37 °C with the conjugated duplex despite no change in T_m. The reduction in $\Delta H°$ indicates that there is a weaker interaction between the two oligomers, most likely a result of steric bulk minimizing the interaction of the base pairs adjacent to the conjugate. However, the conformations of the base pairs or stacking patterns may also have an effect along with counter ion uptake and release and hydration factors may also have an effect. Despite the change in the enthalpy and free energy change for this duplex, the thermal melting temperature remains relatively unaffected overall.

2.2. PNA1, 6-PNA1 and 7-PNA1 inhibit the formation of mutant SOD1 inclusions in neuronal cell lines

We next examined the effect of the PNA compounds in cells expressing mutant SOD1 A4V. Mouse neuronal Neuro2a cells were transfected with a previously generated construct encoding SOD1 A4V,[30] tagged with Enhanced Green Fluorescent protein (EGFP) to aid in visualizing the expressed protein. We previously demonstrated that the presence of the EGFP tag does not affect the activity of the protein.[30] Cells were also treated 4 h post transfection either with dimethyl sulfoxide (DMSO) vehicle as a negative control, unconjugated PNA (**PNA1** or its complementary sequence **PNA2**), menadione-conjugate **6-PNA1** (Fig. 2), previously prepared vitamin E derived-conjugates **7- PNA1**, **8-PNA1** and **9-PNA1** (Fig. 3),[21] or (+/−)-*trans*-1,2-bis(2-mercaptoacetamido)cyclohexane (BMC), which we previously demonstrated significantly reduces the formation of mutant SOD1 A4V inclusions and reduced ER stress.[13]

Cells were transfected for 72 h and then examined by fluorescent microscopy with the percentage of cells bearing fluorescent green mutant SOD1 inclusions quantified. Untransfected cells (UTR) were included as a negative control to specifically determine

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