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INHIBITION OF HUMAN TELOMERASE BY PNA-CATIONIC PEPTIDE CONJUGATES

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Abstract: The inhibition of human telomerase has been explored using peptide conjugated derivatives of a PNA pentamer directed at the RNA template of telomerase. It is demonstrated that the presence of cationic peptides at the N-terminus of the PNA results in enhanced inhibition of telomerase activity. Furthermore, inhibition depended on the specificity of PNA recognition.

Telomeres are guanine-rich regions of DNA located at the ends of all eukaryotic chromosomes. They are essential for preserving the stability of the genome and cell viability by preventing aberrant recombination and degradation of DNA. The human telomeric sequence is $(TTAGGG)_n$ and varies in length from 5 to 12 kB.[1] Telomeres are maintained by telomerase, an RNA-dependent DNA polymerase. Telomerase is a unique reverse transcriptase because it is a ribonucleoprotein that contains its own RNA template as a central part of the enzyme.[2] Telomerase activity has been detected in tumour biopsies, whereas little or no activity is found in normal tissue. For this reason, human telomerase has received much attention as a potential target for anti-cancer chemotherapy.[3]

The RNA-template portion of telomerase has been shown to be a target for inhibition by antisense oligonucleotides.[4] DNA is subject to degradation by nucleases, whereas the nuclease-resistant oligonucleotide mimic, peptide nucleic acid (PNA), was shown to be a more effective antisense inhibitor of telomerase.[5] In PNA, the phosphate backbone has been replaced by (2-aminoethyl)glycine units with the nucleobases attached through methylene carbonyl linkages to the glycine amino group.[6] PNA can hybridize with complementary sequences of DNA or RNA with very high affinity and selectivity. The thermal stability of PNA/DNA hybrids is greater than analogous DNA/DNA hybrids, due to a reduction in electrostatic repulsion[7] and possible entropic effects resulting from restricted rotation about the amide backbone.[8] Furthermore, PNA offers the advantage of greater resistance to degradation by protease or nuclease.[9] Studies by Corey and co-workers have reported nanomolar IC_{50} values for the inhibition of human telomerase by PNAs 11-13 bases in length.[5] Limitations of PNA for direct therapeutic use include poor solubility in aqueous buffer and poor uptake properties, however it remains to be an excellent diagnostic probe.

Here, we describe some initial studies on the inhibition of human telomerase using PNA-peptide conjugates leading to an apparent affinity enhancement. In the case of a known ligand-target binding interaction, it is possible to enhance the affinity by the addition of a second binding interaction. The general concept of enhanced binding by bis-specific inhibitors has been described in a seminal paper by Jencks,[10] and demonstrated for a number of cases that include enzyme inhibition[11] and mRNA binding.[12] We have based the design of the

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inhibitors on a 5-mer antisense PNA component (sequence H_2N-TAGGG-CO_2H), directed at the RNA template of telomerase. This pentameric sequence is a key motif found within the most potent PNA antisense inhibitors.[5] Pendant oligopeptides (7 mer) were connected to the N-terminus of the PNA *via* a linker. The objective being to probe the vicinity of the 3'-end of the RNA template site of telomerase for additional interactions (Figure 1). For this study, we have chosen to synthesise PNA-peptide conjugates that contain five cationic amino acids consisting of lysine, ornithine or arginine, in addition to the fixed alanine, used as a neutral spacer for peptide synthesis[13] and a cysteine residue for coupling.[13,14] We report here that these conjugates appear to be more potent inhibitors of human telomerase than the corresponding unmodified PNA.

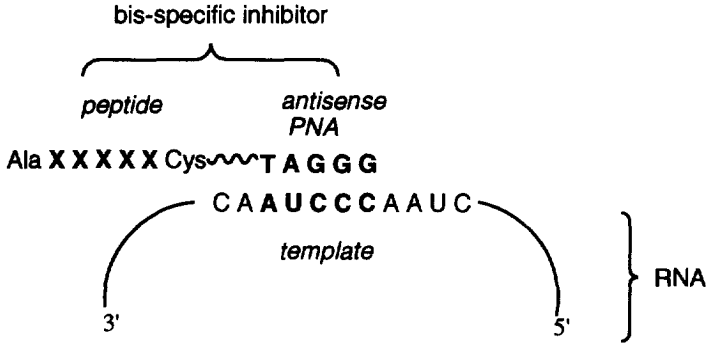
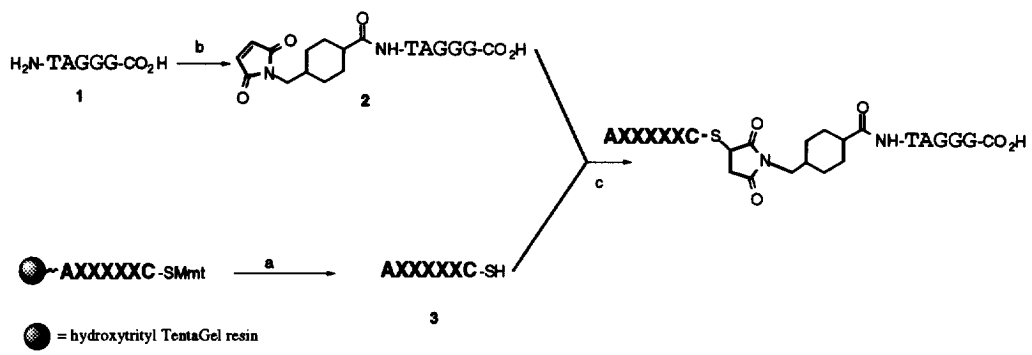


Figure 1. Design of PNA-peptide conjugates for inhibition of human telomerase.

Synthesis of PNA-peptide conjugates

There are numerous methods available in the literature for the synthesis of PNA-peptide conjugates. We have employed a convenient procedure based on our previous work on deoxyoligonucleotide conjugates.[13,14] The methodology involves initial preparation of cysteinyl derivatives of the peptide moieties on solid phase, followed by cleavage and efficient solution phase coupling to the PNA bearing an N-terminal maleimide link (Scheme 1). The peptides were each 7 residues in length with the C- and N- terminal amino acids fixed (alanine and cysteine respectively) with the central 5 positions corresponding to the representative cationic amino acid (lysine, ornithine or arginine). Peptides were prepared as described previously.[14,15] The 5mer PNA (TAGGG, **1**, or the mutated PNA TAAGG) (purchased from Oswel) was converted to the *N*-maleimide derivative (**2**) by coupling to the bifunctional linker, SMCC (Pierce, 10 equivalents in acetonitrile) in potassium phosphate pH 7.8. After 3 hours, the derivative **2** was purified by reverse phase HPLC and was obtained in good yield (70 %). The coupling between the peptide **3** and PNA moiety **2** was accomplished by mixing the modified PNA with an excess of the peptide (5-7 eq.) in 0.1 M potassium phosphate pH 7.0. After 5 minutes reaction time, the products were purified by reverse phase HPLC. The PNA-peptide conjugates were analysed by electrospray mass spectrometry to confirm their identity. Each conjugate gave a mass in good agreement with calculated values. The yield of each coupling, the HPLC retention time and the mass of conjugates are given in Table 1.



Scheme 1[a]. Preparation of PNA-cationic peptide conjugates. Reagents: (a) 95:2.5:2.5 TFA/TIS/water; (b) SMCC (10 eq, in acetonitrile), 0.1 M potassium phosphate (pH 7.8), r.t , 3h, 70% by HPLC; (c) 0.1 M potassium phosphate (pH 7), rt, 5 min.
[a]The identical procedure was employed to prepare a peptide conjugate of the mutated PNA TAAGG.

Table 1. Characterisation of the precursor PNA, the *N*-maleimide PNA and PNA-cationic peptide conjugates by electrospray mass spectrometry and reverse phase HPLC.

PNA and derivatives	HPLC retention time (min)[a]	Yield of peptide-PNA conjugate(%)[b]	Calculated mass (Da)	Observed mass (Da)
H_2N-TAGGG-CO_2H	8.3	-	1431.0	1430.8
SMCC-TAGGG-CO_2H	18.4	-	1650.0	1649.5
AKKKKKC-TAGGG	13.2	87	2483.2	2482.0
AOOOOOC-TAGGG	13.0	91	2413.1	2412.5
ARRRRRC-TAGGG	14.6	85	2623.3	2623.3
H_2N-TAAGG-CO_2H	8.1	-	1416	1415.8
SMCC-TAAGG-CO_2H	18.3	-	1635	1634.6
AOOOOOC-TAAGG	13.1	82	2398.1	2397.7

[a]Reverse phase HPLC was carried out using a Phenomenex RP 5 μm column (4.6 x 150 mm) on a Hewlett Packard series 1100 system. One gradient only was used for all samples and all runs were monitored at 254 nm. Mobile phase A was water with 0.1% of TFA and mobile phase B was HPLC grade acetonitrile with 0.1 % of TFA. The gradient was isocratic 5% B for 5 minutes, 5% B to 30% B in 25 minutes, isocratic 30% B for 5 minutes, 30% B to 100% B in 5 minutes, isocratic 100% B for 3 minutes and 100% B to 5% B in 2 minutes. The flow rate was 1 ml/min.
[b]The quoted yields are based on the absorbance at 260 nm of the gel filtration eluants.

Inhibition of telomerase by PNA-peptide conjugates

Telomerase inhibition experiments were conducted similarly to those described by Hamilton *et al.*[16] Telomerase activity was measured using the TRAPEZE detection kit (Intergen, Purchase, NY), which is a PCR-based assay originally described by Kim *et al.*[3] The source of telomerase was S100 extracts from K562 cells (ATCC No. CCL-243) prepared as described.[17] Inhibitor at varying concentration was pre-incubated with the cell extract (in triplicate) for 10 min at ambient temperature prior to initiating the telomerase reaction by addition of dNTP's (50 μM), telomerase primer, Taq polymerase, and PCR primers as described in the TRAPEZE kit. The reaction mixture was incubated for 60 min at 30 °C, after which samples were transferred to a GENEAMP 2400 thermocycler (Perkin Elmer) for PCR amplification of telomerase products (two-step cycle of 30 s at 94 °C, 30 s

at 60 °C for 27 cycles). Control experiments indicated that PNA-peptide concentrations at or below 8.0 μM did not inhibit the PCR reaction. Samples were analyzed using 12% denaturing polyacrylamide gel electrophoresis and quantitated using a Molecular Dynamics phosphorimager. The telomerase activity and extent of inhibition was determined as described by Hamilton *et al.*[16] and some typical data is shown in Figure 2. Conjugation of cationic peptides to PNA resulted in significantly improved inhibition of telomerase relative to PNA alone (Table 2). The best inhibitor, PNA-Cys(Arg)$_5$Ala, provided an IC_{50} of 0.14 μM as compared to an IC_{50} value of 1.16 μM for the PNA alone. The cationic peptide alone, Ala(Orn)$_5$Cys showed little inhibition even at 8.0 μM (~ 20%) indicating that the the PNA-peptide conjugate was required for strong inhibition. A PNA-peptide containing a single mismatch sequence, TAAGG-Cys(Orn)$_5$Ala, also inhibited poorly, Table 2, consistent with previous results from the Corey lab.[16]

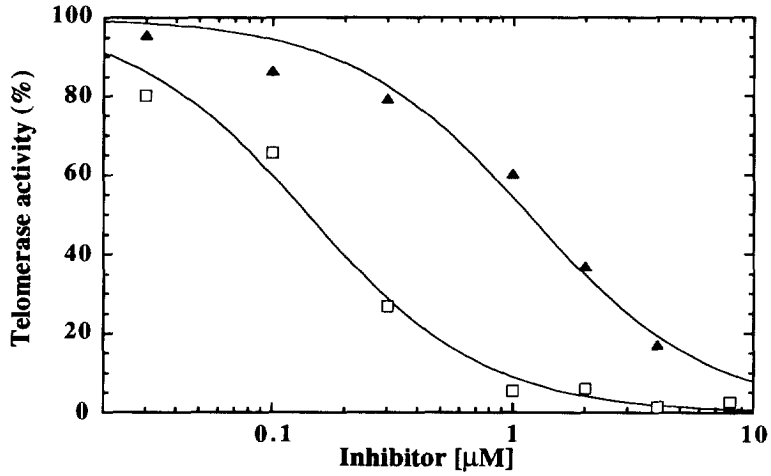


Figure 2. Telomerase activity as a function of varying concentrations of PNA (▲), or PNA-(arg)5 (□). The program Kaleidagraph© was used to fit the data to the equation y = 100/(1 + (I/IC50). The error in the fit was ± 0.05.

Melting temperature analysis of PNA-peptide conjugates

Each PNA-peptide conjugate was analysed for its binding affinity to the complementary DNA 13 mer (sequence 5'-CCCTAACTGAGAA-3') by UV melting. The Tm results are shown in Table 2. The *N*-maleimide PNA had a Tm value of 32°C, close to the Tm of the parent PNA (34 °C) indicating that the presence of the SMCC linker on the PNA doesn't significantly perturb hybridisation. The three PNA-cationic peptide conjugates were observed to have enhanced binding affinity for the 13 mer, which is in accord with the observation that nucleic acid-cationic peptide conjugates do, in general, tend to have enhanced affinities for complementary nucleic acid.[13,18]

Table 2. Melting temperature values of the precursor PNA, the *N*-maleimide PNA and PNA-cationic peptide conjugates with a target 13 mer deoxyoligonucleotide.

PNA and derivatives	Tm value (°C)	IC_{50} (μM)
H_2N-TAGGG-CO_2H	34	1.16
SMCC-TAGGG-CO_2H	32	-
AKKKKKC-TAGGG	40	0.29
AOOOOOC-TAGGG	41	0.45
ARRRRRC-TAGGG	42	0.14
AOOOOOC-TAAGG	-[a]	> 8.0[b]
AOOOOOC	-[a]	> 8.0[b]

The melting analysis was carried out using solutions of 2 μM concentration of precursor PNA oligonucleotides or peptide-PNA conjugates and 13 mer target strand 5'-CCCTAACTGAGAA-3', in 10 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl and 0.1 mM EDTA. The absorbance of the solutions at 260 nm was monitored over a temperature range of 5-80 °C at a heating rate of 1 °C/ min.

[a]Tm was not measurable.

[b]Inhibition at 8.0 μM was ~ 20 % suggesting that the IC_{50} value for each of these compounds was greater than 8.0 μM.

These studies suggest that it is possible to enhance the inhibitory properties of a relatively short PNA oligomer by conjugation to a peptide fragment that may form additional binding interactions with the ribonucleoprotein target. Assuming that the PNA portion of the inhibitor binds to the RNA template,[5] the peptide portion is likely to interact with either protein or RNA towards the anchor site region of telomerase. Both pentamer PNAs, and their peptide conjugates, were used in these inhibition studies at concentrations well below their solubility limits, therefore we rule out changes in solubility as the cause for inhibition patterns.[19] The correlation of IC_{50} values with melting temperature studies suggest that the cationic peptides are probably contributing to inhibition by increasing the stability of the complexes formed with the telomeric RNA template. However, it has been shown that cationic peptides dramatically enhance the kinetics of nucleic acid hybridisation,[20] therefore we cannot rule out this mode of enhancement. Thus, interactions other than base pairing can be exploited to enhance activity of telomerase inhibitors near the active site. We will now be going on to explore the possibility of other interactions with protein using a library approach to generate PNA-peptide conjugates.[13] Issues such as optimisation of linker and conjugation at other sites on the PNA scaffold will be considered.

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