Inhibition of telomerase activity by a cell-penetrating peptide nucleic acid construct in human melanoma cells

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Abstract We investigated the effect of two peptide nucleic acids (PNAs), which are complementary to the RNA component of human telomerase, on the catalytic activity of the enzyme. PNAs induced a dose-dependent reduction of telomerase activity in cell extracts from human melanoma cell lines and surgical specimens. To down-regulate telomerase in intact cells, we generated a chimeric molecule synthesized by coupling the 13-mer PNA to the Antennapedia peptide. The PNA construct induced a dose- and time-dependent inhibition of telomerase activity. However, a 20-day exposure to the PNA construct only caused a slight increase in melanoma cell doubling time and failed to induce any telomere shortening.

Key words: Peptide nucleic acid; Telomerase; Melanoma cell; Telomere

1. Introduction

Human telomerase is a ribonucleoprotein enzyme complex that compensates for telomeric sequence loss, due to the end replication problem [1] by adding 5′-TTAGGG-3′ repeats at the end of linear chromosomes [2]. The essential role of telomerase is to ensure chromosome integrity [3]. Its almost ubiquitous expression in human tumors, and not in somatic cells [4], has supported the hypothesis that the enzyme is involved in cellular immortality and carcinogenesis. The notion that telomere maintenance is essential for the formation of human tumor cells has recently been supported by the findings of Hahn et al. [5], who demonstrated that ectopic expression of the telomerase catalytic subunit in combination with two oncogenes (SV 40 large-T and H-ras) resulted in direct tumorigenic conversion of normal human epithelial and fibroblast cells.

Telomerase is considered as a potential candidate for new targeted therapies with putatively low side effects. In fact, human germ and stem cells that also express telomerase have long telomeres and would be affected by a telomerase inhibitor later than cancer cells.

The RNA component of human telomerase acts as a template for the addition of new telomeric repeats by the reverse transcriptase domain [6] of the enzyme. The inherent accessibility to incoming nucleic acids makes telomerase an ideal target for antisense oligonucleotides. Inhibition of telomerase activity by conventional DNA oligomers [7–9] and phosphorothioate-modified DNA [10] has been reported. However, the poor sequence selectivity observed with such compounds [10] has lead to the search for second generation oligonucleotides able to bind complementary sequences with very high affinity. In this context, an efficient inhibition of telomerase activity has been obtained by 2′-*O*-methyl-RNAs [11] and peptide nucleic acids (PNAs) [10]. Specifically, PNAs are DNA mimics in which the sugar-phosphate backbone has been replaced by a backbone based on amino acids. The uncharged nature of the PNA internucleotide linkage increases the stability of the PNA/DNA (or PNA/RNA) duplex and affords greater resistance to protease and nuclease [12–14] than unmodified DNA oligomers.

Due to the inefficient cellular uptake of oligomers, methods for enhancing their intracellular delivery have been developed, mainly through the use of cationic lipids [15]. PNAs are not taken up spontaneously by cells. Moreover, because of their neutral backbone, PNAs lack the ability to interact with cationic lipids. Cell-penetrating PNA constructs have been generated [16] by coupling PNA oligomers with cellular transporter peptides, which are able to enter cells in an energy- and receptor-independent manner. The 27-amino acid long peptide transportan [16] and the 16-amino acid long peptide corresponding to the third helix of the DNA binding domain of Antennapedia have been used. Specifically, the homeodomain of the transcription factor Antennapedia is able to translocate through biological membranes, and such an internalization does not depend on classical endocytosis [17].

In the present study, we evaluated the inhibitory effect on telomerase activity of 11-mer and 13-mer PNAs complementary to the template region of human telomerase in extracts from human melanoma cell lines and surgical specimens. We also compared inhibition by the 13-mer PNA with that produced by a phosphorothioate oligodeoxynucleotide of analogous sequence. Moreover, with the aim to down-regulate telomerase activity in intact cells, a chimeric molecule, synthesized by coupling the 13-mer PNA to the Antennapedia cell-penetrating peptide, was delivered to growing melanoma cells and evaluated for its potential anti-telomerase activity as well as for its interference with cell proliferation. The possibility to inhibit melanoma cell growth through a negative interference with telomerase appears reasonable since telomerase reactivation has been detected in a high percentage of melanoma lesions [18]. Moreover, melanoma is highly refrac-

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Abbreviations: PNA, peptide nucleic acid; TRAP, telomeric repeat amplification protocol; ITAS, internal TRAP assay standard

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tory to all conventional anti-cancer treatments and thus represents a good model to test innovative therapeutic approaches.

2. Materials and methods

2.1. Synthesis of oligomers, peptide and PNA constructs

PNA monomers were obtained from PerSeptive Biosystems (Framingham, MA, USA). 11-mer (5′-GGGTTAGACAA-3′) and 13-mer (5′-TAGGGTTAGACAA-3′) PNA oligomers were synthesized using the f-moc peptide chemistry according to protocols developed by the manufacturer (PerSeptive). A non-complementary 13-mer (5′-TGTAAGGACTAG) PNA was also synthesized. The phosphorothioate oligodeoxynucleotide (5′-TAGGGTTAGACAA-3′) was synthesized using thioate chemistry and gel-filtered with a Sephadex column. The Antennapedia peptide (QIKIWFQNRRMKWKK, pAntennapedia) was assembled using the f-moc strategy of solid phase peptide synthesis. During the automated synthesis of 13-mer PNA, and with the final purpose to conjugate it with the Antennapedia peptide, two linkers were added to the amino-terminus of the PNA. Biotinylated 13-mer PNA and biotinylated Antennapedia peptide were also prepared. The scheme of synthesis of the cell-penetrating PNA construct was the following: Cys-pAntennapedia+Cys-2 linkers-13-mer PNA (or biotinyl-Cys-2 linkers-13-mer PNA) → pAntennapedia-Cys-S-S-Cys-2 linkers-13-mer PNA (or pAntennapedia-Cys-S-S-(biotinyl)-Cys-2 linkers-13-mer PNA).

Oligomers, peptides and PNA constructs were purified by high-performance liquid chromatography and analyzed by mass spectrometry.

2.2. Tumor material

Two cell lines (JR8 and M14) and four surgical specimens of human cutaneous melanoma were used in the study. The biological characteristics of the cell lines have previously been reported [19]. JR8 and M14 cells were maintained in the logarithmic growth phase at 37°C in a 5% CO_2 humidified atmosphere using RPMI 1640 (Biowhittaker, Vervires, Belgium) supplemented with 10% fetal calf serum, 2 mM L-glutamine and 0.25% gentamicin.

2.3. Telomerase activity detection assay

Cell extracts were obtained as previously described [20]. Telomerase activity was measured by the telomeric repeat amplification protocol (TRAP) [4]. After extension of the substrate by telomerase, the products were amplified by PCR in the presence of ^{32}P-end-labeled TS primer and resolved in 10% polyacrylamide gels. Each reaction product was amplified in the presence of a 36-bp internal TRAP assay standard (ITAS), and each sample extract was tested for RNase sensitivity. A TSR8 quantitation standard (which serves as a standard to estimate the amount of product extended by telomerase in a given extract) was included for each set of TRAP assays. Quantitative analysis was performed with the Image-QuanT software (Molecular Dynamics, Sunnyvale, CA, USA), which allowed densitometric evaluation of the digitized image. Telomerase activity was quantified by measuring the signal of telomerase ladder bands, and the relative telomerase activity was calculated as the ratio to the internal standard using the following formula:

$$\text{relative telomerase activity} : ((X-X_0)/C)\times((R-R_0)/Cr)^{-1}$$

where X is the untreated sample, X_0 is the RNase-treated sample, C is the internal control of untreated samples, Cr is the internal control of TSR8, R is the TSR8 quantitation control and R_0 is the negative control.

The effect of the PNA and phosphothioate oligomers as well as of the cell-penetrating PNA construct on telomerase was expressed as the percentage inhibition of enzyme activity in samples exposed to individual oligomers compared to untreated controls. The extent of inhibition as a function of the oligomer concentration was plotted, and the graphs were utilized to derive the IC_{50} values.

2.4. Immunofluorescence assay

JR8 cells were exposed to the biotinylated PNA construct (10–50 μM) for 1 h or 4 h at 37°C. After incubation, the cells were extensively washed with ice-cold phosphate-buffered saline (PBS) and fixed in 70% ethanol for 15 min at 4°C, and non-specific binding was blocked with 5% bovine serum albumin. Control cells (grown in control medium without the biotinylated PNA construct) were processed in parallel. The internalized biotinylated PNA construct was detected by fluorescence microscopy using a mouse monoclonal antibody anti-biotin (1:200) (Vector Laboratories, Burlingame, CA, USA) and a secondary anti-mouse IgG–FITC conjugate (1:50) (Sigma Chemical Co., St. Louis, MO, USA).

2.5. Cell proliferation assay

After harvesting in the logarithmic growth phase, cells were seeded in 6-well plates. Cell-penetrating PNA construct (10–50 μM) was added to the culture medium, and cells were incubated at 37°C in a 5% CO_2 humidified atmosphere for 6 or 20 days. At the end of treatment, cells were washed with PBS, then trypsinized and counted in a particle counter (Coulter Counter, Coulter Electronics, Luton, UK). The results were expressed as the number of cells in treated samples compared to control samples. For each experimental point, a sample of cells was stained with propidium iodide and analyzed under fluorescence microscopy to evaluate the presence of cells with the characteristic nuclear morphology of apoptosis.

2.6. Telomere length measurement

Total DNA was isolated using DNAzol (Life Technologies, Gaithersburg, MD, USA), digested with 40 U of *Hin*fI, and then electrophoresed on 0.8% agarose gels. Following electrophoresis, gels were denatured, neutralized, transferred to a nylon membrane and then cross-linked with UV light. The membrane was hybridized with a 5′-end [γ-^{32}P]dATP-labeled telomeric oligonucleotide probe $(TTAGGG)_4$ by a standard protocol. Filters were autoradiographed (Hyperfilm-MP; Amersham) with an intensifying screen at −80°C for 12–24 h. Autoradiographs were scanned (ScanJet IIcx/T; Hewlett Packard) and the peak TRF length was calculated as previously reported [21].

3. Results

Exposure of melanoma cell extracts to different concentrations (1–1000 nM) of 11-mer and 13-mer PNAs for 30 min at 37°C induced a dose-dependent inhibition of telomerase activity, with a progressive decline of TRAP signals starting from

Table 1
IC_{50} values for the inhibition of telomerase activity by PNA and PS oligomers in cell extracts and permeabilized cells

		IC_{50} (nM)		
		11-mer PNA	13-mer PNA	PS
Cell extracts				
Cell lines:	JR8	67 ± 6	35 ± 4	500 ± 15
	M14	95 ± 8	70 ± 7	1000 ± 30
Tumors:	ML1342	n.d.	79 ± 6	n.d.
	ML1256	n.d.	77 ± 7	n.d.
	ML1258	n.d.	100 ± 6	n.d.
	ML1259	n.d.	85 ± 4	n.d.
Permeabilized cells				
Cell line:	JR8	490 ± 29	360 ± 23	> 1000

Data represent mean values ± S.E.M. from three independent experiments. PS, phosphorothioate oligodeoxynucleotide. n.d., not determined.

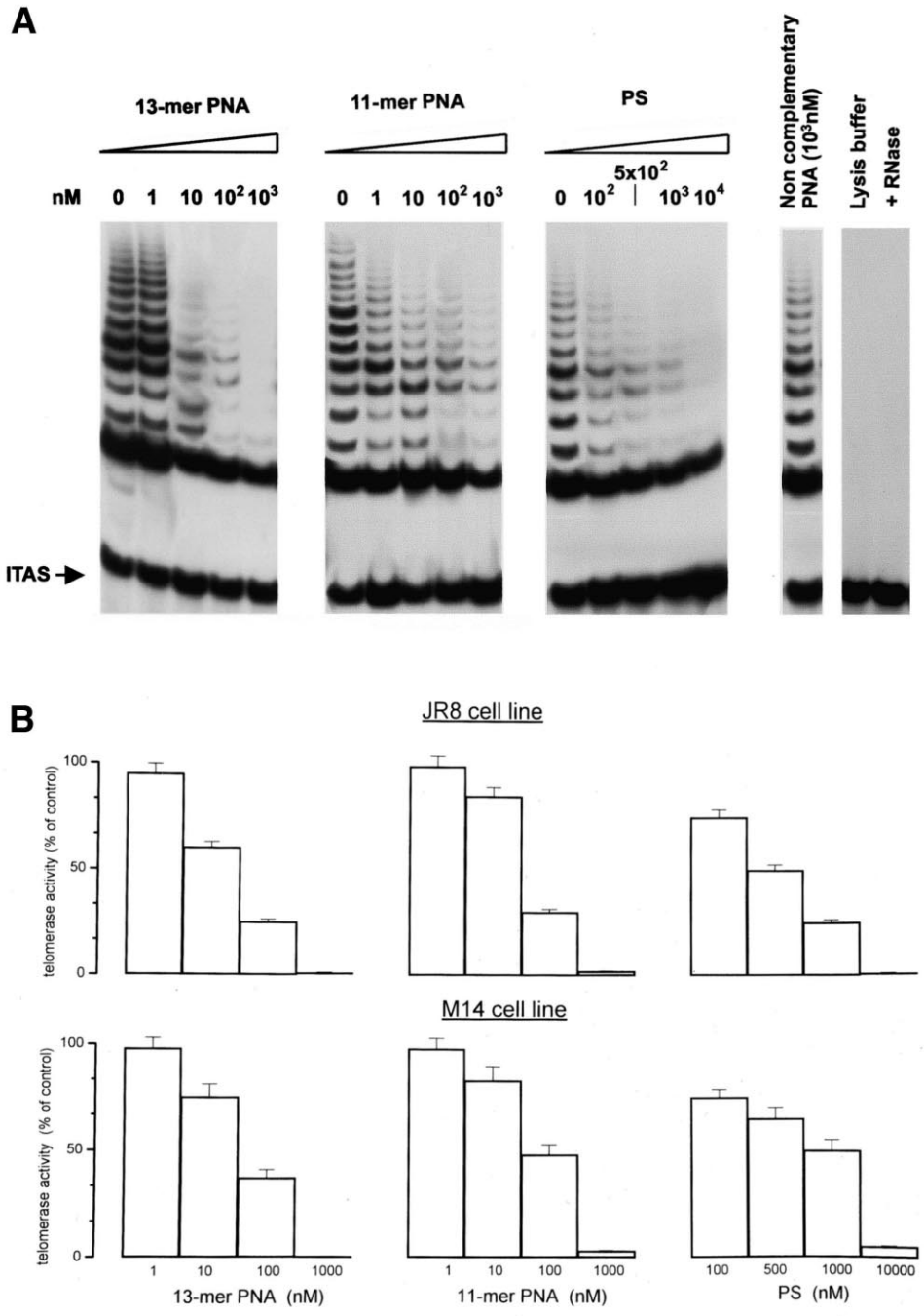

Fig. 1. (A) TRAP assay showing inhibition of telomerase activity by different concentrations of PNA and phosphorothioate (PS) oligomers in JR8 melanoma cell extract. The lane denoted Lysis buffer represents a negative control to which no cell extract was added. The lane denoted +RNase represents a negative control containing the cell extract pretreated with RNase. The location of the internal amplification standard (ITAS) is noted. (B) Quantitation of PNA and PS oligomer-mediated telomerase inhibition in JR8 and M14 melanoma cell extracts. The effect of inhibitors on telomerase is expressed as the percentage inhibition of enzyme activity compared to control. The data represent mean values ± S.E.M. from three independent experiments for each cell line.

the concentration of 10 nM. Results of a representative experiment on JR8 cell extract are shown in Fig. 1A. The 13-mer PNA was more efficient than the 11-mer PNA in inhibiting the enzyme (Fig. 1B), as demonstrated by the lower IC_{50} values (Table 1). Such a finding was particularly evident in the JR8 cell line ($P < 0.05$, Student's t-test for paired samples). No inhibition of telomerase activity was observed when cell extracts were incubated in the presence of the non-complementary PNA. The relative efficiency of inhibition by the 13-mer PNA was then compared to that by a phosphorothioate oligodeoxynucleotide of analogous sequence (Fig. 1A). A substantially lower effect of the phosphorothioate oligomer ($P < 0.001$) was observed (Fig. 1B and Table 1).

A dose-dependent inhibition of telomerase activity by the 13-mer PNA was also confirmed in extracts obtained from four melanoma surgical specimens (Fig. 2A,B). The 13-mer PNA IC_{50} values obtained in the different tumors ranged from 77 to 100 nM (Table 1). When applied to mildly per-

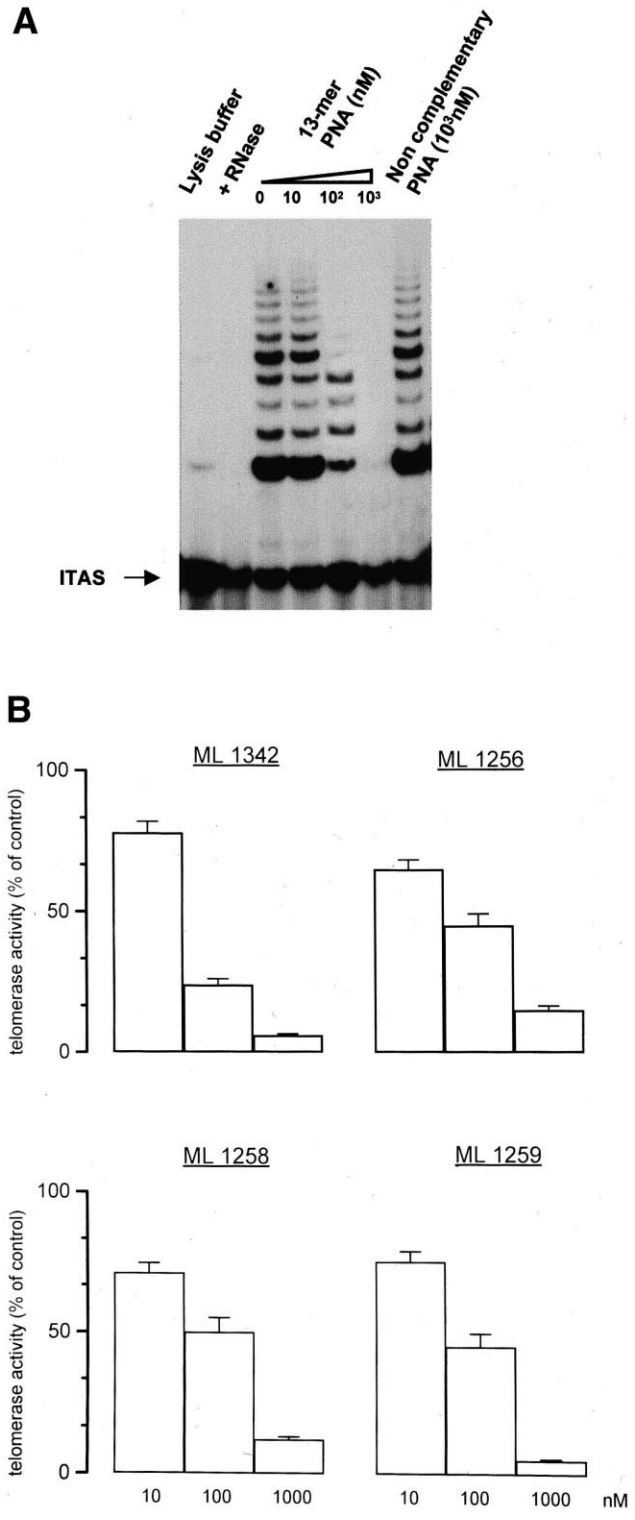

Fig. 2. (A) Inhibition of telomerase activity by different concentrations of 13-mer PNA in an extract from a melanoma surgical specimen. The lane denoted Lysis buffer represents a negative control to which no cell extract was added. The lane denoted +RNase represents a negative control containing the cell extract pretreated with RNase. The location of the internal amplification standard (ITAS) is noted. (B) Quantitation of 13-mer PNA-mediated telomerase inhibition in cell extract from four melanoma surgical specimens. The effect of 13-mer PNA on telomerase is expressed as the percentage inhibition of enzyme activity compared to control. The data represent mean values ± S.E.M. from three replicated samples for each tumor specimen.

meabilized JR8 melanoma cells, PNAs continued to inhibit telomerase activity but with IC_{50} values markedly higher than those required for inhibition in purified extracts (Table 1). Again, under such experimental conditions, the 13-mer PNA was more efficient than the 11-mer PNA as a telomerase inhibitor. As regards the phosphorothioate oligomer, the IC_{50} for the compound was not reached at any of the tested concentrations (Table 1).

For intracellular delivery to intact, growing JR8 melanoma cells, the 13-mer PNA was coupled to the Antennapedia cell-penetrating peptide. Biotin labeling of the PNA construct was instrumental to detect the compounds within cells by using an immunofluorescence method. As shown in Fig. 3, after a 4-h exposure, the biotinylated PNA construct was present and distributed homogeneously within the cells. Moreover, an increase in fluorescence signal was observed by increasing the time of exposure and the concentration of the biotinylated PNA construct (Fig. 3A,B). Absence of a significant fluorescence signal after exposure of cells to the biotinylated 13-mer PNA indicated a lack of internalization of the molecule (data not shown), whereas a strong signal was observed after a 4-h exposure to 50 μM biotinylated Antennapedia peptide (Fig. 3C). Exposure of JR8 cells to different concentrations (10–50 μM) of the PNA construct for 4 and 8 h induced a negligible effect on telomerase activity (data not shown). Conversely, a dose- and time-dependent inhibition of enzyme activity was observed after longer exposure times (Fig. 4A,B). Specifically, the mean IC_{50} value obtained for a 24-h treatment was 36 ± 6 μM, and it dropped to 7.5 ± 4 μM when the exposure time was prolonged to 144 h. No interference with telomerase activity was observed after exposure of cells to concentrations of up to 50 μM of the Antennapedia peptide for 24–144 h (Fig. 5) or of the biotinylated 13-mer PNA for 144 h (Fig. 6).

Next we assessed the effect of treatment with the cell-penetrating PNA construct (10–50 μM) on the proliferation of JR8 cells. After a 144-h exposure, a negligible effect on cell growth was induced by the PNA construct, even in the presence of a markedly reduced telomerase activity (Fig. 4). In fact, the reduction of cell number in treated samples compared to controls ranged from 1% to 8% for the different concentrations of the PNA construct. Moreover, fluorescence microscopy analysis of cells stained with propidium iodide failed to evidence cells with an apoptotic nuclear morphology. In parallel, the analysis of telomere length performed by Southern blot hybridization did not show any telomere shortening in cells treated with the PNA construct with respect to control cells (Fig. 7). When the treatment with the PNA construct (50 μM) was prolonged for 20 days, cells were still able to proliferate even though they showed a significantly ($P < 0.01$) longer doubling time than that of untreated control cells (30.3 ± 0.97 h vs. 24.0 ± 1.08 h). Moreover, fluorescence microscopy analysis revealed the presence of a small but significant percentage of apoptotic cells in treated samples (5.8 ± 1.35% of the overall cell population). However, even at this time point, no telomere shortening was observed in treated cells compared to controls (data not shown).

4. Discussion

Telomerase is potentially an important therapeutic target in the treatment of human malignancies. In fact, the differential expression of telomerase in normal and tumor cells suggests

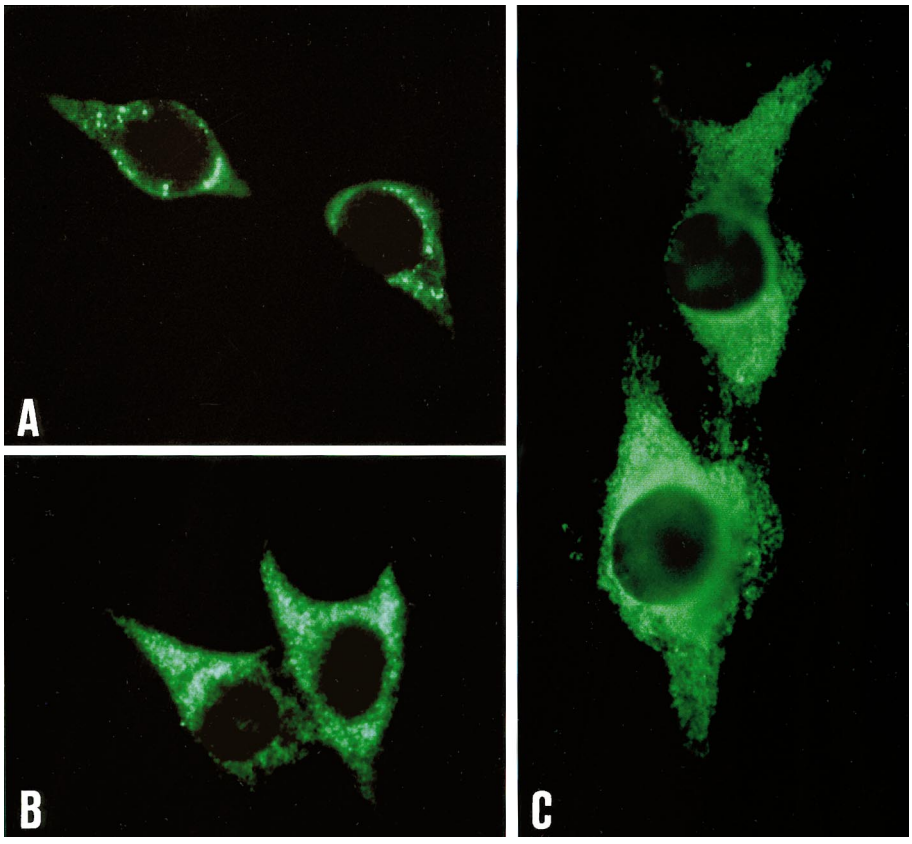

Fig. 3. Intracellular localization of the biotinylated PNA construct and biotinylated Antennapedia peptide in JR8 melanoma cells visualized by immunofluorescence. Cells were exposed to 10 μM of the biotinylated PNA construct for 1 h at 37°C (A), to 50 μM of the biotinylated PNA construct for 4 h at 37°C (B), or to 50 μM of the biotinylated Antennapedia peptide for 4 h at 37°C (C).

that telomerase inhibitors may have relative specificity for malignant cells. Since telomerase utilizes an 11-base sequence belonging to its RNA component to add telomeric repeats to the end of chromosomes, one strategy for inhibiting enzyme activity may be physical blockage of the RNA template. Moreover, due to its necessary accessibility to incoming nucleic acids, telomerase should be highly susceptible to oligonucleotide binding.

In the present study, we evaluated the ability of two PNAs, an 11-mer and a 13-mer DNA analogues designed to cover the template and the 5′-proximal region of human telomerase, to inhibit enzyme activity in extracts of cutaneous melanoma cell lines. PNAs lowered telomerase activity in a dose-dependent fashion, with IC_{50} values in the nanomolar range. Such results are in agreement with previous findings of Norton et al. [10], who demonstrated a PNA-mediated inhibition of telomerase activity in cell extracts of a human immortal breast epithelial cell line. Moreover, in a direct comparison, we observed that the 13-PNA was more potent in inhibiting telomerase activity than a phosphorothioate oligodeoxynucleotide of analogous sequence, thus supporting evidence of a higher affinity and selectivity of PNA through base pairing and not electrostatic interactions for its target RNA [22]. An inhibitory activity by the 13-mer PNA comparable to that observed on melanoma cell line extracts was also recorded on cell extracts directly obtained from four melanoma surgical specimens. Moreover, PNAs continued to inhibit telomerase activity in mildly permeabilized melanoma cells, although at concentrations higher than those required in cell extracts.

PNAs are very potent molecules in vitro but they are poorly transported across cell membranes [23–25], even though it has recently been shown that naked PNAs are taken up by human myoblasts [26]. However, in order to increase PNA uptake, methods for their intracellular delivery have been developed, mainly through conjugation with transport peptides. In particular, it has been reported that a bioconjugate, generated by coupling a PNA antisense for the translation initiation region of prepro-oxytocin mRNA with a retro–inverso delivery peptide, was efficiently internalized by cerebral cortex neurons and depressed the amount of prepro-oxytocin message in the cells [27]. Moreover, PNAs directed against inducible nitric oxide synthase mRNA, and covalently linked to a hydrophobic peptide moiety, were successfully internalized in mouse macrophages and induced a marked decrease in the catalytic activity of the target enzyme [28]. Again, a 21-mer PNA complementary to the human galanin receptor type 1 mRNA, coupled to cellular transport peptides transportan or Antennapedia, was efficiently taken up by Bowes human melanoma cells, where it blocked the expression of galanin receptors [29].

By following such a strategy, and with the final aim to down-regulate telomerase activity in growing melanoma cells, we generated a chimeric molecule by coupling the 13-mer PNA (which was selected as the best inhibitor of telomerase in melanoma cell extracts and mildly permeabilized cells) with the delivery peptide Antennapedia. The cysteine-containing transport peptide was linked by a disulfide bond to cysteine linker-extended PNA. The disulfide bond is thought to be quickly reduced in the intracellular milieu, leading to the dis-

sociation of the PNA from the carrier peptide, thereby permitting PNA to associate with target RNA. However, the PNA was extended with two linkers, in order to space out the internalization peptide from the telomerase RNA template after PNA binding in the case of a lack of disulfide bond reduction.

An inhibition of telomerase activity was observed in intact melanoma cells exposed to the cell-penetrating PNA construct, thus indicating that the chimeric molecule was taken up by cells and that the PNA was able to specifically interact with its complementary RNA sequence. Internalization of the PNA construct was also confirmed by fluorescence microscopy analysis using a biotinylated chimeric molecule. However, the inhibitory effect on telomerase activity was only appreciable at PNA concentrations much higher than those required to induce telomerase inhibition in extracts and for exposure times of at least 24 h. Such a difference could be related to multiple factors including the efficiency of PNA construct internalization as well as the inaccessibility of target RNA due to its subcellular compartmentalization and sequestration.

The inhibition of telomerase activity in cells treated with the PNA construct was not reflected by a significant decline in

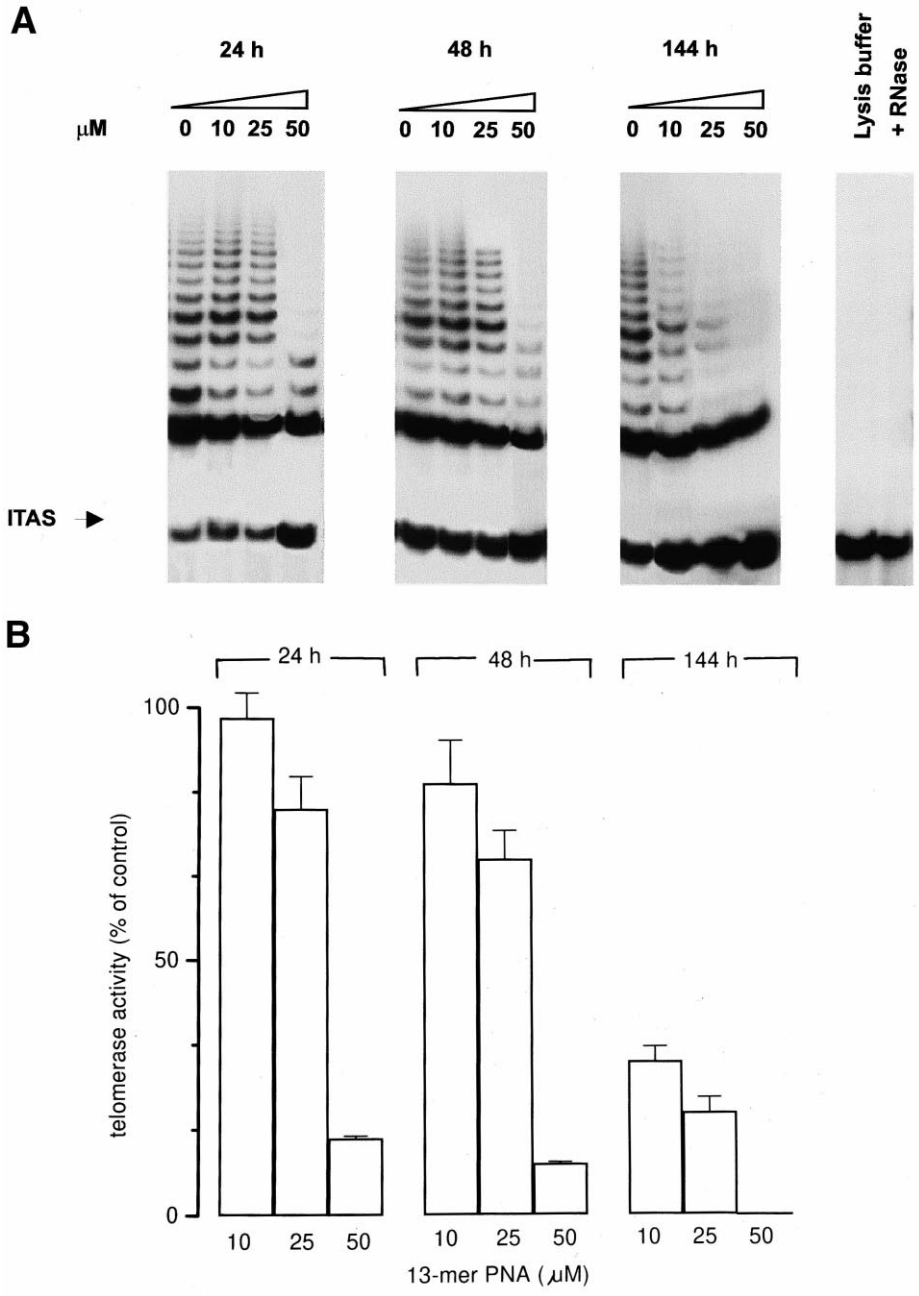

Fig. 4. (A) Inhibition of telomerase activity after exposure of JR8 growing cells to different concentrations of the cell-penetrating PNA construct for different intervals. The lane denoted Lysis buffer represents a negative control to which no cell extract was added. The lane denoted +RNase represents a negative control containing the cell extract pretreated with RNase. The location of the internal amplification standard (ITAS) is noted. (B) Quantitation of telomerase inhibition in JR8 cells after exposure to different concentrations of the cell-penetrating PNA construct for different intervals. The effect of the cell-penetrating PNA construct on telomerase is expressed as the percentage inhibition of enzyme activity compared to control. The data represent mean values ± S.E.M. from three independent experiments.

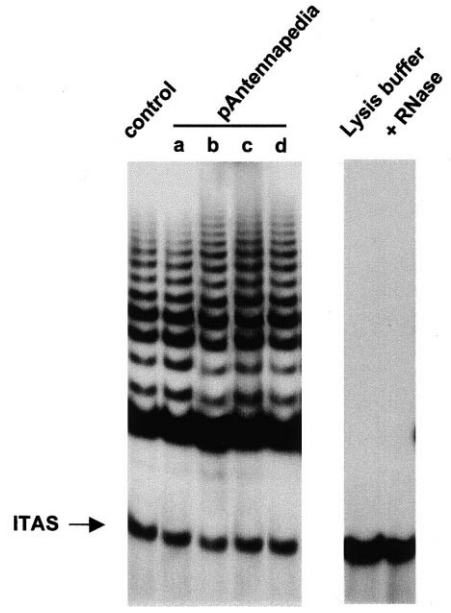

Fig. 5. Telomerase activity of JR8 growing cells after exposure to 50 μM of the Antennapedia peptide for different intervals: 24 h (lane a), 48 h (lane b), 96 h (lane c) or 144 h (lane d). The lane denoted Control represents a positive control for 100% telomerase activity to which no Antennapedia peptide was added. The lane denoted Lysis buffer represents a negative control to which no cell extract was added. The lane denoted +RNase represents a negative control containing the cell extract pretreated with RNase. The location of the internal amplification standard (ITAS) is noted.

proliferation. In fact, after 20 days of continuous treatment with the PNA construct, JR8 melanoma cells were still able to proliferate although they showed a longer doubling time than that of control cells. Moreover, after a long-term exposure to the PNA construct, we observed the presence of a small but significant percentage of cells with a nuclear apoptotic morphology, in agreement with previous reports indicating apoptosis as one of the possible pathways induced by telomerase inhibitors [30].

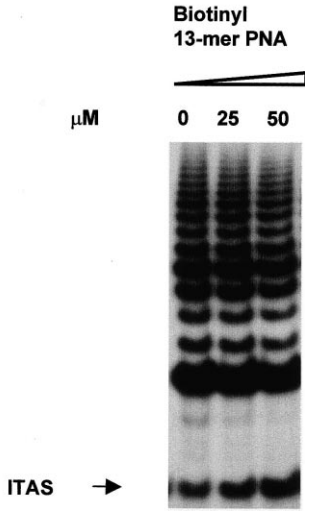

Fig. 6. Telomerase activity of JR8 growing cells after exposure to 25 or 50 μM of the biotinylated 13-mer PNA for 144 h. The location of the internal amplification standard (ITAS) is noted.

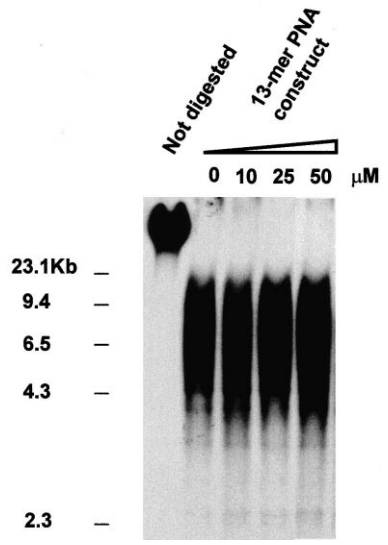

Fig. 7. Telomere length of JR8 cells as determined by Southern blot hybridization. Cells were exposed to different concentrations of the cell-penetrating PNA construct for 144 h.

In testing telomerase inhibitors, the kinetics of cell killing are difficult to predict. In fact, anti-telomerase agents might not have an immediate cytotoxic effect because cells would theoretically need to divide until their telomeres became critically short before succumbing to chromosomal instability. Such a hypothesis implies the opportunity to deliver telomerase inhibitors chronically and suggests that such inhibitors might be most effective in the setting of minimal residual disease.

In PNA-treated cells, we failed to observe any shortening of telomere length. However, it should be emphasized that in the cells, telomerase activity was markedly reduced but not completely abrogated. The lack of telomere shortening might tentatively be explained by the emergence of resistant cells through the activation of alternative lengthening of telomeres (ALT) mechanisms, which are responsible for the maintenance of telomeres and have been demonstrated to be present in a few tumors [31], even though our PNA-treated cells did not show the very long and heterogeneous telomeres typical of ALT cells [31]. Moreover, we have previously reported that, in the same melanoma cell system, the inhibition of telomerase activity by a hammerhead ribozyme targeting the RNA component of telomerase did not result in any telomere shortening [32]. Similarly, other attempts aimed to inhibit telomerase through the interference with its RNA template did not demonstrate telomere shortening [30] or cell growth arrest [33].

Overall, our results suggest that PNA oligomers, which are directed against the RNA template of telomerase, represent a new and potentially powerful method of specifically affecting enzyme activity in growing cells. Although PNAs have major advantages over other nucleotide analogues in terms of binding affinity, chemical stability and resistance to nucleases, efficient strategies for their intracellular delivery must be developed before PNAs can progress toward therapeutic use.

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References

[1] Blackburn, E. (1991) Nature 350, 569–573.
[2] Counter, C.M., Avilion, A., LeFeuvre, C.E., Stewart, C.E., Greider, C.W., Harley, C.B. and Bacchetti, S. (1992) EMBO J. 11, 1921–1929.
[3] Greider, C.W. (1996) Annu. Rev. Biochem. 65, 337–365.
[4] Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L.C., Coviello, G.M., Wright, W.E., Weinrich, S.L. and Shay, J.W. (1994) Science 266, 2011–2015.
[5] Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W. and Weinberg, R.A. (1999) Nature 400, 464–468.
[6] Nakamura, T.M., Morin, G.B., Chapman, K.B., Andrews, W.H., Lingner, J., Harley, C.B. and Cech, T.R. (1997) Science 277, 955–959.
[7] Collins, K., Kobayashi, R. and Greider, C.W. (1995) Cell 81, 677–686.
[8] Mata, J.E., Joshi, S.S., Palen, B., Pirruccello, S.J., Jackson, J.D., Elias, N., Page, T.J., Medlin, K.L. and Iversen, P.L. (1997) Toxicol. Appl. Pharm. 144, 189–197.
[9] Glukhov, A.I., Zimnik, O.V., Gordeev, S.A. and Severin, S.E. (1998) Biochem. Biophys. Res. Commun. 248, 368–371.
[10] Norton, J.C., Piatyszek, M.A., Wright, W.E., Shay, J.W. and Corey, D.R. (1996) Nat. Biotechnol. 14, 615–619.
[11] Pitts, A.E. and Corey, D.R. (1998) Proc. Natl. Acad. Sci. USA 95, 11549–11554.
[12] Nielsen, P.E., Egholm, M. and Buchardt, O. (1994) Gene 149, 139–145.
[13] Demidov, V.V., Potaman, V.N., Frank-Kamenetskii, M.D., Egholm, M., Buchardt, O., Sonnichsen, S.H. and Nielsen, P.E. (1994) Biochem. Pharmacol. 48, 1310–1313.
[14] Lomakin, A. and Frank-Kamenetskii, M.D. (1998) J. Mol. Biol. 276, 57–70.
[15] Lewis, J.G., Lin, K.Y., Kothavale, W.M., Matteucci, M.D., DePrince, R.B., Mook Jr., R.A. and Hendren, R.W. (1996) Proc. Natl. Acad. Sci. USA 93, 3176–3181.
[16] Pooga, M., Hallbrink, M., Zorko, M. and Langel, U. (1998) FASEB J. 12, 67–77.
[17] Derossi, D., Calvet, S., Trembleau, A., Brunissen, A., Chassaing, G. and Prochiantz, A. (1996) J. Biol. Chem. 271, 18188–18193.
[18] Taylor, R.S., Ramirez, R.D., Ogoshi, M., Chaffins, M., Piatyszek, M.A. and Shay, J.W. (1996) J. Invest. Dermatol. 106, 759–766.
[19] Badaracco, G., Corsi, A., Maisto, A., Natali, P.G., Starace, G. and Zupi, G. (1981) Cytometry 2, 63–69.
[20] Villa, R., Zaffaroni, N., Folini, M., Martelli, G., De Palo, G., Daidone, M.G. and Silvestrini, R. (1998) J. Natl. Cancer Inst. 90, 537–539.
[21] Mehle, C., Ljungberg, B. and Ross, G. (1994) Cancer Res. 54, 236–241.
[22] Egholm, M., Buchardt, O., Christensen, L., Beherens, C., Freier, S.M., Driver, D.A., Berg, R.H., Kim, S.K. and Norden, B. (1993) Nature 365, 566–568.
[23] Buchardt, O., Egholm, M., Berg, R.H. and Nielsen, P.E. (1993) Trends Biotechnol. 11, 384–386.
[24] Wittung, P., Kajanus, J., Edwards, K., Nielsen, P., Norden, B. and Malmstom, B.G. (1995) FEBS Lett. 375, 27–29.
[25] Gray, G.D., Basu, S. and Wickstrom, E. (1997) Biochem. Pharmacol. 53, 1465–1476.
[26] Taylor, R.W., Chinnery, P.F., Turnbull, D.M. and Lightowlers, R.N. (1997) Nat. Genet. 15, 212–215.
[27] Aldrian-Herrada, G., Desarmmenien, M.G., Orcel, H., Boissin-Agasse, L., Mery, J., Brugidou, J. and Rabie, A. (1998) Nucleic Acids Res. 26, 4910–4916.
[28] Scarfis, S., Giovine, M., Gasparini, A., Damonte, G., Millo, E., Pazzolini, M. and Benatti, U. (1998) FEBS Lett. 451, 264–268.
[29] Pooga, M., Soomets, U., Hallbrink, M., Valkna, A., Saar, K., Rezaei, K., Kahl, U., Hao, J., Wiesenfeld-Hallin, Z., Hokfelt, T. and Langel, U. (1998) Nat. Biotechnol. 16, 857–861.
[30] Kondo, S., Tanaka, Y., Kondo, Y., Hitomi, M., Barnett, G.H., Ishizaka, Y., Liu, J., Haqqi, T., Nishiyama, A., Villeponteau, B., Cowell, J.K. and Barna, B.P. (1998) FASEB J. 12, 801–811.
[31] Bryan, T.M., Englezou, A., Dalla Pozza, L. and Reddel, R.R. (1997) Nat. Med. 3, 1271–1274.
[32] Folini, M., Colella, G., Villa, R., Lualdi, S., Daidone, M.G. and Zaffaroni, N. (2000) J. Invest. Dermatol. 114, 259–267.
[33] Yokoyama, Y., Takahashi, Y., Shinohara, A., Wan, X., Niwwa, K. and Tamay, A. (1998) Cancer Res. 59, 5406–5410.