Photochemically enhanced delivery of a cell-penetrating peptide nucleic acid conjugate targeting human telomerase reverse transcriptase: effects on telomere status and proliferative potential of human prostate cancer cells

M. Folini*, R. Bandiera*, E. Millo†, P. Gandellini*, G. Sozzi*,
P. Gasparini*, N. Longoni*, M. Binda*, M. G. Daidone*,
K. Berg‡ and N. Zaffaroni*

*Department of Experimental Oncology, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy, †Department of Experimental Medicine, Biochemistry section, Center of Excellence for Biomedical Research, University of Genoa, Genoa, Italy, and ‡Department of Radiation Biology, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo, Norway

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Abstract. *Objectives:* Peptide nucleic acids (PNAs) are DNA mimics that have been demonstrated to be efficient antisense/antigene tools in cell-free systems. However, their potential as *in vivo* regulators of gene expression has been hampered by their poor uptake by living cells, and strategies need to be developed for their intracellular delivery. This study has aimed to demonstrate the possibility (i) of efficiently delivering a PNA, which targets mRNA of the catalytic component of human telomerase reverse transcriptase (hTERT), into DU145 prostate cancer cells through a combined approach based on conjugation of the PNA to Tat internalizing peptide (hTERT-PNA-Tat) and subsequent photochemical internalization, and (ii) to interfere with telomerase function. *Materials and methods:* Treated cells were analysed for telomerase activity, hTERT expression, growth rate, ability to undergo apoptosis and telomere status. *Results:* After exposure to light, DU145 cells treated with hTERT-PNA-Tat and the photosensitiser TPPS_{2a} showed dose-dependent inhibition of telomerase activity, which was accompanied by marked reduction of hTERT protein expression. A dose-dependent decline in DU145 cell population growth and induction of caspase-dependent apoptosis were also observed from 48 h after treatment. Such an antiproliferative effect was associated with the presence of telomeric dysfunction, as revealed by cytogenetic analysis, in the absence of telomere shrinkage, and with induction of DNA damage response as suggested by the increased expression of γ -H2AX. *Conclusions:* Our results (i) indicate

M. Folini and R. Bandiera contributed equally to the work.

Correspondence: Nadia Zaffaroni, Department of Experimental Oncology, Unit 10, Fondazione IRCCS Istituto Nazionale dei Tumori, 20133 Milan, Italy. Tel.: +39.02.23903260; Fax: +39.02.23903052; E-mail: nadia.zaffaroni@istitutotumori.mi.it

photochemical internalization as an efficient approach for intracellular delivery of chimaeric PNAs, and (ii) corroborate earlier evidence suggesting pro-survival and anti-apoptotic roles of hTERT, which are independent of its ability to maintain telomere length.

INTRODUCTION

Human telomeres are specialized structures composed of repetitive DNA sequences and associated proteins that cap chromosome ends and protect them from degradation and recombination processes, which would counteract genomic stability (de Lange 2002). In normal cells, telomeric repeats are progressively eroded at each round of cell division as a consequence of incomplete DNA lagging-strand replication (Levy *et al.* 1992), which results in critically short telomeres that lead to replicative senescence and ultimately cell death (Hayflick 2000). To compensate for telomere attrition and to bypass replicative senescence, most tumour cells reactivate telomerase, an RNA-dependent DNA polymerase that is able to maintain telomere length (Autexier & Lue 2006). The main core of telomerase consists of a ubiquitously expressed RNA template component, human telomerase RNA (hTR) (Hiyama & Hiyama 2002), and a catalytic retrotranscriptase subunit, human telomerase reverse transcriptase (hTERT), which is the limiting factor for the enzyme's activity (Janknecht 2004). It has been recently reported that hTERT plays a role in protection of genome stability by contributing to telomere capping (Chan & Blackburn 2002) and chromatin resetting during DNA replication (Masutomi *et al.* 2005). In addition, hTERT was found to be able to cross-link telomeres and enhance genomic stability and DNA repair (Sharma *et al.* 2003) as well as to maintain tumour cell survival and proliferation (Rahman *et al.* 2005) *via* enzymatic activity-independent mechanisms such as intermolecular interactions involving p53 and poly(ADP-ribose) polymerase (Cao *et al.* 2002). Based on these findings, telomerase has been proposed as a promising selective target for the development of new anticancer therapies, and a variety of inhibitory strategies has been successfully developed (Olaussen *et al.* 2006) including those relying on the use of conventional or modified antisense oligonucleotides, ribozymes and siRNAs, to target either the hTR or the hTERT component (Folini & Zaffaroni 2005).

Peptide nucleic acids (PNAs) belong to the third generation of antisense oligonucleotides and are DNA mimics in which a pseudopeptide backbone composed of N-(2-aminoethyl) glycine units replaces the phosphate backbone (Hyrup & Nielsen 1996). Owing to their flexible and neutral backbone, PNAs have excellent hybridization properties and are extremely stable in biological systems, due to their resistance to nucleases and peptidases (Koppelhus & Nielsen 2003). However, although PNAs have been demonstrated to be efficient antisense/antigene tools in cell-free systems, their potential as *in vivo* regulators of gene expression has been hampered by their poor uptake by living cells (Koppelhus & Nielsen 2003). A variety of approaches have therefore been developed for intracellular delivery of such molecules (Koppelhus & Nielsen 2003). In this context, cell-penetrating PNA conjugates have been generated by coupling PNAs with naturally occurring peptides, which have the capacity to transport molecules across the biological membranes (Vives 2005). It has recently been found that cellular uptake of several cell-penetrating peptide conjugates occurs predominantly by an endocytotic pathway and that trapping of such conjugates within the endosomal/lysosomal compartment is one of the major rate-limiting steps of their intracellular delivery (Console *et al.* 2003; Richard *et al.* 2003; Vives 2003; Vives *et al.* 2003). Various strategies have been proposed to enhance cellular delivery of cell-penetrating PNA conjugates. One of these strategies is photochemical internalization (PCI),

Figure 1. Schematic representation of hTERT-PNA-Tat and the photochemical internalization approach. Representation of the combined approach employed to deliver PNA targeting hTERT into DU145 cells: schematic structure of hTERT-PNA conjugated to the Tat-internalizing peptide along with a draft of the photochemical internalization (PCI) technique is depicted. Right panel summarizes principle of PCI: (I) endocytosis of the photosensitizer (S) and the therapeutic molecule (e.g. PNA); (II) localization of photosensitizer and the therapeutic molecule in the same endocytic vesicles; (III) rupture of endosomal membrane upon light exposure and subsequent release of the therapeutic molecule into the cytosol.

a technique that relies on the properties of photosensitive molecules (photosensitisers) to allow light-induced permeabilization of endocytic vesicles, leading to the release of endocytosed macromolecules into the cytoplasm (Hogset *et al.* 2004).

In this study, we have delivered a 15-mer PNA targeting hTERT efficiently to DU145 human prostate cancer cells, by means of a combined approach based on conjugation of the PNA to HIV1-Tat internalizing peptide and subsequent PCI of the conjugate (Fig. 1); in addition, we have extensively characterized the effects of PNA-mediated down-regulation of hTERT on telomerase activity, telomere status and cell proliferative potential.

MATERIALS AND METHODS

Cell lines and culture conditions

The DU145 androgen-independent human prostate adenocarcinoma cell line (American Type Culture Collection, Rockville, MD, USA), the U2-OS hTERT-negative human osteogenic sarcoma cell line and the U2-OS/hTERT cell line (kindly provided by R. Weinberg, Whitehead Institute for Biomedical Research, Cambridge, MA, USA), obtained by transduction of the

U2-OS human osteogenic sarcoma cell line with hTERT cDNA (Vonderheide *et al.* 1999), were used in this study. DU145 and U2-OS cells were grown in Roswell Park Memorial Institute 1640 and McCoy's 5A Media, respectively, supplemented with 10% foetal calf serum and 0.1% gentamycin. Cells were maintained as a monolayer in their logarithmic growth phase at 37 °C in a 5% CO₂ humidified atmosphere.

Chemical reagents and peptide nucleic acid synthesis

Photosensitisers TPPS_{2a} (meso-tetraphenylporphine with two sulfonate groups on adjacent phenyl rings) and AlPcS_{2a} (aluminium phthalocyanine with two sulfonate groups on adjacent phthalates) were kindly provided by PCI Biotech (Oslo, Norway) as a solution of 0.35 mg/mL in dimethyl sulfoxide and were stored at -20 °C in the dark, until use. A 15-mer antisense PNA (NH₂-CCAGCCGCCAGCCCT-CONH₂), directed against hTERT mRNA (nucleotides 156–170, GeneBank accession no. AF015950) was synthesized and was modified chemically by introduction of a 9-amino-acid-long peptide derived from the internalizing domain of HIV1-Tat protein (RKKRRQRRR) (hTERT-PNA-Tat). The PNA chimaera was manually synthesized using the standard method of solid-phase peptide synthesis, which follows tert-butoxycarbonyl (Boc) strategy (Christensen *et al.* 1995) with minor modifications (Chiarantini *et al.* 2006). Similarly, the same PNA sequence was labelled by direct coupling of rhodamine B on the solid support to the amino terminal of the protected PNA sequences. In addition, the same unconjugated 15-mer PNA sequence (uPNA) and a control PNA (sPNA), which does not have homology with other sequences in the human genome, were synthesized and were used throughout the study. These compounds were purified by reversed-phase high-performance chromatography on a Shimadzu LC-9A preparative HPLC, equipped with a C18 RP Luna column (21.20 × 250 mm, Phenomenex, Torrance, CA, USA). Molecular weights were then confirmed by electrospray ion trap mass spectrometry (ESI-TRAP-MS). PNAs were dissolved in sterile water and were stored at -20 °C.

Fluorescence microscopy

For internalization and relocation experiments, DU145 cells were seeded at a density of 1×10^5 cells per dish in Falcon 3001 dishes. After 24-h incubation at 37 °C in humidified atmosphere, cells were treated for 18 h with 5 µg/mL AlPcS_{2a} and 2 µM hTERT-PNA. Cells were then washed three times with 1 mL complete medium, were incubated for 4 h in fresh medium and were subsequently washed three times with phosphate-buffered saline (PBS) before being exposed to microscope light (Zeiss Axioplan fluorescence and phase-contrast microscope, Oberkochen, Germany) filtered through a 360–390-interference filter for 90 s. One minute later, fluorescence from rhodamine-labelled PNA was detected with a 546-nm band-pass excitation filter, a 580-nm beam splitter and a 590-nm long-pass emission filter. To cut off the fluorescence signal due to excitation of the photosensitizer, a 610–650-nm band-pass filter was also used on the emission side when fluorescence from rhodamine B was detected. Photographs from each sample were taken by means of a cooled charge-coupled device camera (TE2; Astromed 3200, Cambridge, UK).

Photochemical internalization

Cells were seeded at the appropriate density (DU145, 7.5×10^4 cells/well; U2-OS, 5×10^4 cells/well; U2-OS/hTERT, 8×10^4 cells/well) in six-well plates. After 24-h incubation at 37 °C in humidified atmosphere, DU145 cells were exposed for 18 h to 0.7 µg/mL TPPS_{2a} in the presence of hTERT-PNA-Tat, uPNA or sPNA. Cells were then washed three times with complete medium and were incubated for 4 h in fresh medium. Finally, cells were washed one more time and were exposed, or not, to blue light for 90 s. Light was irradiated from an appropriate lamp equipped

with four fluorescent tubes (Osram 18 W/67), provided by PCI Biotech, which emit mainly between 390 and 450 nm, with a peak of emission at around 405 nm. Fluence rate was 7 mW/cm². Following light exposure, cells were harvested and were further analysed after having been incubated at 37 °C for different time intervals, ranging from 48 to 96 h. Experimental controls were cells that had received the same treatment, but with the exception of light exposure. A similar treatment schedule was applied to U2-OS and U2-OS/hTERT cells with the exception that were exposed only to the highest concentration (2 µM) of hTERT-PNA-Tat and were collected 48 h after light exposure.

Telomerase activity detection assay

Telomerase activity was measured on 0.1 µg of protein by the telomeric-repeat amplification protocol (TRAP) (Fajkus 2006) using the TRAPeze kit (MP Biomedicals, Cambridge, UK). Each reaction product was amplified in the presence of a 36-bp internal TRAP assay standard. A TSR8 quantification standard (standard for estimation of 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 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, X₀ is the RNase-treated sample, C is the internal control of untreated samples, Cr is the internal control of TSR8, R is the TSR8 quantification control and R₀ is the negative control.

Cell growth assay

Cells exposed to photochemical treatment with hTERT-PNA-Tat, uPNA or sPNA were harvested after light exposure and were counted in a particle counter (Coulter Counter, Coulter Electronics, Luton, UK). Cell viability was determined by trypan blue dye exclusion test. Results were expressed as percentage variation in number of viable cells in treated compared to control cells.

Apoptosis analysis

Cells were fixed in pre-cooled 70% ethanol, were washed with PBS, stained with a solution containing 50 µg/mL propidium iodide, 50 mg/mL RNase and 0.05% Nonidet P40 for 30 min at 4 °C and then were analysed by a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). A minimum of 1 × 10⁴ events was measured for each sample. Presence of a sub-G₁ peak suggestive of apoptosis (Darzynkiewicz *et al.* 1997) was detected on DNA plots by CellQuest software, according to the ModFit model (Becton Dickinson). An aliquot of propidium iodide-stained cells was spotted onto glass slides and was examined under a fluorescence microscope for the presence of nuclei with apoptotic morphology. Percentages of apoptotic cells were determined by scoring at least 500 cells for each sample.

Cells were also analysed for caspase-3 catalytic activity by means of the APOPCYTO/caspase-3 assay kit (Medical & Biological Laboratories, Naka-ku Nagoya, Japan) according to the manufacturer's instructions. Briefly, total protein extracts and the specific fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA) were mixed and incubated for 1 h at 37 °C. Hydrolysis of specific substrates for caspase-3 was monitored by spectrofluorometry at 460 nm.

Telomere length measurement

Total DNA was isolated from control and from treated cells by using DNAzol (Invitrogen, Gaithersburg, MD, USA), digested with 40 U of *Hinf*I, gel-electrophoresed and transferred to a nylon membrane. The nylon filter was then hybridized with a 5'-³²P-end-labelled telomeric oligonucleotide probe (TTAGGG)₄ according to standard protocols. Filters were autoradiographed and autoradiographs were scanned (ScanJet IICx/T; Hewlett Packard, Roseville, CA, USA). Mean terminal restriction fragment (TRF) length was calculated as previously reported (Villa *et al.* 2000).

Cytogenetic analysis

To obtain metaphase spreads, cells were incubated with 0.05 µg/mL of Colcemid® (Invitrogen, San Giuliano Milanese, Milan, Italy) for 1 h. Hypotonic treatment, fixation and GTG banding of metaphase chromosomes were performed using standard methods. Frequency of telomere dysfunctions was evaluated in 100 metaphases using an Olympus BX51 microscope (Olympus, Tokyo, Japan). Metaphase preparations of cells with contrasting treatment schedules were performed simultaneously under the same experimental conditions.

Western immunoblotting

Total cellular lysates were separated on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to nitrocellulose. Filters were blocked in PBS with 5% skimmed milk and were incubated overnight with primary antibodies specific for hTERT (Rockland, Gilbertsville, PA, USA), H2AX (Abcam, Cambridge, UK) and γ-H2AX (Upstate, Lake Placid, NY, USA). Filters were then probed with horseradish peroxidase-conjugated antirabbit IgG (GE Healthcare Europe GmbH, Cologno Monzese, Milan, Italy) for 1 h. Bound antibodies were detected using the enhanced chemoluminescence Western blotting detection system (GE Healthcare Europe). Anti-β-actin monoclonal antibody (Abcam) was used on each blot to ensure equal loading of protein on the gel.

Statistical analysis

Student's *t*-test was used to analyse differences between untreated, TPPS_{2a}- and TPPS_{2a} + PNAs-treated cells in terms of telomerase activity, γ-H2AX protein expression levels, cell growth, apoptotic rate and *in vitro* catalytic activity of caspase-3. The χ^2 test was applied to cytogenetic analysis results. All tests were two-sided. *P*-values of < 0.05 were considered statistically significant.

RESULTS

Photochemically internalized hTERT-PNA-Tat inhibits telomerase activity in DU145 cells

Because naked PNAs taken up by cells are relegated to the endocytic compartment (Folini *et al.* 2003), we first investigated whether photochemical treatment could allow release of PNAs from endocytic vesicles to the cytosol in DU145 cells. For this purpose, cells were exposed to appropriately filtered microscope light for 90 s, and, after 1 min in the dark, fluorescence generated from the photosensitizer AlPcS_{2a} and rhodamine B-labelled PNA was detected. For fluorescence microscopy, AlPcS_{2a} was used because the compound has the same biological effects with respect to PCI as TPPS_{2a} but with spectral properties less overlapping those of rhodamine B than TPPS_{2a} (Folini *et al.* 2003). In keeping with our previous observation (Folini

Figure 2. Photochemical internalized hTERT-PNA-Tat inhibits telomerase activity in DU145 cells. (a) Representative telomeric repeat amplification protocol (TRAP) assays carried out 48 h after an 18-h exposure of DU145 cells to increasing concentrations of hTERT-PNA-Tat, 2 μ M sPNA or uPNA and 0.7 μ g/mL TPPS_{2a} in the absence or presence of light. (b) Quantification of telomerase activity inhibition in DU145 cells 48 h after an 18-h exposure to increasing concentrations of hTERT-PNA-Tat, 2 μ M sPNA or uPNA and 0.7 μ g/mL TPPS_{2a} in the absence or presence of light. Data are reported as the percentage of enzyme activity in treated compared to untreated samples and represent mean values $\pm$ SD of at least three independent experiments. * $P < 0.01$ as compared to cells exposed to TPPS_{2a} and light (Student's *t*-test). (c) Representative Western immunoblotting experiment showing hTERT protein expression levels in DU145 as a function of different treatment modalities. (d) Quantification of telomerase activity from time-course experiments assessed in DU145 cells as a function of the different treatment modalities. Data are reported as the percentage of enzyme activity in treated compared to untreated samples and represent mean values $\pm$ SD of at least three independent experiments. * $P < 0.01$ as compared to cells exposed to TPPS_{2a} and light (Student's *t*-test).

et al. 2003), results confirmed that light exposure induced relocalization of PNAs and photosensitiser from endocytic vesicles to cytosol (data not shown).

The ability of photochemically internalized hTERT-PNA-Tat to inhibit telomerase activity was then investigated. TRAP results obtained 48 h after treatment of DU145 cells with different concentrations of hTERT-PNA-Tat, in the presence of the photosensitiser TPPS_{2a} for 18 h and exposure to blue light for 90 s, showed dose-dependent inhibition of the enzyme's activity, which reached its maximum at 2 μ M concentration ($96.0 \pm 2.9\%$ with respect to untreated cells; $P < 0.01$) (Fig. 2a,b). Conversely, negligible changes in telomerase activity were observed in cells treated with the same concentrations of hTERT-PNA-Tat and TPPS_{2a} but not exposed to light (Fig. 2a,b), in cells treated with 2 μ M hTERT-PNA-Tat in the absence of the photosensitiser and independently of light exposure (Fig. 2a,b), as well as in cells exposed to 2 μ M sPNA regardless of photochemical internalization. Even though to a lesser extent as compared to that observed in cells exposed to photochemically internalized hTERT-PNA-Tat, a decline in telomerase activity was also observed in cells treated with TPPS_{2a} and exposed to light ($33.1 \pm 6.6\%$ with respect

to untreated cells), and in cells exposed to 2 μM of photochemically internalized uPNA ($60 \pm 5.1\%$, $P < 0.01$ compared to untreated cells) (Fig. 2a,b). However, Western blot analysis of hTERT expression carried out 48 h after photochemical treatment showed no changes in protein abundance in cells treated with TPPS_{2a} and exposed to light, and a modest decrease of hTERT expression levels in cells exposed to 2 μM of photochemically internalized uPNA. In contrast, marked down-regulation of hTERT protein was appreciable in cells treated with 2 μM hTERT-PNA-Tat and TPPS_{2a} and exposed to light (Fig. 2c).

In accordance with the high biostability of PNAs, we also found that inhibition of telomerase activity in cells exposed to 2 μM photochemically internalized hTERT-PNA-Tat was persistent with time and was still appreciable, although to a lesser extent, at 96 h ($65 \pm 8\%$ with respect to untreated cells; $P < 0.01$). Conversely, no appreciable decline in the enzyme's activity was detectable at this time point in cells treated with TPPS_{2a} and exposed to light as well as in cells treated with 2 μM hTERT-PNA-Tat and TPPS_{2a} without light exposure (Fig. 2d).

Photochemically internalized hTERT-PNA-Tat impairs DU145 cell growth and induces apoptosis

To investigate whether PNA-mediated targeting of hTERT affects the proliferative potential of DU145 cells, cell population growth was evaluated 48 h after photochemical treatment with PNAs. Specifically, proliferation was found to be reduced in a dose-dependent fashion in cells exposed to different concentrations of photochemically internalized hTERT-PNA-Tat, and this reduction was at its maximum at a concentration of 2 μM ($80.3 \pm 2.2\%$ with respect to untreated cells; $P < 0.001$) (Fig. 3a). Although to a lesser extent, a decline of cell growth was also observed in DU145 cells exposed to 2 μM of photochemically internalized uPNA ($55 \pm 12.9\%$ with respect to untreated cells, data not shown). Conversely, no significant inhibition of cell population growth was found in cells that had received the same treatment with the exception of light exposure, in cells treated with TPPS_{2a} alone and exposed to light (Fig. 3a), as well as in cells exposed to 2 μM of photochemically internalized sPNA (data not shown), suggesting that inhibition of DU145 cell population growth was specifically attributable to hTERT down-regulation. Moreover, time-course experiments indicated that the growth of cells exposed to 2 μM of photochemically internalized hTERT-PNA-Tat remained significantly inhibited compared to that observed up to 96 h in cells treated with hTERT-PNA-Tat and exposed to light in the absence of the photosensitizer (Fig. 3b).

To further verify specificity of the effects induced by hTERT-PNA-Tat on telomerase activity and cell population growth, we took advantage of the availability of telomerase-negative U2-OS osteosarcoma cell line and its U2-OS/hTERT derivative, which ectopically expresses hTERT (Vonderheide *et al.* 1999). Results of TRAP assay performed 48 h after photochemical treatment with 2 μM hTERT-PNA-Tat indicated marked inhibition of telomerase activity in U2-OS/hTERT cells (Fig. 3c). Even though U2-OS/hTERT cells were more sensitive than DU145 cells to the photochemical treatment alone ($\sim 50\%$ inhibition of cell growth with respect to untreated cells, Fig. 3c), a significant reduction of cell growth was observed in cells 48 h after photochemical treatment in the presence of 2 μM hTERT-PNA-Tat ($P < 0.05$, as compared to cells exposed to TPPS_{2a} and light). Conversely, photochemically internalized hTERT-PNA-Tat failed to significantly affect the growth of hTERT-negative U2-OS cells. In fact, as shown in Fig. 3d, a similar extent of cell growth inhibition was observed after photochemical treatment in the presence of 2 μM hTERT-PNA-Tat with respect to cells exposed to TPPS_{2a} and light.

Impairment of cell growth in DU145 cells was accompanied by induction of apoptosis (Fig. 4). Flow cytometric analysis of DNA content revealed the presence of a sub-G₁ peak, in

Figure 3. Photochemically internalized hTERT-PNA-Tat specifically impairs DU145 cell population growth.

(a) DU145 cell growth curves at 48 h after an 18-h exposure to increasing concentrations of hTERT-PNA-Tat and 0.7 $\mu\text{g/mL}$ TPPS_{2a} in the absence (Δ) or presence ($\blacktriangle$) of light. Data are reported as the percentage of cell number in treated compared to untreated samples and represent mean values $\pm$ SD from at least three independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001, as compared to cells exposed to hTERT-PNA-Tat and TPPS_{2a} in the absence of light (Student's t -test). (b) Time-course experiment showing cell population growth curves of DU145 cells exposed to light after 18-h treatment with 2 μM hTERT-PNA-Tat ($\circ$) or the combination of 2 μM hTERT-PNA-Tat and 0.7 $\mu\text{g/mL}$ TPPS_{2a} ($\blacktriangle$). Data are reported as the percentage of cell number with respect to cultures exposed to TPPS_{2a} and light and represent mean values $\pm$ SD from at least three independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001, as compared to cells exposed to hTERT-PNA-Tat and light (Student's t -test). (c) Telomerase activity and cell growth assessed 48 h after the exposure of the hTERT-expressing U2-OS osteosarcoma cell line to 2 μM hTERT-PNA-Tat and 0.7 $\mu\text{g/mL}$ TPPS_{2a} in the absence or presence of light. Data are expressed as percentage of cell number in treated compared to untreated samples and represent mean values $\pm$ SD from at least three independent experiments. * P < 0.05 as compared to cells exposed to TPPS_{2a} and light (Student's t -test). (d) hTERT-negative U2-OS osteosarcoma cell growth assessed 48 h after exposure to 2 μM hTERT-PNA-Tat and 0.7 $\mu\text{g/mL}$ TPPS_{2a} in the absence or presence of light. Data are expressed as the percentage of cell number in treated compared to untreated samples and represent mean values $\pm$ SD from at least three independent experiments.

cells exposed to 2 μM of photochemically internalized hTERT-PNA-Tat (Fig. 4a, panel *iv*) but not in untreated cells (panel *i*), in cells treated with photosensitizer in the presence of light (panel *ii*), in cells exposed to hTERT-PNA-Tat and light in the absence of the photosensitizer (panel *iii*), as well as in cells exposed to photochemically internalized uPNA (panel *v*) or sPNA (panel *vi*). Moreover, fluorescence microscopy analysis of propidium iodide-stained

Figure 4. Photochemically internalized hTERT-PNA-Tat induces apoptosis in DU145 cells. (a) Flow cytometric detection of a sub-G₁ peak 48 h after light exposure in DU145 cells untreated (i) or exposed for 18 h to 0.7 μg/mL TPPS_{2a} (ii), 2 μM hTERT-PNA-Tat (iii) and 2 μM hTERT-PNA-Tat (iv), 2 μM uPNA (v) or 2 μM sPNA (vi) in combination with 0.7 μg/mL TPPS_{2a}. Arrow indicates the sub-G₁ peak. (b) Quantification of cells with apoptotic nuclear morphology in DU145 cells 48 h after exposure to different treatment modalities. Apoptosis was assessed by fluorescence microscopy on cells stained with propidium iodide and the percentage of apoptotic cells was determined by scoring at least 500 cells in each sample. Data represent mean values ± SD of three independent experiments. **P* < 0.05 as compared to untreated cells (Student's *t*-test). (c) Caspase-3 catalytic activity was assessed by hydrolysis of fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA) in DU145 cells 48 h after exposure to different treatment modalities. Data represent mean values ± SD of three independent experiments; r.f.u., relative fluorescence unit. ***P* < 0.02 as compared to cells exposed to light only (Student's *t*-test).

cells showed significant increase in percentage of cells with apoptotic nuclear morphology in DU145 cultures exposed to 2 μ M of photochemically internalized hTERT-PNA-Tat compared to that observed in all other control samples ($< 5\%$ versus $15.0 \pm 5.0\%$, $P < 0.05$) (Fig. 4b). In addition, significant ($P < 0.02$) increase in caspase-3 catalytic activity was observed in DU145 cells exposed to photochemically internalized hTERT-PNA-Tat compared to all other cultures (Fig. 4c).

Photochemically internalized hTERT-PNA-Tat induces telomere dysfunction in the absence of telomere shortening in DU145 cells

To verify whether growth inhibitory effects on DU145 cells observed a few days after treatment with photochemically internalized hTERT-PNA-Tat are attributable to critical telomere attrition or to interference with telomere integrity, TRF and cytogenetic analyses were performed. As shown in Fig. 5a, TRF analysis, performed by Southern blotting, at 48 h, showed no appreciable difference in mean telomere length of DU145 cells exposed to photochemically internalized hTERT-PNA-Tat as compared to other controls. Cytogenetic analysis (Fig. 5b, Tables 1 and 2) revealed ~3-fold increase in frequency of telomere dysfunction including end-to-end fusions, dicentrics and ring chromosomes, in cells exposed to photochemically internalized hTERT-PNA-Tat compared to cells treated with TPPS_{2a} and exposed to light (0.41 versus 0.18; $P < 0.01$). Conversely, frequency of telomere dysfunction observed in cells exposed to photochemically internalized uPNA or sPNA was superimposable on that found in cells treated with TPPS_{2a} and exposed to light (0.17 versus 0.18). Occurrence of telomere dysfunction in cells exposed to photochemically internalized hTERT-PNA-Tat was paralleled by induction of a DNA damage response, as suggested by a significant ($P < 0.01$) increase in levels of phosphorylated H2AX (γ -H2AX) with respect to control cells. Although to a lesser extent, enhanced expression of γ -H2AX was also observed in cells exposed to photochemically internalized uPNA compared to control cells (Fig. 5c).

DISCUSSION

In the present study, we have analysed consequences of interference with telomerase function accomplished through PNA-mediated down-regulation of hTERT on proliferative potential and telomere status of DU145 human prostate cancer cells. Because low uptake of PNAs in living cells has hampered full exploitation of potential of such antigene/antisense tools, great efforts have been made to develop strategies to allow PNAs to easily cross the cell membrane and to colocalize with their specific targets (Koppelhus & Nielsen 2003). Among these strategies, PCI represents a new approach to achieve photochemically inducible release of endocytosed PNAs into the cytoplasm.

Here, PCI of a Tat-conjugated PNA-targeting hTERT was pursued to inhibit telomerase in DU145 human prostate cancer cells. We demonstrated that the combined modality (i.e. conjugation to Tat peptide followed by PCI) represents a more efficient approach for delivery of PNAs than simple conjugation to an internalizing peptide or photochemical internalization of an unconjugated PNA. In fact, even though the photochemical internalization of a 15-mer unconjugated PNA targeting hTERT quickly (within 48 h) resulted in marked reduction of telomerase catalytic activity (60% compared to untreated cells) and cell growth (55% compared to untreated cells) in DU145 cells, significant enhancement of these biological effects was obtained when cells were exposed to the same concentration of an identical PNA conjugated to Tat after photochemical internalization. Specifically, our data showed that photochemically internalized hTERT-PNA-Tat

Figure 5. Photochemically internalized hTERT-PNA-Tat induces telomere dysfunction in the absence of telomere shortening in DU145 cells. (a) Southern blot hybridization carried out 48 h after light exposure in DU145 cells untreated or exposed for 18 h to 0.7 μg/mL TPPS_{2a} alone or in combination with 2 μM of hTERT-PNA-Tat, uPNA or sPNA. (b) Representative metaphase spread showing occurrence of telomere dysfunction as assessed 48 h after exposure of DU145 cells to 2 μM of photochemically internalized hTERT-PNA-Tat. Arrows indicate typical telomere dysfunctions such as end-to-end fusions, dicentric and ring chromosomes. (c) A representative Western immunoblotting experiment showing expression of γ-H2AX protein, as assessed 48 h after light exposure in DU145 cells untreated or treated for 18 h with 0.7 μg/mL TPPS_{2a} in the absence or presence of 2 μM hTERT-PNA-Tat, uPNA or sPNA. Data are reported as γ-H2AX relative expression levels after normalization to the housekeeping β-actin. **P* < 0.05; ***P* < 0.02 as compared to cells exposed to light only (Student's *t*-test).

led to almost complete inhibition of telomerase activity and cell population growth. These results are corroborated by recent findings from Shiraishi & Nielsen (2006), who demonstrated that antisense effects of different PNA conjugates (Tat, Arg₇, KLA) were significantly enhanced, at both cytosolic and nuclear levels, in HeLa pLuc 705 and p53R cell systems as a result of the PCI approach, and that the most efficient conjugate was with Tat, where PCI enhanced antisense effects by two orders of magnitude. Similar results have also reported for PCI of two PNAs linked to cationic peptides and directed against S100A4 (Boe & Hovig 2006).

Table 1. Summary of telomere dysfunctions in metaphase spreads of DU145 cells 48 h after photochemical treatment in the presence or absence of hTERT-PNA-Tat

	No. of metaphases with telomeric fusions (100 cells analysed)				Total telomere dysfunctions	Frequency of telomere dysfunction
	0†	1†	2†	≥ 3†		
TPPS _{2a}	89	8	1	2	18	0.18
TPPS _{2a} + sPNA	88	7	4	1	17	0.17
TPPS _{2a} + uPNA	90	6	2	2	17	0.17
TPPS _{2a} + hTERT-PNA-Tat	77	12	4	7	41	0.41*

* $P < 0.01$, χ^2 test.

†Number of events per metaphase.

Table 2. Metaphases with telomeric fusions subdivided according to observed fusion pattern

Sample	No. of events/ metaphase	No. of metaphases with telomeric fusions		
		End-to-end fusions	Dicentrics	Rings
TPPS _{2a}	1	3	2	3
	2	—	—	—
	≥ 3	1	1	—
TPPS _{2a} + sPNA	1	5	6	—
	2	3	—	—
	≥ 3	—	—	—
TPPS _{2a} + uPNA	1	4	3	1
	2	—	1	—
	≥ 3	—	—	—
TPPS _{2a} + hTERT-PNA-Tat	1	5	8	4
	2	8	1	—
	≥ 3	1	—	—

Strong inhibitory effect on telomerase activity exerted by photochemically internalized hTERT-PNA-Tat, which was paralleled by marked reduction of hTERT protein expression, led to dose-dependent impairment of cell proliferation and to induction of caspase-mediated apoptosis in DU145 cells. To rule out possibility that the effect on cell population growth observed in prostate cancer cells was the consequence of PNA-mediated side-effects, we exposed hTERT-negative U2-OS osteosarcoma cells to photochemically internalized hTERT-PNA-Tat; no appreciable inhibition of cell proliferation was found. Conversely, the same treatment was able to induce significant decline in cell population growth in U2-OS/hTERT derivative that ectopically expresses hTERT.

PNA-mediated antiproliferative and pro-apoptotic effects observed in our study occurred within 2 days from the end of the treatment and did not correlate with telomere attrition, as TRF analysis failed to reveal any telomere shortening. Such effects could be ascribed to treatment-induced interference with telomerase function independent of telomere elongation and related to telomere capping. Furthermore, our results suggest that PNA-mediated inhibition of hTERT expression induced an acute DNA damage response, as revealed by increase in expression

levels of the phosphorylated form of histone H2AX – a major player in repair of double-stranded DNA lesions (Foster & Downs 2005; Fillingham *et al.* 2006) – and which resulted in significant enhancement of telomeric fusion.

Results of this study corroborate earlier evidence obtained by us on the same cell line following exposure to a 2'-O-methyl-RNA phosphorothioate oligonucleotide, targeting a splicing site within hTERT pre-mRNA, which induced almost complete inhibition of telomerase activity as a consequence of marked reduction of hTERT mRNA expression level, early decline of cell population growth, and apoptotic cell death, without any appreciable telomere shortening (Folini *et al.* 2005). Conversely, exposure of DU145 cells to a 2'-O-methyl-RNA phosphorothioate oligonucleotide targeting the template region of hTR failed to interfere with cell proliferation, in spite of the almost complete abrogation of telomerase activity (Folini *et al.* 2005). Moreover, when we down-regulated hTERT by siRNA-mediated approach in this cell line, we caused rapid decline of cell growth and induction of an apoptotic response, without any evidence of telomere shrinkage (Gandellini *et al.* 2007). Such results, together with those reported in other studies dealing with the use of antisense-mediated approaches to target hTERT (Folini & Zaffaroni 2005), point to pro-survival and anti-apoptotic roles of hTERT, independent of telomerase's ability to guarantee cell proliferation *via* its telomere elongating activity. In this context, it has been demonstrated that lowering hTERT expression in PMC42 breast cancer cells induced apoptosis independently of telomere shortening, and that use of an hTERT mutant lacking telomerase activity rescued such cells from entering apoptosis (Cao *et al.* 2002). In addition, RNAi-mediated down-regulation of endogenous hTERT in MCF-7 cells markedly increased apoptosis induced by both the 4625 Bcl-2/Bcl-X_L bispecific antisense oligonucleotide and the HA14-1 Bcl-2 inhibitor (Del Bufalo *et al.* 2005). Conversely, ectopic expression of hTERT blocked Bcl-2-dependent apoptosis, suggesting that hTERT is involved in mitochondrial regulated apoptosis. The role of hTERT as an anti-apoptotic factor has recently been highlighted by Massard *et al.* (2006). Specifically, this study showed that hTERT suppression through RNAi sensitizes cancer cells to mitochondrial apoptosis induced by DNA-damaging agents and reactive oxygen species by increased activation of Bax protein (Massard *et al.* 2006). Interestingly, no relationship was found between hTERT depletion and expression of Bax itself or of Bax-regulatory proteins, suggesting that post-translational modification in the Bax interactome accounted for enhanced activation of Bax in hTERT-depleted cells (Massard *et al.* 2006).

However, in accord with the classical model in which telomerase inhibitors impair cancer cell growth as a result of prolonged inhibition of telomere-lengthening activity of the enzyme, it has been recently reported that only long-term suppression of hTERT by siRNA-expressing retroviral vector affected the proliferative and tumourigenic potential of HeLa cells as a consequence of telomerase activity inhibition, telomere shortening and loss of telomeric-3' overhangs (Nakamura *et al.* 2005). Moreover, in Barrett's adenocarcinoma SEG-1 cells, inhibition of telomerase activity was associated with erosion of telomeres, dysregulation of genes involved in cell cycle control and programmed cell death, and induction of both senescence and apoptosis only after 3–4 weeks from exposure to a mixture of weekly administered synthetic siRNAs targeting hTERT (Shammas *et al.* 2005).

Overall, our results indicate that photochemical internalization is an efficient approach for intracellular delivery of PNA-internalization peptide conjugates, allowing reduction in amount of PNA required to induce the biological effect compared to other delivery systems, and they suggest that the combined approach could be exploited for *in vivo* applications of PNAs (Ray & Norden 2000). Moreover, by showing rapid impairment of cell proliferation following hTERT down-regulation, our data reinforce earlier evidence suggesting a pro-survival role of hTERT, which is independent of its ability to maintain telomere length.

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