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Telomerase inhibition, telomere shortening, cell growth suppression and induction of apoptosis by telomestatin in childhood neuroblastoma cells

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Abstract

Neuroblastoma is a tumour derived from primitive cells of the sympathetic nervous system and is the most common extracranial solid tumour in childhood. Unfavourable tumours are characterised not only by structural changes, including 1p deletion and amplification of the MYCN proto-oncogene, but also by high telomerase activity. Telomeric G-rich single-stranded DNA can adopt *in vitro* an intramolecular quadruplex structure, which has been shown to inhibit telomerase activity. In this study, we examined telomestatin, a G-quadruplex interactive agent, for its ability to inhibit telomere maintenance of neuroblastoma cells. Telomere length was determined by the terminal restriction fragment method, telomerase activity was measured by a quantitative telomeric repeat amplification protocol, and the expression of human telomerase by quantitative real-time polymerase chain reaction (RT-PCR). Short-term treatment with telomestatin resulted in dose-dependent cytotoxicity and induction of apoptosis. Long-term treatment with telomestatin at non-cytotoxic, but still telomerase activity-inhibiting, concentrations resulted in telomere shortening, growth arrest and induction of apoptosis. These results suggest that the effect of telomestatin is dose-dependent and at least 2-fold. Prolonged low-dose treatment with telomestatin limits the cellular lifespan of NB cells through disruption of telomere maintenance.

Keywords: Neuroblastoma; Telomerase; Novel therapies

1. Introduction

Neuroblastoma (NB) is a tumour derived from primitive cells of the sympathetic nervous system and is the most common extracranial solid tumour in childhood. Recent advances in understanding the biology and genetics of NB have allowed classification into low-, intermediate- and high-risk groups [1]. Unfavourable tumours are characterised not only by structural changes, including deletions of 1p or 11q, unbalanced gain of 17q and/or amplification of the MYCN proto-oncogene, but also by high telomerase activity [2]. These patients are usually older than 1 year, have more advanced stages of disease and a much worse prognosis, even with aggressive treatment [1].

Unlimited replicative potential is an important acquired capacity of cancer [3]. Telomere maintenance is evident in virtually all types of malignant cells; 85–90% of them show upregulated expression of the enzyme telomerase, which adds hexanucleotide repeats onto the ends of telomeric DNA [4,5], while most of the remainder use one or more different mechanisms known as alternative lengthening of telomeres (ALT) [6–8].

Telomerase, or telomere terminal transferase, is a ribonucleoprotein that catalyses the *de novo* synthesis and elongation of telomeric repeats at chromosomal

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ends by using the RNA segment within its molecule as a template [9–11]. While functionally immortal germline cells express telomerase and maintain adequate telomeric repeats, most human somatic cells do not express telomerase. They fail to acquire telomerase activity in successive cultures and become senescent. By contrast, telomerase activity has been detected in numerous cancer cells and tissues, including NB [12–16]. In NB, increased expression of the telomerase RNA component was found to be associated with advanced stage [14,15] and poor outcome [16]. Moreover, telomerase activity in NB was found to correlate with poor survival outcome [17–20].

Optimal telomerase activity requires the non-folded single-stranded telomere overhang and G-quadruplex formation has been shown to inhibit telomerase elongation *in vitro* [21]. Hence, stabilisation of telomeric G-quadruplexes by small molecule ligands has emerged as a strategy to achieve anti-tumour therapy [22,23]. Among those, telomestatin, a natural G-quadruplex-intercalating agent isolated from *Streptomyces anulatus* 3533-SV4 [24] appears very promising due to its high selectivity towards quadruplexes compared with all other nucleic acid confirmations [25,26]. Telomestatin induces delayed growth arrest and/or apoptosis in different tumour cell types and displays an interesting selectivity towards cancer cells compared with normal progenitors [27–31].

In this study, we examined the short- and long-term effects of telomestatin in a representative set of NB cells, where we assessed telomerase expression, telomerase activity and telomere length.

2. Materials and methods

2.1. Human NB cell lines

CHP134, IMR5, LAN1, LAN2, LAN5, NB69, SH-EP007, SH-SY5Y, SK-N-AS and WAC2 human NB cells were kindly provided by Dr. Brodeur, Children's Hospital of Philadelphia, PA, USA. All NB cells were cultured in RPMI 1640 with 10% foetal bovine serrum (FBS) and 1% Penstrept (all reagents from Gibco Invitrogen, Basel, Switzerland). All cell cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Real-time quantitative polymerase chain reaction

Isolation of total RNA, cDNA synthesis and kinetic real-time polymerase chain reaction (RT-PCR) quantification of *hTERT* mRNA was performed as described previously [32]. Experiments were performed in triplicate for each data point. Each PCR run included the five points of the standard curve (serially diluted LAN1 human NB cell line cDNA), a no-template control, and the calibrator cDNA (normal human adrenal). We quantified transcripts of the 18S rRNA housekeeping gene as endogenous controls and each sample was normalised on the basis of its 18S rRNA content. Relative expression of *hTERT* mRNA was calculated using the comparative threshold cycle (CT) method [33]. The amount of *hTERT*, normalised to 18S rRNA and relative to the calibrator (normal human adrenal; Stratagene, Amsterdam, The Netherlands), is then given by 2-ΔΔCT, where $\Delta\Delta CT = \Delta CT(\text{sample}) - \Delta CT(\text{calibrator})$, and ΔCT is the CT of the target gene (*hTERT*) subtracted from the CT of the housekeeping gene.

2.3. Telomerase activity

Telomerase activity in human NB cell lines was measured using the TeloTAGGG telomerase PCR enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics, Basel, Switzerland) as described previously [32]. This assay combines the highly specific amplification of telomerase DNA products (TRAP assay; [12] with the non-radioactive detection of those PCR products by ELISA). For this procedure, 6×10^6 cells were lysed and homogenised in 200 μl of lysis buffer. After 30 min incubation on ice, the lysates were centrifuged at 16,000*g* for 20 min at 2–8 °C. In the telomerase-catalysed primer elongation and PCR amplification step, 1 μl cell extract (2 μg protein/μl) was incubated in a total volume of 50 μl reaction mixture at 25 °C for 30 min to allow the telomerase to add telomeric repeats (TTAGGG) to the end of the biotin-labelled synthetic P1-TS primer. The products extended by telomerase were then amplified by PCR in the presence of the biotin-labelled P1-TS primer and another primer, P2. Amplification products from the PCR were next immobilised at 4 °C. A 5 μl volume of each amplification product was denatured in 20 μl of denaturation reagent for 10 min at room temperature and hybridised for 2 h at 37 °C on a thermoshaker to a digoxigenin (DIG)-labelled probe specific for the telomeric repeat sequence. This step was followed by a 1-h incubation at 37 °C in the wells of a streptavidin-coated microplate. Finally, the DIG-labelled hybrids were visualised with a peroxidase-conjugated anti-DIG antibody and a colourimetric peroxidase substrate and quantified photometrically using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, United States of America (USA)). The level of telomerase activity was calculated by subtracting the mean of the absorbance readings of the negative controls from those of the samples. In this assay, samples are regarded as telomerase-positive if the difference in absorbance is higher than 0.2. The telomerase activity measured by this assay does not reflect *hTERT* enzyme activity in a rigorous biochemical sense, but may also depend on *hTERT* enzyme concentration.

2.4. Telomere length

Telomere length in NB cells was determined using the TeloTAGGG telomere length assay (Roche Diagnostics, Basel, Switzerland) for measuring the length of telomere restriction fragments (TRF) as described previously [32]. In brief, genomic DNA was isolated using the QIAamp DNA Mini Kit (Qiagen AG, Basel, Switzerland) according to procedures recommended by the manufacturer. Genomic DNA (1 μg) was digested overnight with the restriction enzymes *HinfI* and *RsaI* that do not digest within telomeric repeats. Following DNA digestion, the DNA fragments were separated by 8% agarose gel electrophoresis and transferred to a nylon membrane by Southern blotting. The blotted DNA fragments were hybridised with a DIG-labelled probe that recognises TRF. Probe-TRF complexes were detected with alkaline phosphatase-conjugated anti-DIG antibody and CDP-Star (Applied Biosystems, Rotkreuz, Switzerland) chemiluminescent alkaline phosphatase substrate. The mean length for TRF was determined by comparing each fragment with a known molecular weight standard and by quantifying the intensity of the chemiluminescent signal from that fragment with the Lumi-Imager F1 workstation (Roche Diagnostics, Basel, Switzerland).

2.5. Cytotoxicity assay

Cell viability was quantified using a colourimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium inner salt (MTS) assay (Promega, Wallisellen, Switzerland) as described previously [32,34]. Briefly, 100 μl of target cell suspension was added to each well of 96-well microtitre plates, and each plate was incubated at 37 °C in a humidified 5% CO_2 atmosphere. Following 72 h of telomestatin incubation at various concentrations (0, 0.5, 1, 5, 10 μM), 10 μl of MTS working solution was added to each culture well, and the cultures were incubated for 1–4 h at 37 °C in a humidified 5% CO_2 atmosphere. Each condition was performed in triplicate. The absorbance values of each well were measured with a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 490 nm.

2.6. Apoptosis assay

A colourimetric enzyme-immunoassay (Cell Death Detection ELISAPLUS; Roche Diagnostics, Basel, Switzerland) was used for the quantitative determination of cytoplasmic histone-associated DNA fragments after telomestatin induced cell death, as described previously [34]. For short-term incubation, NB cells were incubated for 72 h with telomestatin concentrations as for the cell viability assay (0, 0.5, 1, 5, 10 μM). In addition, apoptosis was also assessed periodically after prolonged telomestatin treatment at non-cytotoxic concentrations (0.2 μM for SK-N-AS, and LAN5 NB cells, 0.5 μM for LAN1 and WAC2 NB cells).

2.7. Drugs

Telomestatin, isolated from *Streptomyces anulatus* 3533-SV4, was provided by Dr. K. Shin-ya, Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan. A stock solution of telomestatin (1 mM) was dissolved in dimethylsulphoxide (DMSO), and diluted to working concentrations with distilled water.

3. Results

3.1. hTERT mRNA expression

Using real-time quantitative RT-PCR, we determined *hTERT* mRNA expression in 10 NB cell lines. Compared with normal human adrenal, all NB cell lines had upregulated *hTERT* mRNA expression (defined as an *hTERT* expression level ⩾5-fold normal human adrenal; Fig. 1). LAN1 (56-fold), SK-N-AS (50-fold), IMR5 (49-fold), SH-SY5Y (47-fold) and WAC2 (43-fold) human NB cells had *hTERT* mRNA expression levels >40-fold human adrenal, whereas *hTERT* mRNA expression levels of LAN5 (17-fold), LAN2 (18-fold), SH-EP007 (23-fold), CHP134 (24-fold) and NB69 (34-fold) human NB cells were 17–34-fold human adrenal. No significant correlation was found between *hTERT* mRNA expression and MYCN amplification (present in NB cell lines CHP134, IMR5, LAN1, LAN2 and LAN5). However, the number of NB cell lines tested is too small to draw firm conclusions.

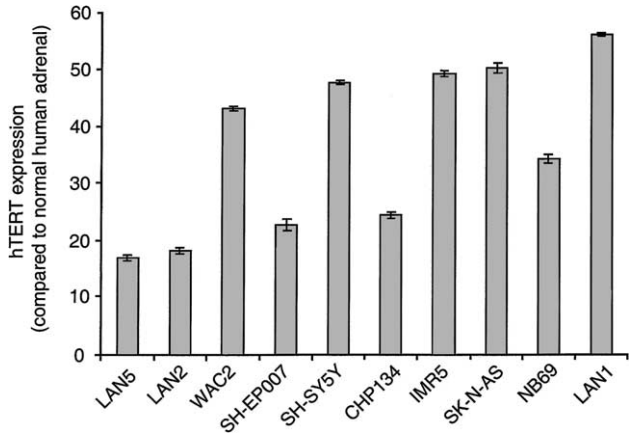

Fig. 1. Human telomerase reverse transcriptase (*hTERT*) mRNA expression in 10 human neuroblastoma cell lines. *hTERT* mRNA expression was determined by quantitative real-time polymerase chain reaction (RT-PCR) using 18S rRNA as an endogenous control and normal human adrenal as a calibrator. All tested NB cell lines had upregulated *hTERT* mRNA (defined as an expression level ⩾5-fold normal human adrenal).

3.2. Telomerase activity

To determine the enzymatic activity of telomerase in NB cells, we performed a modified TRAP assay with the cell lysates of 10 human NB cell lines. After polyacrylamide gel electrophoresis separation of the amplification products from the TRAP assay, we observed typical 6-nucleotide ladders in all NB cells (data not shown). To quantify telomerase activity, we then measured amplification products from the TRAP assay photometrically (Fig. 2). Four NB cell lines (WAC2, SH-SY5Y, NB69 and LAN1) showed high (>3.0 absorbance {A450–A690 nm}) telomerase activity, five NB cell lines (LAN2, SH-EP007, CHP134, IMR5 and SK-N-AS) showed intermediate (1.0–3.0) and one NB cell line (LAN5) had low (<1.0) telomerase activity.

3.3. Telomere length

Telomere lengths of human NB cells were analysed by measuring the TRF length using Southern blotting with a (TTAGGG)4 probe (Fig. 3). LAN5 (7.4 kbp) and WAC2 (6.5 kbp) NB cells had the longest TRF and NB69 (3.7 kbp) and LAN2 (2.9 kbp) NB cells the shortest, whereas CHP134 (3.6 kbp), IMR5 (3.8 kbp), SH-EP007 (4.5 kbp), LAN1 (4.3 kbp), SH-SY5Y (4.4 kbp) and SK-N-AS (5.4 kbp) were intermediate. This indicates greater telomere length in LAN5 and WAC2 than in other NB cells.

3.4. Comparison of telomerase activity, hTERT mRNA expression and TRF length

We then compared telomerase activity with *hTERT* mRNA expression and telomere length. While a significant correlation between telomerase activity and *hTERT* mRNA expression was detected in NB cell lines (Pearson's product-moment correlation $\rho = 0.76$, $P = 0.01$; Fig. 4(a)), no significant correlation was found between telomerase activity and telomere length ($\rho = -0.36$, $P = 0.31$; Fig. 4(b)). NB cells with high telomerase activity (WAC2, SH-SY5Y, NB69, LAN1) had a high *hTERT* mRNA expression, while the LAN5 NB cell line with the lowest telomerase activity had the lowest *hTERT* mRNA expression. Interestingly, LAN5 NB cells with the lowest telomerase activity had a greater telomere length than most other NB cells. This indicates that upregulated telomerase is functionally active in NB cells and that telomere maintenance in NB cells is not always the result of upregulated telomerase expression and/or high telomerase activity, but may also be the result of ALT.

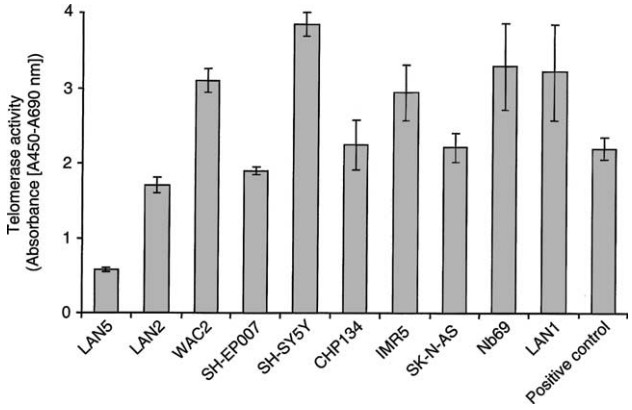

Fig. 2. Telomerase activity in 10 human neuroblastoma (NB) cell lines as determined by the TeloTAGGG telomerase polymerase chain reaction (PCR) enzyme-linked immunosorbent assay (ELISA) kit. Controls included cell extract prepared from immortalised telomerase-expressing human kidney cells (positive control). While telomerase activity was high in WAC2, SH-SY5Y, NB69 and LAN1 NB cells, intermediate in LAN2, SH-EP007, CHP134, IMR5 and SK-N-AS NB cells, telomerase activity was low in LAN5 NB cells.

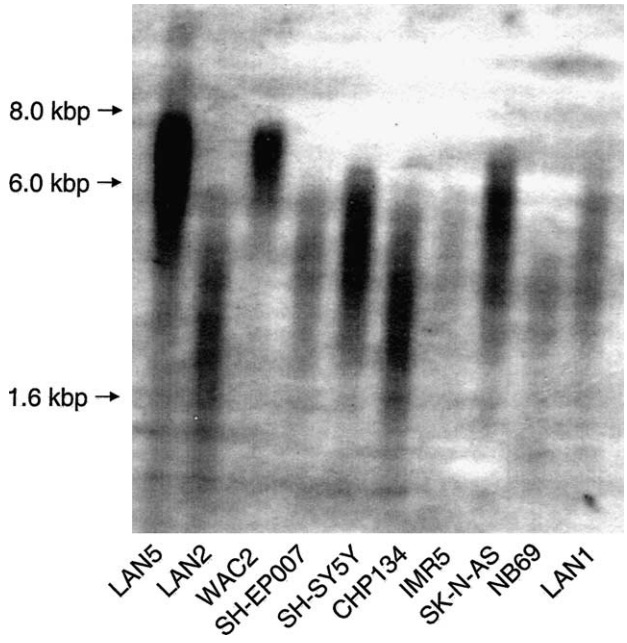

Fig. 3. Telomere length in 10 human neuroblastoma (NB) cell lines as determined by the TeloTAGGG telomere length assay. The length of the telomere restriction fragment (TRF) in each sample was determined by comparing each fragment with a known molecular weight standard. This length, together with the intensity of the chemiluminescent signal from each fragment, was used to calculate the mean TRF length for each NB cell line. LAN5 (7.4 kbp) and WAC2 (6.5 kbp) NB cells had the longest TRF, LAN2 (2.9 kbp) and NB69 (3.7 kbp) NB cells the shortest.

3.5. In vitro telomerase inhibition by telomestatin

To test whether telomestatin inhibits telomerase activity in NB cells, TRAP assays were performed on cell lysates from two NB cell lines with high telomerase activity (LAN1, WAC2), one NB cell line with intermediate telomerase activity (SK-N-AS) and one NB cell line with low telomerase activity (LAN5) treated with various concentrations of telomestatin for 5 d. Telomerase activity

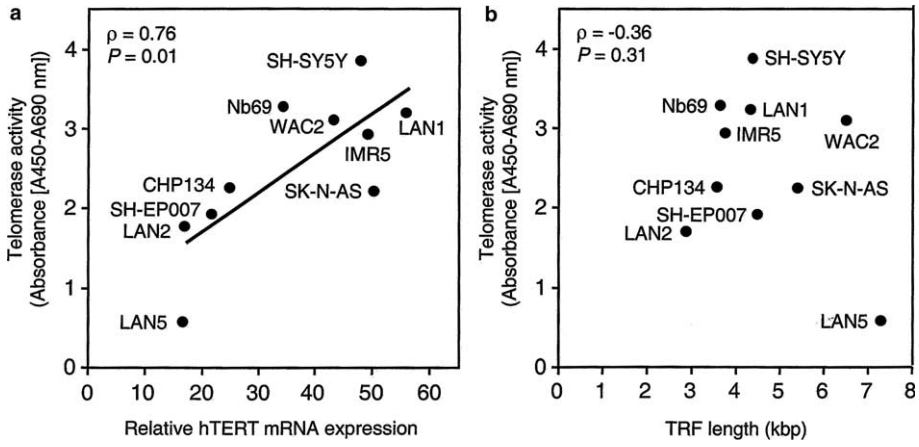

Fig. 4. Comparison of telomerase activity with (a) *hTERT* mRNA expression and (b) telomere length in 10 neuroblastoma (NB) cell lines. While telomerase activity correlated significantly with *hTERT* mRNA expression, it did not correlate with telomere length.

was found to be inhibited in a dose-dependent manner, with $\geqslant 50\%$ inhibition at 1–10 μM of telomestatin (Fig. 5). In LAN1 and WAC2 NB cells (high telomerase activity), the effect of telomestatin was less pronounced when compared with the two other NB cell lines with lower telomerase activity.

3.6. Suppression of NB cell proliferation and induction of apoptosis by telomestatin

To test whether treatment with telomestatin alters proliferation of NB cells, we incubated the same four NB cell lines (LAN1, WAC2, SK-N-AS and LAN5) with various concentrations of telomestatin for 72 h and assessed cell viability by the MTS assay (Fig. 6(a)). Treatment with telomestatin resulted in dose-dependent cytotoxicity in all NB cell lines tested with an IC50 at telomestatin concentration of 0.8–4.0 μM (SK-N-AS, 0.8 μM; LAN5, 2.5 μM; WAC2, 3.2 μM; LAN1, 4.0 μM). In NB cell lines with high telomerase activity (LAN1 and WAC2), the cytotoxic effect of telomestatin was less pronounced when compared with the cytotoxic effect of telomestatin in the other NB cell lines with lower telomerase activity (SK-N-AS and LAN5).

Apoptotic cell death upon treatment with telomestatin was assessed using the cell death ELISA. In all NB cell lines tested, telomestatin induced apoptosis in a dose-dependent manner (Fig. 6(b)). In SK-N-AS and LAN5, this effect was more pronounced when compared

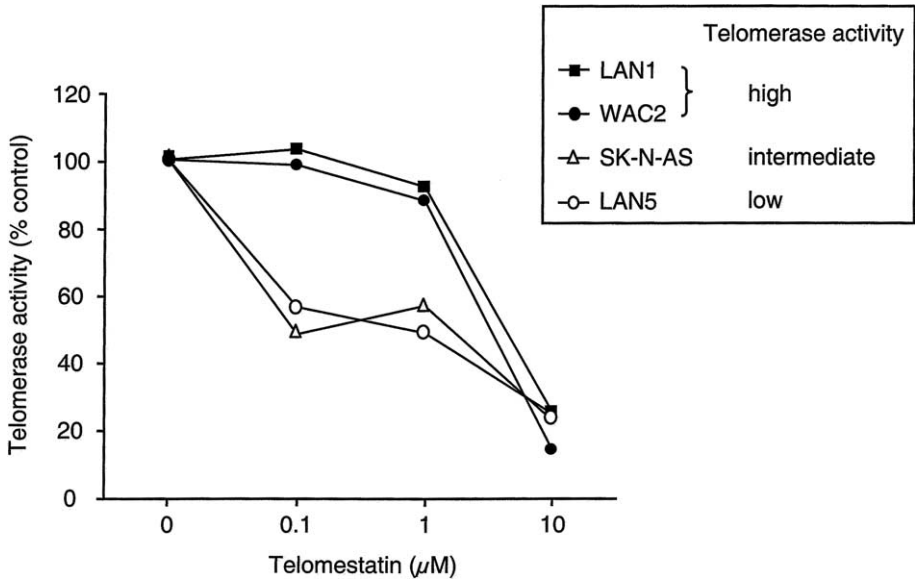

Fig. 5. Dose-dependent inhibition of telomerase activity by telomestatin in four neuroblastoma (NB) cells as determined by the TeloTAGGG telomerase polymerase chain reaction (PCR) enzyme-linked immunosorbent assay (ELISA) kit. Relative telomerase activity was inhibited in a dose-dependent manner with $\geqslant 50\%$ inhibition at 1–10 μM telomestatin.

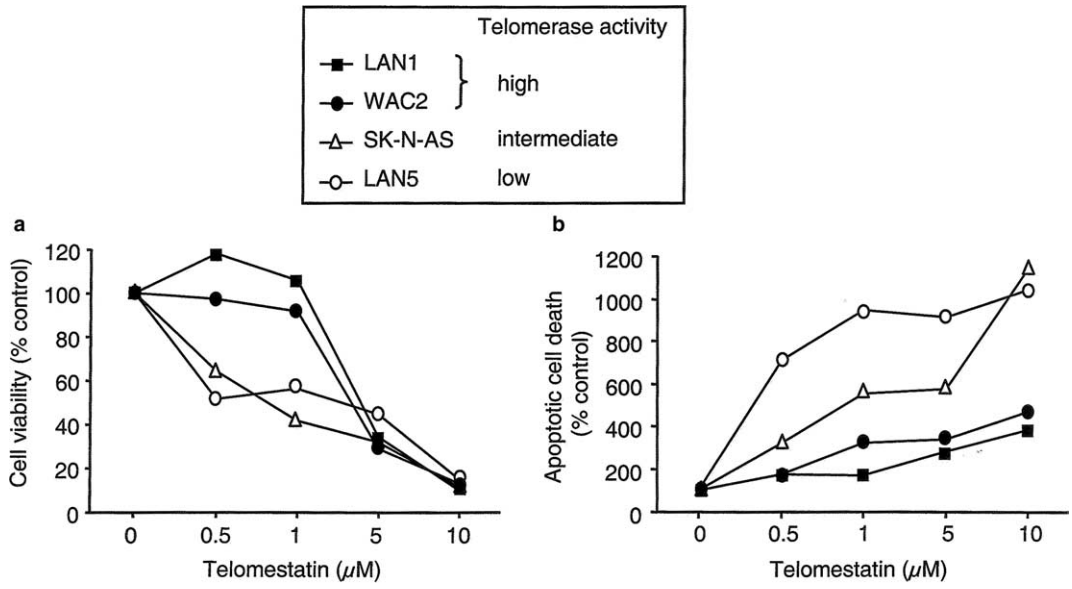

Fig. 6. (a) Dose-dependent suppression of neuroblastoma (NB) cell proliferation by telomestatin. Cell proliferation was determined after 72-h telomestatin treatment by the MTS assay. Values represent the mean percentage of survival compared with control NB cells ($n = 3$). Standard deviations were less than 10%. (b) Apoptotic cell death of telomestatin treated NB cells. Apoptotic cell death was quantified using a cell death ELISA showing enrichment of nucleosomes in the cytoplasmic fraction of telomestatin treated (72 h) human NB cells. Values represent the mean percentage of increase compared with untreated control NB cells ($n = 3$). Standard deviations were less than 10%.

with the two other NB cell lines with high telomerase activity (LAN1 and WAC2).

3.7. Effects of long-term exposure with non-toxic concentrations of telomestatin on cell growth, telomere length and apoptotic cell death

Four NB cell lines (LAN1, LAN5, WAC2 and SK-N-AS) were treated with non-cytotoxic but still telomerase activity inhibiting concentrations of telomestatin for prolonged periods. Constant numbers of cells were plated in 6 well plates (10,000 cells/well) and treated with telomestatin (WAC2, 0.5 μM; LAN1, 0.5 μM; LAN5, 0.2 μM; SK-N-AS, 0.2 μM). NB cells were passaged every 7 d to maintain log-phase growth, and counted to calculate the growth rate. At the moment of cell passage, aliquots of cells were harvested for molecular assays and the living cells were counted and were replated (10,000 cells/well) and treated with telomestatin at same concentrations.

The growth kinetics of telomestatin-treated NB cells did not differ significantly from those of untreated control NB cells in the first week (Fig. 7(a)). After 2 weeks, cell growth of telomestatin-treated NB cells decreased. In SK-N-AS NB cells, this effect was most prominent. After 5 weeks of telomestatin treatment, SK-N-AS NB cells ceased to grow completely.

Periodically, total DNA samples were prepared from telomestatin-treated and control NB cells, and the telomere length was examined using the TeloTAGGG Telomere Length Assay. In SK-N-AS NB cells, low-dose telomestatin treatment resulted in a continuous decrease of TRF from 5.4 kbp (week 0) to 3.8 kbp (week 6). Shortening of TRF was also detectable in the other NB cells, and it became evident that telomere shortening resulted in apoptosis as demonstrated using the Cell Death Detection ELISA (Roche Diagnostics, Basel, Switzerland) (Fig. 7(b)). SK-N-AS NB cells with the most pronounced telomestatin-mediated decrease of TRF, the increase of the apoptotic rate was highest when compared with the other NB cell lines. These data indicate that telomestatin treatment at low concentrations induces apoptotic NB cell death predominantly through its effect on telomerase function, impairing its ability to extend telomeres.

4. Discussion

In the present study, we systematically evaluated the telomere maintenance system in a representative set of NB cell lines. While a significant correlation between telomerase activity and *hTERT* mRNA expression was detected in NB cell lines, no significant correlation was found between telomerase activity and telomere length. This indicates that upregulated telomerase is functionally active in NB cells and that telomere maintenance in NB may also be the result of either ALT or regulation by other factors including the telomeric repeat binding factors TRF1 and TRF2 [35,36].

Telomere maintenance impairing agents might represent an interesting avenue for novel cancer therapies [37]. In this group, telomestatin is a promising novel

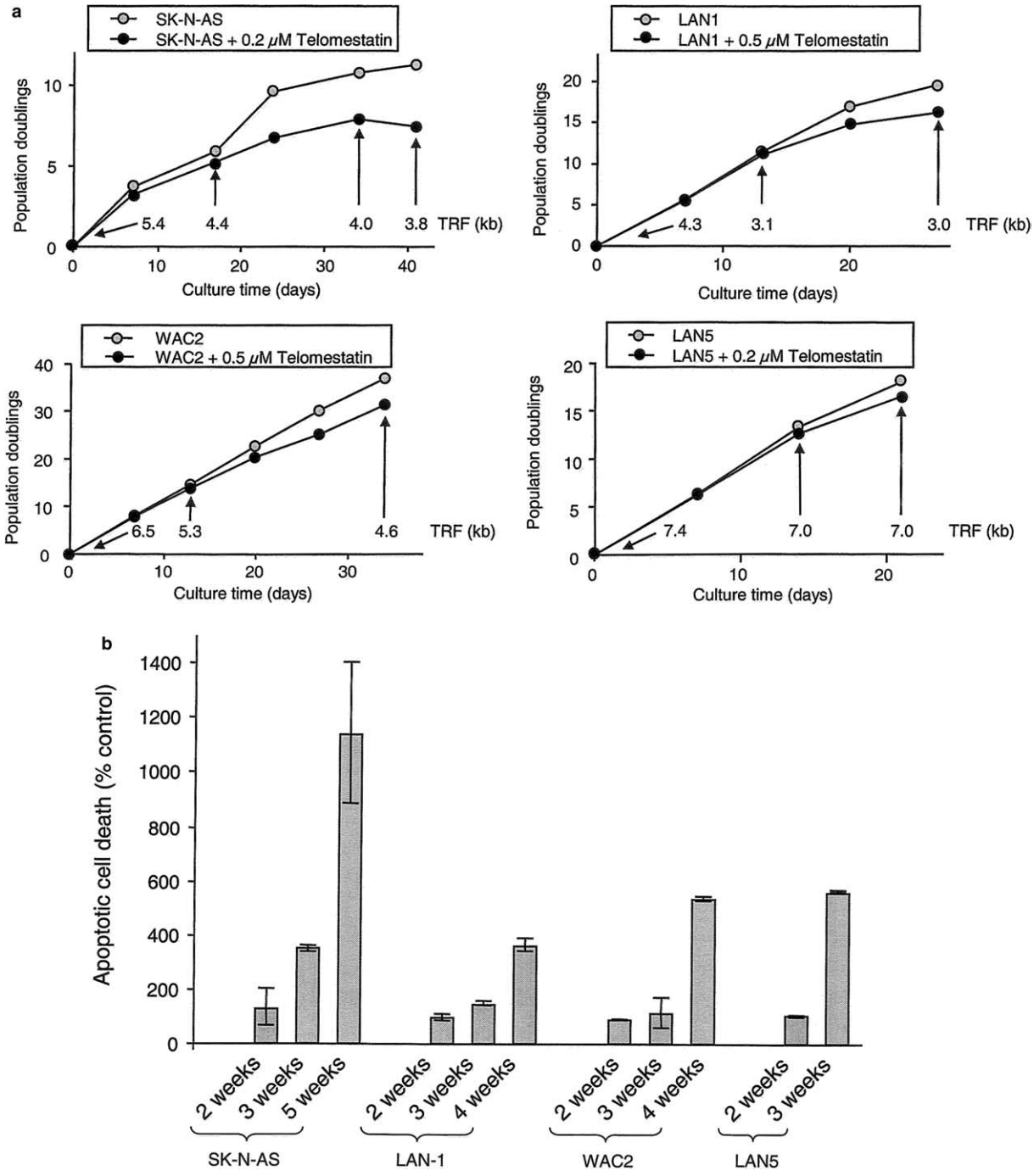

Fig. 7. Long-term treatment with non-toxic concentrations of telomestatin. (a) Effect of telomestatin on telomere length and cell growth. SK-N-AS, LAN1 and WAC2 demonstrated time-dependent telomere shortening and decrease of cell growth after 2 weeks. (b) Effects of telomestatin on cell death. All NB cells tested demonstrated increases of apoptotic cell death with prolonged telomestatin treatment. In SK-N-AS NB cells, this effect was most prominent. Values represent the mean percentage of increase compared with untreated control NB cells ($n = 3$).

agent that induces telomere intramolecular G-quadruplex structures within minutes [28] and stabilises it strongly [27]. The folding of telomeric DNA into quadruplex configuration alters the telomere secondary structure. Such altered structure has been proven to be a poor substrate for telomerase [24], and it has been found that telomestatin treatment results in cell growth inhibition [38]. Moreover, telomestatin treatment has been found to reduce *hTERT* mRNA expression, which is the necessary template for synthesis of telomerase [30].

In all NB cell lines examined, treatment with telomestatin reproducibly inhibited telomerase activity. The effective dose of telomestatin on reducing telomerase activity appears to be associated with the level of telomerase activity [24,31]. In NB cells with intermediate

and low levels of telomerase activity, relatively low concentrations (0.1 μM) of telomestatin were sufficient to decrease telomerase activity to less than 60%. However, NB cells with high TRAP activity required 5 μM telomestatin for a similar level of inhibition. Therefore, it has to be investigated *in vivo*, whether high-risk neuroblastomas with high levels of telomerase activity require higher doses of telomestatin than low-risk neuroblastomas in order to inhibit telomerase activity.

Short-term treatment (72 h) with telomestatin at telomerase-activity-inhibiting concentrations resulted in dose-dependent cytotoxicity and induction of apoptosis. Such rapid effects were unexpected because telomerase inhibitors usually lead to delayed inhibition of cell growth only after sufficient telomere shortening upon proliferation [39]. There is increasing evidence to suggest that changes in the structure of the telomere complex, rather than telomere shortening alone, can also cause uncapping of the telomeric ends, resulting in DNA damage and induction of growth arrest [40,41]. Such uncapped telomeres activate the single-strand-specific nucleases S1 and the telomere structure-specific endonuclease ERCC1/XPF complex, which leads to the telomere cleavage [28,42]. The DNA damage response cascade will therefore be activated, leading to chromosome fusion and to the induction of apoptosis or senescence [43,44]. Hence, it is possible that high-dose telomestatin led to high level of intramolecular G-quadruplex formation in NB cells. This does not only suppress the activity of telomerase by formation of intramolecular G-quadruplexes, which is a poor substrate for telomerase, but also alters telomere capping and leads to the fast activation of single-strand-specific nucleases, resulting in accelerated telomere dysfunction and cancer cell death. In addition, high-dose telomestatin may also not only induce dysfunctions of the telomere maintenance system but also other DNA damages [45]. Moreover, it has been described that interference with telomerase activity in human fibroblast cells restricts cell lifespan without changing the rate of overall telomere shortening [46].

Prolonged treatment with telomestatin at non-toxic concentrations resulted in time-dependent telomere shortening, growth arrest, and induction of apoptosis in 3 of 4 NB cell lines tested (LAN1, WAC2 and SK-N-AS). In LAN5 NB cells, which possess the longest telomere length and the lowest telomerase activity, there was a very limited shortening of telomeres. Nevertheless, cell growth slowed down and the rate of apoptosis increased. Although telomestatin is an intramolecular G-quadruplex-interactive compound with high specificity for telomeric G-quadruplex structures [31], it may affect expression of other genes, which can also account for the anti-proliferative activity of this agent [22,47–49].

In summary, our results suggest that the effect of telomestatin is dose-dependent and at least 2-fold. Prolonged low-dose treatment with telomestatin limits the cellular lifespan of NB cells through disruption of telomere maintenance. The paradigm of requiring prolonged exposure to telomerase inhibitors associated with erosion of telomeres to a critical length before cellular senescence develops is questioned by the presented results of short-term effects of telomestatin. Clearly, further research is needed to elucidate the nature of the short-term effects of telomestatin. In addition more knowledge about the *in vivo* pharmacokinetics and efficacy of telomestatin is required before this promising new drug may be considered for clinical testing.

Conflict of interest statement

None declared.

References

1. Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* 2003, **3**(3), 203–216.
2. Riley RD, Heney D, Jones DR, *et al*. A systematic review of molecular and biological tumor markers in neuroblastoma. *Clin Cancer Res* 2004, **10**(1 Pt 1), 4–12.
3. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000, **100**(1), 57–70.
4. Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. *Eur J Cancer* 1997, **33**(5), 787–791.
5. Bryan TM, Cech TR. Telomerase and the maintenance of chromosome ends. *Curr Opin Cell Biol* 1999, **11**(3), 318–324.
6. Bryan TM, Englezou A, Gupta J, *et al*. Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J* 1995, **14**(17), 4240–4248.
7. Neumann AA, Reddel RR. Telomere maintenance and cancer – look, no telomerase. *Nat Rev Cancer* 2002, **2**(11), 879–884.
8. Henson JD, Neumann AA, Yeager TR, *et al*. Alternative lengthening of telomeres in mammalian cells. *Oncogene* 2002, **21**(4), 598–610.
9. Blackburn EH. Structure and function of telomeres. *Nature* 1991, **350**(6319), 569–573.
10. Feng J, Funk WD, Wang SS, *et al*. The RNA component of human telomerase. *Science* 1995, **269**(5228), 1236–1241.
11. Bosoy D, Peng Y, Mian IS, *et al*. Conserved N-terminal motifs of telomerase reverse transcriptase required for ribonucleoprotein assembly *in vivo*. *J Biol Chem* 2003, **278**(6), 3882–3890.
12. Kim NW, Piatyszek MA, Prowse KR, *et al*. Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994, **266**(5193), 2011–2015.
13. Mu J, Wei LX. Telomere and telomerase in oncology. *Cell Res* 2002, **12**(1), 1–7.
14. Maitra A, Yashima K, Rathi A, *et al*. The RNA component of telomerase as a marker of biologic potential and clinical outcome in childhood neuroblastic tumors. *Cancer* 1999, **85**(3), 741–749.
15. Reynolds CP, Zuo JJ, Kim NW, *et al*. Telomerase expression in primary neuroblastomas. *Eur J Cancer* 1997, **33**(12), 1929–1931.
16. Krams M, Hero B, Berthold F, *et al*. Full-length telomerase reverse transcriptase messenger RNA is an independent prognostic factor in neuroblastoma. *Am J Pathol* 2003, **162**(3), 1019–1026.

17. Poremba C, Willenbring H, Hero B, *et al*. Telomerase activity distinguishes between neuroblastomas with good and poor prognosis. *Ann Oncol* 1999, **10**(6), 715–721.
18. Nozaki C, Horibe K, Iwata H, *et al*. Prognostic impact of telomerase activity in patients with neuroblastoma. *Int J Oncol* 2000, **17**(2), 341–345.
19. Poremba C, Scheel C, Hero B, *et al*. Telomerase activity and telomerase subunits gene expression patterns in neuroblastoma: a molecular and immunohistochemical study establishing prognostic tools for fresh-frozen and paraffin-embedded tissues. *J Clin Oncol* 2000, **18**(13), 2582–2592.
20. Choi LM, Kim NW, Zuo JJ, *et al*. Telomerase activity by TRAP assay and telomerase RNA (hTR) expression are predictive of outcome in neuroblastoma. *Med Pediatr Oncol* 2000, **35**(6), 647–650.
21. Zahler AM, Williamson JR, Cech TR, *et al*. Inhibition of telomerase by G-quartet DNA structures. *Nature* 1991, **350**(6320), 718–720.
22. Neidle S, Parkinson G. Telomere maintenance as a target for anticancer drug discovery. *Nat Rev Drug Discov* 2002, **1**(5), 383–393.
23. Mergny JL, Riou JF, Mailliet P, *et al*. Natural and pharmacological regulation of telomerase. *Nucleic Acids Res* 2002, **30**(4), 839–865.
24. Shin-ya K, Wierzba K, Matsuo K, *et al*. Telomestatin, a novel telomerase inhibitor from *Streptomyces anulatus*. *J Am Chem Soc* 2001, **123**(6), 1262–1263.
25. Rosu F, Gabelica V, Shin-ya K, *et al*. Telomestatin-induced stabilization of the human telomeric DNA quadruplex monitored by electrospray mass spectrometry. *Chem Commun (Camb)* 2003, **21**, 2702–2703.
26. Gomez D, Paterski R, Lemarteleur T, *et al*. Interaction of telomestatin with the telomeric single-strand overhang. *J Biol Chem* 2004, **23**, 23.
27. Kim MY, Vankayalapati H, Shin-Ya K, *et al*. Telomestatin, a potent telomerase inhibitor that interacts quite specifically with the human telomeric intramolecular g-quadruplex. *J Am Chem Soc* 2002, **124**(10), 2098–2099.
28. Kim MY, Gleason-Guzman M, Izbicka E, *et al*. The different biological effects of telomestatin and TMPyP4 can be attributed to their selectivity for interaction with intramolecular or intermolecular G-quadruplex structures. *Cancer Res* 2003, **63**(12), 3247–3256.
29. Gomez D, Aouali N, Renaud A, *et al*. Resistance to senescence induction and telomere shortening by a G-quadruplex ligand inhibitor of telomerase. *Cancer Res* 2003, **63**(19), 6149–6153.
30. Tauchi T, Shin-Ya K, Sashida G, *et al*. Activity of a novel G-quadruplex-interactive telomerase inhibitor, telomestatin (SOT-095), against human leukemia cells: involvement of ATM-dependent DNA damage response pathways. *Oncogene* 2003, **22**(34), 5338–5347.
31. Shammas MA, Reis RJ, Li C, *et al*. Telomerase inhibition and cell growth arrest after telomestatin treatment in multiple myeloma. *Clin Cancer Res* 2004, **10**(2), 770–776.
32. Didiano D, Shalaby T, Lang D, *et al*. Telomere maintenance in childhood primitive neuroectodermal brain tumors. *Neuro-oncol* 2004, **6**(1), 1–8.
33. Giulietti A, Overbergh L, Valckx D, *et al*. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* 2001, **25**(4), 386–401.
34. Grotzer MA, Eggert A, Zuzak TJ, *et al*. Resistance to TRAIL-induced apoptosis in primitive neuroectodermal brain tumor cells correlates with a loss of caspase-8 expression. *Oncogene* 2000, **19**, 4604–4610.
35. Oh BK, Kim YJ, Park C, *et al*. Up-regulation of telomere-binding proteins, TRF1, TRF2, and TIN2 is related to telomere shortening during human multistep hepatocarcinogenesis. *Am J Pathol* 2005, **166**(1), 73–80.
36. Iwano T, Tachibana M, Reth M, *et al*. Importance of TRF1 for functional telomere structure. *J Biol Chem* 2004, **279**(2), 1442–1448.
37. Shay JW, Wright WE. Telomerase: a target for cancer therapeutics. *Cancer Cell* 2002, **2**(4), 257–265.
38. Risitano A, Fox KR. The stability of intramolecular DNA quadruplexes with extended loops forming inter- and intra-loop duplexes. *Org Biomol Chem* 2003, **1**(11), 1852–1855.
39. White LK, Wright WE, Shay JW. Telomerase inhibitors. *Trends Biotechnol* 2001, **19**(3), 114–120.
40. Karlseder J, Smogorzewska A, de Lange T. Senescence induced by altered telomere state, not telomere loss. *Science* 2002, **295**(5564), 2446–2449.
41. El-Daly H, Kull M, Zimmermann S, *et al*. Selective cytotoxicity and telomere damage in leukemia cells using the telomerase inhibitor BIBR1532. *Blood* 2004.
42. Zhu XD, Niedernhofer L, Kuster B, *et al*. ERCC1/XPF removes the 3′ overhang from uncapped telomeres and represses formation of telomeric DNA-containing double minute chromosomes. *Mol Cell* 2003, **12**(6), 1489–1498.
43. Smogorzewska A, Karlseder J, Holtgreve-Grez H, *et al*. DNA ligase IV-dependent NHEJ of deprotected mammalian telomeres in G1 and G2. *Curr Biol* 2002, **12**(19), 1635–1644.
44. Takai H, Smogorzewska A, de Lange T. DNA damage foci at dysfunctional telomeres. *Curr Biol* 2003, **13**(17), 1549–1556.
45. Lemarteleur T, Gomez D, Paterski R, *et al*. Stabilization of the c-myc gene promoter quadruplex by specific ligands' inhibitors of telomerase. *Biochem Biophys Res Commun* 2004, **323**(3), 802–808.
46. Masutomi K, Yu EY, Khurts S, *et al*. Telomerase maintains telomere structure in normal human cells. *Cell* 2003, **114**(2), 241–253.
47. Rezler EM, Bearss DJ, Hurley LH. Telomeres and telomerases as drug targets. *Curr Opin Pharmacol* 2002, **2**(4), 415–423.
48. Riou JF, Gomez D, Lemarteleur T, *et al*. G-quadruplex DNA: myth or reality? *Bull Cancer* 2003, **90**(4), 305–313.
49. Darnell JC, Jensen KB, Jin P, *et al*. Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. *Cell* 2001, **107**(4), 489–499.