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European Journal of Cancer 38 (2002) 1792–1801

European Journal of Cancer

www.ejconline.com

Inhibition of telomerase activity by a distamycin derivative: effects on cell proliferation and induction of apoptosis in human cancer cells

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Received 24 January 2002; received in revised form 12 March 2002; accepted 30 April 2002

Abstract

In this study, we evaluated the potential of the distamycin derivative MEN 10716 as a telomerase inhibitor. Exposure of human melanoma cell extracts to MEN 10716 induced a dose-dependent inhibition of telomerase activity, with an IC_{50} of 24 ± 3 μM. When intact JR8 melanoma cells were chronically exposed to the drug (200 μM every other day for 50 days), a marked inhibition (>80%) of the enzyme's catalytic activity was consistently observed starting from day 1. At later points in time, MEN 10716 inhibited melanoma cell proliferation and induced apoptosis. Cells surviving MEN 10716 exposure were characterised by a higher melanin content and a greater expression of $p16^{INK4A}$ protein than control cells. The effects of MEN 10716 were subsequently evaluated in different tumour cell systems. In particular, even in the H460 non-small cell lung cancer cell line, chronic exposure of the cells to the drug (100 μM every other day for 50 days) induced a consistent inhibition (>85%) of telomerase activity, a reduction of cell proliferation potential, and apoptosis. Conversely, MEN 10716 treatment did not appreciably inhibit cell proliferation in the U2-OS telomerase-negative human osteogenic sarcoma cell line. Interestingly, no variation in the mean telomere length was observed in MEN 10716-treated JR8 melanoma cells, whereas an appreciable increase in the mean telomere length was found in H460 lung cancer cells after drug exposure. Overall, the results of the study indicate that MEN 10716 is a possible telomerase inhibitor and suggest that abrogation of telomerase activity can affect cell proliferation even through pathways that are not dependent on telomere erosion.

Keywords: Distamycin derivative; Telomerase; Telomere; Apoptosis; Melanoma; Non-small cell lung carcinoma; Osteogenic sarcoma

1. Introduction

Human telomerase is a ribonucleoprotein enzyme complex that maintains the telomeric structures at the chromosome termini by adding 5′-TTAGGG-3′ repeats [1,2]. Several lines of evidence indicate that the enzyme is involved in the attainment of immortality in cancer cells and therefore may contribute to tumorigenesis and neoplastic progression [3]. The notion that telomerase is reactivated in 80–90% of human cancers [4] has led to the proposal of telomerase as a promising therapeutic target for novel anticancer interventions [5].

An evolving understanding of the composition and functions of telomerase has prompted the formulation of distinct rationales for the development of inhibitors. These include antisense-based oligonucleotide inhibitors targeting the RNA component of human telomerase (hTR) that acts as a template for the addition of new telomeric repeats by the reverse transcriptase domain of the enzyme (hTERT) [6,7]. In fact, the inherent accessibility to incoming nucleic acids makes telomerase an ideal target for antisense oligonucleotide strategies. Physical blockage of the RNA template by conventional DNA oligomers and phosphorotioate-modified DNA has been reported [8,9]. Telomerase inhibition was also obtained by using second generation oligonucleotides, such as 2-*O*-methyl-RNAs, 2′-5′ linked oligoadenylates and peptide nucleic acids [10–13], or hammerhead ribozymes [14–16].

The reverse transcriptase activity of telomerase has led to the appraisal of nucleotide-based molecules with established activity against viral reverse transcriptases [17–19]. However, the lack of specificity for telomerase in

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0959-8049/02/$ - see front matter
PII: S0959-8049(02)00139-9

comparison to other polymerases represents the major limitation with this approach. A different approach based on the use of dominant-negative alleles of telomerase reverse transcriptase has been reported to be a successful strategy for enzyme inhibition [20].

A strategy of indirect telomerase targeting, via the telomere primer strand, has also been actively pursued by using compounds capable of interacting with telomeric DNA tetraplex structures to prevent enzyme access [21–23]. Finally, a potential antitelomerase activity has also been demonstrated for conventional antitumour agents such as cisplatin [24] or for small molecules, including isothiazolone derivatives [25], rhodacyanine FJ5002 [26] and a catechin component of green tea [27]. However, the mechanisms underlying telomerase inhibition by these compounds are still unknown. More recently, a selective telomerase inhibition was obtained by using non-nucleoside small molecules [28].

In the present study, we evaluated the ability of a distamycin derivative, MEN 10716 (Fig. 1a), to inhibit telomerase activity. MEN 10716 is a penta(*N*-methylpyrrole carboxamide) analogue of distamycin in which the N-terminal formylamino group is replaced by a carbamoyl moiety [29].

2. Materials and methods

2.1. Cell lines

The JR8 human melanoma cell line [30], the H460 human non-small cell lung carcinoma cell line and the

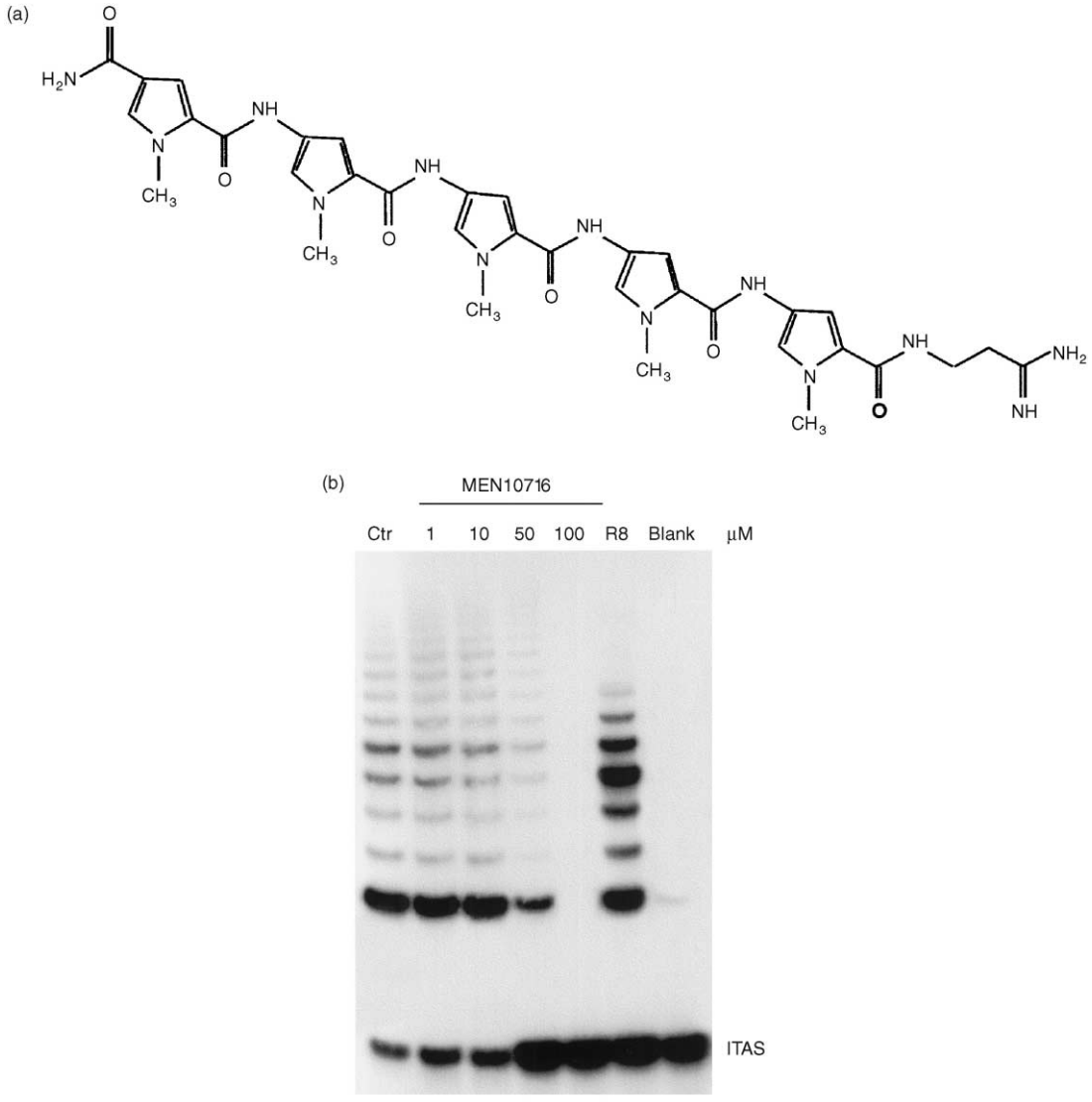

Fig. 1. (a) Chemical structure of MEN 10716; (b) a representative TRAP assay showing inhibition of telomerase activity in JR8 cell extracts exposed to MEN 10716 for 90 min at 37 °C. Ctr, control. R8 is an external quantitative standard (TSR8). The location of the internal amplification standard (ITAS) is indicated.

U2-OS human osteogenic sarcoma cell line (American Type Culture Collection) were used in this study. The cell lines were maintained as a monolayer in the logarithmic growth phase at 37 °C in a 5% CO_2 humidified atmosphere in air, using Roswell Park Memorial Institute (RPMI)-1640 (for JR8) or McCoy's (for H460 and U2-OS) medium supplemented with 10% fetal calf serum, 2 μM L-glutamine and 0.1% gentamycin.

2.2. Drug

The distamycin derivative MEN 10716 was obtained from Menarini Ricerche (Pomezia, Italy). The drug was reconstituted in sterile water and then diluted with 0.9% (w/v) sodium chloride to the desired concentration immediately before each experiment.

2.3. Cell growth and drug treatment conditions

After harvesting in the logarithmic growth phase, cells were seeded in T75 plastic flasks. Six hours after seeding, MEN 10716 was added to the culture medium. (The drug concentrations used throughout the study were 200 μM for the JR8 cell line and 100 μM for the H460 and U2-OS cell lines. These concentrations were defined on the basis of preliminary experiments indicating a lack of cytotoxic activity of the drug after 4 days of exposure.) Every 48 h, the culture medium was removed and fresh medium containing MEN 10716 was added to the cultures. Cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere for 6 days, following which they were trypsinised and counted in a particle counter (Coulter Counter, Coulter Electronics, Luton, UK). Cells were then reseeded in plastic flasks and the drug was added to the cultures. At different intervals after seeding, samples of both adherent and floating cells were collected separately, counted and stored for further determinations.

2.4. Telomerase activity detection assay

Cell extracts were obtained as previously described in Ref. [31]. Telomerase activity was measured by the polymerase chain reaction (PCR)-based telomeric-repeat amplification protocol (TRAP) [32], with some modifications [12]. Samples containing 1 μg of protein were analysed in the TRAP reaction by the TRAPeze kit (Intergen Company, Oxford, UK), according to the manufacturer's protocol. After extension of the substrate oligonucleotide thymidylate synthase (TS) (5′-AATCCGTCGAGCAGAGTT-3′) oligonucleotide by telomerase, the telomerase products were amplified by PCR in the presence of 5′[^{32}P]-end-labelled TS primer for 28 cycles and resolved on 10% (w/v) 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 digitalised image. Telomerase activity was quantified by measuring the signal of telomerase ladder bands and calculated as the ratio to the internal standard, as previously described in Ref. [12].

2.5. Flow cytometric analysis

Samples of 1×10^6 of cells were fixed in 70% (v/v) ethanol. Flow-cytometric sub-$G_{0/1}$ peak was detected on DNA plots after staining of cells in the solution A containing 50 μg/ml propidium iodide, 50 mg/ml RNAse, and 0.05% (v/v) Nonidet P40 (NP40) for 30 min at 4 °C. The fluorescence of stained cells was measured by a fluorescent activated cell sorting (FACS)can flow cytometer (Becton Dickinson, Sunnyvale, CA, USA).

2.6. Evaluation of apoptotic morphology by fluorescence microscopy

Cells were washed in phosphate buffered solution (PBS) and stained with the solution A. After staining, the slides were observed under a fluorescence microscope. The percentage of apoptotic cells was determined by scoring at least 500 cells in each sample.

2.7. Relative melanin content of cells

Melanoma cell pellets were dissolved in 1 ml of 1 N KOH at 80 °C for 1 h. The hydrolysate was centrifuged at 12 000*g* for 10 min. The relative melanin concentrations of the supernatants were determined by measuring the optical density (O.D.) at 492 nm [33] and comparing with a standard curve prepared from synthetic melanin (Sigma Chemical Co., St. Louis, MO, USA) dissolved in the same solution.

2.8. Detection of DNA polymerase α activity

The catalytic activity of DNA polymerase α was determined by using the method previously described by Spiro and colleagues in Ref. [34].

2.9. Pulsed-field gel electrophoresis

Pelleted melanoma cells were resuspended in L-buffer (0.5 M Tris–HCl, pH 7.6, 1 M NaCl, 0.5 M ethylene diamine tetraacetic acid (EDTA), pH 8) and mixed with molten 1% (w/v) low melting point agarose at 65 °C. Fractions of lysate mixture were cast into precooled 85-μl block molds and allowed to solidify at 4 °C. The

agarose-embedded lysates were then treated with L-buffer containing 2.8% (w/v) *N*-lauryl sarcosine and proteinase K (125 μg/ml) (Sigma Chemical Co.) at 37 °C for 15 h. Deproteinated lysate plugs were rinsed in Tris–EDTA (TE) buffer, pH 8, and loaded directly onto the wells of pulsed-field gels. The wells were sealed with 1% (w/v) agarose gel (Qbiogene Inc., Carlsbad, CA, USA), and pulsed-field gel electrophoresis was carried out using a Clamped Homogeneous Electric Field-DR (CHEF-DR) III Electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) at 4.5 V/cm for 15 h in 0.5×Tris borate/EDTA buffer at 14 °C, through a 1% (w/v) agarose gel. Pulse intervals were ramped from $T_1 = 1$ s to $T_2 = 13$ s. Molecular size markers were run on each gel. Gels were stained in 0.5×Tris borate/EDTA buffer containing 0.5 μg/ml ethidium bromide, and DNA fragments were visualised by ultraviolet transillumination and photographed.

2.10. Cell lysis and immunoblotting

One hundred μg of total cellular lysate [35] was dissolved in 2× sample loading buffer, separated on 15% (w/v) sodium dodecyl sulphate (SDS)-polyacrylamide gel and transferred to nitrocellulose. The filters were blocked in PBS with 5% (w/v) skim milk and then incubated overnight with the primary monoclonal antibody anti-$p16^{INK4A}$ (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The filters were then incubated with the secondary anti-mouse Ig horseradish peroxidase-linked whole antibody (Amersham Pharmacia Biotech, Uppsala, Sweden). Bound antibody was detected using the enhanced chemoluminescence western blotting detection system (Amersham). An anti proliferating cell nuclear antigen (PCNA) monoclonal antibody (Santa Cruz Biotechnology) was used on each blot to ensure equal loading of protein on the gel.

2.11. Telomere length measurement

Total DNA (obtained from cells growing attached to the plastic) was isolated using DNAzol (Life Technologies, Gaithersburg, MD, USA), digested, electrophoresed, transferred to a nylon membrane and hybridised with a 5′-end [γ-^{32}P]deoxyadenosine(dATP)-labelled telomeric oligonucleotide probe $(TTAGGG)_4$ by a standard protocol. Filters were autoradiographed and the autoradiographs were scanned (ScanJet IIcx/T; Hewlett Packard, Milan, Italy) and digitalised by Image Quant (Molecular Dynamics, Sunnyvale, CA, USA); the mean telomere restriction fragment (TRF) length was calculated as previously reported in Ref. [36].

3. Results

The effect of MEN 10716 on telomerase activity was initially assessed by exposing cellular extracts of the JR8 human melanoma cell line to different concentrations of the drug (from 1.0 to 100 μM) for 90 min at 37 °C. In the JR8 cell-free system, a dose-dependent decline of the TRAP signal was observed (Fig. 1b), with an IC_{50} (drug concentration that inhibited telomerase activity by 50% with respect to the control) of 24±3 μM.

We next examined whether MEN 10716 was capable of inhibiting telomerase activity in intact JR8 melanoma cells. For this purpose, cells were chronically exposed to MEN 10716 (200 μM of drug every other day for 50 days), and a marked inhibition (>80% with respect to the control) of telomerase activity (Fig. 2a) was consistently observed starting from day 1 (Fig. 2b). The inhibition appeared to be specific for telomerase, since no appreciable interference of MEN 10716 with the catalytic activity of other DNA polymerases, such as DNA polymerase α, was detected (data not shown). Interestingly, the effect of MEN 10716 on telomerase activity was observed at earlier time points than that on

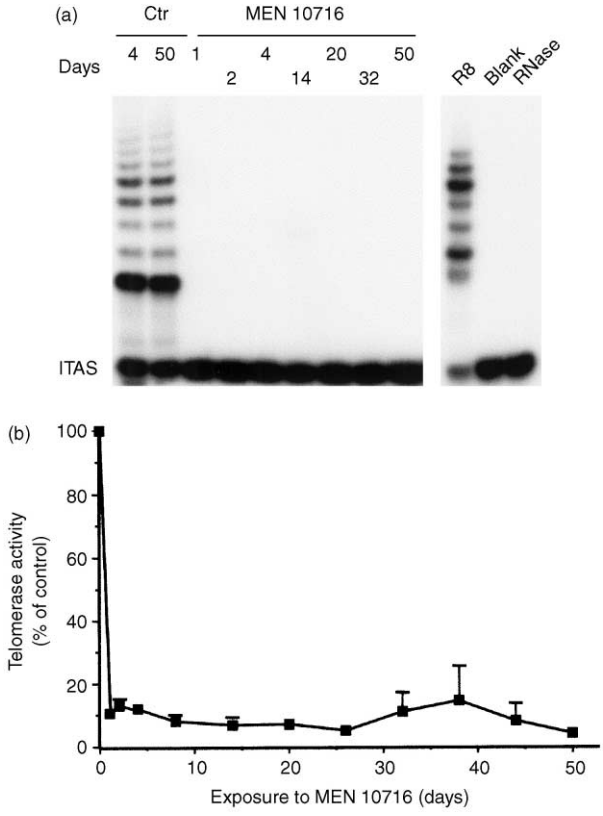

Fig. 2. (a) A representative TRAP assay showing inhibition of telomerase activity in JR8 cells at different days of exposure to MEN 10716. Ctr, control cells. R8 represents an external quantitative standard (TSR8). Blank represents a negative control to which no cell extract was added. RNase represents a negative control containing JR8 cell extract pretreated with RNase. The location of the internal amplification standard (ITAS) is indicated. (b) Quantitation of MEN 10716-mediated telomerase inhibition in JR8 melanoma cells. The effect of MEN 10716 is expressed as the percentage of the enzyme's activity compared with control. The data represent mean values + standard deviations (S.D.) of three independent experiments.

the JR8 cell growth potential. In fact, the numbers of treated cells, assessed at the end of different rounds of drug treatment, remained superimposable to those of controls for the first 8 days of treatment and then declined precipitously to ~2% of the previous cell number at day 20. At later intervals, the number of surviving cells increased, but remained approximately 20–30% of that of control cells until day 50 (Fig. 3).

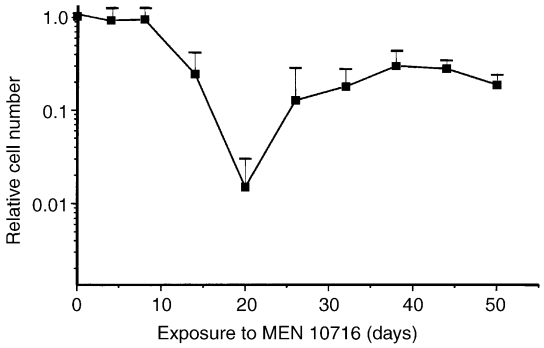

Fig. 3. Effect of MEN 10716 on the proliferative potential of JR8 cells. Cell numbers of treated cells are shown on a logarithmic scale, relative to control cells. The data represent mean values + standard deviation (S.D.) of three independent experiments.

To assess the mode of cell death affecting MEN 10716-treated cells, we investigated the induction of apoptosis in floating JR8 cells by considering several endpoints. When the presence of cells with an apoptotic morphology was determined by fluorescence microscopy after cell staining with propidium iodide (Fig. 4a), a negligible percentage of apoptotic cells (1–3%) was consistently observed in control samples at the different time points considered. This percentage progressively increased in treated samples as a function of treatment time (Fig. 4b) reaching its maximum (38%) by day 20. Until day 38, approximately 25% of the apoptotic cells were still appreciable in drug-treated samples, whereas the value had dropped to less than 5% by day 50. Similar results were obtained by flow cytometric analysis. Specifically, no hypodiploid DNA peak appeared in untreated JR8 cells, whereas marked pre-G_1 apoptotic cell peaks were observed in samples exposed to MEN 10716 (Fig. 4c). The percentage of drug-treated cells with a hypodiploid DNA content progressively increased

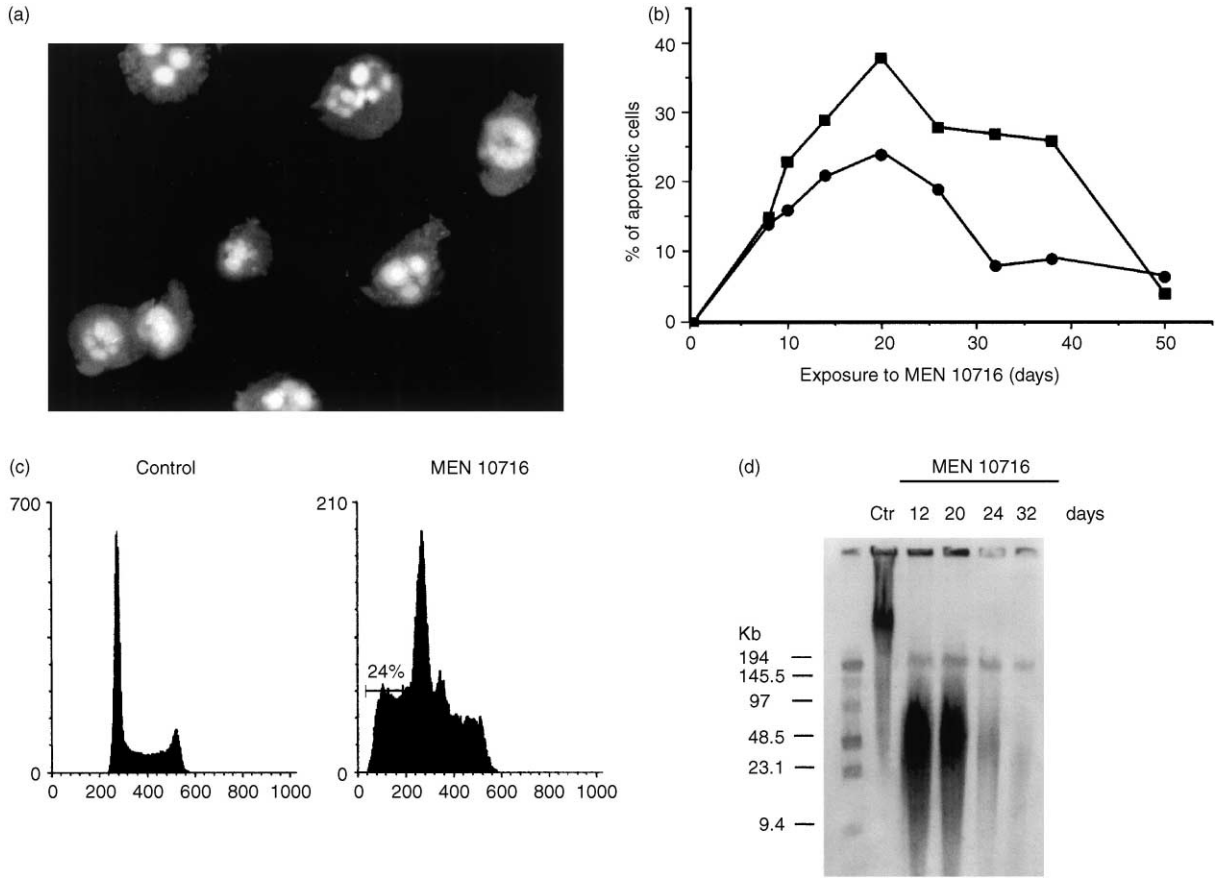

Fig. 4. (a) Propidium iodide staining of JR8 cells treated with MEN 10716 for 20 days. (b) Quantitation of apoptosis in JR8 cells treated with MEN 10716 for different intervals, as detected by fluorescence microscopy (■) or flow cytometry (●). Data represent mean values of two independent experiments. (c) Flow-cytometric analysis of JR8 cells stained with propidium iodide. The percentage of pre-G1 population is reported in the bottom left-hand corner of the histogram. (d) Pulsed-field gel electrophoresis analysis of DNA degradation in JR8 cells untreated (Ctr) or exposed to MEN 10716. The first lane is the low range pulsed-field gel electrophoresis molecular weight marker set.

with time, reaching its maximum (24%) by day 20 and then decreasing to 6% by day 50 (Fig. 4b). Pulsed-field gel electrophoresis analysis of DNA revealed the presence of large (i.e. 50-kilobase) fragments in JR8 cells exposed to MEN 10716 (Fig. 4d). The extent of DNA fragmentation was maximum after 12–20 days of treatment in concomitance with the highest accumulation of apoptotic cells, as detected by fluorescence microscopy and flow cytometric analysis (Fig. 4b).

Non-apoptotic JR8 cells that survived MEN 10716 exposure were characterised by an increased level of endogeneous melanin content (+56, +100 and +100% with respect to the control after 12, 24 and 36 days of treatment, respectively). Moreover, the expression of the cyclin-dependent kinase inhibitor $p16^{INK4A}$ was markedly enhanced in these cells compared with control cells (Fig. 5). The difference was appreciable from days 12 to 28 of drug treatment, whereas at later intervals in culture a tendency to an increased expression of $p16^{INK4A}$ protein was also observed in control cells. The analysis of telomere length performed in parallel by Southern blot hybridisation in JR8 cells exposed to MEN 10716 for different intervals did not show any appreciable reduction in the average telomere length compared with control cells (Fig. 6).

The effects of MEN 10716 were then evaluated in a different tumour cell system, i.e. the H460 non-small cell lung carcinoma cell line. Chronic exposure of cells to the drug (100 μM every other day for 50 days) induced a marked inhibition of telomerase activity (>85% with respect to controls), which was appreciable starting from day 2 and remained almost constant until day 50 (Fig. 7). In H460 cells, treatment with MEN 10716 also caused a decrease in cell number. Specifically, the number of treated cells remained similar to that of controls for the first 4 days of treatment, whereas a 65% inhibition with respect to control cells was observed by day 6. At later intervals, the number of surviving cells slightly increased, but remained approximately 50% of that of controls until day 50. Fluorescence microscopy analysis revealed the presence of an apoptotic morphology in a small fraction of drug-treated H460 cells, with a peak (13%) of apoptotic cells after 20 days of treatment. Interestingly, analysis of telomeres in H460 cells exposed to MEN 10716 for different intervals showed an increase in mean telomere length in drug-treated cells with respect to controls (mean TRF values: ~7 kb versus ~5 kb) (Fig. 8).

To better understand the relevance of telomerase inhibition for the cytotoxic activity of MEN 10716, the U2-OS telomerase-negative human osteogenic sarcoma cell line (Fig. 9a) was chronically treated with the drug (100 μM of drug every other day for 24 days). In this cell system, MEN 10716 caused a very modest reduction in the cell number, which remained constant for the entire observation period (Fig. 9b). Moreover, the presence of cells with apoptotic morphology, as detected by fluorescence microscopy after cell staining with propidium iodide, was occasionally observed after MEN 10716 treatment and accounted for less than 2% of the overall cell population.

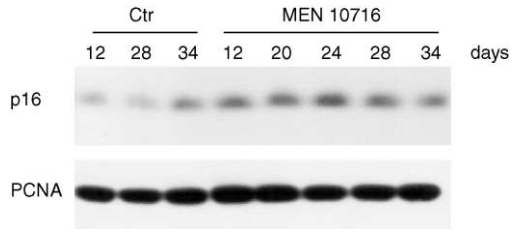

Fig. 5. A representative experiment illustrating the effect of MEN 10716 on the expression of $p16^{INK4A}$. Western blots were probed with the monoclonal antibody for $p16^{INK4A}$. Anti-PCNA monoclonal antibody was used to ensure equal loading of protein on the gel. Ctr, control cells; MEN 10716, JR8 cells exposed to the drug.

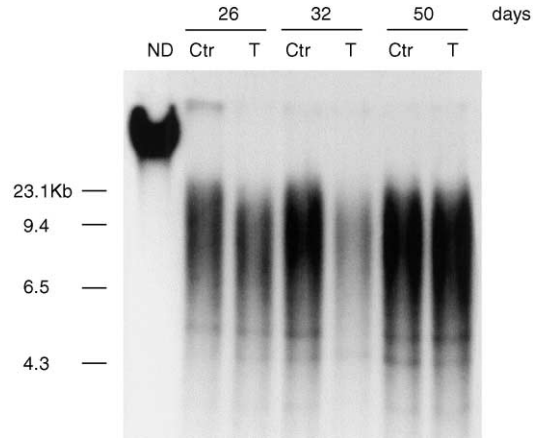

Fig. 6. Telomere length, detected by Southern blotting, of JR8 cells untreated (Ctr) or exposed to 200 μM MEN 10716 (T). ND, non-digested DNA.

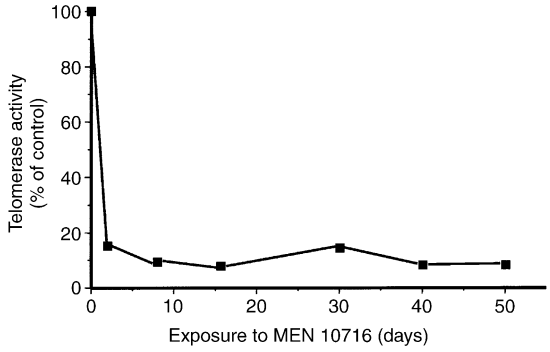

Fig. 7. Quantitation of MEN 10716-mediated telomerase inhibition in H460 non-small cell lung carcinoma cells. The effect of MEN 10716 is expressed as the percentage of the enzyme's activity compared with control.

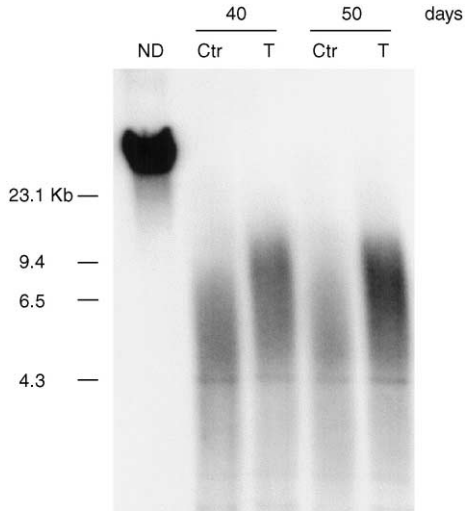

Fig. 8. Telomere length, detected by Southern blotting, of H460 cells untreated (Ctr) or exposed to 100 μM MEN 10716 (T). ND, non-digested DNA.

4. Discussion

Our study demonstrates the ability of the distamycin derivative MEN 10716 to inhibit telomerase activity. Specifically, a marked attenuation of telomerase activity was observed in intact melanoma and non-small cell lung carcinoma cells exposed to the drug. Such an inhibition does not seem to represent a secondary event of drug-induced tumour cell growth arrest, since the effect of MEN 10716 on telomerase activity was observed at earlier time points than that on cell proliferation in both cell systems. Moreover, the inhibitory effect of the drug appeared to be specific for telomerase, since another DNA polymerase, DNA polymerase α, was not affected by treatment. Inhibition of telomerase activity was not accompanied by any reduction in the expression levels of the telomerase catalytic subunit (*hTERT*) mRNA and the RNA component *hTR* (data not shown). This finding together with the results obtained in the JR8 cell-free system suggests that the MEN 10716-induced loss of telomerase activity was a direct effect of the drug on the enzyme, although it cannot be excluded that the decline of telomerase activity was the consequence of the interference of MEN 10716 with other cellular functions. However, considering the MEN 10716 concentration required to inhibit telomerase by 50% in cell extracts, it appears that the compound is less potent as a telomerase inhibitor than recently proposed small molecule inhibitors [28,37] or quadruplex interactive compounds (such as, for example, polycyclic acridines [23,38]) which are able to efficiently inhibit telomerase at submicromolar levels.

Chronic exposure to MEN 10716 effectively inhibited JR8 cell proliferation with a peak of cell growth arrest after 20 days of treament. Cells then partially recovered, however, their growth potential remained markedly reduced compared with that of control cells. Although less pronounced, a decrease in the proliferative potential of drug-treated H460 cells was also observed. The results obtained in JR8 cells with a variety of assays indicated that MEN 10716 produced the decrease in melanoma cell proliferation through the induction of apoptosis. Specifically, in the drug-treated JR8 cell line, a significant percentage of cells showed an apoptotic nuclear morphology and the presence of a hypodiploid DNA content. Moreover, in JR8 cells exposed to MEN 10716 DNA degradation with the presence of large

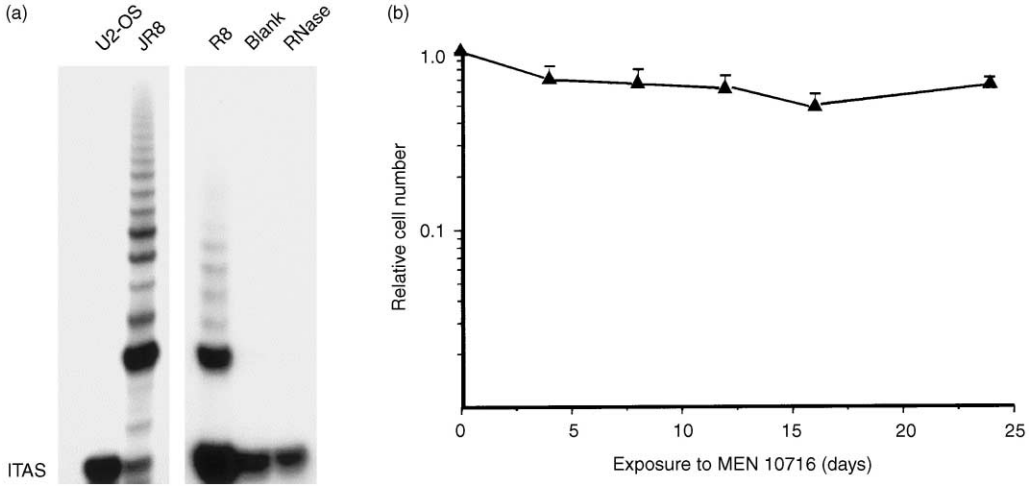

Fig. 9. (a) A representative TRAP assay showing the absence of telomerase activity in U2-OS human osteogenic sarcoma cells. JR8 cells were used as a telomerase-positive control. R8 represents an external quantitative standard (TSR8). Blank represents a negative control to which no cell extract was added. RNase represents a negative control containing JR8 cell extract pretreated with RNase. The location of the internal amplification standard (ITAS) is indicated. (b) Effect of MEN 10716 on the proliferative potential of U2-OS cells. Cell numbers of treated cells are shown on a logarithmic scale, relative to control cells. The data represent mean values + standard deviation (S.D.) of triplicate samples.

DNA fragments was observed, indicating an endonucleolytic activity associated with apoptotic cell death [36]. A small fraction of cells with an apoptotic nuclear morphology was also detected in the H460 lung carcinoma cell line after exposure to MEN 10716.

In the JR8 melanoma cell line, we previously demonstrated the occurrence of apoptosis after treatment with specific telomerase inhibitors such as a hammerhead ribozyme [15] and a cell-penetrating peptide nucleic acid construct [12], both targeting the RNA template of telomerase. Specifically, exposure of JR8 cells to these molecules, besides inducing a marked attenuation of the enzyme's catalytic activity, caused a reduction of cell proliferative potential, as evidenced by longer doubling times, and the appearance of small, but significant, percentages of apoptotic cells.

In the current study we found that non-apoptotic JR8 cells surviving MEN 10716 exposure were characterised by increased melanin content and enhanced expression of the cyclin-dependent kinase inhibitor $p16^{INK4A}$. Although accumulation of $p16^{INK4A}$ has been correlated with the onset of senescence in human fibroblasts [39] and a possible association between senescence and $p16^{INK4A}$ expression has been observed in human melanocytes [40], recent reports would exclude a direct role of the cyclin-dependent kinase inhibitor in senescence [41]. Accordingly, in cells exposed to MEN 10716, senescence-associated β-galactosidase activity, a surrogate marker of senescence [42], was comparable to that of control cells when measured after different rounds of *in vitro* treatment (data not shown).

In JR8 melanoma cells surviving MEN 10716 exposure, we failed to observe any significant variation in the mean telomere length. Although we were unable to visualise the length of individual short telomeres in a distribution of terminal restriction fragments (ranging from 3 to 23 kb) on a Southern blot, it is possible that drug treatment selectively killed cells characterised by the shortest telomere. In fact, it was recently observed that the shortest telomere, rather than the average telomere length, elicits a cellular response and is critical for cell viability [43]. We have previously reported that in the JR8 cell system the inhibition of telomerase activity by different antisense approaches targeting the RNA component of the holoenzyme did not result in any telomere shortening [12,15].

Interestingly, we found an appreciable increase in the mean telomere length in MEN 10716-treated H460 cells. This finding might be tentatively explained by the emergence, under treatment pressure, of a cell population characterised by the presence of alternative lengthening of telomeres (ALT) mechanisms, which are responsible for the maintenance of telomeres—probably through recombination events [44]—and have been demonstrated to be present in a small fraction of tumours [45]. It has previously been shown that some human tumours exhibit both telomere maintenance mechanisms [45]. Moreover, results obtained by Perrem and colleagues [46] in hybrid somatic cell clones generated by fusion of the ALT cell line GM847 with telomerase-positive cells indicate that in such hybrids telomerase is maintained and ALT is repressed, even though the factor(s) responsible for ALT repression has still to be identified. However, the authors did not exclude that ALT repression in hybrids is indirectly mediated by telomerase, probably in concert with other telomerase-associated factors [46]. On the basis of such evidence, as regards our results obtained in H460 non-small cell lung carcinoma cells, it could be hypothesised that the marked and prolonged inhibition of telomerase activity following MEN 10716 treatment was responsible for the reactivation of ALT mechanisms in these cells.

Considering that MEN 10716 is able to interact with DNA and, as a consequence, to induce cellular effects other than telomerase inhibition, it is challenging to define the relative contribution of the attenuation of telomerase catalytic activity to the overall antiproliferative effect of the drug. However, the very modest antiproliferative effect we observed after chronic exposure to MEN 10716 in the U2-OS telomerase-negative human osteogenic sarcoma cell line [47], which maintains telomeres through ALT mechanisms, suggests that telomerase is a relevant target for the overall MEN 10716 cytotoxic activity.

In the telomerase-positive tumour cell models used in this study, we found that MEN 10716 was able to induce apoptotic cell death after a few days of treatment. This finding is in accordance with previous reports showing a rapid loss of cell viability with concomitant apoptosis after a few days of treatment with telomerase inhibitors in different tumour cell lines [48,49]. Notably, there have also been reports of antisense treatments leading to a rapid apoptotic cell death in tumour models growing in mice. Specifically, a significant reduction in the growth of human malignant glioma [13] and prostate [50] xenografts at one week after treatment was reported with repeated direct intratumoral injection of an antisense oligonucleotide linked to a 2′,5′-oligoadenylate. More recently, Saretzki and colleagues [51] transduced four ovarian cancer cell lines with an adenovirus expressing a ribozyme targeting *hTERT* mRNA and observed a massive cell loss in mass culture from all cell lines 3 days after transduction. The results of their study cannot be explained by the classical model which predicts that long-term exposure of tumour cells to telomerase inhibitors should induce telomere shortening after a certain number of rounds of cells division (the number being dependent upon the initial telomere length) and growth arrest. In fact, in the aforementioned studies, it is unlikely that cell death was related to telomere erosion since the cells would not have undergone enough divisions to significantly shorten their telomeres. Overall, these data suggest two different mechanisms by which telomerase

inhibition could lead to cell growth arrest and apoptosis. The first, which does not require telomere shortening, is probably due to the interference of telomerase inhibitors with the capping functions of telomerase, while the second is the classical mechanism based on slow telomere shortening. Recent evidence suggests that telomeres normally exist in a capped state, but may switch to an uncapped state. The appropriate response to the uncapping of a telomere is action by telomerase to protect the telomere from signalling into cell-cycle arrest/apoptosis pathways [52]. Based on these findings, it could be hypothesised that in presence of a marked inhibition of telomerase activity, as in the case of cells exposed to MEN 10716, the enzyme is no longer able to protect the telomere and cells can die through a mechanism independent of telomere length.

Overall, the results of the study indicate that MEN 10716 is a possible telomerase inhibitor and suggest that abrogation of telomerase activity can affect cell proliferation even through pathways that are not dependent on telomere erosion.

Acknowledgements

The study was supported by grants from the Italian Association for Cancer Research (AIRC) and the National Research Council (CNR), Target Project on Biotechnology (no. 01.00709.PF49). The authors thank B. Johnston and B. Canova for their editorial assistance.

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