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Cis-[Pt(Cl)$_2$(pyridine)(5-SO$_3$H-isoquinoline)] complex, a selective inhibitor of telomerase enzyme[a]

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Abstract

Since it has been widely demonstrated that platinum-based drugs, like cisplatin, carboplatin and oxaliplatin, bind preferentially to guanine in N7 position and that telomerase assemblage includes a RNA portion rich in guanine, we previously designed and synthesized a series of new complexes with a cytotoxic [Pt(II)Cl$_2$] moiety, with the aim of selecting carrier ligands able to inhibit telomerase enzyme. Among these compounds, [*cis*-dichloropyridine-5-isoquinolinesulfonic acid Pt(II)], named Ptquin8, showed the most significant inhibition of telomerase in a cell-free biochemical assay. In this paper, we report the biological effects of Ptquin8 on *in vitro* tumor model (MCF-7). This complex is able to reduce telomerase activity from 12 to 46%, in a concentration range between 10^{-9} and 10^{-5} M after 24 h continuous treatment. Moreover, Ptquin8 shows significant cytotoxicity after 10 days of continuous treatment only at concentrations higher than 10^{-5} M. The determination of residual telomere length confirmed the inhibition of telomerase action. This induced a progressive reduction of the cell proliferative capacity, and the appearance of an elevated number of apoptotic cells after 18 days. RT-PCR analysis of telomerase RNA components excluded any interaction of the compound at genomic level. The biochemical effects of Ptquin8 were also evaluated on non-neoplastic NIH3T3 cells, that are able to down-regulate telomerase activity as a consequence of the confluence contact inhibition. In this cell model, the reactivation of telomerase due to re-seeding at lower density was significantly inhibited by Ptquin8 in a dose-dependent manner. These results highlight a possible role of Ptquin8 as a selective anti-telomerase tool for cancer treatment.

Introduction

Telomerase is deputed to the elongation of chromosomal ends. This mechanism confers an unlimited proliferative potential to the cells that become able to escape replicative senescence (Parkinson *et al.*, 1997). A number of experimental evidences demonstrate a strong telomerase activity in most human tumors and their metastasis, a mild activity in germinal cells, and undetectable levels in normal somatic tissues (Faraoni *et al.* 1997; Ogino *et al.* 1998). This enzyme, that is a specific tumor marker, represents an ideal pharmacological target, too. The inhibition of telomerase, in fact, could cause a progressive and critical reduction of telomeres, a potent signal for cell proliferation blockage and for the induction of apoptosis, especially for highly proliferative cells, like metastasis (Hahn *et al.* 1999).

An ideal anticancer strategy should be free of systemic toxicity, typical for actual chemotherapy. Telomerase-directed therapy neither affects somatic

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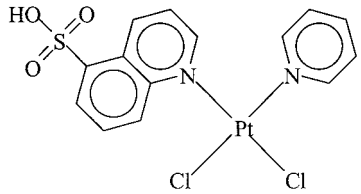


Fig. 1. Sketch of structure of Ptquin8.

cells, since they are telomerase-negative, nor compromises the survival of staminal and germinal cells, that possess low levels of the enzyme and are able to regulate its activation (Campisi 1997).

In the last years, different authors have carried out some experimental strategies to inhibit telomerase with promising results (Campisi 1997; Liu 1999; Yegorov *et al.* 1996; Strahl & Blackburn 1994; Melana *et al.* 1998; Strahl & Blackburn 1996; Lingner *et al.* 1997).

Platinum(II)-based antiproliferative drugs possess high affinity for nucleotides, especially for guanine in N7 position (Sherman *et al.* 1987; Wong *et al.* 1999). Telomerase catalytic activity is based on its RNA template, rich in guanine (Norton *et al.* 1996; Kim *et al.* 1994). Thus, platinum(II)-based compounds seem to offer a good model for telomerase-blocker design. Earlier, we have described the synthesis of a series of platinum complexes with carrier groups suitable for interacting with telomerase (Cavigiolio *et al.* 2000) in a cell-free biochemical assay. The aim of the present work is to further investigate the biological effects of the most promising molecule, i.e., [*cis*-dichloropyridine-5-isoquinolinesulfonic acid Pt(II)], hereafter Ptquin8 (Figure 1), on cell models.

Materials and methods

Cell culture

An established cell line, the human mammary adenocarcinoma, MCF-7, has been used for the *in vitro* experiments (ATCC HTB-22, American Type Culture Collection, Rockville, MD, USA). In addition, for comparison studies a non-tumoral model (NIH3T3 cell line from Swiss albino mouse fibroblasts) was adopted. This cell line maintains the contact-inhibition of growth. NIH3T3 cells were plated at high density, in order to become confluent within 48 h, and grown for at least two weeks. The medium was replaced every 36 h. All the cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U ml penicillin, 100 μg/ml streptomycin and 0.25 μg ml amphotericin B. Culture reagents were purchased from Sigma. Cells were cultured at 37 °C in humidified 5% CO_2 atmosphere, and cell monolayers were periodically screened for mycoplasma contamination.

Ptquin8 was dissolved in culture medium freshly before use. For long term treatments (up to 4 weeks), the MCF-7 cells were detached with 0.05% trypsin − 0.02% EDTA solution (Sigma) every 72 h and were plated in complete medium conditioned with the compound. Every 36 h the growth medium was replaced with fresh medium containing appropriate concentration of Ptquin8.

Chemicals

Ptquin8 was synthesized according to the previously published method (Cavigiolio *et al.*, 2000).

Unless otherwise indicated, all reagents and salts were purchased from Sigma (Sigma Chemical Co, St. Louis, MO, USA).

Cell viability assay

The tetrazole-based vitality test was adopted using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). Converted dye was dissolved in HCl-acidified isopropanol and measured spectrophotometrically at 570 nm (background subtraction at 630 and 690 nm). All experiments were conducted at least in triplicate. The results are presented as mean ± standard deviation (SD) and subjected to student's *t*-test statistical analysis.

Determination of the residual telomerase activity

The effects of the Ptquin8 on cell proliferation were correlated with telomerase activity. Cells were washed and the extraction of active residual telomerase was performed according to Kim *et al.* (1994). Briefly, after the treatments, cells were washed with cold phosphate-buffered saline (PBS), pH 7, rinsed with a buffer containing 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl and 1 mM DTT, and incubated with 3 volumes of a cold solution containing 10 mM Tris-HCl, 1 mM EGTA, 0.5% CHAPS, 1 mM $MgCl_2$, 10% glycerol, 0.1 mM PMSF and 5 mM 2-mercaptoethanol. Cell lysate was fractionated by centrifugation at 10 000 *g* for 30 min. Supernatants were collected quickly and stored at −80 °C until use. Total cellular protein was determined by Bradford method (1976).

A range of 0.5–1 μg total cellular protein extract was employed for the TRAP (Telomeric Repeat Amplification Protocol) reaction. Determination of telomerase activity by this semi-quantitative method was performed as previously described (Kim *et al.* 1994) The untreated cell extract was used as the positive control and a lysis buffer as the negative control.

Reaction products were resolved on an 8% polyacrylamide gel. Densitometry was performed using a GS-250 Molecular Imager (BioRad) with BI screen type and Phosphor Analist 1.1 program for densitometric analysis.

Apoptosis

The effects of Ptquin8 on apoptotic response were evaluated by double staining of the treated cell monolayers. Cells were grown in plates and were treated continuously with Ptquin8 at 10^{-9} and 10^{-7} M concentrations. Every 3 days cells were re-seeded with fresh conditioned medium and assayed for apoptotic pattern by staining with Hoechst 33342 and propidium iodide. Nuclear morphological changes and cell membrane damage were analyzed by confocal microscopy (BioRad MRC 600 coupled to a Nikon Diaphot 200 microscope).

Reverse transcription-PCR (RT-PCR)

In order to discriminate between non-specific action of the compound at the transcription level (thus interfering also with the synthesis of the telomerase) and its ability to interact exclusively with the synthesized enzyme, we evaluated the expressions of the RNA for three telomerase subunits, hTERT (human telomerase reverse transcriptase) hTERC (human telomerase RNA component), and TEP-1 (telomerase protein). Total RNA was extracted (Quiagen) and selected sequences were retrotranscribed and amplified with specific primers (AMV-RT, Sigma). We adopted 60 °C annealing temperature, 28 cycles for hTERT, hTERC, and TEP-1 and 21 cycles for β-actin. The specific primers for the three sequences were previously described by other authors (Yokoyama, 1998). The primer sets were as follows: for hTERT, 5′-TGAACTTGCGGAAGACAGTGG-3′ (forward) and 5′-ATGCGTGAAACCTGTACGCCT-3′ (reverse); for hTERC, 5′-TTTGTCTAACCCTAACTGAGAAG-3′ (forward) and 5′-TTGCTCTAGAATGAACGGTGGA-3′ (reverse); for TEP-1, 5′-TCAAGCCAAACCTGA-ATCTGAG-3′ (forward) and 5′-CCCCGAGTGAAT-CTTTCTACGC-3′ (reverse); for β-actin, 5′-GTGGG-GCGCCCCAGGCACCA-3′ (forward) and 5′-CTCC-TTAATGTCACGCACGATTTC-3′ (reverse). The products were resolved by 1.5% agarose electrophoresis and the data were normalized on β-actin expression using Gel Doc 1000 for acquisition and Quantity One program 4.0.3 (BioRad) for quantification analysis.

Determination of residual telomere length

For the telomere length determination, treated cells were detached, included in agarose plugs (10^7 per ml), and digested with proteinase K at 50 °C for 48 h. This procedure was adopted to facilitate the DNA restriction step, conducted with Hinf I enzyme (60 U per 10^7 cells). This reaction provided terminal DNA restriction fragments (TRF, 10^6 bp fragments), preserving the telomeric portion of DNA (10^3-10^4 bp). The samples were resolved with pulse field agarose gel electrophoresis (0.7%, 600 Vh). Resolved DNA was blotted on Nylon membranes (Hybond-N+, nylon), UV cross-linked and characterized with Southern blot analysis. Membranes were hybridized with specific probes for telomeres $(TTAGGG)_4$, ^{32}P-labeled, then analyzed with autoradiography and densitometry (PhosphorImager). Ladders were referred to the Hind III standard.

Results

As previously described by our group, Ptquin8 (Figure 1) was able to inhibit the enzyme in a cell-free biochemical assay in a dose-dependent manner in a concentration range of 10^{-11}–10^{-7} M (Cavigiolio *et al.* 2000).

The effect of this compound has been than evaluated directly on cells. The treatment of log-phase growing MCF-7 monolayers for 24 h with Ptquin8 evidenced a maximum inhibition of ca. 44% in 10^{-7}–10^{-4} M interval. This inhibition progressively decreased at lower concentrations (Figure 2). A typical dose-response TRAP assay is shown Figure 3.

The toxicity profile of Ptquin8 was evaluated by culturing the cells for 10 days (with administration interval of 24 h) with complete medium containing 10^{-9}–10^{-4} M of the compound. Results obtained from MTT assay showed that this compound had significant cytotoxic effects only at concentrations higher than 10^{-5} M. Therefore, in further experiments, we employed Ptquin8 at 10^{-9} and 10^{-7} M concentrations in order to keep significant anti-telomerase activity and avoid any aspecific cytotoxic effects.

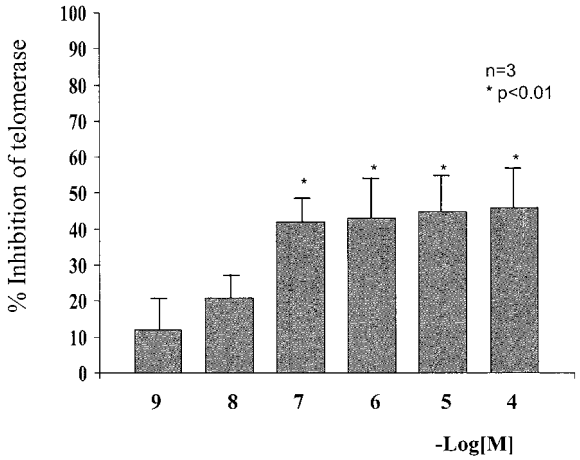


Fig. 2. Antitelomerase activity of Ptquin8 on tumoral cells. MCF-7 were treated for 24 h with Ptquin8 in a concentration range of 10^{-4}–10^{-9} M. The average reduction of residual telomerase activity extracted from the cells was 46% for 10^{-4}–10^{-7} M range. Data are expressed as the mean ± SD.

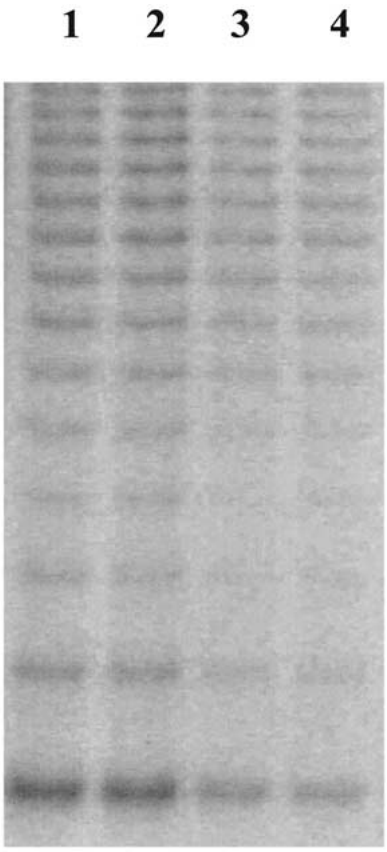


Fig. 3. Typical TRAP assay resolved with PAGE. Lane 1: untreated MCF-7 (control), Lane 2 to 4: MCF-7 cells treated with 10^{-9}, 10^{-7} and 10^{-5} M Ptquin8 for 24 h.

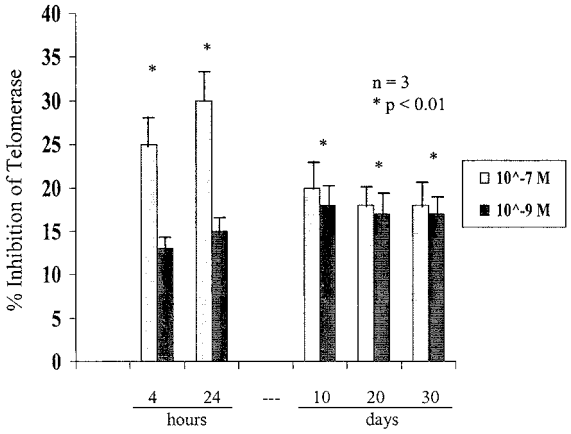


Fig. 4. Long term effects (30 day continuous treatment with conditioned media renewal every 36 hours) on telomerase activity of Ptquin8 at two concentrtations, namely 10^{-7} and 10^{-9} M. Data are expressed as the reduction of activity respect to the untreated control at the same time point.

To investigate the effect of long term treatments on telomerase and the relative consequences on cell growth pattern, a 30-day continuous incubation with Ptquin8 at 10^{-9} and 10^{-7} M concentrations was performed. At given time intervals, telomerase activity, cell vitality, apoptosis, and telomere length were evaluated.

The analysis of telomerase activity with TRAP assay at 10^{-7} M Ptquin8 showed a significative decrease (ca. 30%) in the first 24 h, and a stable reduction (ca. 20%) for the remaining period (Figure 4). Incubation at 10^{-9} M led to a lower, but constant (ca. 15%) reduction of telomerase activity. Starting from day 15, Ptquin8 was able to reduce the proliferation of the cells with respect to the untreated control (Figure 5), even if the visual assessment of the culture or the trypan blue dye exclusion did not reveal a consistent number of dead cells. However, apoptotic nuclei were visible from day 18. This phenomenon was followed by a progressive cell death from day 21, and matched the vitality assay results.

In order to demonstrate that the reduction of telomerase activity was not due to a reduction of the transcription of the components of telomerase, we measured their expression levels, as shown in Figure 6. Ptquin8 (10^{-7} M) after 4, 20, 30 and 48 h of continuous treatment, caused no differences in expression of mRNA of telomerase components, namely hTERT, hTERC and TEP-1, compared to the untreated control. These data were obtained by RT-PCR and normalized with the housekeeper β-actin expression.

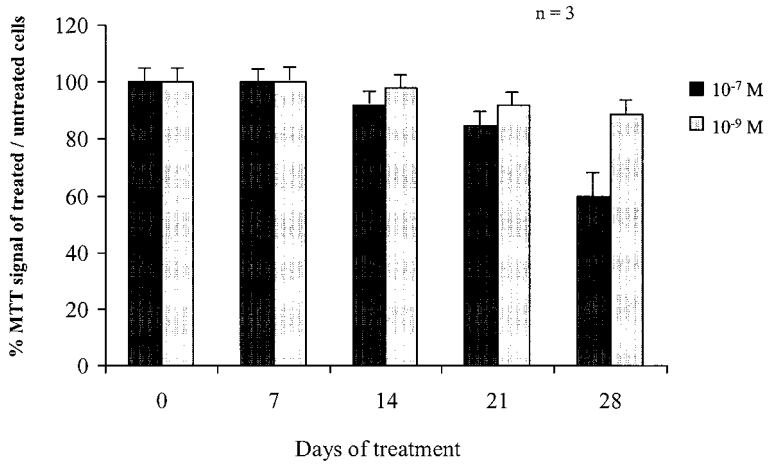


Fig. 5. Long term effects of Ptquin-8 on proliferation of MCF-7. Cells were continuously incubated with Ptquin8, 10^{-7} and 10^{-9} M, freshly replaced every 36 h. After 72 h, the cells were detached and re-seeded at 1/3 density. Data are expressed as the mean ± SD.

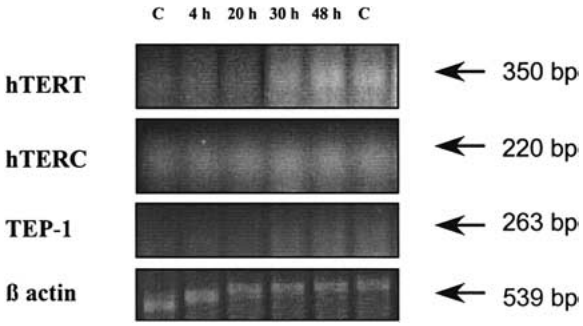


Fig. 6. Effects of Ptquin8 on the transcription of the different components of telomerase. Ptquin8 10^{-7} M for 4, 20, 30 and 48 h continuous treatments induced no differences on the expression of the mRNA of hTERT, hTERC and TEP-1, respect to the untreated controls (C). The expression of the components was measured by RT-PCR and normalized with b-actin. No differences in the expression could be appreciated.

The telomere analysis revealed that the continuous exposure of cells with Ptquin8 and the relative telomerase inhibition caused a progressive reduction of their length (Figure 7). The overall experimental protocol has been applied also to the non-tumoral fibroblastic NIH3T3 cell line. The cytotoxic profile of Ptquin8 (10^{-7} M) on these cells, revealed no effects up to 14 days of continuous treatment (data not shown). These cells, when confluent, naturally down-regulate telomerase activity, as already demonstrated by other authors (Holt *et al.* 1996). The continuous exposure to Ptquin8 contributed to down-regulate the enzyme activity (Figure 8). Re-seeding of confluent cells at lower density led to a fast increase in telomerase activity within 24 h. Treatment of re-seeded cells with Ptquin8

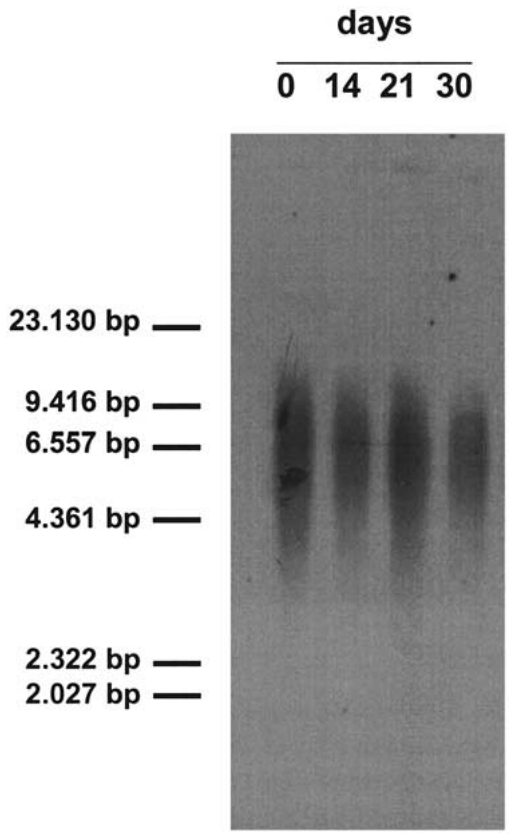


Fig. 7. Effects of Ptquin8 on telomere length. Long term treatments induced a progressive reduction of telomere length, evident from day 14. Lane 0 days represents the untreated control. Hind III was the reference bp ladder.

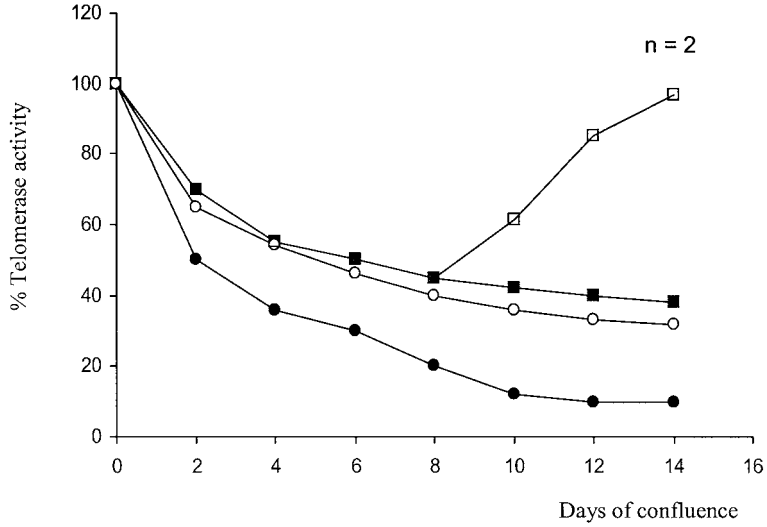
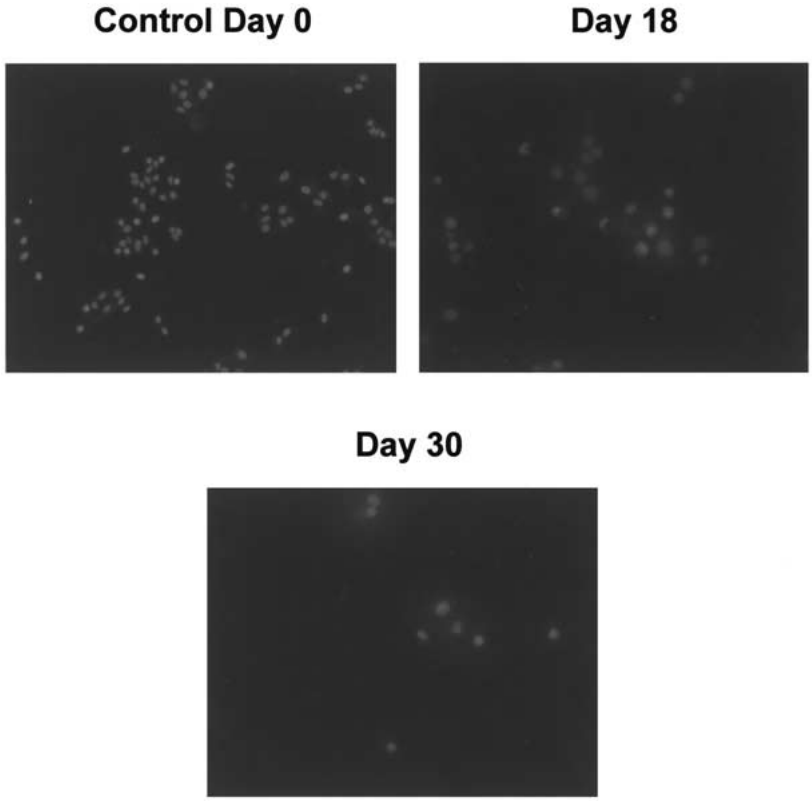


Fig. 8. Down-regulation of telomerase activity induced by contact inhibition of the growth in NIH3T3. Data points represent telomerase activity. Cells were grown to confluence and kept without media renewal for up to 14 days (filled squares). Ptquin8 10^{-7} M (filled circles) or 10^{-9} M (empty circles) treatments induced a faster disappearance of telomerase activity. Re-seeding of the cells at lower density at day 8, promoted a fast recovery of telomerase activity in 24–48 h (empty squares). Ptquin8 abolished this re-activation.



Panel showing the effects of long term continuous treatments with Ptquin8 on MCF-7 cell-line. Cells were kept under standard culture conditions, passed every 4 days, and fresh complete medium conditioned with 10^{-7} M Ptquin8 was renewed every 48 h. Untreated cells represented day 0 control. Control picture is characterized by the presence of normal living cells as evidenced by blue spots due to Hoecht 33342, and is representative of day 7, 21, 30 controls. Starting from day 7, the presence of late apoptotic cells could be appreciated, and was exponentially increasing after day 21, as demonstrated by the red staining due to propidium iodide. At day 30 no living cells could be detected.

(10^{-7} M) strongly inhibited the recovery of the telomerase activity. Moreover, neither the proliferative rate nor the level of confluence of the monolayers were affected, when compared to the untreated controls. In this cell line, Ptquin8 did not cause the appearance of apoptotic nuclei even after 30 day continuous exposure (data not shown).

Discussion

Among a number of molecules synthesized by our group, Ptquin8 has been selected because of its ability to reduce telomerase activity in a cell-free system (Cavigiolio *et al.* 2000). In this paper, we show that Ptquin8 is also capable of reducing telomerase activity in living cells to a similar extent.

The observation that Ptquin8 induced a fast decrease of telomerase activity suggests a direct interaction between the compound and the existing pool of telomerase, rather than interference with the synthesis of the constituents of the enzyme. The RT-PCR data lead support to this finding, and suggest that the complex is not able to affect genomic DNA, at least in the concentration interval employed. On the contrary, the telomerase inhibition caused by cisplatin seems the result of genomic alteration (Burger *et al.* 1997; Zhang *et al.* 2002). Although Ptquin8 is not able to completely abolish the enzyme activity at any concentration tested, the inhibition obtained is sufficient to destabilize the telomeric homeostasis, causing the telomere shortening and the appearance of apoptotic pattern. This correlates with the impaired proliferative potential of the cells, as expressed by a progressive reduction of their replication.

In the non-tumoral NIH3T3 cell line, where neosynthesis of telomerase is down-regulated in confluent cells, Ptquin8 induces a faster reduction of the enzyme, thus strengthening the hypothesis of a Ptquin8-telomerase direct interaction.

However, Ptquin8 does not induce apoptosis in these cells, even for long term treatments. Indeed, NIH3T3 cells derived from mouse where telomeres are much longer compared to those of human cells (Lejnine *et al.* 1995).

Interestingly, Ptquin8 is active at very low concentrations (10^{-9}–10^{-7} M) where no aspecific cytotoxic effects have been observed. Therefore, this molecule can represent a new tool for cancer chemotherapy, where long term treatments, selectivity, and low systemic toxicity are required.

Studies to fully characterize the mechanism of action of Ptquin8, and its effect on cisplatin-resistant cell lines are in progress.

Acknowledgements

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