Potent Inhibition of Telomerase by Small-Molecule Pentacyclic Acridines Capable of Interacting with G-Quadruplexes

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ABSTRACT

A novel pentacyclic acridine, 3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl]acridinium methosulfate (RHPS4), has been identified as a potent inhibitor of telomerase in the cell-free telomeric repeat amplification protocol (TRAP). Modeling and biophysical studies suggest that RHPS4 inhibits telomerase through stabilization of four-stranded G-quadruplex structures formed by single-stranded telomeric DNA. In contrast to G-quadruplex interactive telomerase inhibitors described previously, RHPS4 inhibited telomerase at submicromolar levels (50% inhibition in the TRAP assay at 0.33 $\pm$ 0.13 μM). Moreover, RHPS4 exhibited a wide differential between this potent inhibition of telomerase and acute cellular cytotoxicity (mean IC$_{50}$ value of 7.02 μM in 4-day growth inhibition assay). RHPS4, when added to 21NT breast cancer cells at nonacute cytotoxic concentrations (200 nM) every 3 to 4 days, induced a marked cessation in cell growth after 15 days. Similar effects were observed using another cell line possessing relatively short telomeres, A431 human vulval carcinoma cells, but not in a human ovarian carcinoma cell line (SKOV-3) possessing relatively long telomeres. In 21NT cells, growth cessation was accompanied by an increase in cells in the G$_2$/M phase of the cell cycle, a reduction in cellular telomerase activity, and a lower expression of the $hTERT$ gene. These effects occurred in the absence of a detectable reduction in telomere length as measured by slot blotting. RHPS4 also induced a cessation of growth of GM847 cells that maintain telomeres by a nontelomerase alternative mechanism for lengthening telomeres (ALT) after 15 days. RHPS4 represents a promising G-quadruplex interactive small molecule that is a potent cell-free inhibitor of human telomerase and induces growth inhibitory effects in human tumor cell lines after prolonged (2-week) exposure to nonacute cytotoxic drug concentrations.

In recent years, telomerase, a reverse transcriptase responsible for adding hexanucleotide TTAGGG telomeric repeats onto the ends of chromosomes, has emerged as a highly promising novel target for therapeutic intervention in the treatment of cancer (Pitts and Corey, 1999; Kelland, 2000). In normal somatic cells, telomeres shorten by 50 to 200 bases on each round of replication as a consequence of the "end-replication problem": the inability of DNA polymerase to fully replicate the 3′ end of linear molecules during discontinuous lagging strand synthesis (Harley et al., 1990). In approximately 85% of human cancers, however, telomerase is reactivated and acts to maintain telomere length, usually at relatively short lengths in the region of 3 to 8 kb (Kim et al., 1994; Holt and Shay, 1999; Chu et al., 2000). Moreover, aside from a few normal cells [such as hematopoietic T and B cells, which have longer telomeres in the region of 15 kb (Kolquist et al., 1998)] the vast majority of normal cells do not possess

telomerase activity. Telomerase activation has been shown to represent a key step in human epithelial tumor oncogenesis (Hahn et al., 1999a; Gonzalez-Suarez et al., 2000), and studies of telomerase-negative knockout mice have revealed its role in maintaining high renewal organs (Lee et al., 1998). A small proportion of human tumors seems to maintain telomere length by a mechanism not involving telomerase (Bryan et al., 1997). These ALT cells have recently been shown to maintain telomeres by end-to-end recombination events (Dunham et al., 2000).

Telomerase is known to comprise at least three components: an RNA template (hTR) (Feng et al., 1995); a catalytic protein domain (hTERT, hTRT, hEST2) (Meyerson et al., 1997; Harrington et al., 1997a); and a further protein (TP1, hTEP1) (Harrington et al., 1997b). In addition, various proteins have recently been shown to associate with telomeres and may also contribute to the folding of telomeres into higher-order t-loop structures (Griffith et al., 1999) and/or the regulation of telomere length. These include TRF1 (van

ABBREVIATIONS: kb, kilobase pair(s); TRAP, telomeric repeat amplification protocol; RHPS4, 3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl] acridinium methosulfate; SRB, sulforhodamine B; PCR, polymerase chain reaction; CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; bp, base pair(s); ALT, alternative mechanism for lengthening telomeres; PIPER, N,N'-bis[2-(1-piperidine)ethyl]-3,4,9,10-perylenetetracarboxylic diimide.

Steensel and de Lange, 1997), TRF2 (van Steensel et al., 1998), and tankyrase (Smith et al., 1998). Telomerase may also contribute to capping telomere ends, thereby providing protection from activating DNA damage-response pathways (Blackburn, 2000).

Target validation studies using dominant negative constructs of hTERT transfected into cells of varying telomere length have shown telomerase inhibition with inhibition of cell growth and elimination of tumorigenicity in nude mice; the onset of effects is related to the initial cellular telomere length (Hahn et al., 1999b; Zhang et al., 1999). A number of approaches have been described previously to inhibit telomerase and/or telomeres (Neidle and Kelland, 1999; Pitts and Corey, 1999). These mainly include antisense molecules directed at the RNA template (e.g., oligonucleotides, ribozymes, or peptide nucleic acids) (Kondo et al., 1998; Yokoyama et al., 1998; Hamilton et al., 1999) and reverse transcriptase inhibitors [e.g., azidothymidine (Strahl and Blackburn, 1996)]. However, neither of these approaches is ideal from a pharmaceutical drug perspective; thus far, antisense molecules have proven relatively difficult to selectively deliver to tumors in vivo, and reverse transcriptase inhibitors have problems of specificity for telomerase versus other reverse transcriptases. Our strategy has been to design small molecules capable of interacting with higher-order structures formed by the G-rich single-stranded overhang of telomeres, namely four-stranded G-quadruplexes. Because telomerase requires a single-stranded telomeric primer, agents that stabilize the folding of telomeric DNA into four-stranded G-quadruplexes have been shown to be effective telomerase inhibitors (Zahler et al., 1991; Mergny et al., 1999). Classes of G-quadruplex inhibitors described to date by us include the first reported small-molecule telomerase inhibitors, disubstituted anthraquinones (Sun et al., 1997; Perry et al., 1998), fluorenones (Perry et al., 1999a), acridines (Harrison et al., 1999), and a tetracyclic-based compound (Perry et al., 1999b). Other groups have described porphyrin-based G-quadruplex inhibitors (Wheelhouse et al., 1998; Izbicka et al., 1999) and those based on a perylenetetracarboxylic diimide (Fedoroff et al., 1998). These have typically shown telomerase inhibition in vitro using the cell-free enzyme-based telomeric repeat amplification protocol (TRAP) (Kim et al., 1994) in the region of 2 to 20 μM. A general goal, largely unrealized by the above series of compounds, is to achieve potent telomerase inhibition in the TRAP assay (ideally at submicromolar concentrations) without acute cytotoxicity (believed to be indicative of effects on duplex rather than quadruplex DNA) (Neidle and Kelland, 1999).

The aim of this study was to evaluate the telomerase inhibitory activity and molecular pharmacological properties of a pentacyclic acridine-based compound, 3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl]acridinium methosulfate (RHPS4) (Fig. 1). This compound was selected from a small series of analogs that, because of their structural similarities to previously identified G-quadruplex interactive telomerase inhibitors (see above), were proposed to inhibit the enzyme. Initial studies showed that particular compounds in this series, such as RHPS4, exhibited the desired profile of potent submicromolar inhibition of telomerase in the TRAP assay but with acute cytotoxicity only at concentrations significantly greater than these levels. Consequently, further whole-cell studies have been performed using nonacute cyto-

toxic concentrations of drug, along with measurements of telomerase inhibition, telomere length, effects on the cell cycle and growth inhibition, and effects on various telomerase and telomere-associated genes.

Materials and Methods

Cell Lines and Reagents. The human breast cancer line 21NT (kindly provided by R. Newbold, Brunel University, Uxbridge, UK) was used for initial whole-cell experiments. This line possesses relatively short telomeres and has been used previously in studies of telomerase repression and growth arrest by chromosome-3 transfer (Cuthbert et al., 1999). Other cell lines used were A431 human vulval carcinoma cells that also possess relatively short telomeres (Zhang et al., 1999), A2780 and SKOV-3 human ovarian carcinoma (Beale et al., 2000), and the immortal human cell line GM847, which maintains telomeres by the ALT mechanism (Hahn et al., 1999b). Lines were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum, 0.5 μg/ml hydrocortisone, and 2 mM L-glutamine in a humidified 6% CO_2/94% air atmosphere. In addition, for 21NT cells, insulin (1 μg/ml) and epidermal growth factor (12.5 ng/ml) were added to support growth. Chemicals were from Sigma (St. Louis, MO) unless otherwise stated. Oligonucleotide primers were from Oswel Ltd. (Southampton, UK). Chemical synthesis and molecular modeling details for RHPS4 will be reported separately (Heald et al., in preparation).

Growth Inhibition Assay. Acute growth inhibitory effects were assessed using the sulforhodamine-B assay as we described previously (Perry et al., 1999a). Briefly, between 3000 and 6000 cells/well were seeded into 96-well microtiter plates and allowed to attach overnight. Drug (freshly dissolved at a concentration of 500 μM in water) was then added at a range of concentrations to quadruplicate wells and left in contact for 4 days. At this point, cell numbers were compared in treated versus control wells by fixing in ice-cold 10% w/v trichloroacetic acid (30 min) and staining with 0.4% SRB in 1% v/v acetic acid (15 min). The mean absorbance at 540 nm for each drug concentration was expressed as a percentage of the control untreated well absorbance.

Taq **Polymerase Assay.** As discussed previously (Neidle and Kelland, 1999; Kelland, 2000), because the TRAP (see below) for assessing inhibitory activity against telomerase is PCR-based, it is important to ensure that positive inhibition in this assay is mediated through the effects on telomerase rather than via nonspecific inhibition of the PCR. Therefore, RHPS4 was added at concentrations of 1, 5, 10, and 20 μM to a PCR 50-μl master mix containing 10 ng of pCI-neo mammalian expression vector (Promega, Southampton, UK) and forward (GGAGTTCCGCGTTACATAAC) and reverse (GTCTGCTCGAAGCATTAACC) primers (200 nmol) as described previously (Perry et al., 1999a). The product of approximately 1 kb was visualized with the use of ethidium bromide after separation by
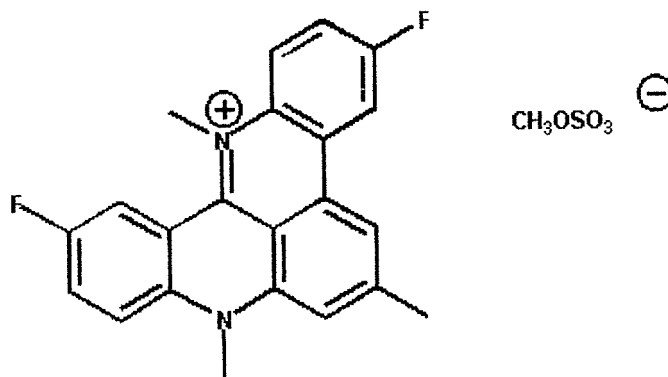

Fig. 1. Chemical structure of the pentacyclic acridine RHPS4.

electrophoresis on a 2% w/v agarose gel after amplification (30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2.5 min) using an MBS thermal cycler (Thermo Hybaid, Ashford, Middlesex, UK).

TRAP. The ability of RHPS4 to inhibit telomerase in a cell-free assay was assessed using the TRAP assay as described previously (Perry et al., 1998; Harrison et al., 1999). Briefly, telomerase was prepared from extracts of exponentially growing A2780 cells by lysing for 30 min on ice in a CHAPS-based buffer [0.5% w/w CHAPS, 10 mM Tris-HCl, pH 7.5, 1 mM MgCl$_2$, 1 mM EGTA, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol] with 0.1 mM AEBSF freshly added. The lysate was then centrifuged at 12,000 rpm for 30 min at 4°C, and the supernatant was collected and stored frozen in aliquots at −80°C for up to 3 months. Total cellular protein was then determined, and the TRAP was performed in two steps using 20 ng of protein. Step 1 consisted of the telomerase-mediated extension of a nontelomeric oligonucleotide forward telomerase substrate primer (5′-AATC-CGTCGAGCAGAGTT) (0.1 μg) contained in a 40-μl reaction mix comprising TRAP buffer (20 mM Tris-HCl, pH 8.3, 68 mM KCl, 1.5 mM MgCl$_2$, 1 mM EGTA, 0.05% Tween 20), 0.05 μg of bovine serum albumin, 50 μM deoxynucleotide triphosphate, and 3 μCi of [α-^{32}P]dCTP (Amersham Pharmacia Biotech UK, Ltd., Little Chalfont, Buckinghamshire, UK). Protein was then incubated with the reaction mix, with or without RHPS4, at various final concentrations, for 20 min at 25°C. A lysis buffer (no protein) control, heat-inactivated (85°C for 10 min) protein control, and 25% protein control (10 ng) were included in each experiment. In step 2, samples were heated at 85°C for 5 min to inactivate telomerase; during this time, 0.1 μg of reverse CX primer (3′-AATCCCATTCCCATTCCCAT-TCCC) and 2 units of *Taq* DNA polymerase ("red hot"; Advanced Biotechnologies, Columbia, MD) were added. A three-step PCR was then performed: 31 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. Telomerase-extended PCR products, with or without RHPS4, were then measured either by electrophoretic separation [8% (w/w) acrylamide denaturing gels; Sequagel, National Diagnostics, Atlanta, GA] and visualization by phosphoimaging or by harvesting on filters (25 mm; Whatman, Maidstone, UK) by trichloroacetic acid precipitation and then analyzing by liquid scintillation counting.

Long-Term Exposure Studies. Cells were seeded in growth medium into T80 tissue culture flasks at 1.25 × 10^5 cells per flask and exposed to a nonacute cytotoxic concentration of RHPS4 (200 nM) or an equivalent volume of water (drug vehicle control) every 3 to 4 days. Every 7 days, the cells in control and drug-treated flasks were trypsinized and counted using a hematocytometer, and flasks were reseeded with 1.25 × 10^5 cells. Remaining cells were collected and used for measurements described below. This weekly process, with twice-weekly drug addition, was continued until such time that there were fewer than 1.25 × 10^5 cells available for reseeding. For measurements of telomerase activity within treated or control cells, protein extracts were prepared as described above for the TRAP, and then known amounts of protein were included in the above-described TRAP.

Measurements of Telomere Length. As an approximation of telomere length, (TTAGGG)$_4$ repeats were measured using slot-blotting from cell pellets collected weekly within the long-term exposure experiments. Pellets were washed in PBS and were DNA-extracted using a QIAamp blood kit (QIAGEN, Dorking, Surrey, UK) according to the manufacturer's instructions. DNA content was determined using Genequant (Amersham Pharmacia Biotech), and 25 and 50 ng from control or treated cells, respectively, were loaded onto Hybond-XL nylon membranes (Amersham Pharmacia Biotech). Membranes were air-dried, denatured (using 0.5 M NaOH, 1.5 M NaCl), neutralized (0.5 M Tris-HCl, pH 8, 1.5 M NaCl), and cross-linked (Stratalinker; Stratagene, Cambridge, UK). Probes for telomere length (TTAGGGTTAGGGTTAGGGTTAGGG) and for centromeric loading controls (GTTTGAAACACTCTTTTTGTAGAATCTGC) were end-labeled using [γ^{32}P]ATP. Slot blots were prehybridized with Rapidhyb (Amersham Pharmacia Biotech) for 30 min, and denatured probes were added and hybridized at 42°C for 3 h. Blots were exposed

to PhosphorImager screen for 1 h and visualized with the use of a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Blots were then stripped and checked clean by PhosphorImager and then hybridized with the centromeric probe as for the telomeric probe. Image analysis was performed using ImageQuant software (Molecular Dynamics, Sevenoaks, UK).

Flow Cytometry. Samples were collected weekly, resuspended in PBS and ice-cold 70% ethanol, and stored at 4°C until analysis. Samples were then centrifuged at 15,000 rpm for 5 min, and cell pellets were resuspended in 100 mg/ml RNase A (Sigma) and 40 μg/ml propidium iodide (Sigma) and incubated at 37°C for 30 min. Samples were then analyzed on a Coulter Elite flow cytometer (Beckman Coulter Inc., Buckinghamshire UK) equipped with a Spectra Physics (San Jose, CA) argon-ion laser with an output of 200 mW at 488 nm. Typically, data from 2 × 10^4 cells were analyzed for forward and orthogonally scattered light together with red fluorescence (peak and integrated area). Data were then analyzed using WinMDI2.8 Windows Multiple Document Interface Flow Cytometry Application (http://www.uwcm.ac.uk/study/medicine/haematology/cytonetuk/index.htm).

Reverse Transcriptase-PCR. Gene expression for *hTERT, tankyrase,* and *c-myc* were analyzed from 21NT cells exposed to RHPS4 or water by reverse transcriptase-PCR. Total RNA was extracted from drug-treated or control cells using RNeasy kit (QIAGEN). Total RNA (600 ng) was reverse-transcribed using cDNA cycle kit (Invitrogen). Then 2 μl of cDNA, 2 μM MgCl2, 200 μM dNTPs, 1.25 units of red hot DNA polymerase (ABgene, Epsom, Surrey, UK), 1× buffer, and 10 μM forward and reverse primers were used for amplification by PCR for the following genes: *hTERT*: forward, 5′ (GCCAAGTTCCTGCACTGGCTGATG); reverse, 3′ (GTTCT-GGGGTTTGATGATGCTGGCG); 34 cycles of 94°C for 1 min, 61°C for 1 min, 72°C for 1 min, and product size of 588 bp; *tankyrase*: forward, 5′ (GCAGCGGGCTACAACAGAGT); reverse, 3′ (GCGC-CATGGCTAAGTAACAAAGA); 28 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 2 min, and product size of 251 bp; *c-myc*: forward, 5′ (ATGCCCCTCAACGTTAGCTTC); reverse, 3′ (CTTACGCACAA-GAGTTCCGTA); 26 cycles of 94°C for 1.5 min, 45°C for 2 min, 72°C for 2 min, and product size of 1300 bp; and *GAPDH* (control): forward, 5′ (CGGGAAGCTTGTGATCAATGG), reverse, 3′ (GGCAGT-GATGGCATGGACTG); 19 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and product size of 358 bp. PCR products were then visualized and expressed as a percentage of expression in control water-treated 21NT cells using ImageQuant software.

Results

Initially, RHPS4 was evaluated in a PCR to determine whether the compound was a nonspecific inhibitor of the reaction and would thereby score as a "false-positive" telomerase inhibitor in the TRAP. Results indicated that there was no decrease in PCR product compared with water controls in the presence of 1, 5, or 10 μM RHPS4. There was some inhibition of the reaction at a drug concentration of 20 μM.

Subsequently, RHPS4 was added to the cell-free TRAP at concentrations ranging from 50 nM to 10 μM (Fig. 2A). Results showed that RHPS4 caused potent inhibition of telomerase-mediated telomere extension at concentrations greater than 100 nM, approaching 100% inhibition at concentrations greater than 1 μM. Visualization of telomerase inhibition is shown in the TRAP gel (Fig. 2B). Again, 2.5 μM and especially 5 μM RHPS4 led to a marked reduction in the appearance of telomerase-produced hexanucleotide repeats compared with protein controls. Hence, RHPS4 inhibited telomerase in the TRAP at concentrations well below those shown to cause nonspecific inhibition of PCR; the concentration causing 50% inhibition was 330 ± 135 nM.

A goal in the search for "second-generation" G-quadruplex interactive telomerase inhibitors is to increase selectivity for binding to quadruplex versus duplex DNA and thereby widen the differential between telomerase inhibition and acute cytotoxicity. The acute growth inhibitory effects of RHPS4 were assessed against a small panel of human tumor cell lines. A dose-response relationship for 21NT cells showed that RHPS4 induced cytotoxicity only at concentrations greater than 3 μM [i.e., 10-fold greater than that required for telomerase inhibition in the TRAP (Fig. 2A)]. Across a panel of six cell lines (21NT, A431, CH1, A2780, SKOV-3, and GM847), the mean IC_{50} value for RHPS4 in the 4-day SRB assay was 7.02 μM, more than 20 times higher than the concentration required for inhibition in TRAP. IC_{50} values were 6.5 μM for A431, 0.5 μM for CH1, 1.1 μM for A2780, 18 μM for SKOV-3, and 8 μM for GM847.

These data show that, in contrast to many G-quadruplex–based telomerase inhibitors described previously [e.g., anthraquinones (Perry et al., 1998)], RHPS4 possesses a sufficient differential between cell-free telomerase inhibition and acute cell killing in five of six of the cell lines. The cellular pharmacological effects of RHPS4 in terms of cell growth, cell cycle, telomerase, and telomeres were investigated using 21NT cells (chosen on the basis of their possession of relatively short telomeres (Cuthbert et al., 1999). In addition, long-term effects were investigated in three other cell lines of predetermined telomere length (Fig. 2C): A431, which also possesses relatively short telomeres (also as shown by Zhang et al., 1999); SKOV-3, which possesses relatively long telomeres; and the ALT line GM847. RHPS4 was used at 200 nM, a concentration sufficient to cause marked inhibition of te-

lomerase in the TRAP assay but well below that causing acute cytotoxicity (Fig. 2A). Results showed that RHPS4 caused a small reduction in 21NT cell growth at 8 days but a marked cessation in growth after 15 days of incubation (Fig. 3A). This was maintained at 22 days, whereupon cell numbers were too low to continue. A similar effect was observed in another human tumor cell line possessing relatively short telomeres (A431) (Fig. 3B), in which marked growth inhibition was observed at 12 and 19 days postincubation. In contrast, the growth of SKOV-3 cells (which possess longer telomeres and are inherently more resistant to the cytotoxic effects of RHPS4) was unaffected over 29 days of drug incubation (Fig. 3C). It may be hypothesized that a telomerase-inhibitory strategy that targets the substrate telomere may also confer growth inhibitory effects in cells that maintain telomeres by the ALT nontelomerase mechanism. In support of this concept, RHPS4 caused a marked reduction in cell growth of GM847 cells at 15 days postincubation. However, in contrast to results for 21NT and A431 cell lines (Fig. 3, A and B), there was some recovery of GM847 cells by day 29 (Fig. 3D).

Finally, the effect of RHPS4 on 21NT cells was assessed in terms of identifying possible pharmacodynamic markers of response through a telomerase/telomere-related mechanism. First, as observed for porphyrin-based G-quadruplex inhibitors (Izbicka et al., 1999), long-term exposure of 21NT cells to noncytotoxic concentrations of RHPS4 resulted in a marked reduction in cellular telomerase activity at similar times (days 15 and 22) during which growth inhibition was observed (Fig. 4A). RHPS4 also induced cell cycle changes after 22 days of exposure: there was an increase in the population

A)

C)

B)
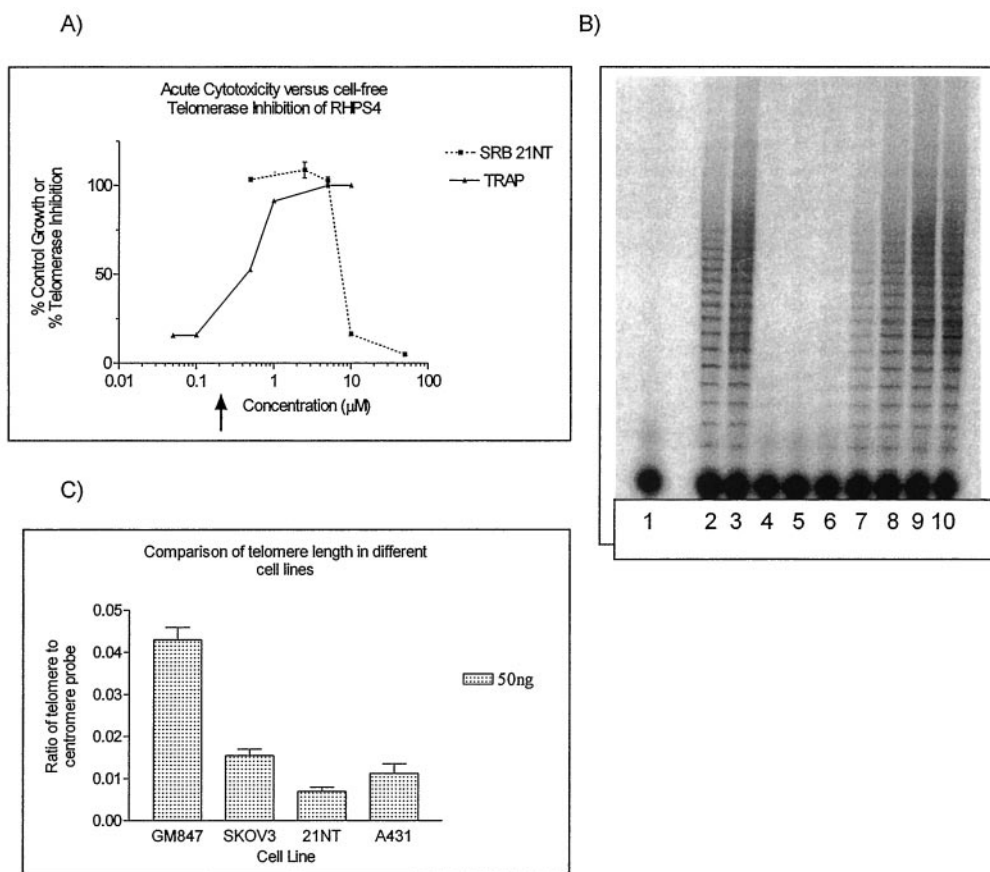
Fig. 2. A, cell-free inhibition of telomerase versus acute cytotoxicity against 21NT cells (SRB assay) by RHPS4 at differing concentrations. Arrow indicates the concentration (200 nM) used in subsequent long-term exposure experiments. B, representative TRAP results: lane 1, lysis buffer control; lane 2, A2780 extract 5 ng of protein; lane 3, A2780 extract 20 ng of protein; lane 4, 20-ng A2780 extract heat-inactivated; lane 5, 20-ng extract plus 5 μM RHPS4; lane 6, 20-ng extract plus 2.5 μM RHPS4; lane 7, 20-ng extract plus 1.0 μM RHPS4; lane 8, 20-ng extract plus 0.5 μM RHPS4; lane 9, 20-ng extract plus 0.1 μM RHPS4; lane 10, 20-ng extract plus 0.05 μM RHPS4. C, relative telomere length in 21NT, A431, SKOV-3, and GM847 cells determined by slot-blotting telomere versus centromere probes.

of cells in the G_2 phase of the cycle concomitant with a decrease in S phase (Fig. 4B). The effects on telomerase activity and cell cycle changes were not accompanied by any reduction in telomere length over the same period (up to 22 days), as measured using a slot-blot method detecting $(TTAGGG)_4$ repeats (Fig. 5A). The effect of long-term RHPS4 on the expression of three genes that have been shown to relate to telomerase expression or maintenance of telomeres (*hTERT*, *tankyrase,* and *c-myc*) showed that the drug induced a small (nonsignificant) reduction in *hTERT* expression but no changes in either *tankyrase* or *c-myc* expression (Fig. 5B).

Discussion

Since the compelling biological data linking the reactivation of telomerase with human cancer formation first came to light, there has begun an intensive search to discover and develop potent inhibitors of this reverse transcriptase. Much has been published on inhibition by various antisense molecules targeted to hTR (e.g., oligonucleotides, peptide nucleic acids, ribozymes, and 2′-*O*-methyl RNA) (Pitts and Corey, 1998; Yokoyama et al., 1998; Hamilton et al., 1999; Kondo et al., 2000). However, our alternative approach has focused on the discovery of more "drug-like" small-molecule inhibitors that are capable of interacting with and stabilizing G-quadruplex DNA. We have described previously several such chemical classes of telomerase inhibitor, such as disubstituted anthraquinones, fluorenones, and acridines (Perry et al., 1998, 1999a; Harrison et al., 1999). However, the most potent of these inhibited telomerase in the TRAP assay only in the 2- to 5-μM range; moreover, they caused short-term
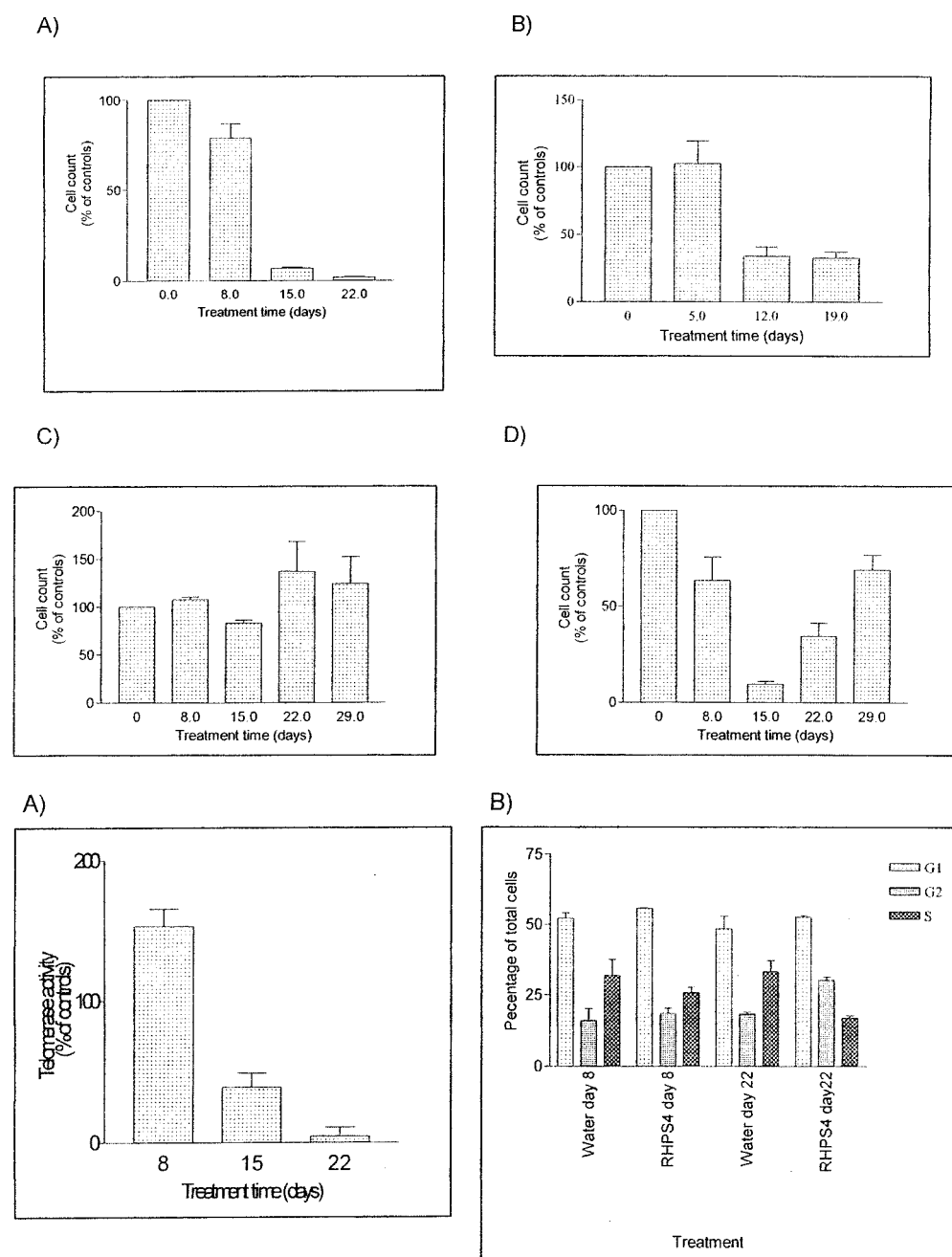
Fig. 3. The effect of long-term exposure of 21NT breast cancer cells (A), A431 vulval carcinoma cells (B), SKOV-3 human ovarian carcinoma cells (C), and GM847 cells (D) to nonacute cytotoxic concentrations (200 nM added every 3–4 days) of RHPS4 on cell growth.
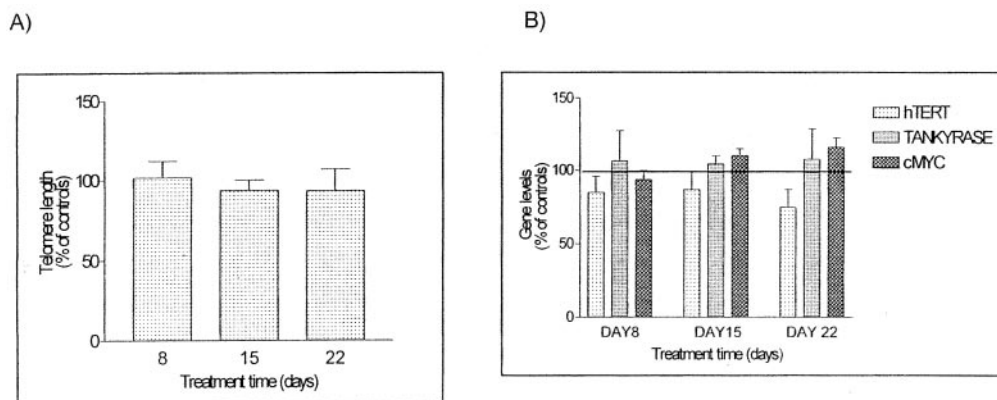

Fig. 4. The effect of long-term exposure of 21NT cells to nonacute concentrations (200 nM added every 3–4 days) on telomerase activity in cell extracts (A) and cell cycle as determined by flow cytometry (B).

cytotoxicity at similar concentrations. Such cytotoxicity is presumed to be a result of binding to duplex DNA and, consequently, low relative affinities for quadruplex versus duplex DNA. We therefore sought second-generation compounds that possessed a wider differential between inhibition in the TRAP assay and low acute cytotoxicity. RHPS4 represents an advance in the discovery of telomerase inhibitors that exhibit cell-free enzyme inhibition at concentrations well below those leading to acute cytotoxicity. RHPS4 is a potent telomerase inhibitor (50% inhibition in the TRAP assay of only 330 nM), whereas in most cell lines, the IC_{50} value for acute cytotoxicity was 20-fold higher at approximately 6 to 8 μM. A comparison of concentrations involving cell-free versus whole-cell assays is not ideal, but this particularly promising property allowed the study of RHPS4 in long-term exposure whole tumor-cell studies without the complication of acute cytotoxicity occurring. Very recently, a series of ethidium derivatives has been shown to bind to G-quadruplexes and also inhibit telomerase at concentrations of 18–100 nM (Koeppel et al., 2001). However, no cell-based data were presented.

According to molecular modeling calculations (to be reported elsewhere; Heald et al., in preparation), RHPS4 is hypothesized to also interact with and stabilize G-quadruplexes. Furthermore, biophysical studies using competition dialysis reveal that RHPS4 possesses a greater selectivity for higher-ordered DNA structures (quadruplex) than duplex DNA (Heald et al., in preparation). Finally, binding constants for RHPS4 measured by fluorescence quenching confirm its selectivity for quadruplex (K = 2.25×10^5) over duplex (K = 3×10^4) DNA (Heald et al., in preparation). Although there is no absolute evidence that G-quadruplexes exist in vivo, our results with RHPS4, as with those obtained previously by us (Read et al., 2001) and others with porphyrins and PIPER (Fedoroff et al., 1998; Wheelhouse et al., 1998; Izbicka et al., 1999; Read et al., 1999; Han et al., 2000), are all strongly suggestive of a telomerase inhibitory mechanism via G-quadruplex stabilization. Aside from these modeling- and NMR-based studies, another piece of biological evidence to support this is provided by observations from TRAP gels. Here, as with other quadruplex inhibitors such as porphyrins (Wheelhouse et al., 1998), even relatively high concentrations of inhibitor still result in the formation of two to three hexanucleotide repeats. This fits with a model whereby telomerase inhibition cannot occur until telomerase has added two to three hexanucleotides to the oligonucleotide template; only at this stage is quadruplex formation, and

thus inhibition, permissible. Hence, the working hypothesis is that RHPS4 probably inhibits telomerase through interaction with the substrate single-stranded telomeric overhang rather then via direct effects on the enzyme. Specificity for telomerase over other polymerases (such as *Taq* polymerase) or other DNA-processing enzymes (such as topoisomerases) is provided by data showing that RHPS4 did not inhibit *Taq* polymerase until concentrations of 20 μM were reached and that the pattern of growth inhibition for RHPS4 across the NCI 60 cell-line panel did not compare with any topoisomerase inhibitors (to be reported fully in Heald et al., in preparation).

Long-term exposure of 21NT or A431 cell lines to nonacute cytotoxic concentrations of RHPS4 resulted in a marked decrease in cell growth after 15 days. This fits with data using mutant hTR constructs transfected into human cells in which an altered telomere structure occurred, causing telomere malfunction and cell death (Marusic et al., 1997;Guiducci et al., 2001). However, a concomitant reduction in telomerase activity was a surprising finding, because RHPS4 is proposed to act on telomeric overhangs rather than on telomerase itself. However, this effect has also been observed with porphyrin-based G-quadruplex interactive telomerase inhibitors (Izbicka et al., 1999). A possible mechanism for this observation is through a secondary down-regulation of telomerase activity via interference with telomeric overhangs. Notably, we also observed a reduction in *hTERT* gene levels in 21NT cells exposed to RHPS4 for 15 days. Alternatively, the reduction in telomerase may occur via effects on c-myc, which possesses a quadruplex-sensitive region in its promoter (Rangan et al., 2001) and has been shown to regulate hTERT (Wang et al., 1998). However, we did not detect changes in *c-myc* gene expression in RHPS4-treated 21NT cells. Another possibility is that the decrease in telomerase activity is a consequence of direct cytotoxicity, because some, but not all, studies, have shown telomerase activity to decrease after exposure to high concentrations of cytotoxic drugs (Burger et al., 1997; Akiyama et al., 1999; Faraoni et al., 1999). However, we used a drug concentration markedly below that required for acute cytotoxicity in the long-term exposure studies; moreover, reduced telomerase activity has also been reported after long-term exposure to the porphyrin class of G-quadruplex interactive telomerase inhibitors (Izbicka et al., 1999). A final possibility is via the carryover of RHPS4 from the treated cellular extracts into the TRAP assay, although this seems unlikely because of the washing steps present before telomere extension within the assay. What-

A)

B)

Fig. 5. The effect of long-term exposure of 21NT cells to nonacute concentrations (200 nM added every 3–4 days) on "telomere" length (expressed as a percentage of controls using slot-blotting with centromere probe to control loading) (A) and gene expression of *hTERT*, *tankyrase,* and *c-myc* (B).

ever the mechanism, the reduction in telomerase activity concomitant with decreased transcription of hTERT mRNA may provide a sensitive pharmacodynamic marker of response for telomerase inhibition by G-quadruplex interactive drugs.

The original paradigm of telomerase inhibition leading to a cessation in cell growth is through gradual telomere erosion until such time that telomeres become critically short. In support of this, the transfection experiments performed with dominant-negative hTERT showed that the cessation in cell growth in various human tumor cell lines correlated with initial telomere length (Hahn et al., 1999b; Zhang et al., 1999). The abundance of telomeric sequences might provide a surrogate measure of drug effects on telomeres. However, our data show that over the 15-day period required for cell growth to be inhibited in 21NT cells, which already possess short telomeres, there was no detectable shortening of telomeres as measured by a slot-blot method to detect $(TTAGGG)_4$ repeats. This apparent finding may be the case because subtle changes in telomere length have occurred but are outside of the sensitivity limits of the slot-blot method (over 15 days, less than 1 kb of telomeric DNA would be eroded at a rate of 50 bases lost per division of 21NT cells). The method may also detect TTAGGG repeats in other parts of the genome. Alternatively, the strategy of direct targeting of telomeres rather than the telomerase enzyme may induce effects (e.g., on telomere capping) independent of initial telomere length. However, in keeping with the original model, no growth inhibitory effects were observed over 29 days in SKOV-3 cells that possess relatively longer telomeres. Finally, it may be hypothesized that a substrate-targeted approach to telomerase inhibition may confer retention of activity in cells that maintain telomere length by the ALT mechanism. In support of this, we observed that RHPS4 also conferred a growth inhibitory effect against GM847 ALT cells after 15 days of exposure, even though these cells possess very long telomeres. In common with effects observed in human cells, including ALT lines, using mutant telomerase hTR (Guiducci et al., 2001) (which resulted in telomere malfunction), we observed a small increase in 21NT cells in the G_2 phase of the cell cycle after 22 days of exposure to RHPS4.

In summary, a novel class of potent G-quadruplex inhibitors of human telomerase derived from pentacyclic acridines has been discovered. The lead molecule in this series inhibits telomerase at submicromolar concentrations and, moreover, exhibits a good differential between potent telomerase inhibition and low acute cytotoxicity. RHPS4, when added to 21NT or A431 human tumor cells at nonacute cytotoxic concentrations, caused a marked decrease in cell growth at 14 days concomitant with a reduction in telomerase activity. The original paradigm of requiring prolonged exposure to telomerase inhibitors concomitant with erosion of telomeres to a critical length before cellular senescence ensues is questioned by this telomere-targeted approach. RHPS4 represents a good lead from which to develop analogs with further improved telomerase inhibition and optimized in vivo pharmacological properties.

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