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Pharmacodynamics of the G-Quadruplex-Stabilizing Telomerase Inhibitor 3,11-Difluoro-6,8,13-trimethyl-8*H*-quino[4,3,2-*kl*]acridinium methosulfate (RHPS4) in Vitro: Activity in Human Tumor Cells Correlates with Telomere Length and Can Be Enhanced, or Antagonized, with Cytotoxic Agents

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ABSTRACT

Telomeric integrity is required to maintain the replicative ability of cancer cells and is a target for the G-quadruplex–stabilizing drug 3,11-difluoro-6,8,13-trimethyl-8*H*-quino[4,3,2-*kl*]acridinium methosulfate (RHPS4). We report a senescent-like growth arrest in MCF-7 breast cancer cells, within 14 to 17 days, and a reduction in telomere length (from 5.2 kilobases (kb) to 4.7 and 4.3 kb after 17 days of treatment at 0.5 and 1 μM, respectively). These effects occurred at noncytotoxic drug concentrations (doses $<$ 1 μM over a 14-day exposure) compatible with long-term drug dosing. The telomere length of cancer cells influences their sensitivity to growth inhibition by RHPS4: mutant (*mt*) human telomerase reverse transcriptase (hTERT)-expressing MCF-7 cells [short telomere restriction fragment (TRF) length, 1.9 kb; IC_{50}, 0.2 μM] were 10 times more sensitive to RHPS4 compared with wild-type (*wt*) hTERT-expressing, vector-transfected control cells (longer TRF-length 5.2 kb; IC_{50} 2 μM) in the 5 day SRB assay. This relationship was corroborated in a panel of 36 human tumor xenografts grown in vitro showing a positive correlation between telomere length and growth inhibitory potency of RHPS4 (15-day clonogenic assay, $r = 0.75$). These observations are consistent with loss of the protective capping status of telomeres mediated by RHPS4 G-quadruplex-stabilization, thus leading to greater susceptibility of cells with shorter telomeres. In combination studies, paclitaxel (Taxol), doxorubicin (Adriamycin), and the experimental therapeutic agent 17-(allylamino)-17-demethoxygeldanamycin, which inhibits the 90-kDa heat shock protein, conferred enhanced sensitivity in RHPS4 treated MCF-7 cells, whereas the DNA-interactive temozolomide and cisplatin antagonized the action of RHPS4. Our results support the combined use of certain classes of cytotoxic anticancer agents with RHPS4 to enhance potential clinical benefit.

Telomeric repeat sequences at the chromosome termini confer protection against exonucleases and ligases, thus maintaining chromosomal stability and preventing end-joining or fusion (Blackburn, 1991). Telomerase counteracts the progressive telomere shortening that occurs with each cell division because of the end-replication problem and ultimately leads to replicative senescence or apoptosis (Counter et al., 1992). Telomerase activation is considered the single most frequent alteration found with malignancy (Kim et al., 1994)—it is essential in the propagation of the immortal phenotype and to the survival and proliferation of cancer cells. Moreover, expression of mutant telomerase catalytic subunit (hTERT) has been shown to inhibit tumor growth thus validating the enzyme as a target for the development of new anticancer drugs (Hahn et al., 1999).

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This article is part 18 in a series entitled Antitumor Polycyclic Acridines; for part 17, see Cookson et al. (2005).

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ABBREVIATIONS: hTERT, human telomerase reverse transcriptase; RHPS4, 3,11-difluoro-6,8,13-trimethyl-8*H*-quino[4,3,2-*kl*]acridinium methosulfate; TRAP, telomeric repeat amplification protocol; TRF, terminal restriction fragment; kb, kilobase(s); *mt*, mutant; *wt*, wild-type; 17-AAG, 17-(allylamino)-17-demethoxygeldanamycin; PBS, phosphate-buffered saline; CI, combination indices; β-gal, β-galactosidase; DSB, DNA double strand breaks; SRB, sulforhodamine B.

The discovery that interference with telomere architecture and maintenance (telomere capping) can rapidly initiate senescence has alleviated fears that benefits of telomerase inhibition would involve a substantial lag-time necessary to shorten telomeres to a critical length to realize a reduction in tumor burden (Karlseder et al., 2002). The telomere capping model proposes that an uncapped telomere state evokes cell cycle arrest and senescence, in part through a DNA damage signal (Blackburn, 2000). Consistent with this hypothesis is the observation that G-quadruplex–stabilizing agents, which disrupt telomere maintenance, rapidly induce senescence in melanoma (Leonetti et al., 2004) and prostatic tumor cells (Incles et al., 2004). The human telomeric DNA sequence has a propensity to form an intramolecular G-quadruplex structure in vitro because of its guanine-rich nature (Wang and Patel, 1993; Parkinson et al., 2002); however, the nonfolded, single-stranded telomeric overhang is required for optimal access to the telomerase machinery. G-quadruplex DNA structures formed within the telomeric repeat sequence have been shown to inhibit telomerase activity (Zahler et al., 1991), and ligands stabilizing these higher-ordered isoforms are effective inhibitors of telomerase and exert growth inhibitory effects on tumor cells in vitro and in vivo (Gowan et al., 2002; Grand et al., 2002; Riou et al., 2002; Burger et al., 2005).

A series of pentacyclic acridines synthesized at the University of Nottingham (Nottingham, UK) showed preference for binding to and stabilizing, G-quadruplex DNA isoforms over duplexes (Gavathiotis et al., 2003). The lead compound, 3,11-difluoro-6,8,13-trimethyl-8*H*-quino[4,3,2-*kl*]acridinium methosulfate (RHPS4) (Fig. 1) was found to be a potent telomerase inhibitor in the TRAP assay (IC_{50}, 0.33 μM) and to cause irreversible cessation of growth after long-term culture at noncytotoxic concentrations in cancer cells with relatively short telomeres but not in a cell line with longer telomeres (Gowan et al., 2001). This effect was accompanied by an increase in the number of 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. A recent study in melanoma cell lines showed induction of apoptosis as well as generation of a senescent phenotype after challenge with RHPS4 (Leonetti et al., 2004). In addition, the occurrence of telomere dysfunction in terms of presence of telomeric fusions, polynucleated cells, and anaphase bridges were seen. Because the latter effects occurred at growth-inhibitory doses (i.e., 1–10 μM over a 2- to 5-day exposure) after short-term exposure to drug, they were proposed to result from telomere-capping alterations. However, previously published studies describing the biological effects of RHPS4 have failed to demonstrate telomere length reduction in tumor cells by this drug (Gowan et al., 2001; Leonetti et al., 2004).

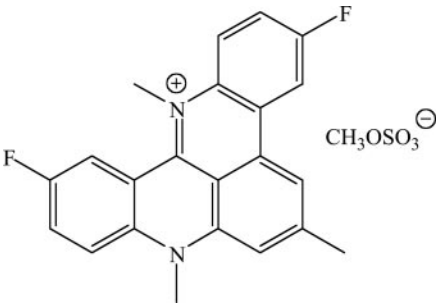

Fig. 1. RHPS4 molecular structure

We now report the following new features of the in vitro pharmacodynamics of RHPS4, which will aid the design of in vivo animal studies:

1. We show that breast MCF-7 tumor cells rapidly exhibit a senescent phenotype when challenged even with noncytotoxic doses of the drug, consistent with effects on telomere maintenance.
2. For the first time with this class of drugs, we show a strong relationship between telomere length and potency of RHPS4 in a panel of 36 different patient-derived xenografts and human tumor cell lines grown as xenografts in vitro. We further used the breast cancer cell line MCF-7 [telomere restriction fragment (TRF) length, 5.2 kb] and an isogenic subclone MCF-7 c81 that expresses *mt*-hTERT (TRF length, 1.9 kb) as a model to dissect subtle effects on telomere length and to evaluate whether such drug-induced changes are concomitant with effects on proliferation and induction of cellular senescence.
3. We have explored whether the consequences of telomere capping alteration after short-term exposure to cytotoxic concentrations of RHPS4 could sensitize tumor cells to anticancer agents that act via disparate mechanisms to identify a possible clinical strategy involving combination chemotherapy.

Materials and Methods

Drugs. RHPS4 was synthesized in our lab as described previously (Heald et al., 2002) (Fig. 1). 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG) was kindly provided by the US National Cancer Institute Central Repository, Biological Testing Branch, Developmental Therapeutics Program. All other drugs were purchased as clinical formulation from the University of Freiburg Hospital Pharmacy. RHPS4 stock solutions were prepared in PBS, 17-AAG in dimethyl sulfoxide, and the clinical formulations were used as provided.

Cell Culture. MCF-7, MCF-7 vector control, and MCF-7 c81 cells were grown in 25-cm^2 tissue culture flasks in RPMI 1640 tissue culture medium supplemented with L-glutamine and 10% fetal bovine serum and maintained at 37°C, 5% CO_2 in a humidified atmosphere. MCF-7 vector control and MCF-7 c81 cultures were cultured in the presence of G418 (300 μg/ml).

Long-Term Effect of RHPS4 on Cell Proliferation/Population Doubling. MCF-7 cells (3×10^4 per 25-cm^2 flask) were seeded in 10 ml of tissue culture medium, and drug was added to a final concentration of 0, 0.2, 0.5, or 1 μM RHPS4. After 7 days, dead cells in the supernatant were discarded and viable attached cells were harvested with trypsin, counted using a hemocytometer and the original seeding density, then passaged on in the same manner and recounted after a further 7 days until there were less than 3×10^4 cells available for re-seeding. The number of population doublings that had occurred over the 7 days (n) could be calculated from the total cell count by using the equation $n = (\log P_n - \log P_0)/\log 2$, where P_n is the number of cells after n doublings; P_0 is the initial seeding density (i.e., 3×10^4). The cumulative number of population doublings was plotted against time. The data represent the extent of proliferative/replicative capacity of viable MCF-7 cells in the presence of RHPS4.

Senescence-Associated β-Galactosidase Staining. Senescence was assayed with the β-galactosidase expression procedure (Dimri et al., 1995). Ten thousand cells were seeded in six-well plates in either 5 ml of vehicle control (containing PBS in a concentration equal to the highest drug dose) or RHPS4 in concentrations ranging

from 0.0001 to 1 μM for 15 days. Drug and medium were replaced every 4 days. At day 15, cells were washed with PBS, fixed in 2% formaldehyde/0.2% glutaraldehyde, and stained. The total number of β-galactosidase positive (blue-green) cells per well and the total number of cells per well were counted. The mean value of three wells was generated, and the number of β-galactosidase positive cells per 100 cells was calculated.

Telomere Length. Mean TRF length was determined using the Telo-TAGGG-telomere length kit from Roche (Penzberg, Germany), following the manufacturer's instructions. Genomic DNA was isolated from pellets of permanent cell lines and primary cells grown in culture with 0.5 and 1 μM RHPS4 for 15 days and cells grown in vehicle-treated medium (PBS). DNA (2 μg) digested with HinfI and RsaI (2 h at 37°C) was separated on a 0.8% agarose gel in 1× Tris-acetate/EDTA buffer. Telomere length for tumors available as xenograft material only was measured using DNA derived from primary cultures.

Creation of Mutant hTERT and Transfection of MCF-7 Cells. *mt*-hTERT (dominant-negative) was generated from the full-length hTERT gene provided as TERT-pcDNA3.1-Myc-His plasmid by Dr. G. Hagen (Bayer AG, Leverkusen, Germany) (Wick et al., 1999). Point mutations in the essential telomerase catalytic subunit hTERT were created in its reverse transcriptase motif 5 (DD/868–869 to AA/868–869) as described previously (Zhang et al., 1999). *mt*-hTERT containing the C-terminal Myc/His tags was then subcloned into pIRESneo plasmid (BD Biosciences Clontech, Palo Alto, CA). Empty and *mt*-hTERT vectors were transfected into logarithmically growing MCF-7 cells using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Positive clones were selected with G418-containing media (300 μg/ml) and confirmed by green fluorescent protein detection and His-tag expression.

Clonogenic Assay. The clonogenic assay was performed with xenograft tissues only. Xenografts growing s.c. in nude mice were removed when an average diameter of 1.5 cm was reached. They were then mechanically disaggregated and subsequently incubated with collagenase (123 U/ml), DNase (375 U/ml), and hyaluronidase (290 U/ml) in RPMI 1640 medium at 37°C for 30 min. Cells were washed and passed through sieves. The clonogenic assay was performed in 24-well plates according to a modified two-layer soft agar assay (Hamburger and Salmon, 1977). Cells were added in 0.2 ml of Iscove's medium/20% fetal bovine serum containing 0.4% agar and plated on top of the base layer (0.75% agar). After 24 h, drug was added (0.01–100 μM) in an additional 0.2 ml of medium. Cultures were incubated at 37°C, 7% CO_2 for 15 days and monitored closely for colony growth. Vital colonies were stained with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (1 mg/ml) 24 h before evaluation, and colonies >50 μm were counted with an automated image analysis system (Omnicon FAS IV; BIO-SYS GmbH, Karben, Germany). Drug effects were assessed in terms of growth inhibitory concentrations 50 and 70% (IC_{50} and IC_{70} values).

COMPARE and Statistics. The Freiburg cancer drug screening program has developed a COMPARE algorithm based on the differential activity of drugs against human tumor cell lines and human tumors growing in soft agar in vitro analogous to the National Cancer Institute-DTP COMPARE computer program (Paull et al., 1989; Phillips et al., 2000). This tool was used to compare TRF length in human tumor cell lines and xenografts in relation to their in vitro sensitivity by employing the Spearman rank coefficient test (Sachs, 1997). The significance of drug effect in growth assays was tested using the Student *t* test.

Sulforhodamine B (SRB) Short-Term (5-Day) Proliferation Assay and Combination Studies. Two thousand cells were seeded into 96-well plates in 0.1 ml of RPMI 1640 medium supplemented with 10% fetal calf serum. Cells were grown overnight at 37°C/5% CO_2, and RHPS4 was added in 0.1 ml of medium to obtain final drug concentrations between 0.01 and 10 μM. Cell proliferation was determined 5 days after continuous exposure to drug by SRB staining (Skehan et al., 1990). The plates were read at 515 nm with a Millipore Cytofluor 2350-microplate reader. For combination studies, IC_{50} values from SRB data for the individual combination partners were calculated and divided, resulting in a drug A versus B ratio. Drugs were then combined at this fixed ratio in six concentrations, ranging from 0.01 to 10 μM for RHPS4, and assays were performed as described above. The plates were incubated for 72 h at 37°C, 5% CO_2, fixed, and stained with SRB. Fractions of affected cells were calculated from the readouts and entered into the Calcusyn program (Chou and Talalay, 1984); combination index values were extracted.

Results

RHPS4 Induces a Senescent-like Growth Arrest in MCF-7 Cells. The cumulative antiproliferative effect exerted by RHPS4 on the MCF-7 tumor cell line over 14 to 21 days in passage at noncytotoxic drug concentations: ≤1 μM is illustrated in Fig. 2. Our observations are consistent with a cytostatic rather than cytotoxic mode of action at the doses examined [i.e., lack of detaching or floating cells and occurrence of senescence-like changes in morphology (enlarged, flattened cells with a higher ratio of cytoplasm to nucleus and increased granularity) (Fig. 3B) (Campisi, 2001)]. The experiment manifested an antiproliferative effect of RHPS4 with the challenging of 3×10^4 cells to repopulate every 7 days: as the viable proportion of the population decreases, a higher reseeding density is required to maintain a viable/growing population. At RHPS4 concentrations of 1.0 and 0.5 μM, MCF-7 cells could not be maintained beyond 14 days in culture (Fig. 2). Induction of the senescent phenotype was confirmed by positive β-galactosidase staining accompanying complete cessation in growth of MCF-7 cells treated with noncytotoxic drug concentrations of RHPS4 (≤1 μM) for 15 days (Fig. 3). The extent of β-galactosidase positive cells was highest at 1 μM (Fig. 3, B and C), but significant fractions of senescent cells were also observed at 0.1 to 0.01 μM (Fig. 3C).

RHPS4 Induces a Telomere Length Reduction at Subtoxic Doses. Reductions in TRF length of 0.5 and 0.9 kb were observed in MCF-7 cells treated for 17 days with 0.5 and 1 μM RHPS4, respectively (Fig. 4A). It was not possible to collect cell material beyond that period under continuous exposure to 0.5 and 1 μM RHPS4 because the cells ceased

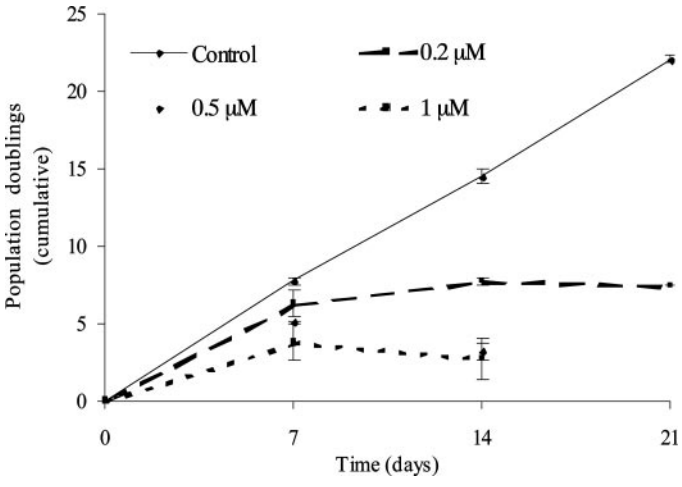

Fig. 2. Effect of RHPS4 on long-term proliferation of MCF-7 cells. The cumulative number of population doublings was calculated every 7 days (see *Materials and Methods*). Each value shows the mean and S.D. of three independent experiments. Cessation of growth on day 14 at 0.5 and 1 μM and reduction in proliferative capacity/population doubling at 0.2 μM RHPS4 is caused by replicative senescence.

growth. With a population doubling time of 1 day, the observed TRF shortening over 17 population doublings corresponds to loss of 30 to 50 base pairs per round of cell division.

Telomere Length Correlates with Sensitivity of Human Tumor Cells to RHPS4. Stably *mt*-hTERT-transfected MCF-7 cells possessing short telomeres were 10-fold more sensitive to RHPS4 (*mt*-hTERT: TRF, 1.9 kb; IC_{50}, 0.2 μM) as their vector only-transfected MCF-7 counterparts possessing longer telomeres (*wt*-hTERT: TRF 5.2 kb; IC_{50}, 2 μM) in the short-term SRB proliferation assay (Fig. 4, B and C). Compared with parental MCF-7 cells, *mt*-hTERT transfected MCF-7 were 3-fold more sensitive (IC_{50}, 0.2 versus 0.6 μM, respectively) (Fig. 4, B and C). The differences in sensitivity of *mt*-hTERT expressing MCF-7 cells to RHPS4 versus vector control and parental cells were statistically significant with a $p < 0.0003$ in the case of *mt*-hTERT/vector and $p < 0.05$ for *mt*-hTERT/parental MCF-7 (Fig. 4C). There was no statistical difference between the effect of RHPS4 on parental and vector-transfected MCF-7 ($p > 0.1$). This suggested to us a not-surprising relationship between short telomeres and greater sensitivity to RHPS4. To corroborate the latter conclusion, tumor cell growth inhibition by RHPS4 was assessed for concentrations ranging from 0.01 to 200 μM across a panel of 36 permanent human tumor cell lines grown as xenografts and patient-derived xenograft tissues in vitro using the long-term soft agar colony-forming assay (15 days); telomere lengths of 15 lines could be determined (Table 1, examples shown in Fig. 5A). RHPS4 showed a markedly differential sensitivity profile in this panel in the clonogenic assay with a mean IC_{50} over all 36 tumors of 11.3 μM and for individual lines with IC_{50} values ranging from 20 nM to 155 μM. For the 15 available comparisons of TRF length and clonogenic assay response, low IC_{50} values correlated with shorter telomeres resulting in a Spearman rank coefficient of 0.75 (Fig. 5B, Table 1). This can be illustrated by, for example, the sensitive human prostate cancer cell line PC3 (IC_{50}, 20 nM), which possesses short telomeres (average TRF, 2.5 kb), compared with the resistant small-cell lung tumor LXFS 650 (IC_{50}, 155 μM), which possesses longer telomeres (average TRF, 5.7 kb) (Fig. 5, A and B; Table 1, note that PC3 was assayed from tumor cells grown as xenografts in mice).

Combination Studies with RHPS4. To explore the potential for interaction of RHPS4 with clinically used cytotoxic agents, RHPS4 was combined with doxorubicin (Adriamycin), gemcitabine, cisplatin, temozolomide, 17-AAG, and paclitaxel (Taxol). Drugs were added at a fixed ratio deduced from their respective IC_{50} values in MCF-7 cells. Combination indices (CI) at doses effecting 50% and 75% reduction in cell viability (ED_{50}

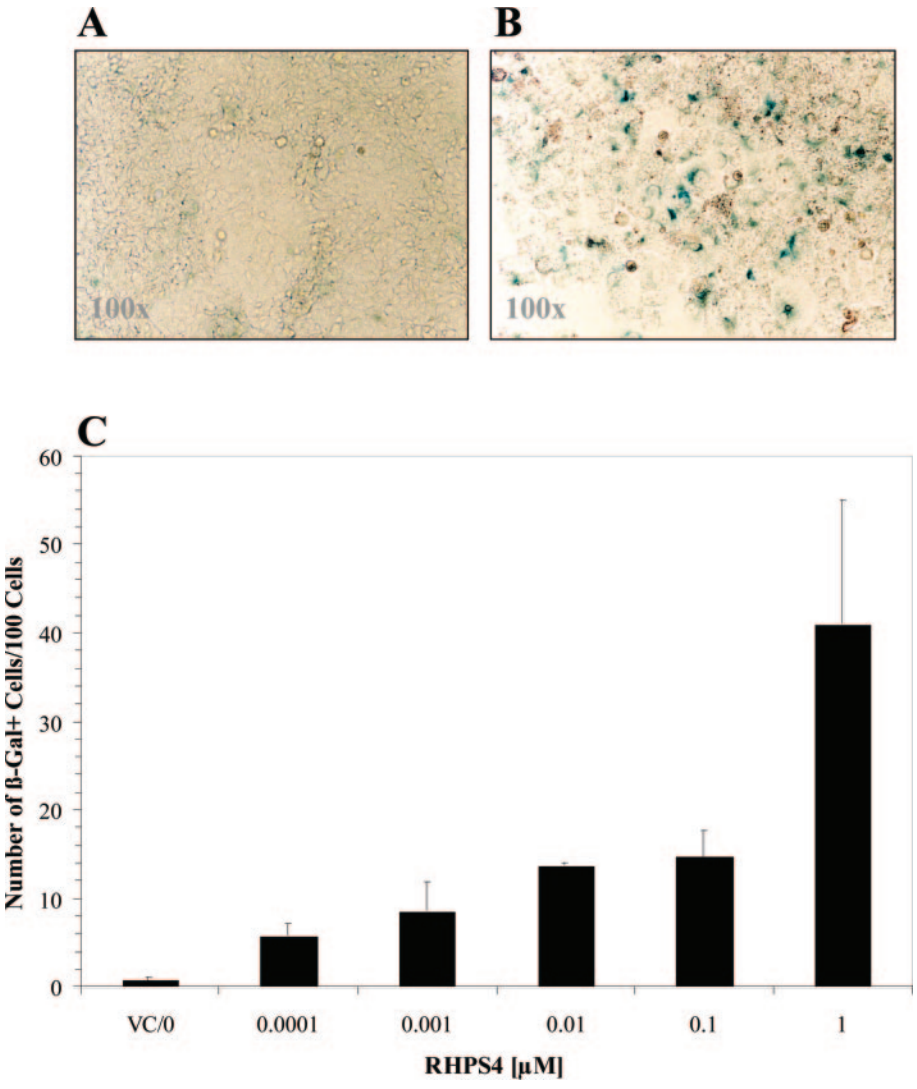

Fig. 3. Staining for induction of senescence by RHPS4. A, untreated MCF-7 control cells do not express senescence associated β-galactosidase. B, β-Galactosidase staining at 1 μM RHPS4 after 15 days in culture (mean, 41 β-galactosidase positive cells per 100 cells). Magnification, 100×. C, number of β-galactosidase positive (blue-green) cells/100 cells in a six-well format for MCF-7 cells treated with 0.0001 to 0.1 μM RHPS4. The S.E. for no treatment = 0.4; 0.0001 μM = 1.4; 0.001 μM = 3.3; 0.01 μM = 0.4; 0.1 μM = 3; 1 μM = 14.

and ED_{75}, respectively) were calculated according to the mathematical algorithm that allows prediction of synergism, additive effects or antagonistic effects based on the CI value (Chou and Talalay, 1984). A CI < 1 is considered to reflect synergistic effects, CI $= 1$ is additive, and CI > 1 the drugs might act antagonistically. Four independent experiments were performed for each combination of RHPS4; the results are summarized in Table 2. Gemcitabine, cisplatin, and temozolomide showed a CI average greater than 1, at both ED_{50} and ED_{75}, which indicates that they antagonize RHPS4 activity; doxorubicin was, to the contrary, additive with RHPS4. 17-AAG was on average at least additive with RHPS4, but slight synergism CI < 1 ($= 0.96$) was found at the mean ED_{75} (Table 2). Paclitaxel, however, was synergistic at both ED_{50} and ED_{75} with CI values of 0.88 and 0.41, respectively (Table 2).

Discussion

Previous work has confirmed the affinity and selectivity of the pentacyclic acridinium salt RHPS4 for G-quadruplex DNA, and its ability to inhibit telomerase in the TRAP assay at concentrations more than a log-fold below those producing short-term cytotoxicity (Gowan et al., 2001; Heald et al., 2002). Here, we examined molecular and cellular effects of RHPS4 at concentrations inhibiting telomerase without causing short-term cell death (≤ 1 μM), hence modeling pharmacodynamics of long-term drug dosing that is a putative scenario in the translation of telomerase inhibitors and/or telomere-modulating agents into clinical application. Nonetheless, it has to be emphasized that long-term toxicities can be as problematic as short-term toxicities in cancer patients. Because long-term exposure to RHPS4 is likely to be needed to demonstrate any anticancer efficacy; animal models and clinical studies will finally have to show whether long-term RHPS4 administration has a therapeutic window and can reach pharmcodynamically active plasma concentrations.

Our studies of RHPS4 biological effects in tumor cells in vitro showed that the drug exerts cumulative antiproliferative effects in MCF-7 breast cancer cells, that a senescence-

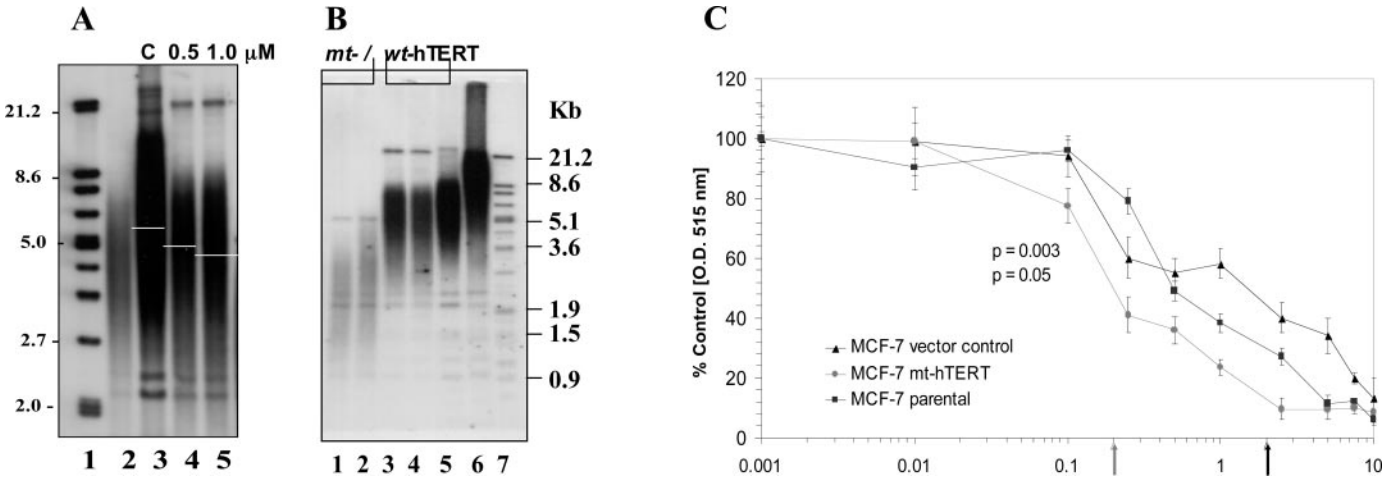

Fig. 4. A, TRF length analysis by Southern blot of MCF-7 cells treated with noncytotoxic RHPS4 concentrations for 17 days. Dose-dependent telomere shortening is seen under RHPS4 treatment. The average TRF-length (indicated by a horizontal line in lanes 3–5) of MCF-7 cells decreased from 5.2 to 4.7 kb (0.5 μM) and 4.3 kb (1 μM) respectively. Lane 1, molecular weight marker; lane 2, low molecular weight standard; lane 3, control MCF-7; lane 4, MCF-7 + RHPS4 0.5 μM, 17 days; lane 5, MCF-7 + RHPS4 1 μM, 17 days. B, TRF length of *wt*-hTERT and *mt*-hTERT MCF-7 cells measured by Southern blot. Lanes 1 and 2, *mt*-hTERT MCF-7 clone 81; lanes 3 and 4, *wt*-hTERT MCF-7 vector-transfected; lane 5, parental MCF-7 cell line; lane 6, high molecular weight TRF standard; lane 7, molecular weight marker. C, growth inhibition by RHPS4 in relation to TRF-length in isogenic MCF-7 cells with short (*mt*-hTERT) and long (*wt*-hTERT: vector control and parental lines) telomeres, measured by SRB assay.

TABLE 1
Telomere restriction fragment length (TRF) in human tumor cells versus potency of RHPS4 in the clonogenic assay

Tumor Designation	Tumor Type	Tumor Origin	IC_{50}	TRF	Rank IC_{50}	Rank TRF
			μM	*kb*	*μM*	*kb*
CXF 1103	Colon	Patient	111	5.5	14	9
LXFA 289	Lung	Patient	100	3.8	13	6
LXFL 529	Lung	Patient	2.93	5.9	9	10
LXFS 650	Lung	Patient	155	5.7	15	11
MAXF 401	Breast	Patient	2.37	3.9	7	7
MAXF 857	Breast	Patient	6.91	3.9	11	7
MCF-7	Breast	Cell Line	6.81	5.2	10	8
OVXF 899	Ovary	Patient	8.11	3.5	12	5
PAXF 736	Pancreas	Patient	0.44	3.7	3	4
DU145	Prostate	Cell Line	1.9	3.7	5	4
PC3M	Prostate	Cell Line	2.3	3.2	6	3
PC3	Prostate	Cell Line	0.02	2.5	1	1
RXF 944	Renal	Patient	2.7	7	8	11
UXF 1138	Uterus	Patient	0.2	2.5	2	1
GXF 251	Stomach	Patient	1.2	3	4	2

XF, xenograft established in Freiburg; PC3M, metastatic subclone of PC3, established by the US National Cancer Institute; LXFA, adenocarcinoma of the lung; LXFL, large-cell lung cancer; LXFS, small-cell lung cancer.

like growth arrest is induced (positive senescence-associated β-gal staining and morphological changes) and that a reduction in telomere length is seen. Telomere shortening is an important observation providing good evidence of telomerase inhibition and/or displacement of the enzyme from the telomere after G-quadruplex stabilization by RHPS4 in these cells (Burger et al., 2005).

We further observed an influence of telomere length on sensitivity to the antiproliferative effect of RHPS4, which is strongly suggested by the increased sensitivity of *mt*-hTERT cells (possessing short telomeres) to the short-term cytotoxic effects of RHPS4 compared with the wild-type and parental cells (relatively longer telomeres) in the SRB assay. Further confirmation of the positive correlation between telomere length and resistance to RHPS4 growth inhibitory properties is indicated in a long-term soft agar growth assay across a panel of cell line- and patient-derived human tumor xenografts.

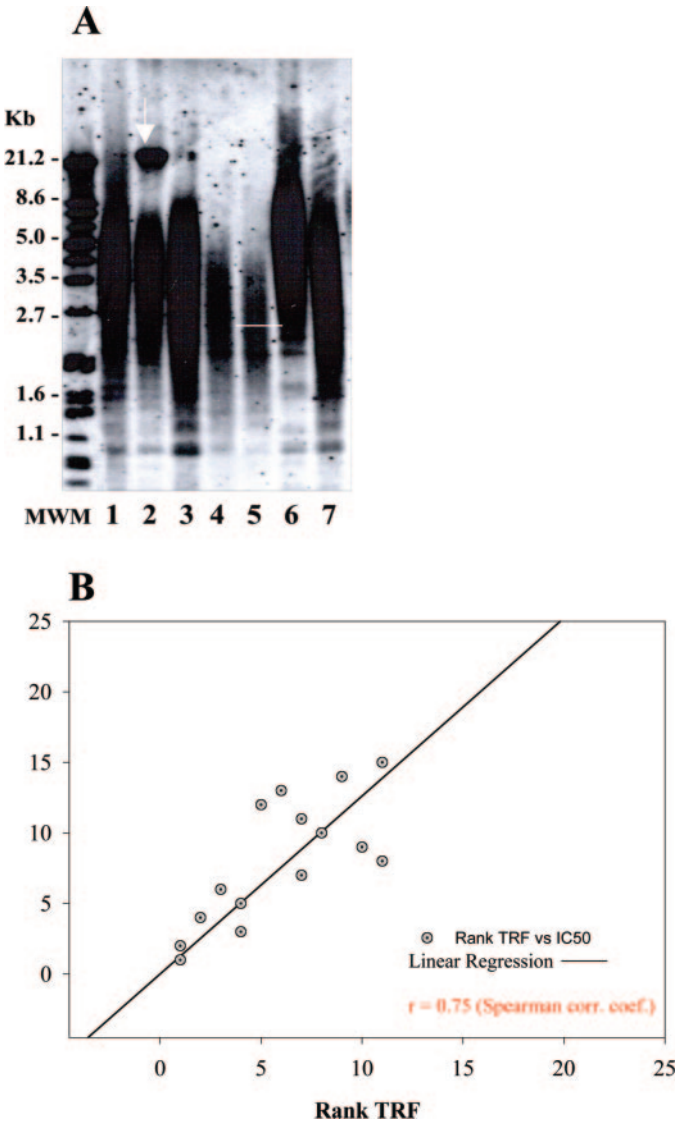

Fig. 5. Tumors with shorter telomeres are more sensitive to RHPS4. A, Southern blot detection of mean telomere length in human tumor cell lines and xenografts. The arrow in lane 2 indicates residual mouse telomere signal from a xenograft primary culture, which contained mouse fibroblasts. The mean TRF length of the human telomere signal was determined relative to a molecular weight standard and taken as the mean of the high-density telomere smear (e.g., indicated in lane 5 as a horizontal line). MWM, molecular weight marker; lane 1, low TRF standard; lane 2, LXFA 289; lane 3, RXF 393; lane 4, OVXF 899; lane 5, UXF 1138; lane 6, LXFL 529; lane 7, DU145. B, correlation between telomere length and chemosensitivity to RHPS4. Mean TRF values and IC_{50} values were ranked for available comparisons, Spearman rank analyses were performed, $r = 0.75$ (r = correlation coefficient). Because of the wide range of actual IC_{50} values (Table 2), the correlation analysis had to be performed using the Spearman rank statistics. The Spearman rank correlation coefficient is also a better indicator that a relationship exists between two variables when the relationship is nonlinear. The data are presented as scatterplot with regression line.

These results support a model wherein an equilibrium between G-quadruplex and canonical DNA forms in telomeric DNA naturally exists, perhaps as a means to regulate telomerase activity and telomere length and is shifted by RHPS4 in favor of G-quadruplex formation, through enhanced stability of such structures. Consequently, access of telomerase and telomere-binding proteins to the telomeres is impeded. This would progressively precipitate uncapping and invoke a growth arrest, the nature of which would depend on the genetic/checkpoint status of the cell. It has been shown that antibodies specific for telomeric G-quadruplex DNA reacted specifically with *Stylonychia lemnae* macronuclei (Schaffitzel et al., 2001), providing experimental evidence that the telomeres of the macronuclei adopt, *in vivo*, a G-quadruplex structure, thus suggesting a role in telomere functioning. Support for a G-quadruplex–induced displacement of telomere-binding proteins comes from electrophoretic mobility shift assay studies in our laboratories, in which increasing concentrations of RHPS4 progressively mediate the loss of protein binding to the telomeric DNA sequence in vitro (data not shown). Moreover, Leonetti et al. (2004) have proposed that short-term effects in human melanoma cell lines, such as telomeric fusions, polynucleated cells, and occurrence of anaphase bridges elicited by short-term cytotoxic RHPS4 drug concentrations, are consistent with telomere capping alterations. Telomere targeting and uncapping have emerged as viable concepts for cancer treatment. The G-quadruplex–interactive agent BRACO19, which produced growth arrest and senescence in long-term cell assays and showed antitumor activity in vivo (Gowan et al., 2002; Incles et al., 2004; Burger et al., 2005), is proposed to elicit telomere uncapping. The formation of the G-quadruplex complex disrupts D- and T-loops, exposing 3′ telomere ends, and triggers senescence (Harrison et al., 2003; Burger et al., 2005). Telomere dysfunction, rather than telomere length, has also been proposed as the principal determinant governing the enhanced chemosensitivity of acute myeloid leukemic cells to certain DSB-inducing agents by pretreatment with the G-quadruplex–interactive agent telomestatin, in that

TABLE 2
Combination indices for RHPS4 and anticancer agents in MCF-7 cells
Values are presented as mean ± S.D.

Drug Combination	FixedRatio	CI	
		ED_{50}	ED_{75}
RHPS4 + doxorubicin	12.5:1	1.0 ± 0.15	1.0 ± 0.17
RHPS4 + gemcitabine	25:1	1.9 ± 0.7	1.9 ± 0.6
RHPS4 + cisplatin	1:4	1.2 ± 0.27	1.3 ± 0.13
RHPS4 + temozolomide	1:750	1.6 ± 0.16	1.16 ± 0.11
RHPS4 + 17-AAG	20:12	1.0 ± 0.32	0.96 ± 0.24
RHPS4 + paclitaxel	250:1	0.88 ± 0.05	0.41 ± 0.01

such effects were observed before telomere shortening (Sumi et al., 2004).

A role for telomerase in suppressing or processing DNA damage in the genome has been suggested, favoring cell survival and proliferation; the presence of telomerase activity signals cells to continue dividing (Blasco, 2002). Therefore, although the molecular mechanisms are not yet identified, telomerase inhibition may be a useful tool to sensitize cancer cells to other agents. Chemotherapeutic responses in normal and neoplastic cells derived from telomerase RNA-null mice were assessed (Lee et al., 2001), and telomere dysfunction, rather than telomerase per se, was found to be the principal determinant governing chemosensitivity, specifically to agents that induced DSB such as doxorubicin. Enhanced chemosensitivity in the telomere dysfunctional cells was linked to therapy-induced fragmentation and multichromosomal fusions, whereas telomerase reconstitution restored genomic integrity and chemoresistance.

To investigate whether RHPS4 can sensitize tumor cells to DNA-damaging drugs, we combined the drug with the antimetabolite gemcitabine, the cross-linking agent cisplatin, the alkylating agent temozolomide, and the DSB-inducing agent doxorubicin. In addition, we examined the efficacy of combining RHPS4 with the experimental therapeutic agent 17-AAG, currently in phase I/II clinical trials, which has been reported to inhibit telomerase activity by depleting the 90-kDa heat shock protein, an essential chaperone for the telomerase catalytic subunit (Holt et al., 1999; Villa et al., 2003). Among the DNA-interactive agents, only the DSB-inducing drug doxorubicin was effectively combined with RHPS4 in MCF-7 cells (additive effects). Gemcitabine, cisplatin, and temozolomide seemed to antagonize RHPS4 activity; 17-AAG, however, was additive to synergistic with RHPS4. This latter observation is in agreement with our findings that MCF-7 cells expressing a dominant-negative form of hTERT (*mt*-hTERT), and hence lack or have little functional telomerase activity, were also more sensitive to RHPS4 compared with *wt*-hTERT cells. Paclitaxel acts clearly synergistically with RHPS4. Owing to its mechanism as a microtubule-stabilizing agent, it is possible that paclitaxel enhances the mitotic defects that have been reported to be caused by RHPS4, such as telophase bridges and telomeric fusions (Schiff and Horwitz, 1980; Leonetti et al., 2004).

Antagonistic drug effects observed between RHPS4 and cisplatin or temozolomide are perhaps unsurprising considering that all three agents prefer to react with guanine-rich tracts of DNA, albeit by different mechanisms—RHPS4 in G-quadruplex stabilization, cisplatin by cross-linking guanines, and temozolomide by methylation of DNA at the O^6 position of guanine. With this sharing of potential target sites, the action of one drug could be sterically preventing the action of the other. Thus guanine residues methylated at the guanine O^6-position by temozolomide would be unable to participate in G-quadruplex formation because of impaired Hoogsteen hydrogen bonding within the G-quartets. Likewise, one may anticipate protection of G-quadruplex–involved guanines from the methylating effects of temozolomide, which prefers to methylate guanine residues in runs of three or more guanines (Clark et al., 1995; Arrowsmith et al., 2002).

The pharmacodynamic properties of RHPS4 reported here, in relation to the modulation of its target, the telomeric G-quadruplex, and with respect to its interaction with other anticancer agents, convey important conclusions: 1) RHPS4 can shorten telomeres and its cellular effects are consistent with an alteration of the telomere capping status; 2) human tumors with a shorter mean telomere length are more susceptible to RHPS4 treatment, and this could be used as a criteria for tumor model selection for preclinical in vivo studies; 3) certain cytotoxic anticancer agents that are currently used in the clinic (paclitaxel, doxorubicin, and 17-AAG) show enhanced activity if combined with RHPS4, but drug combinations with RHPS4 require careful consideration of the particular mechanistic class of cytotoxic because antagonism might occur (e.g., with cisplatin and temozolomide). Moreover, the outcome of the combination studies might be different in cultured cells compared with animal models and man. The Chou and Talalay combination index used for our short-term in vitro tests, is not an applicable experimental design for in vivo studies, other statistical approaches need to be used (Tan et al., 2003). Hence, synergism for paclitaxel and additive effects of RHPS4 with doxorubicin and 17-AAG warrant confirmation in carefully designed xenograft experiments (Tan et al., 2003).

Our results, together with those of other studies (Gowan et al., 2001; Grand et al., 2002; Incles et al., 2004; Leonetti et al., 2004; Burger et al., 2005) represent an accumulating body of evidence supporting G-quadruplex–interactive agents as potentially exciting anticancer compounds to take into clinical trial. To this end, we have recently completed a pharmaceutical profiling of RHPS4 and related pentacyclic acridinium salts, confirming that this novel agent possesses suitably robust pharmaceutical properties for potential parenteral use in animals and clinical trials (Cookson et al., 2005).

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