Simultaneous Targeting of Telomeres and Telomerase as a Cancer Therapeutic Approach

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ABSTRACT

Telomeres, which are important for maintaining chromosome integrity and functions, shorten with each cell division. Telomerase, responsible for telomere synthesis, is expressed in ~90% of human tumor cells but seldom in normal somatic cells. This study evaluated the hypothesis that simultaneous shortening of telomeres and inhibition of telomerase results in synergistic and tumor-selective cytotoxicity. In telomerase-positive human pharynx FaDu tumor cells, paclitaxel caused telomere erosion (first detected at 1 h) and apoptosis. Expression of antisense to the RNA component of human telomerase (hTR) inhibited telomerase activity, shortened telomere length, reduced cell growth rate, and resulted in a significant higher sensitivity to paclitaxel. Another telomerase inhibitor, 3’-azido-3’-deoxythymidine (AZT), at a concentration that produced little or no cell detachment or apoptosis, inhibited the telomerase activity and enhanced the paclitaxel-induced cell detachment and apoptosis. AZT also enhanced the activity of paclitaxel in mice bearing well-established s.c. FaDu xenograft tumors (i.e., reduced residual tumor size, enhanced apoptotic cell fraction, and prolonged survival time), without enhancing host toxicity. In contrast, AZT did not enhance the paclitaxel activity in the telomerase-negative osteosarcoma Saos-2 cells nor in FaDu cells where telomerase was already suppressed by antisense hTR, confirming that the AZT effect in parent FaDu cells is mediated through telomerase inhibition. These results demonstrate that combined use of agents targeting both telomere and telomerase yielded synergistic activity selective for tumors that depend on telomerase for telomere maintenance.

INTRODUCTION

Telomeres are specific DNA structures at the ends of chromosomes that protect chromosomes from end-to-end fusion, maintain chromosome integrity, reversibly repress transcription of neighboring genes, and play a role in chromosome positioning in the nucleus (1). Because of the inability to replicate the 3’ end of chromosomes by DNA polymerases, telomeres are shortened by 50–200 bp per cell division in normal somatic cells. Loss of telomeres to below a threshold value is believed to induce senescence. Telomerase is a ribonucleoprotein DNA polymerase that synthesizes telomeric repeats de novo and is involved in multiple cellular processes, including cell differentiation, proliferation, inhibition of apoptosis, tumorigenesis, and possibly DNA repair and drug resistance (2–5). Telomerase is present in nearly all immortal cell lines, germ-line cells, stem cells, and ~90% of human tumors but seldom in normal somatic cells (6).

The selective expression of telomerase in tumor cells makes telomerase an attractive therapeutic target. However, telomerase inhibition results in cytotoxicity only after depletion of preexisting telomeres that occur over a significant lag time. For example, telomerase inhibitors resulted in cytotoxicity in HeLa cells after 23–26 cell doublings (7). The long lag time may also allow for activation of the telomerase-independent ALT (8). The requirement of telomere depletion limits the therapeutic potential of telomerase inhibitors.

We hypothesized that simultaneous shortening of telomeres and inhibition of telomerase may provide synergistic antitumor activity against tumor cells that depend on telomerase for telomere maintenance. This hypothesis was tested using paclitaxel that causes telomere erosion (9) and two telomerase inhibitors. Antisense to the RNA portion of hTR, which blocks the template for telomere synthesis (7), was used as the specific telomerase inhibitor. Because of the difficulty in delivering antisense under in vivo conditions, the merit of the telomere/telomerase-targeting approach in vivo was investigated using a small molecule, AZT. AZT has multiple pharmacological actions, including inhibition of reverse transcriptase, human telomerase reverse transcriptase component, integrase, DNA polymerase γ, and thymidine kinase, and is preferentially incorporated into telomeric DNA and Z-DNA-containing regions of Chinese hamster ovary cells (10–15). To determine the telomerase-directed selectivity in this approach, the effect of telomerase inhibition was compared in telomerase-positive human pharynx cancer FaDu cells and telomerase-negative human osteosarcoma Saos-2 cells that use ALT for telomere maintenance (8).

MATERIALS AND METHODS

Chemicals and Reagents. Paclitaxel was obtained from Hande Tech. (Houston, TX), Aldrich Chemicals (Milwaukee, WI), and Bristol Myers Squibb, Inc. (Princeton, NJ); AZT from the National Cancer Institute (Bethesda, MD); sulfonfodahamine B and bichinonic acid kit from Sigma (St. Louis, MO); cefotaxime sodium from Hoechst Marion Roussel (Somerville, NJ); γ-32P-ATP from ICN (Costa Mesa, CA); and all other culture supplies and Lipofectamine from Life Technologies, Inc. (Grand Island, NY). All other biological reagents, except those noted otherwise, were purchased from Roche (Indianapolis, IN). Stock solutions of paclitaxel were dissolved in 100% ethanol, G418, and IPTG in PBS, and AZT was dissolved in distilled water. Human pharynx cancer FaDu and osteosarcoma Saos-2 cells, purchased from American Type Culture Collection (Manassas, VA), were maintained in minimum essential medium and McCoy’s medium, respectively.

Construction of Recombinant Expression Vector Containing Antisense or Sense hTR. Total RNA was isolated from FaDu cells and cdNA was synthesized. A 185-bp hTR fragment was obtained using reverse transcriptase-PCR with the primers 5’-CAGCTGACATTTTGTGTGCTCTA-3’ and 5’-GGTTGGCGAGGTTGGCCT-3’. The 185-bp fragment was isolated using Agarose Gel DNA Extraction Kit and ligated with pGEM-T Easy Vector (Promega, Madison, WI). The recombinant plasmid was digested with NotI, and the resulting 219-bp DNA fragment was subcloned into the NotI site of the expression vector pOPRSV/MCS (LaSbitch II Inducible Mammalian Expression System; Stratagene, La Jolla, CA). The presence and orientation of the insert in the recombinant plasmid were verified by: (a) digestion with EcoRI and selecting the five clones that contained the insert; (b) digestion with PvuII.

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2 Equal contribution.

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4 The abbreviations used are: ALT, alternative telomere lengthening; AZT, 3’-azido-3’-deoxythymidine; FISH, fluorescence in situ hybridization; Flow-FISH, quantification of fluorescence signal from FISH using flow cytometry; hTR, human telomerase RNA; IPTG, isopropyl-1-thio-β-D-galactopyranoside; TALA, telomere amount and length assay; TRAP, telomeric repeat amplification protocol.
which produced fragments of 2856, 1296, 1106, and 608 bp for antisense recombinant plasmids and fragments of 2856, 1481, 1106, and 423 bp for sense recombinant plasmids. The results demonstrated one clone as sense and four clones as antisense orientations; and (c) confirmation of the antisense and sense orientation by sequencing with T7 or T3 promoter primer (finol DNA Sequencing System; Promega).

**Transfection of FaDu Cells with Recombinant Expression Vectors.** FaDu cells were stably transfected using the Lipofectamine method, first with the repressor vector pCMVLacI (LacSwitch II Inducible Mammalian Expression System) and subsequently with the pPORSV/MLCS-antisense or sense hTR recombinant plasmid DNA. The clones expressing both the Lac repressor and antisense or sense hTR were selected and maintained in 200 μg/ml hygromycin B and 400 μg/ml G418. IPTG (3 mM), which decreases the binding affinity of Lac repressor protein to the operator vector and triggers transcription and expression of the inserted gene, was used to induce the expression of antisense or sense hTR.

**Measurement of Telomere Activity and Telomere Length.** A modified nonradioactive TRAP was used to measure telomerase activity in all experiments, except for the AZT experiments, which used intracellular TRAP (16, 17). Telomere length was measured by three methods. The mean length of terminal restriction fragments in total cells was measured using our recently developed solution hybridization-based method, i.e., TALA (18). This method measured both the amount and length of telomere (18). In brief, genomic DNA was isolated, and 10 μg of DNA were digested at 37°C overnight with 10 units each of HindIII/CfoI/HindIII. The oligonucleotide probe (TTAGGG)₅ was labeled by γ-³₂P-ATP with polynucleotide kinase. Three ng of the probe was added to 2.5 μg of DNA solution. After denaturation at 98°C for 5 min, hybridization was performed at 55°C overnight. The resulting samples were electrophoresed on 0.7% agarose gel. After drying under vacuum without heating, the gel was exposed to phosphorimaging screen, and the result was analyzed using the area-under-curve method of the ImageQuant software from Molecular Dynamics (Sunnyvale, CA). The point representing 50% of the area-under-curve was the mean telomere length.

FISH was used to measure the telomere signals in individual cells (18). Flow-FISH was used to measure the fluorescence signal and the DNA content of a cell and to calculate the average telomere signals normalized to DNA contents in total cells. Flow-FISH was performed as previously described (19), with the exception that cells were fixed in 10% formaldehyde followed by three washes with PBS containing 0.1% BSA.

**In Vitro Drug Activity Evaluation.** Drug treatment was initiated after cells were allowed to attach to the growth surface. AZT treatment was initiated 24 h before paclitaxel treatment to allow for the conversion of AZT to AZT-triphosphate and expression of antisense or sense hTR. Drug effects were measured as (a) a reduction of the number of cells that remained attached to the growth surface, using the sulforhodamine B assay (20); (b) an increase in the fraction of cells that detached from the growth surface, i.e., ratio of (detached cells that were the cells in the supernatant of the culture medium and in two subsequent Versene rinses) to (attached cells that were harvested by trypsinization after Versene rinses), and (c) an increase in the extent of apoptosis as indicated by the release of DNA-histone complex from the nucleus to the cytoplasm (20).

The effect of antisense hTR on paclitaxel activity was additionally evaluated using a clonogenic assay as described previously (21). Briefly, 2.5 × 10⁶ cells were seeded on 6-well plates for 24 h before dosing with different concentrations (0, 0.5, 1.0, 2.5, 5.0, and 10.0 nM) of paclitaxel. After treatment for 96 h, the numbers of colonies with >16 cells, in five randomly selected microscopic fields/well at 25× magnification, were counted.

M-phase cells were identified morphologically after Giemsa staining by the disappearance of nuclear envelope and appearance of chromosomes.

**In Vivo Treatment with Paclitaxel and/or AZT.** FaDu cells were implanted s.c. (10⁶ viable cells in 0.1-ml physiological saline) in the right and left flanks of male Balb/c nu/nu mice (6–8 weeks old, National Cancer Institute). Mice were cared for in accordance with institutional guidelines. Drug treatment was initiated 2 weeks after tumor implantation or when tumors in all animals reached a size of >3 mm in diameter. The paclitaxel group received 5 consecutive daily i.v. injections of 10 mg/kg/day via the tail vein; the dosing solution (200 μl) consisted of 1 volume of paclitaxel dissolved in Cremophor/ethanol and 9 volumes of physiological saline. The control group received 5 consecutive daily treatment of 200-μl physiological saline. The AZT group received a 7-day infusion of AZT at a rate of 200 μg/h, delivered by a 1002D minipump (Alzet Corp., Palo Alto, CA) s.c. implanted on the back of the animal and removed at the end of infusion. The combination group received both drugs, with the paclitaxel treatment initiated 1 day after the initiation of AZT treatment. Some animals were euthanized on day 10, and the tumors were removed and processed for histological evaluation. The number of apoptotic cells and nonapoptotic cells in tumors, in four randomly selected microscopic fields at ×400 magnification, were determined using image analysis procedures as described previously (22). A separate experiment determined the surviving fraction and the survival time where animals were monitored for 100 days or until death or moribundity. Moribundity was reached when the length of the tumor was ≥1 cm.

**RESULTS**

**Cytotoxicity of Paclitaxel in FaDu Cells.** The maximal reduction in the number of cells attached to the growth surface, because of paclitaxel treatment, was ~40% (Fig. 1A). This was achieved at 100-1000 nM paclitaxel. Subsequent studies used 200 nM, which resulted in cell detachment from the growth surface and apoptosis. The fraction of detached cells increased with the duration of paclitaxel treatment from <1% before treatment to ~14% at 12 h and ~40% at 48 h (Fig. 1B). Morphological evaluation indicated that >60% of the attached cells were interphase cells, and >80% of the detached cells were M-phase cells.

Paclitaxel-induced apoptosis was first detected at 24 h and increased with drug treatment time by 2-fold at 36–48 h, whereas no apoptosis was detected in untreated controls (P < 0.01; Fig. 1C).

**Telomere Erosion in FaDu Cells by Paclitaxel.** The TALA results indicate that paclitaxel treatment for up to 48 h did not affect the telomeric DNA. Telomere erosion was monitored by FISH. The telomere signals were measured in individual cells using a point representing 50% of the area-under-curve method of the ImageQuant software from Molecular Dynamics (Sunnyvale, CA).

**Fig. 1.** Synergism between paclitaxel and AZT on FaDu cells. AZT treatment was initiated 24 h before paclitaxel treatment, then the cells were treated with AZT plus paclitaxel for an additional 24 h (A) and 48 h (B and C). Data represent mean ± SD of two to three experiments with six replicates each. Symbols: □, control with no drug treatment; □, AZT alone; ●, paclitaxel alone; ○, paclitaxel plus 1 μM AZT; △, paclitaxel plus 10 μM AZT. A, reduction of number of FeDu cells attached to growth surface because of treatment, measured by the sulforhodamine B assay. Results are expressed as percentage of controls. For experiments using only paclitaxel, the controls were without drug treatment. For the experiments using combinations of paclitaxel and AZT, because AZT alone reduced the cell number by ~5% at 1 μM and ~60% at 10 μM, the controls were normalized to the effect of AZT alone. The IC₅₀ of paclitaxel were 12.3 ± 4.7, 8.2 ± 2.9, and 2.0 ± 0.1 μM for paclitaxel, paclitaxel plus 1 μM AZT, respectively. B, detachment of the cells from growth surface because of treatment by paclitaxel alone or paclitaxel plus 10 μM AZT. C, apoptosis was measured by the release of DNA-histone complex from the nucleus to the cytoplasm, using cell death ELISA. Results are expressed as the ratio of A₆0₀ in the drug-treated versus untreated control cells.
telomere length in the attached cells but resulted in significant telomere length reduction in detached cells (Fig. 2A, Table 1). TALA measures the population-average telomere length of all cells and does not indicate the telomere status in individual cells. Hence, we also used FISH to visualize the telomere status in individual cells and used Flow-FISH to quantify the telomere length in individual cells and to standardize the telomere length by the DNA content. This normalization was necessary because paclitaxel is known to increase the fraction of cells in the G2-M phase with tetraploid DNA.

The Flow-FISH results show telomere erosion in the detached cells but no changes in telomere signals in the attached cells after paclitaxel treatment for 48 h (Table 2). These results are consistent with the TALA results (Table 1). No significant differences were observed in the extents of telomere erosion/shortening measured by these two methods ($P > 0.14$), indicating that these methods gave comparable quantitative measurements.

Results of FISH analysis show reduced and/or completely absent telomere signals in chromosomes of individual M-phase cells in either the detached or attached cell fraction as early as 1 h after paclitaxel treatment (Fig. 2B). This effect was observed in some but not all chromosomes. After paclitaxel treatment, the fraction of M-phase cells with eroded telomeres increased from $\sim 40\%$ at 1 h to $\sim 70\%$ at 48 h in the detached cells and from $\sim 20\%$ at 1 h to $\sim 50\%$ at 48 h in the attached cells (Fig. 2C). In comparison, only $\sim 10\%$ of the M-phase cells in the untreated controls showed telomere erosion. No significant changes in telomere signals were observed in interphase cells (data not shown), indicating that the telomere erosion effect of paclitaxel is specific to M-phase cells.

### Table 1 Reduction of average telomere length by paclitaxel with and without AZT: TALA results

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Sample</th>
<th>No drug</th>
<th>Paclitaxel</th>
<th>AZT</th>
<th>Paclitaxel + AZT</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>Attached</td>
<td>106 ± 4.3</td>
<td>100 ± 7.2</td>
<td>101 ± 3.2</td>
<td>100 ± 5.8</td>
</tr>
<tr>
<td></td>
<td>Detached</td>
<td>Not applicable</td>
<td>64.3 ± 17.6*</td>
<td>Not applicable</td>
<td>77.7 ± 13.2*</td>
</tr>
<tr>
<td>48</td>
<td>Attached</td>
<td>93.5 ± 5.0</td>
<td>94.8 ± 4.3</td>
<td>91.2 ± 6.2</td>
<td>80.8 ± 8.9</td>
</tr>
<tr>
<td></td>
<td>Detached</td>
<td>Not applicable</td>
<td>66.9 ± 15.0*</td>
<td>Not applicable</td>
<td>65.7 ± 6.4*</td>
</tr>
</tbody>
</table>

* $P < 0.05$ compared with attached cells (Student’s $t$ test).
Changes in Telomerase Activity after Paclitaxel Treatment. In FaDu cells, paclitaxel induced the telomerase activity at early time points, reaching a peak level of ~150% of the pretreatment level at 12 h. The telomerase activity then returned to the pretreatment level at 24 h and further declined to ~60 and 20% of the pretreatment level at 48 and 96 h, respectively (Fig. 3). Similar changes were observed in attached and detached cells, with greater changes in the detached cells.

Effect of Antisense hTR in FaDu Cells. IPTG induction of cells transfected with the antisense hTR plasmid showed two clones with marked inhibition of telomerase activity, which were selected for further study. The experiments included four controls, i.e., parent FaDu cells, cells treated with IPTG, transfected with sense hTR and treated with IPTG; and cells transfected with antisense hTR but without IPTG induction. Expression of antisense hTR resulted in telomerase inhibition (~74%; Fig. 4A), gradual reduction in telomere length (30–38% after 42 days; Fig. 4B), and slower growth rate (increase of doubling time from ~22 h to ~27 h after 19 days of IPTG induction), whereas the four control groups did not show these changes. After IPTG induction for 55–60 days, most antisense-expressing cells began to detach from the flask and showed apoptotic morphology. The remaining cells stopped growing and showed morphological characteristics of cell senescence (23), including enlarged and flattened morphology with increased granularity (data not shown).

Effect of AZT in FaDu Cells. AZT at 1 and 10 μM resulted in ~5 and ~60% cell growth inhibition at 48 h, a slight increase in the detached cell fraction (<7.5% at 24 and 48 h, Fig. 1B) and no measurable apoptosis at up to 48 h (Fig. 1C). Fig. 4A shows that AZT inhibited telomerase, with ~40 and ~100% inhibition of telomerase activity at 1 and 10 μM AZT, respectively. Table 2 shows that 48-h treatment with 10 μM AZT did not affect the telomere length of the parent cells. If untreated control cells; Student’s t test.

Table 2 Telomere erosion by paclitaxel: Flow-FISH results

<table>
<thead>
<tr>
<th>Cells</th>
<th>Treatment time (h)</th>
<th>Parent FaDu</th>
<th>Sense + IPTG</th>
<th>Antisense + IPTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Attached</td>
<td>24</td>
<td>112.3 ± 26.6</td>
<td>87.2 ± 14.8</td>
<td>83.3 ± 8.6</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>107.4 ± 26.9</td>
<td>91.7 ± 27.2</td>
<td>89.5 ± 30.5</td>
</tr>
<tr>
<td>Detached</td>
<td>24</td>
<td>40.8 ± 13.7a</td>
<td>48.6 ± 6.4a</td>
<td>42.2 ± 28.1a</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>44.1 ± 15.5a</td>
<td>42.7 ± 26.9a</td>
<td>27.0 ± 15.1a</td>
</tr>
</tbody>
</table>

P < 0.05 as compared with untreated control cells; Student’s t test.

Fig. 3. Alteration of telomerase activity in FaDu cells after paclitaxel treatment. FaDu cells were treated with 200 nM paclitaxel. Telomerase activity in the attached and detached cells was detected by modified TRAP. IS, internal standard; M, DNA molecular weight marker (pBR322 DNA cleaved with HaeIII); NC, negative control. Data are mean ± SD of three experiments.

Fig. 4. Effect of antisense hTR and AZT on telomerase activity and telomere length. A, telomerase activity as measured by modified TRAP. AZT treatment was for 48 h. IS, internal standard; M, DNA molecular weight marker (pBR322 DNA cleaved with HaeIII); NC, negative control. B, telomere length after 14, 21, and 42 days of culture or IPTG induction, as measured by TALA. Parent FaDu cells (Lane 1), cells treated with IPTG (FaDu + IPTG; Lanes 2, 6, and 10), cells transfected with antisense hTR but without IPTG induction (antisense-IPTG; Lanes 3, 7, and 11), FaDu cells where the expression of antisense hTR was induced by IPTG (antisense + IPTG; Lanes 4, 5, 8, 9, 12, and 13), and cells transfected with sense hTR and treated with IPTG (sense + IPTG; Lane 14). M, DNA molecular weight marker (A-DNA, cleaved with EcoRI and HindIII). Horizontal white bars depict the locations of the mean telomere length. The mean telomere lengths were 2.9, 2.8, 2.8, 1.9, 2.7, 2.6, 1.7, 1.6, 2.7, 2.2, 1.6, 1.6, and 2.7 Kb for Lanes 1 through 14.
attached cells. The detached fraction was not analyzed because of inadequate cell numbers.

Effect of Telomerase Inhibition on Paclitaxel Activity in FaDu Cells. Measurements of cytotoxicity by cell number reduction and clonogenic assays show similar results; cotreatment with antisense hTR significantly increased paclitaxel activity (Fig. 5, A and B). The IC_{50} of paclitaxel in antisense hTR-expressing cells (i.e., cells stably transfected with antisense hTR and induced by IPTG treatment) was significantly lower than the values in the control cells that were either not transfected or transfected with sense hTR (P < 0.01; Student’s t test). The telomere signal in antisense-expressing cells was also significantly lower than the signal in control cells (Table 2).

Cotreatment with AZT, at 1 and 10 μM, significantly decreased the IC_{50} of paclitaxel (3–8-fold), increased cell detachment (2–5-fold) and apoptosis (1.5–5-fold; Fig. 1A–C), and accelerated the appearance of apoptosis (12 versus 24 h; Fig. 1C). The differences between paclitaxel alone and paclitaxel plus AZT were statistically significant (P < 0.05; Student’s t test) for the IC_{50} (after 48-h treatment), cell detachment (after 12–36-h treatment), and apoptosis (after 12–48-h treatment).

Measurement of the mean telomere length using TALA indicates that addition of AZT to paclitaxel did not significantly enhance the extent of paclitaxel-induced telomere erosion in detached cells (Table 1). However, because AZT significantly increased the detached cell fraction induced by paclitaxel (Fig. 1B), AZT in effect enhanced the number of cells with shortened telomere. In attached cells, addition of AZT to paclitaxel enhanced the telomere length reduction from 5 to 20%. However, the difference was not statistically significant (P = 0.09).

Lack of Effect of AZT in Telomerase-negative Cells. AZT did not enhance the paclitaxel effect in the telomerase-negative Saos-2 cells (P = 0.6; Student’s t test), nor in FaDu cells where telomerase activity was already inhibited by expression of antisense hTR (P = 0.37; Fig. 5, C and D).

Effect of AZT on Antitumor Activity of Paclitaxel in Mice Bearing FaDu Xenograft Tumor. AZT enhanced the in vivo activity of paclitaxel. Table 3 shows the size and the morphological data of tumors obtained 3 days after drug treatment was ended. The paclitaxel/AZT combination resulted in a decrease in tumor size, whereas animals in untreated control and single agent groups showed up to 4-fold increase in tumor size. The combination group also showed 2–4-fold higher fraction of apoptotic cells in the residual tumors compared with the other groups. Fig. 6 shows the long-term survival data measured up to 100 days after initiation of treatment. The combination group showed a longer survival time and a higher survival rate.

Table 3 Enhancement of antitumor activity of paclitaxel by AZT in mice bearing FaDu xenograft tumors

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>% apoptotic cells/tumor</th>
<th>End of experiment body weight, % of pretreatment value</th>
<th>End of experiment tumor size, % of pretreatment value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline control (11)</td>
<td>12 ± 2</td>
<td>106 ± 3</td>
<td>457 ± 221</td>
</tr>
<tr>
<td>AZT (10)</td>
<td>10 ± 3</td>
<td>105 ± 6</td>
<td>415 ± 172</td>
</tr>
<tr>
<td>Paclitaxel (12)</td>
<td>30 ± 17</td>
<td>97 ± 6</td>
<td>168 ± 60</td>
</tr>
<tr>
<td>Paclitaxel + AZT (12)</td>
<td>72 ± 26a</td>
<td>99 ± 4</td>
<td>95 ± 24a</td>
</tr>
</tbody>
</table>

* P < 0.05 compared with the control and AZT groups (Student’s t test).

# P < 0.05 compared with all other groups (Student’s t test).
Treatment with single agents or their combination produced minimal toxicity, with no toxicity-related death and <3% body weight loss.

**DISCUSSION**

Our results indicate that paclitaxel treatment resulted in telomere erosion and that inhibition of telomerase, by either antisense hTR or AZT, enhanced the cytotoxicity of paclitaxel in the telomerase-positive FaDu cells. The AZT effect was not observed in telomerase-negative Saos-2 cells that depend on ALT for telomere maintenance, nor in FaDu cells where the telomerase activity was already suppressed by the expression of antisense hTR. AZT also enhanced the in vivo activity of paclitaxel in mice bearing FaDu xenograft tumors without enhancing the toxicity. We additionally observed a transient increase in telomerase activity after paclitaxel treatment. Collectively, these results confirm our hypothesis that simultaneous targeting of telomere and telomerase produces synergistic antitumor activity selective for telomerase-positive tumors. These results also have implications for the pharmacological actions of paclitaxel and the role of telomerase in tumor sensitivity to drugs.

Paclitaxel is one of the most important anticancer drugs developed in the last two decades and has a broad spectrum of activity against multiple human tumors (24). Paclitaxel promotes polymerization and stabilization of microtubules, causes blockade of cells in the M phase, and induces apoptosis (25). Paclitaxel-induced telomere erosion has been observed in metastatic murine melanoma K1735 cells (9) and is confirmed in this study. The TALA and Flow-FISH results show that telomere erosion by paclitaxel was readily detected in the detached cells but not in the attached cells (Tables 1 and 2). This is likely a result of the differences in M phase and interphase cell fractions in the attached and detached cells (i.e., >80% detached cells were M-phase cells and >60% of attached cells were interphase cells), because, as shown by the FISH results, telomere erosion by paclitaxel was specific to M-phase cells.

The mechanism of telomere erosion by paclitaxel is unknown but is not likely attributable to telomerase inhibition for the following reasons. First, telomere erosion by paclitaxel occurred while there was a transient increase in telomerase activity (i.e., or at before 12 h; Fig. 3). Second, telomerase inhibition by antisense hTR or AZT caused gradual and slow reduction of the telomere length, i.e., ≥35% over 42 days for antisense hTR (Fig. 4B) and shortening 15–20 bp per cell doubling for AZT (10). In contrast, telomere erosion by paclitaxel was more abrupt and extensive (i.e., ≤35% after 1 day).

This study indicates telomere shortening/deletion in M-phase cells as an early effect of paclitaxel treatment, occurring at 1 h. This effect precedes other biochemical events during paclitaxel-induced apoptosis. For example, paclitaxel and other microtubule agents activate c-Jun NH2-terminal kinase and inactivate extracellular signal-regulated kinase at 8–16 h, which is followed by caspase-3 activation at 30–48 h in human epidermal cancer KB-3 cells (26). In human prostate cancer PC-3, leukemia HL60, and Jurkat T cells, paclitaxel activates caspase-3 at 12 h and causes cleavage of poly(ADP-ribose) polymerase at 24 h (27–29). The finding that telomere erosion by paclitaxel occurred before the detection of hallmark biochemical events in apoptosis suggests that the early onset telomere erosion is unlikely to be the result of apoptosis.

The consequence of telomere erosion by paclitaxel is not known. Telomere erosion is associated with apoptosis. For example, apoptosis in human HeLa 293 and MW451 cells induced by hydroxyl radicals was associated with telomere erosion and occurred without caspase activation (30). Telomere shortening by telomerase inhibition induced apoptosis in several cell types (7, 31–34), whereas elongation of telomere in human fibroblast IDH4 and prostate DU145 cells resulted in higher resistance to apoptosis induced by serum depletion (35). Furthermore, telomerase inhibitors enhanced the cytotoxicity of paclitaxel, indicating the importance of telomere maintenance (or lack thereof) in the paclitaxel activity. A similar finding has been observed for cisplatin; treatment of cisplatin-resistant human glioblastoma U251-MG cells with an antisense to telomerase reduces the telomerase activity and enhances the cisplatin-induced apoptosis (32). We speculate that telomere erosion contributes to the antitumor activity of paclitaxel. The significance of the transient telomerase induction by paclitaxel is unclear but may be related to the telomere erosion because up-regulation of telomerase activity is associated with telomere shortening (6, 36).

Additional studies are needed to determine the mechanism by which paclitaxel induces telomere erosion, the role of telomere erosion in drug-induced apoptosis, and whether the rapid onset telomere erosion effect is specific to paclitaxel, microtubule-targeting agents, or other drug classes.

An interesting and unexpected finding is the eradication of tumors in 20% (2 of 10) of the mice treated with AZT alone (Fig. 4). It is not clear which of the known or other yet-unknown effects of AZT resulted in its antitumor effect in FaDu xenograft tumor. However, this finding is consistent with the antitumor activity of AZT observed in other tumor systems, e.g., AZT inhibited the growth of breast cancer cell lines (11) and methylgI nosourea-induced rat mammary tumors (37).

In conclusion, our results establish that simultaneous targeting of telomeres and telomerase represents a potential strategy to take advantage of the tumor specificity of telomerase for the treatment of tumor cells that depend on telomerase for telomere maintenance. The tumor selectivity of this therapeutic approach is suggested by the finding that AZT enhanced the activity of paclitaxel without enhancing the toxicity. Studies on the effect of simultaneous telomere/telomerase targeting on germ-line and stem cells are needed to confirm the tumor selectivity. Studies to determine whether this approach is generally applicable to telomerase-positive tumor cells are also warranted.

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SIMULTANEOUS TARGETING OF TELOMERES AND TELOMERASE


Simultaneous Targeting of Telomeres and Telomerase as a Cancer Therapeutic Approach

Yiqun Mo, Yuebo Gan, SaeHeum Song, et al.


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