Consequences of Telomerase Inhibition and Combination Treatments for the Proliferation of Cancer Cells

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ABSTRACT
Telomerase is expressed in most types of tumor cells but not in most somatic cells, suggesting that telomerase inhibitors may be a powerful new approach to cancer chemotherapy. Here we explore this hypothesis by treating cultured human tumor cells with a 2′-O-methoxymethyl oligonucleotide that binds the telomerase RNA template and acts as a potent inhibitor. Treatment of DU145 (Rb', p53') and LNCaP (Rb', p53') cells causes telomeres to shorten and cell proliferation to stop. Decreased cell proliferation in culture is not observed immediately but occurs after several weeks and is accompanied by telomere shortening. Antiproliferative effects are more profound for cells growing in soft agar or in colony formation assays, with 90% reduction in the colony-forming ability of LNCAP cells after less than 2 weeks of exposure to the inhibitor. Decreased growth of DU145 and LNCAP tumors and large reductions in prostate-specific antigen levels are also observed in vivo in xenograft models. Short-term treatment of cells with telomerase inhibitors does not increase the effects of standard antiproliferative agents paclitaxel, doxorubicin, etoposide, cisplatin, or carboplatin. Long-term inhibition and telomere shortening sensitize DU145 cells, but not LNCAP cells, to cisplatin or carboplatin. These results demonstrate that methoxymethyl oligomers directed against the template region of telomerase are potent agents and that significant antiproliferative effects can be observed after 2–3 weeks of treatment. Reduced cell proliferation and tumor growth support the hypothesis that telomerase inhibition can make a useful contribution to chemotherapy and should encourage broad testing of telomerase inhibitors.

INTRODUCTION
Telomerase is a ribonucleoprotein that maintains telomere length (1, 2). Telomerase consists of a protein reverse transcriptase, hTERT (3–5), and a RNA component, hTR (6). These components cooperate to form the telomerase active site and add the repeated sequence TTAGGG to telomere ends. hTERT is responsible for enzymatic elongation, whereas hTR provides a template for binding and extending substrate telomeric DNA.

Telomerase has attracted substantial attention because of the observation of telomerase activity in most types of human tumors, but not in adjacent normal cells (7–9). This correlation has led to two related hypotheses: (a) reactivation of telomerase is necessary for the sustained cell proliferation that characterizes cancer; and (b) telomerase is a promising target for therapeutic development. Testing the latter hypothesis requires development of potent inhibitors of telomerase activity and characterization of the effect of telomerase inhibition on cancer cells (10–12). Inhibitors tested successfully to date include agents that promote formation of G-quadruplex structures (13–15), naphthalene derivatives (16), and oligonucleotides (18–25).

Telomerase is a good target for inhibition by oligonucleotides because a key step in its normal function, binding of the telomere to hTR, can be blocked by hybridization of complementary oligomers. We initially demonstrated that PNA oligomers complementary to hTR could act as potent telomerase inhibitors (18–20). Subsequently, we and others have shown that 2′-O-methyl RNA (19), MOE RNA (21, 22), and thiophosphoramidate DNA (23, 24) can also inhibit telomerase and cause telomeres to shorten and cell proliferation to decrease. Unlike antisense oligonucleotides that inhibit translation by binding to mRNA, the oligonucleotides and PNA function like traditional competitive enzyme inhibitors that bind and block enzyme active sites.

Another advantage of using oligonucleotides as lead compounds for the development of anti-telomerase therapeutics is that there is substantial clinical experience with oligonucleotides as a class of molecule (26). One oligonucleotide is an approved drug, and several others are in clinical trials including two in Phase III trials for cancer therapy. Clinical experiences suggest that, as a class of molecule, oligonucleotides are well tolerated. Protocols for large-scale synthesis have been optimized, and costs are as low as $200 per gram, making oligonucleotides a viable option for systemic administration over extended periods. The potency of anti-telomerase oligonucleotides, combined with their similarity to agents that are already in clinical trials, makes them promising candidates for clinical development (25).

It is clear that oligonucleotides can be potent telomerase inhibitors, but important issues need to be addressed to better understand the potential clinical relevance of telomerase inhibition. Perhaps the most obvious is that telomeres in cancer cells are hundreds or thousands of bases long (10), and if telomerase is fully inhibited, telomeres may erode at a rate of 50–200 bases per population doubling, with variability likely caused by genetic background and growth conditions (27, 28). These facts suggest that there will be a lag period between the initiation of anti-telomerase therapy and the observation of beneficial effects and that the length of this lag period will be a critical factor determining the feasibility of anti-telomerase therapy.

Here we examine telomerase inhibition by MOE RNA, an oligonucleotide chemistry currently in clinical trials that has been demonstrated to decrease immune stimulation, increase binding affinity, and improve pharmacokinetics and oral bioavailability (29–32). We find that the addition of an anti-telomerase MOE oligomer to cells for less than 5 weeks causes substantial decreases in proliferation in culture and in xenograft tumors. Synergistic effects in DU145 cells are observed upon administration of the oligonucleotide with carboplatin or cisplatin. These experiments demonstrate that MOE oligomers are potent agents for limiting cell growth through telomerase inhibition and that significant antiproliferative effects can be achieved relatively rapidly.

MATERIALS AND METHODS

Cell Culture. DU145 and LNCaP cells were obtained from ATCC (Manassas, VA) and grown in recommended media at 37°C under 5% CO2. DU145 cells were grown in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented
with 10% FBS (heated-inactivated FBS; Atlanta Biologicals, Norcross, GA), 20 units/ml penicillin, and 0.02 mg/ml streptomycin; LNCaP cells were grown in RPMI 1640 (ATCC) supplemented with 10% FBS.

Cell Proliferation (MTS) Assay. The cells were seeded at 5000–8000 cells/well (0.1 ml) in 96-well plates and incubated overnight at 37°C. The next day, the cells were exposed to varying concentrations of doxorubicin, etoposide, cisplatin, carboplatin, or paclitaxel (all agents from Sigma-Aldrich) for 72–96 h. At the end of the experiments, 20 μl of CellTitre 96 AQsoultion reagent MTS (Promega, Madison, WI) in 100 μl of Opti-MEM were added to each well, incubating cells for 1–4 h, based on the rate of color change. Cell viability was estimated by measuring the absorbance at 490 nm using a MR5000 microtiter plate reader (Dynatech).

Introduction of Oligonucleotides into Cells. Oligonucleotides were obtained as described previously (21) and introduced into cells by transfection using cationic lipids or by direct addition to culture media. DU145 cells were treated with oligonucleotide in complex with LipofectAMINE (Invitrogen, Carlsbad, CA) every 3–4 days according to the manufacturer’s directions. LNCaP cells could not be transfected using cationic lipid because it caused them to lift off culture dishes. For this cell line, oligonucleotide was added directly to culture media (22). For the long-term treatment of cells with oligonucleotides, 25,000 (for DU145) or 35,000 cells (LNCaP) per well were seeded in a 24-well plate and treated with ISIS 24691 (match) or ISIS 125628 (mismatch) 13-mer MOE RNA. For LNCaP cells, a 5 μM concentration of oligonucleotide was used without lipid, whereas for DU145 cells, a 125 nM concentration of oligonucleotide was used, with lipid. Every 3–4 days, cells were trypsinized, counted using a Coulter Z Series cell counter (Beckman Coulter, Fullerton, CA), and then replated at the same density.

Telomerase Assay. Telomerase activity was monitored using the TRAPEze telomerase detection kit (Intergen Co., Purchase, NY), a variation of the telomere repeat amplification protocol, following the manufacturer’s directions (33).

Telomere Length. Telomere size was evaluated by measuring TRF size (34, 35). Briefly, genomic DNA (1–2 μg) was digested with six restriction enzymes (Alul, CfoI, HaeIII, Hinfl, MspI, and RsaI). The digestion products were subjected to 1% agarose gel electrophoresis, followed by Southern hybridization with a 32P end-labeled telomeric (CCCTAA) probe. Similar quantities of DNA were added to each lane. The hybridized gel was washed in 2X SSC and exposed in a PhosphorImager cassette. The length of the telomere restriction fragment was calculated as described previously (35).

Colony Formation Assay. LNCaP or DU145 cells that had been treated with oligonucleotides for various time periods were trypsinized, counted, and seeded at 500-1000 cells/plate in 100-mm tissue culture dish. Cells were fed with fresh growth media every 4–5 days for 2–3 weeks until the colonies were well formed. Growth media did not contain oligonucleotide. Giemsa Stain (Life Technologies, Inc., Gaithersburg, MD) was used to visualize the colony formation. Growth media did not contain oligonucleotide. Giemsa Stain (Life Technologies, Inc., Gaithersburg, MD) was used to visualize the colony formation.

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Soft Agar Assay. LNCaP or DU145 cells were treated with oligonucleotides as described above for various time periods and then seeded at 1–2 × 104 cells/well in triplicates in 6- or 12-well culture dishes in 0.35% agar over a 0.6% agar layer. Cells were then fed with growth media (100–200 μl/well) once a week until colonies grew to a suitable size for observation (3–4 weeks). Growth media did not contain oligonucleotide. Colonies were counted after they were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (1 mg/ml, 100 μM) overnight for better visualization.

Tumor Xenograft. Male nude mice were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. DU145 cells that had been pretreated with mismatch ISIS 125628 or match ISIS 24691 oligomers for 45 days were harvested, suspended in PBS, and s.c. injected into the right and left flanks (4 × 106 cells/flank) of 5–6-week-old mice. Tumor size was measured every 3–4 days by a caliper. The tumor volumes were determined by the length (L) and the width (W): \( V = LW^{2}/2 \). LNCaP cells were pretreated with mismatch ISIS 125628 for 21 days or with match ISIS 24691 for either 21 days (six mice) or 35 days (three mice), after which they were collected and resuspended in PBS at 2 × 106 cells/50 μl. These cells were mixed with an equal volume of Matrigel Matrix (BD Biosciences, Bedford, MA), and 100 μl of cell suspension were inoculated s.c. into the right and left flanks (2 × 106 cells/flank) of 5–6-week-old mice. PSA levels in the blood of mice implanted with LNCaP cells were determined using an enzymatic immunoassay kit (IMX PSA reagent pack; Abbott IMX, Irving, TX). The samples were analyzed using an IMX MEIA instrument (Abbott Laboratories Inc., Irving, TX).

Cell Cycle Analysis. Cells (1–2 × 106) were suspended in 5 ml of PBS in a centrifuge tube. Cells were centrifuged for 6 min at −200 × g and washed once with PBS. Then the cells were thoroughly resuspended in 500 μl of PBS. Seventy percent ethanol was added to the cells up to 5 ml while gently vortexing. Cells suspended in 70% ethanol were kept at −20°C for at least 24 h. The cells were then washed in 5 ml of PBS and suspended in 500 μl of propidium iodide/Triton X-100 staining solution supplemented with RNase A [0.1% (v/v) Triton X-100 (Sigma) in PBS, 0.2 mg/ml DNase-free RNase A (Sigma), and 200 μl of 1 mg/ml propidium iodide (Molecular Probes, Eugene OR)]. The cell suspension was kept for 30 min at room temperature. The cell cycle distribution was measured by a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ), followed by quantification using CellQuest software.

RESULTS AND DISCUSSION

Experimental Design. The telomerase inhibitor used in these studies was ISIS 24691, a 13-base-long oligonucleotide containing MOE bases with phosphorothioate backbone linkages in which a sulfur atom replaces a nonbridging oxygen (21, 22). Inclusion of MOE bases increases binding affinity, whereas phosphorothioate linkages increase nuclease resistance and cellular uptake (29, 36). ISIS 24691 is complementary to the template region of hTERT, blocks binding of primer DNA, and inhibits telomerase with an IC50 value of 3 nM in cell-free assays (21).

ISIS 24691 can enter several different types of cultured cells spontaneously and inhibit telomerase when added alone or in complex with cationic lipid (22). The oligomer continues to inhibit >75% of telomerase activity for up to 1 week after addition to cells (22). The ability of ISIS 24691 to enter cells without complexation with lipid was critical for experiments with LNCaP cells, because in our hands, LNCaP cells detach upon addition of lipid, preventing use of standard transfection protocols. For studies with DU145 cells, media were removed 6 h after addition of oligonucleotide. Cells were carefully washed and allowed to grow without inhibitor. We observed that >85% inhibition of telomerase activity persists for up to 6 days after addition of inhibitor.

As a control for specificity, we used ISIS 125628, a MOE oligomer that contains two mismatched bases relative to ISIS 24691 and inhibits telomerase activity 200-fold less potently than does ISIS 24691 (21). We have also performed control experiments with a guanine-rich oligomer (ISIS 5320) that can form a G-quartet structure and inhibit HIV replication in vitro by binding to the V3 loop domain of gp120 (37), and we have observed that it does not inhibit telomerase activity or reduce cell proliferation (data not shown).

We characterized the effect of telomerase inhibition on cell proliferation by adding ISIS 24691 and ISIS 125628 to cultured cells for periods varying from 3 to 140 days. During these periods, we monitored telomerase activity, cell proliferation, telomere length, cell cycle distribution, and the effects of coadministration with standard anti-proliferative agents. Cell proliferation was monitored in liquid culture, by colony formation, and by growth in soft agar. For experiments to determine the outcome of combining telomerase inhibition with treatment by standard antiproliferative agents, we used ISIS 24691 or ISIS 125628 in combination with a taxane (paclitaxel), topoisomerase inhibitors (doxorubicin and etoposide), or platinum compounds (cisplatin and carboplatin).

Prostate Cancer Cell Lines. We compared the effects of telomerase inhibition in two prostate cancer cell lines DU145 (p53-)- and LNCaP (p53Rb-) to investigate how different cell types respond to telomerase inhibition and telomere shortening (38–40). Two strains of DU145 cells were used. One, used immediately upon...
obtaining it from the ATCC, had a TRF value of 2.3 kb and will be denoted DU145-ATCC. The other DU145 strain had been cultured in the laboratory for an extended period and had an initial TRF value for our experiments of 2.7 kb. Use of two different strains of DU145 allows us to monitor differences in the effects of telomerase inhibition in cell lines that are closely related but vary slightly in telomere length. LNCaP cells have a TRF value of 2.8 kb and would be expected to behave similarly to DU145 cells if mean telomere length were the only variable affecting the response to inhibitor.

**Effect of Addition of ISIS 24691 on Proliferation of DU145 and LNCaP Cells in Culture.** Addition of ISIS 24691 had no immediate antiproliferative effect on the growth of DU145-ATCC, DU145, or LNCaP cells (Figs. 1 and 2), consistent with the hypothesis that telomeres must shorten significantly to cause reduced cell growth and that telomerase inhibition alone is not sufficient to immediately impede proliferation of human cancer cells. Growth rates, however, did slow significantly before the cultures completely lost viability. For example, after 38 days, DU145-ATCC proliferation had decreased by 51% relative to cells treated with mismatch ISIS 125628 (Fig. 2A), whereas after just 17 days, LNCaP proliferation had decreased 30% (Fig. 2C). These observations are important because they suggest that focusing solely on the time required to completely stop cell growth may underestimate the value of telomerase inhibitors because significant reductions in proliferation occur during early to middle stages of treatment.

The proliferation of cells treated with ISIS 24691 continued to decrease as the experiment progressed, whereas proliferation of cells treated with ISIS 125628 continued at a constant pace that was similar to the rate of proliferation of untreated cells. Treatment of DU145-ATCC, DU145, and LNCaP cells with ISIS 24691 led to total loss of cell viability by 67, 73, and 83 days, respectively (Fig. 1). For DU145-ATCC cells, similar results were subsequently obtained when ISIS 24691 was added to cells without lipid (22).

For a portion of the DU145 cells, treatment was stopped after 67 days of treatment with ISIS 24691 (Fig. 1B), and the rate of proliferation of this group of cells increased to match the rate of cells whose telomerase had never been inhibited. This result supports the hypothesis that the proliferative effects of telomerase inhibition are reversible and is consistent with our previous observation that mean telomere length returns to its original value within 2 weeks after inhibition is stopped (19). For another portion of DU145 cells, treatment was reduced to one transfection every 5–8 days. This culture could be maintained indefinitely, and fluorescence-assisted cell sorting analysis was used to monitor the effects of lengthy treatment times on cell cycle distribution (Table 1).

Our observation that addition of telomerase inhibitor causes proliferation of DU145 cells to cease contradicts previous studies from our laboratory that showed that a 2’-O-methyl RNA oligomer analogous to...
in sequence to ISIS 24691 was not able to stop cell proliferation after incubations as long as 100 days (19). The greater success we report here may be due to better potency of our MOE oligomer, an outcome that would be in agreement with the improved properties of MOE oligomers complementary to mRNA targets that have been noted by others (29-32).

**Effect of Addition of ISIS 24691 on Telomere Length of DU145 and LNCaP Cells.** Inhibition of telomerase in cancer cells is predicted to disrupt telomere length maintenance and cause telomeres to erode. To investigate whether ISIS 24691 is causing telomeres to shorten, we characterized telomere length using the TRF length assay, a modified Southern assay that measures the length of chromosome ends after restriction enzyme digestion. As noted above, the initial TRF length of DU145-ATCC, DU145, and LNCaP cells is 2.3, 2.7, and 2.8 kb, respectively. It is important to realize that TRF values are not equivalent to telomere length because they include some subtelomeric DNA.

Upon treatment with ISIS 24691 for 45 days TRF length of DU145-ATCC cells was reduced to an average of 1.8 kb (Fig. 3A). Treatment of DU145 cells with ISIS 24691 for 65 or 90 days reduced TRF lengths to 1.9 and 1.6 kb, respectively (Fig. 3B). Treatment of LNCaP cells for 55 or 70 days reduced TRF lengths to 2.0 and 1.5 kb, respectively (Fig. 3C). In these assays, it is apparent that the signal intensity of DNA from cells treated with ISIS 24691 was reduced. In most cases equal amounts of genomic DNA were loaded in each lane, and the lower signal intensity is observed because little telomeric DNA remained to hybridize to the probe. For LNCaP cells treated with ISIS 24691 for 70 days (Fig. 3C, Lane 5), twice as much DNA was added for visualization. No significant telomere shortening was observed in cells treated with mismatch-containing oligonucleotide ISIS 125628, supporting the conclusion that telomere shortening is due to a sequence-specific interaction between telomerase and ISIS 24691.

**Cell Cycle Analysis of DU145 and LNCaP Cells Treated with ISIS 24691.** To examine the mechanism of reduced proliferation, the distribution of cells within the cell cycle was examined by fluorescence-assisted cell sorting (Table 1). DU145 cells were sampled after 7, 60, 90, or 120 days of treatment with ISIS 24691. To allow DU145 cells to remain viable and collect enough cells to obtain data at 90 and 120 days, ISIS 24691 was added every 5-8 days, rather than every 3-4 days. LNCaP cells were collected after 30 and 45 days of treatment.

We observed that treatment of DU145 cells with ISIS 24691 for 7 days had no effect on cell cycle distribution relative to cells that were treated with mismatch control ISIS 125628 (Table 1). After 60 days, there was a significant increase in the percentage of cells in G2-M phase when treated with ISIS 24691 relative to ISIS 125628 (40% versus 18%) and the percentage of cells in the sub-G1 population (8% versus 2%). The accumulation of treated DU145 cells in G2-M phase, instead of G1 phase might be due to their lack of functional Rb and p53 because both proteins are key effectors controlling the G1-S phase transition (41, 42). By 90 days, the percentage of apoptotic cells in a culture treated with ISIS 24691 had increased to 29%, with a further increase to 68% after 120 days. These results suggest that prolonged inhibition of telomerase activity produces a shift from G2-M arrest to apoptosis as telomeres shorten in DU145 cells.

For LNCaP cells, substantial cell death was observed only after 45 days of treatment with ISIS 24691 (Table 1). The more rapid cell death in LNCaP cells is consistent with the data shown above from simply counting cells (Figs. 1 and 2). In contrast to DU145 cells that express mutant p53 and Rb proteins, in LNCaP cells with wild-type p53 and Rb, we observed more cell death instead of G2-M arrest. These observations are consistent with the hypothesis that cells ex-

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**Table 1 Cell cycle distribution of DU145 and LNCaP cells treated with match oligomer ISIS 24691 or mismatch oligomer ISIS 125628 compared with untreated cells**

<table>
<thead>
<tr>
<th>Phase</th>
<th>Untreated</th>
<th>ISIS 24691</th>
<th>ISIS 125628</th>
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<tr>
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<td>0.4</td>
<td>0.4</td>
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<tr>
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<td>67</td>
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<tr>
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<td>14</td>
<td>15</td>
</tr>
<tr>
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<tr>
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**Fig. 3.** Effect of telomerase inhibition on telomere length of DU145 and LNCaP cells monitored by the TRF assay. Telomere lengths are noted below each lane. A: Lane 1, mismatch oligonucleotide ISIS 125628 added for 45 days; Lane 2, match oligonucleotide ISIS 24691 added for 45 days; Lane 3, no oligonucleotide added. B: DU145 cells: Lane 1, no oligonucleotide added, cells were cultured for 65 days; Lane 2, cells were cultured in the presence of lipid for 65 days; Lane 3, mismatch oligonucleotide ISIS 125628 added in complex with lipid for 65 days; Lanes 4 and 5, match oligonucleotide ISIS 24691 added for 65 or 95 days, respectively. C: Lane 1, no oligonucleotide added, cells were harvested after 3 passages; Lane 2, no oligonucleotide added, cells were harvested after 17 passages; Lane 3, match oligonucleotide ISIS 24691 added for 55 days; Lane 4, mismatch oligonucleotide ISIS 125628 added for 55 days; Lane 5, match oligonucleotide ISIS 24691 added for 70 days. Size markers are shown on the right.
pressing wild-type Rb and p53 proteins may be more susceptible to telomerase inhibition.

**Effect of Addition of ISIS 24691 on Colony Formation and Growth in Soft Agar.** We used soft agar and colony formation assays to assess the effect of telomerase inhibition on the tumorigenic potential of prostate cancer cells. Such assays are thought to more accurately test the ability of cells to grow tumors in vivo because short-term assays in liquid culture can underestimate the potential for decreased cell proliferation, a discrepancy that has been highlighted for studies of the effects of p53 expression on chemosensitivity of cancer cells (41).

We treated DU145 cells with ISIS 24691 and ISIS 125628 for 4, 16, or 73 days; plated them in tissue culture dishes; and allowed formation of colonies for 14–21 days. No oligonucleotide was added during colony growth, so any observed effects are due to inhibitor that enters cells during the incubation in liquid culture. However, it is important to note that ISIS 24691 continues to inhibit telomerase for up to a week after transfection, so the period during which telomerase is inhibited is a few days longer than the periods mentioned above.

DU145 cells that were treated with oligonucleotide in liquid culture for 4 days formed similar numbers of colonies in soft agar, regardless of whether match oligomer ISIS 24691, mismatch oligomer ISIS125628, or lipid only was added (Fig. 4A). For the 16-day treatment, match oligomer-treated cells formed 55% fewer colonies than untreated control or mismatch-treated cells. This reduction in proliferation of DU145 cells after 16 days of treatment was significantly greater than reductions observed for growth in liquid culture after 14, 20, or 28 days but was similar to the reduction observed after 47 days (Figs. 1A and 2A). For the 73-day treatment, match treated cells formed 80% fewer colonies than controls, and the colonies that did appear were smaller. Colony formation assays revealed similar results (Fig. 5A).

Soft agar and colony formation assays were also performed using LNCaP cells. Treatment of cells with ISIS 24691 in liquid culture for 4 days before plating did not lead to inhibition of colony growth in soft agar (Fig. 4B), consistent with the belief that significant telomere shortening must occur before obtaining reduced cell growth. Treatment of LNCaP cells with ISIS 24691 for 12 days, however, produced a 90% reduction in soft agar growth relative to untreated cells or cells treated with mismatch ISIS 125628 (Fig. 4B). No colonies were formed after 60 days of treatment. Colony formation assays yielded similar results (Fig. 5B).

**Implications of Reduced Growth during Soft Agar/Colony Formation Assays.** Compared with growth in liquid culture, our soft agar and colony formation assays reveal relatively rapid antiproliferative effects, especially for LNCaP cells. These data reinforce the suggestion that the lag phase between starting telomerase inhibition and observation of significant effects may be overestimated if one only considers the time required to completely eliminate cell viability.

Interestingly, diminished colony formation is observed, even though no oligonucleotide was added to the cells after plating. This suggests that the telomere shortening achieved before plating may be sufficient to affect growth and that healing of telomeres upon resumption of telomerase activity may not be adequate to restore the ability of these cells to form colonies. Although it is impossible to extrapolate this result to the treatment of human cancer, it does suggest that even short periods of telomerase inhibition lead to reduction in the proliferation of some tumor cells. Even modest reductions might have a substantial beneficial effect when combined with treatment with the antiproliferative agents used in existing therapeutic regimes.

One explanation for the dramatic decrease in anchorage-independent growth and colony formation of LNCaP cells after only 12 days of treatment with telomerase inhibitors is that the presence of functional p53 and Rb protein may make the cells more susceptible to even modest amounts of telomere shortening. The presence of p53 and Rb is not the only difference between LNCaP and DU145 cells, and those other differences may also play determining roles for the response to ISIS 24691. We note that dramatic reductions in colony size and number had been noted previously after 3 weeks of treatment of
AT-SV1 cells (telomere length, 2.2 kb) with PNA oligomers analogous in sequence to ISIS 24691 (20).

**Effect of Reduced Telomere Length on Growth of Tumor Cells in a Xenograft Tumor Model.** To test whether telomerase inhibition and reduced telomere length would reduce tumor cell growth in an animal model, we treated cultured DU145 or LNCaP cells with ISIS 24691 or ISIS 125628 and then implanted the cells into nude mice. We adopted this protocol because pretreatment of cells with inhibitor before implantation is directly comparable with the soft agar and colony formation assays performed above, yet it allows testing of tumor cell growth *in vivo.*

We implanted DU145 cells that had been treated with oligonucleotide for 45 days in cultured and monitored their growth. We observed only slightly less growth of implanted ISIS 24691-treated DU145 cells relative to DU145 cells that had been treated with ISIS 125628 (Fig. 6, A, B, and E). The treatment of LNCaP cells, by contrast, yielded a much more dramatic effect on tumor growth. Most of the animals implanted with cells treated for 21 days with ISIS 125628 developed large tumors, whereas tumors were much smaller in animals implanted with cells treated for 21 days [mice 1–6 (Table 2; Fig. 6, C, D, and F)]. No tumors were detectable in mice that had been implanted with cells treated with ISIS 24691 for 35 days (mice 7–9; Table 2). The larger effect of telomerase inhibition and telomere shortening in LNCaP cells compared with DU145 cells is consistent with the data in soft agar and colony formation assays reported above.

Efficient *in vivo* tumor formation by LNCaP cells requires that Matrigel be added with the cells upon implantation (43), and clearing of Matrigel probably accounts for the fluctuation in measured tumor size before day 32 in Fig. 6D. At the end of the study (day 55), LNCaP tumors were harvested for histological analysis. Staining with H&E demonstrated that tumors were composed of LNCaP carcinoma cells; no Matrigel residue or mouse fibroblasts were noted.

To confirm the extent of tumor growth, we took advantage of the fact that LNCaP cells secrete PSA and measured PSA levels as a surrogate marker for tumor growth (Table 2; Ref. 43). Of the animals that had been implanted with cells treated with mismatch oligomer ISIS 125628, one had a low PSA level of 0.8, whereas the remaining six animals had PSA levels of 23.9–106.7 (average PSA level of 37 for *n* = 7). Of the nine animals implanted with cells treated with ISIS 24691, six animals exhibited PSA levels of <1, whereas the remaining three had PSA levels of <5 (average PSA level of 1.2 for *n* = 9).

**Effect on Cell Proliferation of Combining Brief Telomerase Inhibition by ISIS 24691 and Standard Chemotherapeutic Agents.** It is important to understand the effects of treating cells with both telomerase inhibitors and standard antiproliferative agents because telomerase inhibitors are unlikely to be used as single agents in the clinic. Previous studies to test this hypothesis have produced conflicting results. Ludwig *et al.* (44) have reported that inhibition of telomerase in combination with treatment with the topoisomerase inhibitor doxorubicin produces synergistic effects on cell prolifera-

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**Fig. 6. Growth of DU145 and LNCaP tumors in nude mice.** The growth of every tumor is shown in A–D. A, tumor growth of DU145 cells treated with ISIS 125628. B, tumor growth of DU145 cells treated with ISIS 24691. C, tumor growth of LNCaP cells treated with ISIS125628. D, tumor growth of LNCaP cells treated with ISIS 24691. LNCaP cells were mixed with Matrigel in a 1:1 ratio and injected s.c. Average tumor sizes with SE are shown for tumors derived from (E) DU145 cells that had been treated with match or mismatch oligonucleotide for 45 days in culture before implantation and (F) LNCaP cells that had been treated with match or mismatch oligonucleotide for 21 days before implantation. Student's *t* test was performed to determine the differences between match and mismatch treatments. Statistical significance was determined at *P* < 0.05 (*) and *P* < 0.001 (**).
tion, whereas Folini et al. (45) failed to show additive effects with platinum compounds, taxanes, and topoisomerase inhibitors. Mo et al. (46) have reported additive effects from a combination of paclitaxel and expression of anti-hTERT antisense RNA.

To determine whether short-term inhibition of telomerase might increase susceptibility of cells to other compounds, we added the antiproliferative agents cisplatin, carboplatin, doxorubicin, etoposide, or paclitaxel to DU145 or LNCaP cells that had been treated with match oligomer ISIS 24691 or mismatch ISIS 125628 for 1 day before initiating treatment with various concentrations of chemotherapy agents for an additional 3 days. We observed no synergistic effects on proliferation with either cell line, regardless of the antiproliferative agents used. These results indicate that inhibition of telomerase activity for brief periods does not sensitize these cells to chemotherapy agents and is consistent with the hypothesis that telomere shortening, not telomerase inhibition, is critical for producing a therapeutically relevant phenotype.

**Effect on Cell Proliferation of Combining Sustained Telomerase Inhibition/Telomere Shortening by ISIS 24691 and Standard Chemotherapeutic Agents.** The experiments described above offer no support for the hypothesis that short-term inhibition of telomerase can act synergistically with antiproliferative agents, but they do not address the possibility that achieving synergistic effects may require longer periods of inhibition and telomere shortening. To test this hypothesis in human cells, we performed quadruplicate experiments in which we added antiproliferative agents for 72 h to DU145 cells that had been treated previously with telomerase inhibitors for 30, 45, 55, or 65 days. In contrast to the lack of synergistic effects obtained with short-term treatment, DU145 cells that had been treated with ISIS 24691 for 55 or 65 days were sensitized to treatment with cisplatin or carboplatin (Fig. 7, A and B). No synergistic effects were observed with etoposide, doxorubicin, or paclitaxel at any time point (data not shown). Mismatch oligomer ISIS125628 did not sensitize the cells to cisplatin or carboplatin.

We also tested combination treatment of LNCaP cells and found that they were not sensitized to any agent (doxorubicin, etoposide, paclitaxel, cisplatin, and carboplatin) after 30, 40, 50, or 70 days of treatment with ISIS 24691 (Fig. 7, C and D). The failure of telomere shortening to sensitize LNCaP cells was surprising because LNCaP cells appear to be more susceptible than DU145 cells to telomerase inhibition alone (Figs. 1, 4, 5, and 6). The discrepancy between the results of treatment of LNCaP and DU145 cells reinforces the sug-

<table>
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<tr>
<th>Mouse/flank</th>
<th>PSA</th>
<th>Tumor volume (mm³)</th>
<th>Mouse/flank</th>
<th>PSA</th>
<th>Tumor volume (mm³)</th>
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<tr>
<td>Mismatch 1/R</td>
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* R, right flank; L, left flank.

**Table 2. PSA levels in mice implanted with LNCaP cells treated with match ISIS 24691 or mismatch ISIS 125628**

All mismatch treated mice and match-treated mice 1–6 were implanted with cultured cells that had been treated with oligonucleotide for 21 days. Match-treated mice 7–9 had been implanted with cultured cells that had been treated with oligonucleotide for 35 days.
gestion that the outcome of telomerase inhibition will vary depending on the genetic background of the targeted cell type.

**The Challenge of the Lag Phase for Therapeutic Development.** Telomerase has attracted wide attention because the linkage between telomerase activity and cancer cell proliferation suggests that anti-telomerase agents may represent a new class of drugs for the treatment of many different cancers. This enthusiasm has been tempered by the fact that, unlike most antiproliferative agents that kill cells within hours or days, the need to erode telomeres suggests that anti-telomerase agents may require weeks or months before cell growth is affected. In an extreme example of this, six generations were required before observation of a phenotype in transgenic mice lacking the RNA component of telomerase (47, 48). This extremely long lag can be explained by the fact that mice have much longer telomeres than those found in human tumor cells (49). Nevertheless, the likelihood of a lag phase in human cancer cells is sobering, and identification of strategies that will shorten it is an important goal for the development of anti-telomerase therapeutics.

In previous studies with anti-telomerase PNAAs and 2′-O-methyl oligonucleotides, we have observed substantial decreases in cell proliferation (19, 20). However, maximal effects required 3 months or more of treatment. For example, HME50-5E cells, a line chosen for its exceptionally short telomeres, required over 100 days of treatment with anti-telomerase 2′-O-methyl RNA to halt cell growth. Similarly, we observed that growth of DU145 cells was slowed during 130 days of treatment but never ceased (19).

To develop more potent inhibitors, we obtained oligomers containing MOE RNA, an oligonucleotide chemistry that has been optimized for better binding, stability, and pharmacokinetics (29–32). In striking contrast to our previous inconclusive results with DU145 cells, we now observe that prolonged treatment with MOE RNA oligomer ISIS 24691 caused DU145-ATCC, DU145, and LNCaP cultures to become growth arrested even 67, 73, and 83 days, respectively.

It is clear that evaluating only the end point for cell growth in culture is a misleading and overly pessimistic indicator of the potential of telomerase inhibition. Both LNCaP and DU145 cells begin to grow more slowly after only 2–5 weeks of treatment (Fig. 2). Decreased tumor cell proliferation was even more striking when measured by colony formation, soft agar growth, and growth of xenograft tumors in nude mice, assays that are thought to be more accurately test the tumorigenic potential of treated cells (Figs. 4–6). Because we do not envision telomerase inhibitors being used alone in the clinic, even partial reductions in growth rates during short treatments may be a valuable outcome for patients.

The combination of telomerase inhibitors with existing chemotherapeutics may produce more rapid effects and provide another strategy for minimizing the lag phase. Telomerase inhibition did not produce acute antiproliferative effects (*i.e.* within 4 days), regardless of whether other antiproliferative agents were present. Long-term telomerase inhibition and telomere shortening sensitized DU145 cells to carboplatin and cisplatin but did not sensitize LNCaP cells. The differing results from combination studies with LNCaP and DU145 cells suggest that the appearance of synergistic effects will vary depending on tumor type. Our observations with DU145 are consistent with experiments using knockout mice (mTrp −/−) that show the telomere shortening, not telomerase inhibition alone, increases the antiproliferative effects of agents that induce double-strand breaks (50).

**Summary.** Based on our data, we conclude that telomerase inhibition may yield significant antiproliferative effects after relatively short treatment periods. Differences in the effects of telomere shortening between LNCaP and DU145 suggest the likelihood that the beneficial effects of telomerase inhibition for treatment of cancer will vary depending on the genetic background of target cancer cells. Our findings suggest that telomerase inhibitors can contribute to cancer therapy as part of a combination with antiproliferative agents that are administered after initial chemotherapy, surgery, or radiation has removed the bulk of tumor mass. As with any treatment, telomerase inhibition may have a greater impact on some cancers than on others. These data support aggressive testing of anti-telomerase oligonucleotides in additional tumor models.

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Consequences of Telomerase Inhibition and Combination Treatments for the Proliferation of Cancer Cells

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