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14. ABSTRACT
We hypothesize that telomerase inhibition (telomere shortening) can sensitize human tumor cells to existing anticancer drugs. During the period of the grant we made the following findings: 1) 2'-methoxy ethyl oligonucleotides inhibit telomerase in prostate cancer cells, cause telomeres to shorten, and cause cell proliferation to decrease; 2) Cell proliferation in culture is more pronounced when cells are grown under conditions that mimic tumor growth; 3) Cell proliferation is dramatically reduced in a xenograft model using LNCAP cells, and 4) We did not observe significant synergy with standard chemotherapy agents. The ability of relatively short-term treatments with telomerase inhibitors to slow tumor growth in vivo suggests that telomerase inhibitors are a reasonable approach to prostate cancer therapy.

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Telomerase, oligonucleotides, chemotherapeutic

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Introduction

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

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Key Words: Telomerase; Oligonucleotides; Antiproliferative; Combination treatment; Telomere.

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-methoxyethyl (MOE) 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. Anti-proliferative 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 two weeks exposure to the inhibitor. Decreased growth of DU145 and LNCaP tumors and large reductions in PSA levels are also observed in vivo in xenograft models. Short-term treatment of cells with telomerase inhibitors does not increase the effects of standard anti-proliferative agents paclitaxel, doxorubicin, etoposide, cisplatin or carboplatin. Long term inhibition and telomere shortening sensitize DU145, but not LNCaP, cells to cisplatin or carboplatin. These results demonstrate that MOE oligomers directed against the template region of telomerase are potent agents and that significant antiproliferative effects can be observed after two to three 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.

Body/Main Text

Telomerase is a ribonucleoprotein that maintains telomere length (1,2). Telomerase consists of a protein reverse transcriptase, hTERT (3-5), and an 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, while 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 1) reactivation of telomerase is necessary for the sustained cell proliferation that characterizes cancer, and 2) 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), napthalene derivatives (16), dietary polyphenols (17), 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 peptide nucleic acid (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), 2'-methoxyethyl RNA (21,22), thiophosphoramidate DNA (23,24), can also inhibit telomerase, cause telomeres to shorten, and cell proliferation to decrease. Unlike antisense oligonucleotides that inhibit translation by binding to mRNA, the oligonucleotides and PNAs 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 suggests 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 fifty to two hundred bases per population doubling, with variability likely caused by genetic background and growth conditions (27,28). These facts suggest that there will be 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 2'-O-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 2'-MOE oligomer for to cells for less than five 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

2'-MOE oligomers are potent agents for limiting cell growth through telomerase inhibition, and that significant antiproliferative effects can be achieved relatively rapidly.

EXPERIMENTAL PROCEDURES

Cell culture. DU145 and LNCaP cells were obtained from American Type Culture Collection (ATCC, Manassas VA) and grown in recommended media at 37^0C under 5 % CO_2. DU145 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis MO) supplemented with 10 % FBS (heated-inactivated fetal bovine serum) (Atlanta Biologics, Nocross GA), 20 U/ml penicillin and 0.02 mg/mL streptomycin; LNCaP cells were in RPMI-1640 (ATCC) supplemented with 10% FBS.

Cell proliferation (MTS) assay. The cells were seeded at 5000-8000/well (0.1 mL) in 96-well plates and incubated overnight at 37^0C. The next day, they were exposed to varying concentrations of doxorubicin, etoposide, cisplatin, carboplatin, or paclitaxel (all agents from Sigma-Aldrich) for 72-96 hours. At the end of the experiments, 20 µL of the CellTiter 96® AQueous One solution reagent MTS (3-4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Promega, Madison WI) in 100 µL Opti-MEM was added to each well, incubating for 1-4 hours based on the rate of color change. Cell viability was estimated by monitoring the absorbance at 490 nm using a MR5000 micro-titer plate reader (Dynatech).

Introduction of oligonucleotides into cells. Oligonucleotides were obtained as described (21) and introduced into cells by transfection using cationic lipids or by direct addition to culture media. DU145 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

cells/well for DU145 or 35,000 cells for LNCaP, were seeded in a 24-well plate and treated with ISIS 24691 (match) or ISIS 125628 (mismatch) 13-mer 2'MOE RNA. For LNCaP cells a 5 µM concentration of oligonucleotide was used without lipid, while 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), then replated at the same density.

Telomerase assay. Telomerase activity was monitored using the TRAPeze telomerase detection kit (Intergene Co., Purchase, NY), a variation of the telomere repeat amplification protocol (TRAP), following the manufacturer's directions (33).

Telomere length. Telomere size was evaluated by measuring TRF (terminal restriction fragment) size (34,35). Briefly, 1-2 µg of genomic DNA was digested with 6 restriction enzymes (Alu I, Cfo I, Hae III, Hinf I, Msp I and Rsa I). The digestion products were subjected to 1% agarose gel electrophoresis, followed by Southern hybridization with a ^{32}P end-labeled telomeric (CCCTAA) probe. Similar quantities of DNA were added to each lane. The hybridized gel was washed in 2 x SCC and exposed in a PhosphorImager cassette. The length of the telomere restriction fragment was calculated as described (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/dish 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, Gaithersburg MD) were used to visualize the colonies.

Soft-agar assay. LNCaP or DU145 cells were treated with oligonucleotides as described above for various time periods and then seeded at $1-2 \times 10^4$ 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 MTT (1 mg/ml, 100 µl/well) overnight for better visualization.

Tumor Xenograft. Male nude mice were maintained in accordance with the Institutional Animal Care and Use Committee (IACUC) procedures and guidelines. DU145 cells that had been pre-treated with mismatch ISIS 125628 or match ISIS 24691 oligomers for 45 days were harvested, suspended in PBS and subcutaneously injected into the right and left flanks (4 x 10^6 cells per flank) of 5-6 weeks 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 pre-treated with mismatch ISIS 125628 for 21 days or match ISIS 24691 for either 21 days (six mice) or 35 days (3 mice), after which they were collected and resuspended in PBS at 2 x 10^6 cells/50 µL. These cells were mixed with an equal volume of Matrigel^{TM} Matrix (BD Biosciences, Bedford, MA), and 100 µL of cell suspension were inoculated subcutaneously into the right and left flanks (2 x 10^6 cells per flank) of 5-6 weeks 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. 1-2 x10^6 cells were suspended in 5 mL PBS in a centrifuge tube. Cells were centrifuged for 6 minutes at ~200 x g and washed with PBS once. Then the cells were thoroughly resuspended in 500 µL PBS. 70 % ethanol was added to the cells up to 5 mL while gently vortexing. Cells suspended in 70 % ethanol were kept at –20 ºC for at least 24 hours. The cells were then washed in 5 mL PBS and then suspended in 500 µL propidium iodide (PI) /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 PI (Molecular Probes, Eugene OR)]. The cell suspension was kept for 30 minutes 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.

Key Research Accomplishments

Experimental Design. The telomerase inhibitor used in these studies was ISIS 24691, a thirteen base long oligonucleotide containing 2'-MOE bases with phosphorothioate backbone linkages in which a sulfur atom replaces a nonbridging oxygen (20, 21). Inclusion of 2'-MOE bases increases binding affinity, while phosphorothioate linkages increase nuclease resistance and cellular uptake (29,36). ISIS 24691 is complementary to the template region of hTR, blocks binding of primer DNA, and inhibits telomerase with an IC_{50} 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 when added in complex with cationic lipid (21). The oligomer continues to inhibit more than 75 % of telomerase activity for up to one week after addition to cells (21). 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 either DU145 or LNCaP cells, media was removed six hours after addition of oligonucleotide. Cells were carefully washed and allowed to grow without inhibitor. We observed that greater than 85 % inhibition of telomerase activity persists for up to six days after addition of inhibitor.

As a control for specificity, we used ISIS 125628, a 2'-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 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 co-administration with standard antiproliferative agents. Cell proliferation was monitored in liquid culture, by colony formation, and growth in soft agar. For experiments to determine the outcome of combining telomerase inhibition with treatment by standard antiproliferative agents we used ISIS 24691 and 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$^-$Rb$^-$) and LNCaP (p53$^+$Rb$^+$) 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 American Type Tissue Culture Collection (ATCC), had a telomere restriction fragment length (TRF) value of 2.3 kB, and will be denoted DU145-ATCC. The other DU145 strain had been cultured in the lab 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 telomere restriction fragment (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 anti-proliferative effect on the growth of DU145-ATCC, DU145, or LNCaP cells (**Figure 1 and Figure 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 (**Figure 2 A**), while after just seventeen days LNCaP proliferation had decreased 30 % (**Figure 2 C**). 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, while 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 (**Figure 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 (**Figure 1 B**) 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 with two 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 FACS 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 lab that showed that a 2'-O-methyl RNA oligomer analogous in sequence to ISIS 24691 was not been 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 2'-MOE oligomer, an

outcome that would be in agreement with the improved properties of 2'-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 terminal restriction fragment length (TRF) 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 are 2.3 kB, 2.7 kB, 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 **(Figure 3 A)**. Treatment of DU145 cells with ISIS 24691 for 65 or 90 days reduced TRF lengths to 1.9 and 1.6 kB respectively **(Figure 3 B)**. Treatment of LNCaP cells for 55 or 70 days reduced TRF lengths to 2.0 and 1.5 kB respectively **(Figure 3 C)**. 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, **(Figure 3 C, 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 (FACS) **(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 to 8 days rather than every 3 to 4 days. LNCaP cells were collected after 30 and 45 days 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 G_2/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 G_2/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 from simply counting cells shown above (**Figure 1 and Figure 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 relative to G2/M arrest. These observations are consistent with the hypothesis that cells expressing 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 (**Figure 4 A**). 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 (**Figures 1 A and 2 A**). 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 (**Figure 5 A**).

Soft agar and colony formation assays were also performed using LNCaP cells. Treatment of cells with ISIS 24691 in liquid culture for four days prior to plating did not lead to inhibition of colony growth in soft agar (**Figure 4 B**), consistent with the belief that significant telomere shortening must occur prior to 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 (**Figure 4 B**). No colonies were formed after the 60 days treatment. Colony formation assays yielded similar results (**Figure 5 B**).

Implications of Reduced Growth During Soft Agar/Colony Formation Assays. Compared to 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 prior to 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. While 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 twelve 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 three weeks of treatment of AT-SV1 cells (telomere length 2.2 kB) with peptide nucleic acid (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 prior to implantation is directly comparable to the soft agar and colony formation assays performed above yet 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 (**Figure 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, while tumors were much smaller in animals implanted with cells treated for 21 days (mice 1-6) (**Table 2, Figure 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 to 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 **Figure 6 D**. At the end of the study (day 55) LNCaP tumors were harvested for histological analysis. Staining with hematocxylin and eosin 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**) (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 while the remaining six animals had PSA levels of 23.9 to 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 under 1, while the remaining three had PSA levels under 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. von Zglinicki and colleagues have reported that inhibition of telomerase in combination with treatment with the topoisomerase inhibitor doxorubicin produces synergistic effects on cell proliferation (44), while Zaffaroni and coworkers failed to show additive effects with platinum compounds, taxanes, and topoisomerase inhibitors (45). Au and colleagues have reported additive effects from a combination of paclitaxel and expression of anti-hTERT antisense RNA (46).

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 one day prior to initiating treatment with varying concentrations of chemotherapy agents were added to cells for an additional three 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 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 hours to DU145

cells that had been previously treated 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 (**Figure 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 (**Figure 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 (**Figure 1, 4, 5, and 6**). The discrepancy between the results of treatment of LNCaP and DU145 cells reinforces the suggestion 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 antitelomerase 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 prior to 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 PNAs and 2'-O-methyl oligonucleotides we had observed substantial decreases in cell proliferation (19,20). However, maximal effects required three 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-meRNA 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 2'-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 2'-MOE RNA oligomer ISIS 24691 caused DU145-ATCC, DU145, and LNCaP cultures to become nonviable after only 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 two to five weeks of treatment (**Figure 2**). Decreased tumor cell proliferation was even more striking when measured by colony formation, soft agar growth, growth of xenograft tumors in nude mice, assays that are thought to be more accurately test the tumorigenic potential of treated cells (**Figures 4, 5, and 6**). Since 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 chemotherapy 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 are 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 knock out mice (mTR$^{-/-}$) that show the telomere shortening, not telomerase inhibition alone, increases the antiproliferative effects of agents   that   induce   double   strand   breaks   (50).

Conclusions

Based on our data we conclude that telomerase inhibition can 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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FIGURE LEGENDS

Figure 1. Effect of telomerase inhibition on cell proliferation. **A.** Inhibition of growth of DU145-ATCC cells (TRF=2.3 kB) treated with ISIS 2469 or mismatch ISIS 125628 compared with untreated cells. **B.** Inhibition of growth of DU145 cells (TRF=2.7 kB) treated with ISIS 24691 or ISIS 125628 compared with untreated cells. After 67 days cells treated with ISIS 24691 were split into three groups. One group was no longer treated with ISIS 24691. One group was treated with ISIS 24691 less frequently to maintain a viable culture. The final group was treated with ISIS 24691 on day 71 and subsequently was no longer viable. **C.** Inhibition of growth of LNCaP cells treated with ISIS 24691 or ISIS 125628 compared with untreated cells. Intracellular delivery of oligonucleotides to LNCaP cells was achieved without added lipid, allowing us to omit the lipid-only control from this time-course.

Figure 2. Inhibition of growth of cells treated with ISIS 24691 at different times relative to cells treated with mismatch ISIS 125628. **A.** DU145-ATCC cells. **B.** DU145 cells. **C.** LNCaP cells.

Figure 3. Effect of telomerase inhibition on telomere length of DU145 and LNCaP cells monitored by the telomere restriction fragment (TRF) assay. Telomere lengths are noted above each lane. **A.** DU145-ATCC Cells; **Lane 1;** mismatch oliognucleotide ISIS 125628 added for 45 days. **Lane 2;** match oligonucleotide ISIS 24691 added for 45 days. **Lane 3;** no oligonucleotide aded. **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 oligonuceotide 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.** LNCaP cells. **Lane 1;** no oligonucleotide added, cells were harvested after three 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 left.

Figure 4. Effect of telomerase inhibition on anchorage-independent growth in soft agar. **A.** DU145 cells. **B.** LNCaP cells. ***, denotes no LNCaP colonies were detected at 60 days.

Figure 5. Effect of telomerase inhibition on colony formation. **A.** DU145 cells. **B.** LNCaP cells.

Figure 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 and **(B)** Tumor growth of DU145 cells treated with ISIS 24691. **(C)** Tumor growth of LNCaP cells treated with ISIS125628 and **(D)** Tumor growth of LNCaP cells treated with ISIS 24691. LNCaP cells were mixed with Matrigel in a 1:1 ratio and injected subcutaneously. Average tumor sizes with standard error are shown for tumors derived from **(E)** DU145 cells that had been treated with match or mismatch oligonucleotide for 45 days in culture prior to implantation and **(F)** LNCaP cells that had been treated with match or mismatch oligonucleotide for 21 days prior to 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 $P < 0.001$ (**).

Figure 7. The effect on proliferation of DU145 and LNCaP cells of combining telomerase inhibition with the antiproliferative agents cisplatin and carboplatin. **A.** Effect of addition of cisplatin on proliferation of DU145 cells that had been treated with ISIS 24691 for 50 days. **B.** Effect of addition of carboplatin on proliferation of DU145 cells that had been treated with ISIS 24691 for 50 days. **C.** Effect of addition of cisplatin on proliferation of LNCaP cells that had been treated with ISIS 24691 for 50 days. **D.** Effect of addition of

carboplatin on proliferation of LNCaP cells that had been treated with ISIS 24691 for 50 days. All data points

are averages of quadruplicate determinations.

TABLES

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.

Phase	Untreated	24691	125628
		DU145 cells, 7 days	
sub-G_1	0.4	0.4	0.4
G_0/G_1	66	67	67
S	15	14	15
G_2/M	19	18	17
		DU145 cells, 60 days	
sub-G_1	1	8	2
G_0/G_1	62	36	67
S	17	16	13
G_2/M	20	40	18
		DU145 cells, 90 days	
sub-G_1	1	29	2
G_0/G_1	62	27	68
S	17	14	13
G_2/M	20	30	17
		DU145 cells, 120 days	
sub-G_1	1	68	1
G_0/G_1	58	7	58
S	21	9	20
G_2/M	20	16	21
		LNCaP cells, 30 days	
sub-G_1	0.5	7	0.3
G_0/G_1	61	64	63
S	21	10	21
G_2/M	17.5	19	16
		LNCaP cells, 45 days	
sub-G_1	1	36	1
G_0/G_1	80	49	80
S	10	8	9
G_2/M	9	7	10

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

Mouse/Flank	PSA	Tumor Vol. (mm)^3	Mouse/flank	PSA	Tumor Vol. (mm)^3
Mismatch 1/R	106.7	394	Match 1/R	0.6	53
/L		515	/L		0
Mismatch 2/R	25.7	235	Match 2/R	0	0
/L		180	/L		0
Mismatch 3/R	44.2	734	Match 3/R	3.2	0
/L		42	/L		147
Mismatch 4/R	29.8	205	Match 4/R	4.8	195
/L		375	/L		76
Mismatch 5/R	23.9	329	Match 5/R	2.3	0
/L		0	/L		133
Mismatch 6/R	0.8	0	Match 6/R	0	0
/L	0		/L	0	
Mismatch 7/R	30.5	0	Match 7/R	0	0
/L		499	/L		0
			Match 8/R	0.1	0
			/L		0
			Match 9/R	0.3	0
			/L		0

R=right flank. L=left flank. 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.
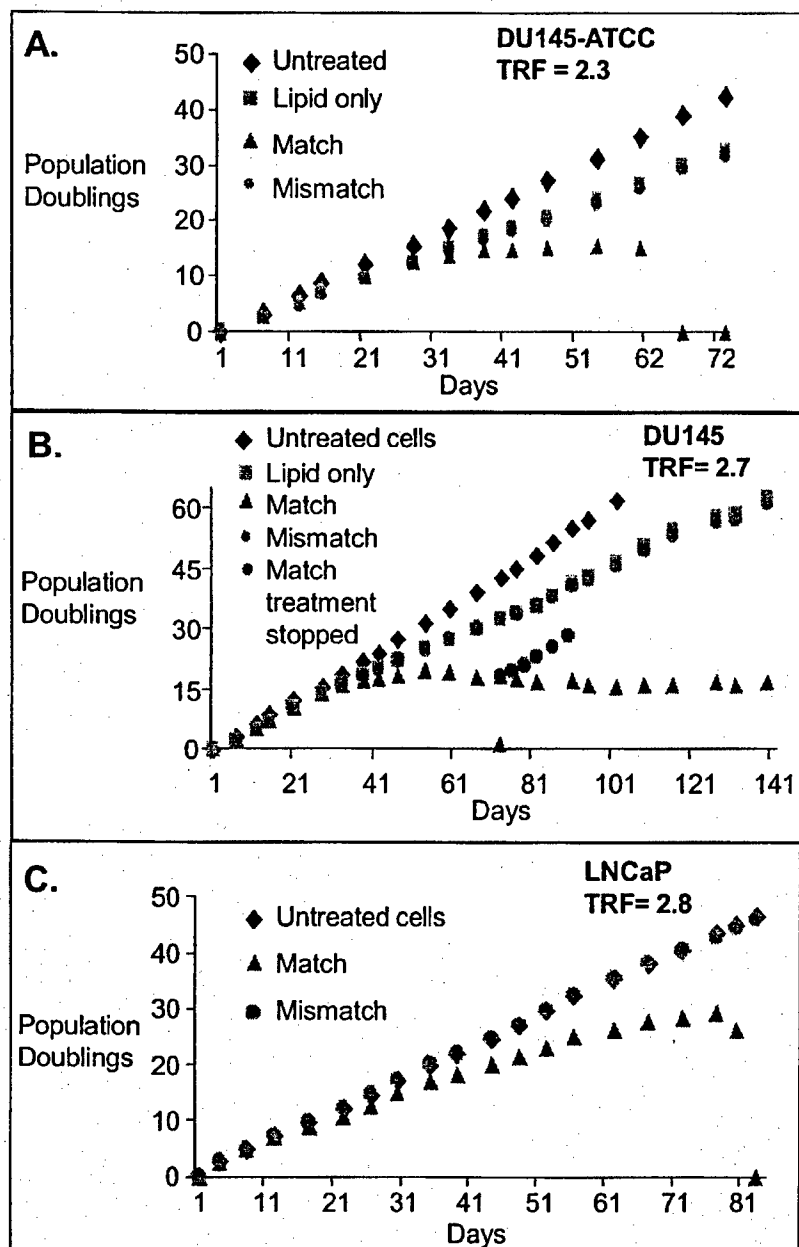
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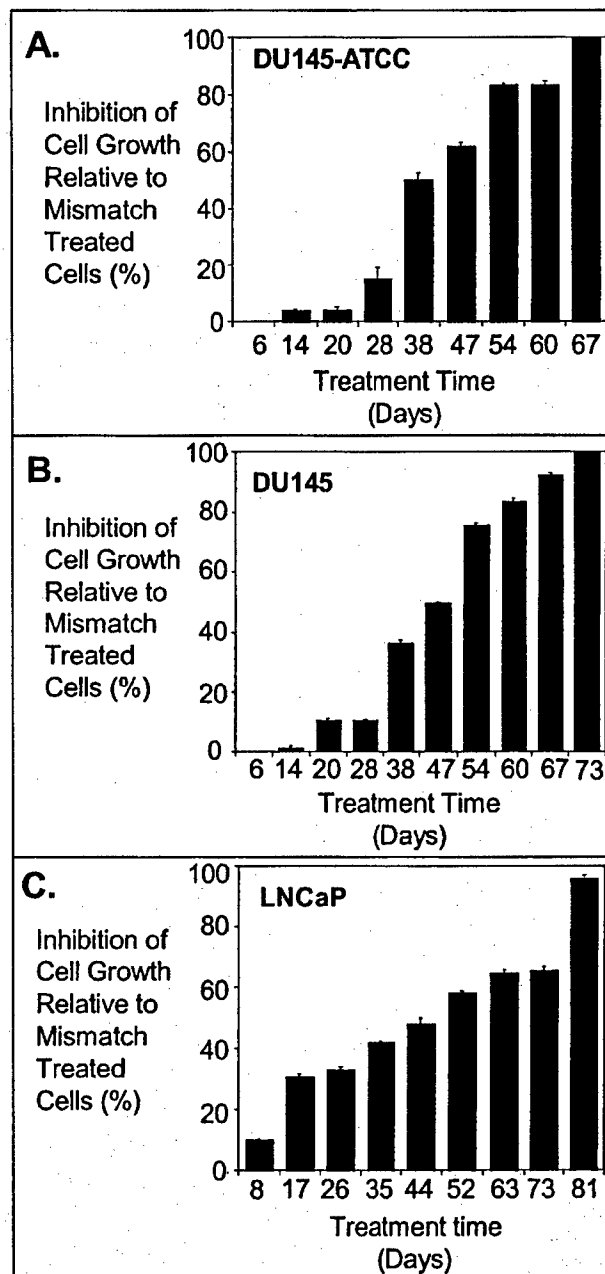
Figure 2. Inhibition of growth of cells treated with ISIS 24691 at different times relative to cells treated with mismatch ISIS 125628. **A.** DU145-ATCC cells. **B.** DU145 cells. **C.** LNCaP cells.
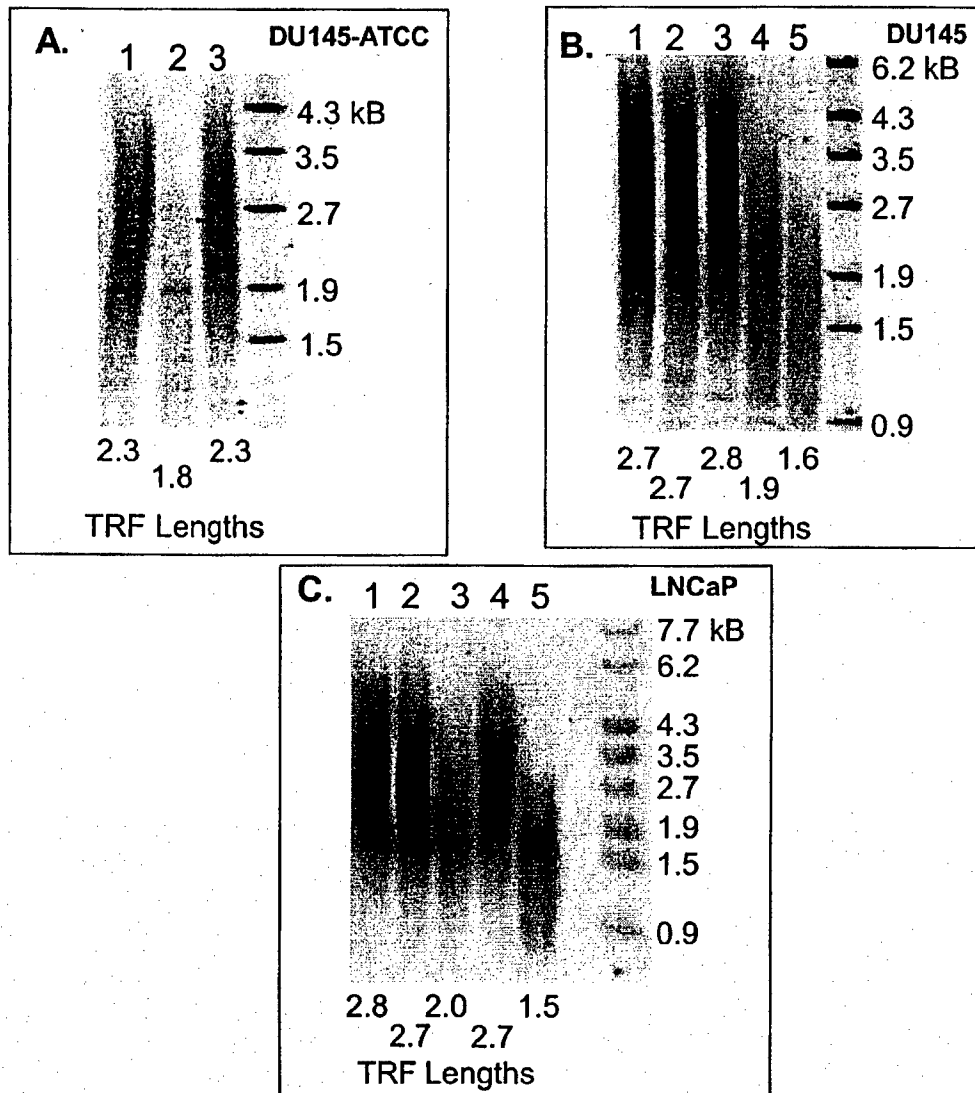
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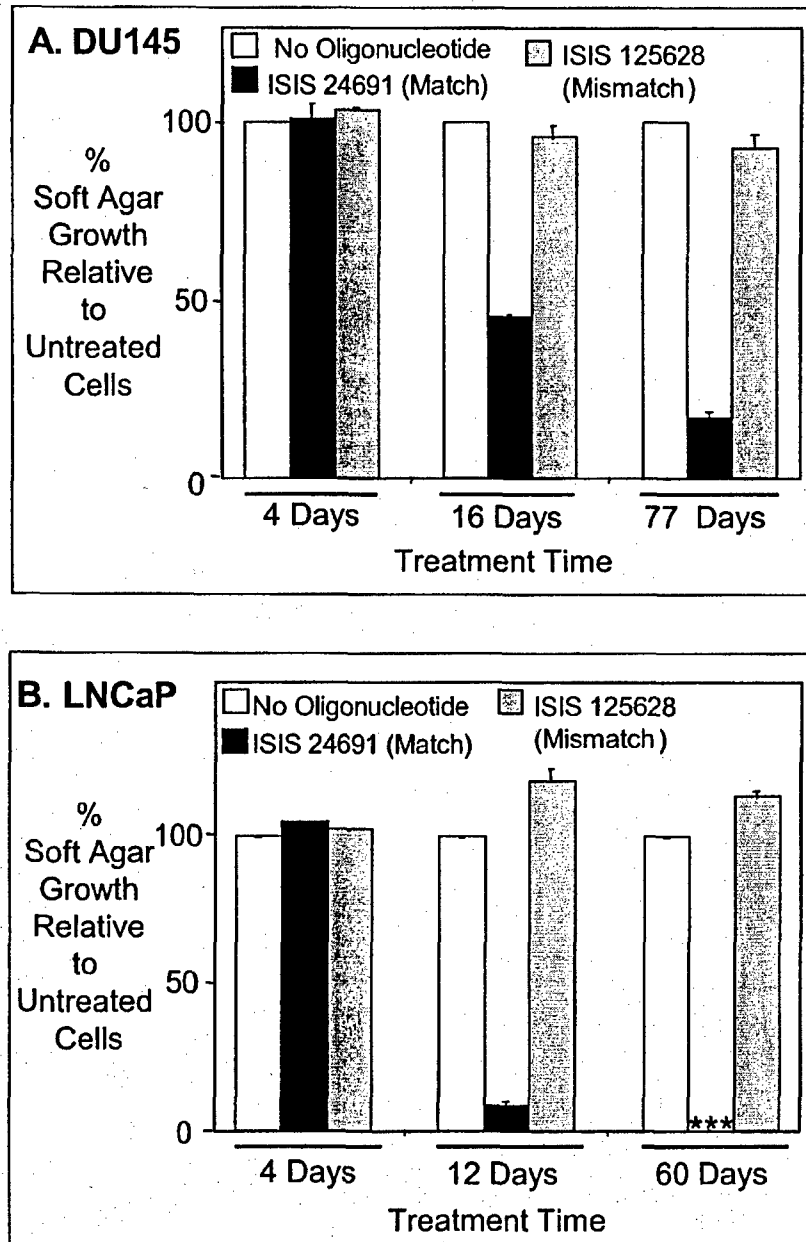
Figure 4. Effect of telomerase inhibition of anchorage-independent growth in soft agar. **A.** DU145 cells. **B.** LNCaP cells. ***, denotes no LNCaP colonies were detected at 60 days. The values shown are averages of at least duplicate determinations.
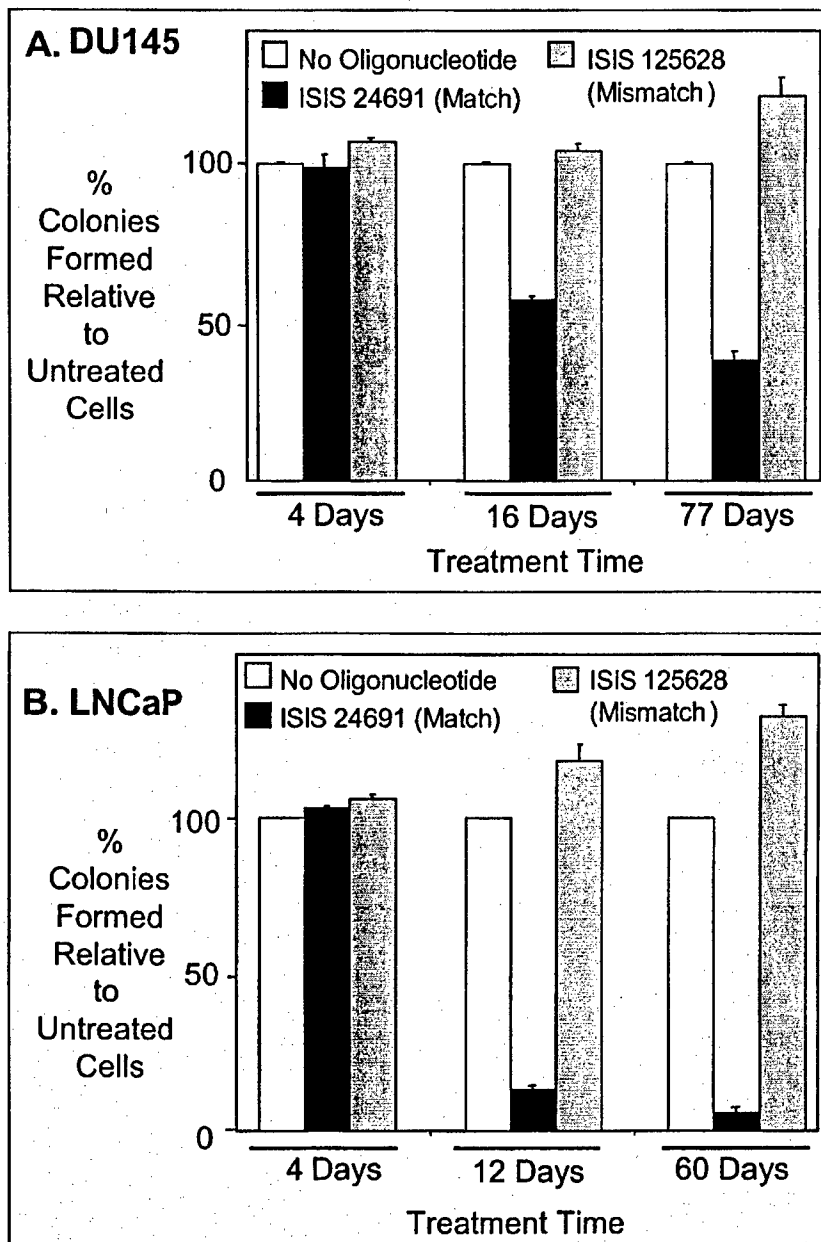
A. DU145

Legend:
- ☐ No Oligonucleotide
- ■ ISIS 24691 (Match)
- ▨ ISIS 125628 (Mismatch)

% Colonies Formed Relative to Untreated Cells

Y-axis: 0, 50, 100

X-axis (Treatment Time): 4 Days, 16 Days, 77 Days

B. LNCaP

Legend:
- ☐ No Oligonucleotide
- ■ ISIS 24691 (Match)
- ▨ ISIS 125628 (Mismatch)

% Colonies Formed Relative to Untreated Cells

Y-axis: 0, 50, 100

X-axis (Treatment Time): 4 Days, 12 Days, 60 Days

Figure 5. Effect of telomerase inhibition on colony formation. **A.** DU145 cells. **B.** LNCaP cells. The values shown are averages of at least duplicate determinations.
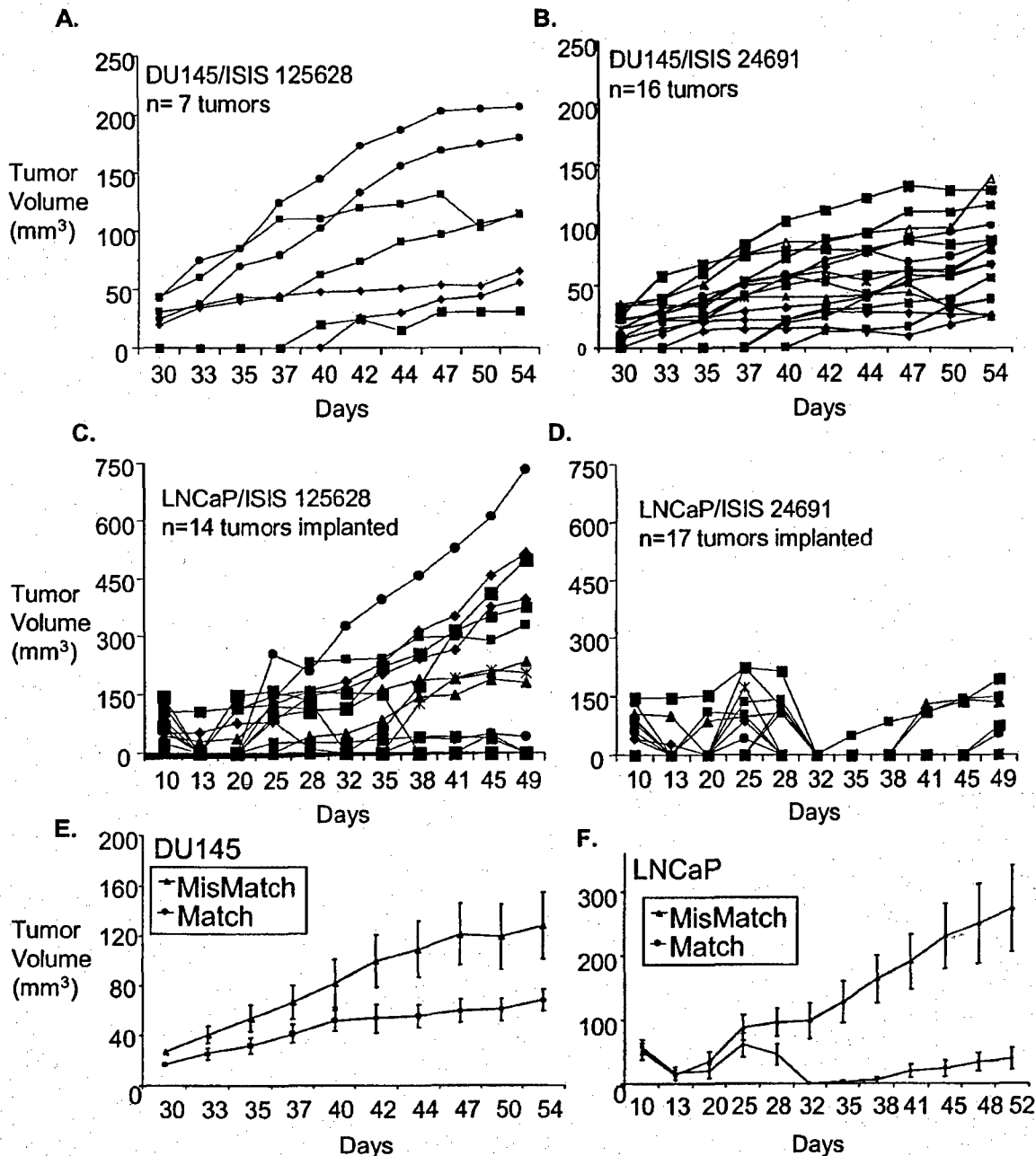
Figure 6. Growth of DU145 and LNCaP tumors in nude mice. **(A)** Tumor growth of DU145 cells treated with ISIS 125628 and **(B)** Tumor growth of cells treated withISIS 24691. **(C)** Tumor growth of LNCaP cells treated with ISIS125628 and **(D)** Tumor growth of cells treated withISIS 24691. LNCaP cells were mixed with Matrigel in a 1:1 ratio and injected subcutaneously. Average tumor size with standard error are shown for **(E)** DU145 (P<0.05) and **(F)** LNCaP (P<0.001).
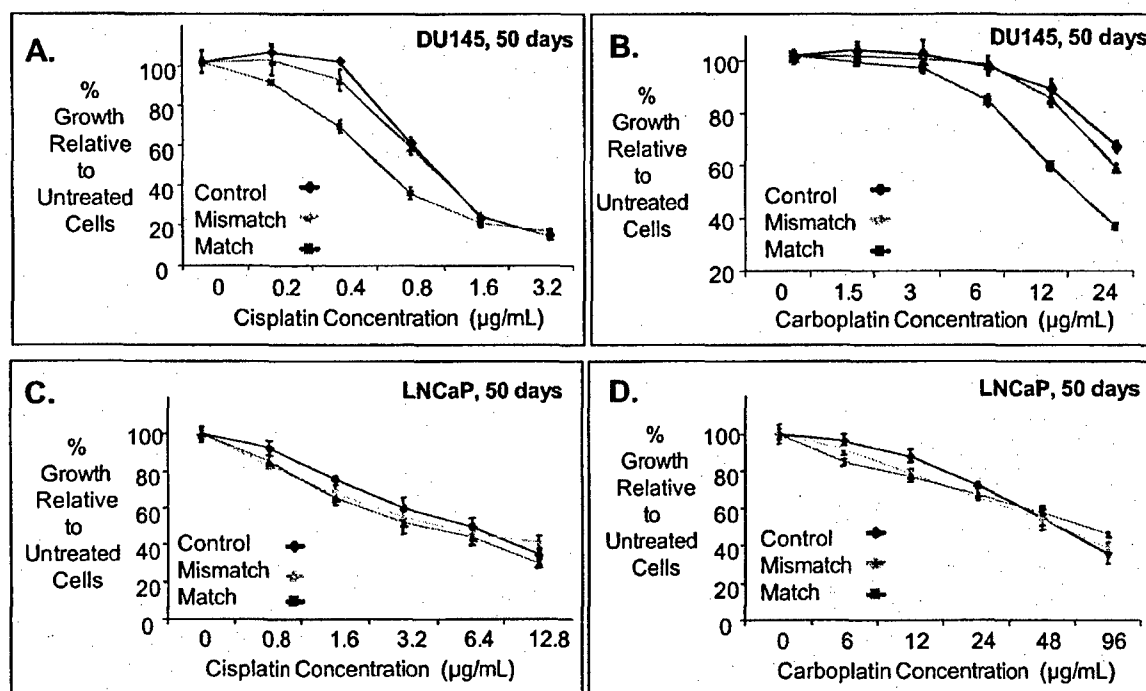
Figure 7. The effect on proliferation of DU145 and LNCaP cells of combining telomerase inhibition with the antiproliferative agents cisplatin and carboplatin. **A.** Effect of addition of cisplatin on proliferation of DU145 cells that had been treated with ISIS 24691 for 50 days. **B.** Effect of addition of carboplatin on proliferation of DU145 cells that had been treated with ISIS 24691 for 50 days. **C.** Effect of addition of cisplatin on proliferation of LNCaP cells that had been treated with ISIS 24691 for 50 days. **D.** Effect of addition of carboplatin on proliferation of LNCaP cells that had been treated with ISIS 24691 for 50 days. All data points are averages of quadruplicate determinations.