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**13. Abstract** ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Telomerase, a ribonucleoprotein reverse transcriptase, has been detected in reproductive cells, immortal cell lines, and most cancer cells. The activity of telomerase has been shown to be absent in normal somatic cells, with the exception of stem cells. The reactivation of telomerase has been seen as an early event in most cancers, especially breast cancer. In the first year I showed that the inhibition of telomerase led to the inhibition of cell growth via telomere-based mechanisms. The inhibition of telomerase was correlated with progressive telomere shortening in these cells and, consequently, inhibition of cell growth via cell death. Furthermore, I was able to show that telomerase inhibitors can prevent the spontaneous immortalization of Li-Fraumeni Syndrome-derived breast epithelial cells, which have not reached cellular crisis, by the prevention of telomerase activation. Tamoxifen also reduced the frequency of spontaneous immortalization in these cells. Since cancer is mostly a disease of epithelial cells, we believe our unique system of normal and spontaneously immortalized human breast epithelial cells should provide a good model system to examine the effects of tamoxifen and telomerase inhibitors. These studies should lead to new insights in preventing the occurrence or recurrence of breast cancer.

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## Introduction

Li-Fraumeni Syndrome (LFS) is characterized by heterozygous mutations in the tumor suppressor gene p53 (1). Alterations in the p53 gene are thought to lead to genomic instability and allow for continued cellular proliferation, leading to further instabilities (2). Studies of the importance of p53, and identification of the LFS germ line mutations of p53, have led to an understanding of the cancer risk to LFS families. Among women in affected LFS families, breast tumors are the most prevalent cancer (afflicting at least 50%), with one quarter of the breast cancers diagnosed before age 30 and 89% diagnosed before age 50 (1,3-4). The molecular mechanism of the specific increased incidence of breast cancer as opposed to other cancers in families affected by LFS is not completely understood (5-6). One of the Li-Fraumeni Syndrome (LFS) cell strains that is used as a model for this project is derived from a patient who had surgery for breast cancer. These human mammary epithelial (HME) LFS cells are telomerase silent, grow in defined medium for approximately 50 population doublings then undergo a crisis stage, reproducibly immortalize and then express telomerase (7).

Cells contain repeated TTAGGG DNA sequences, called telomeres, at the end of chromosomes to provide genomic stability and to provide a source of expendable DNA due to the end replication problem where one DNA strand cannot complete its end during replication (8-10). Telomere length has been shown to decrease with time (and with increasing age). Maintenance of telomeres has been shown to involve telomerase activity, which acts as a reverse transcriptase to add base pairs to the ends of chromosomes (11). It has been proposed that an immortalized cell emerges (a hallmark of most cancer cells) from a stage called crisis when telomerase or another mechanism to maintain telomere stability is activated (12). Once telomerase is activated it stabilizes telomere length and permits continued cell division. Most cancer cells have been shown to contain short but stable telomeres compared to parental cells. Beside germ line and stem cells of renewal tissues, other cells having telomerase activity are cancer cells (90% of those tested) (13).

Telomerase has been shown to become active early in breast cancer progression (13 for review). Thus, the proposed study will determine, using a cell culture model system, if inhibiting telomerase activation has the potential to prevent the occurrence or recurrence of breast cancer. The approach is to examine the effects of telomerase inhibitors, and other putative chemopreventive agents such as tamoxifen, on the growth of immortalized breast epithelial cells and the frequency of spontaneous immortalization in human breast epithelial cell from individuals predisposed to breast cancer.

As telomerase is re-expressed in cancer cells, the critically short telomeres may be favored to be elongated by telomerase. The average telomere length in these cells becomes stable at lengths well below normal cells. These shorter lengths are at a critical length for cell survival. The difference in telomere lengths between normal and cancer cells provides for a mechanism that may specifically target and inhibit the growth of cancer cells. It is proposed that the inhibition of telomerase may lead to the growth inhibition of immortalized human mammary epithelial cells (HMECs) and to the prevention of the spontaneous immortalization of HMECs through telomere shortening.

## Body and Conclusions

In the first year, the "Statement of Work's Task 1" was to inhibit the growth of telomerase expressing, spontaneously immortalized Human Mammary Epithelial Cells (HMECs) via a telomere-based mechanism using telomerase inhibitors. I showed that the inhibition of telomerase led to the inhibition of cell growth via telomere-based mechanisms (14, Appendix A1). To confirm action through a telomere-based mechanism, telomerase inhibitors should (i) reduce telomerase activity, but initially not affect cell growth rates; (ii) lead to progressive shortening of telomeres with each cell division; and (iii) cause cells to die or undergo growth arrest. In addition, the time necessary to observe decreased proliferation should vary depending on initial telomere length, and chemically related molecules that do not inhibit telomerase activity should not cause decreased cell proliferation or telomere shortening. First, the immortalized HMECs were treated with non-toxic concentrations of telomerase inhibitors and tamoxifen. The lifespan of the treated and untreated cells was monitored and aliquots of cells were either saved for DNA, RNA and protein analyses or stored frozen. Cells were lysed and assayed for telomerase activity and telomere length using established laboratory protocols (described in 14). While non-toxic concentrations of tamoxifen did not inhibit telomerase activity, the 2'-O-meRNA specific to the RNA component of telomerase or the dominant negative hTERT did inhibit telomerase activity. Shortening of telomeres and subsequently growth inhibition followed inhibition of telomerase activity. The method of growth inhibition was determined as apoptotic due to critically shortened telomeres. The next task is to understand the mechanism for the induction of cell death via telomere shortening in these cells.

In addition to the tasks outlined for the first year, I was able to show that telomerase inhibitors and tamoxifen can prevent the spontaneous immortalization of Li-Fraumeni Syndrome-derived breast epithelial cells, which have not reached cellular crisis, by the prevention of telomerase activation (15, Appendix A2). The work was originally outlined for the second year as "Statement of Work's Task 2". First, the HMECs were treated prior to crisis with telomerase inhibitors and tamoxifen. The lifespan of the treated and untreated cells was monitored and aliquots of cells were either saved for DNA, RNA and protein analyses or stored frozen. Approximately ten population doublings before cells enter crisis, fluctuation analyses were performed (described in 15). The frequency of spontaneous immortalization of Li-Fraumeni Syndrome HMECs was reduced with tamoxifen and telomerase inhibitors. The finding that treating cells prior to crisis with anti-telomerase agents diminishes the spontaneous immortalization of LFS-derived breast epithelial cells in vitro indicates a potential approach for the development of rational chemoprevention strategies using both clinically and preclinically validated chemopreventive agents for women with a genetic predisposition to breast cancer. Moreover, the prevention of spontaneous immortalization offers a new intermediate endpoint for validating novel chemopreventive agents. The next task is to understand the mechanism for preventing the spontaneous immortalization of these cells.

Since cancer is mostly a disease of epithelial cells, we believe our unique system of normal and spontaneously immortalized human breast epithelial cells should provide a good model system to examine the effects of tamoxifen and telomerase inhibitors. Inhibition and/or reversal of the immortal phenotype and other endpoints such as genomic instability, telomere stability, anchorage independent growth assays, should provide insights into the earliest stages of cancer development, leading to more effective cancer prevention measures.

## Key Research Accomplishments

*Original Statement Task 1.* Inhibit the growth of telomerase expressing, spontaneously immortalized Human Mammary Epithelial Cells (HMECs) via a telomere-based mechanism using telomerase inhibitors, Months 1-12:

- The immortalized HMECs were treated with telomerase inhibitors and tamoxifen.
- The lifespan of the treated and untreated cells was monitored and aliquots of cells were either saved for DNA, RNA and protein analyses or stored frozen.
- Cells were lysed and assayed for telomerase activity and telomere length using established laboratory protocols.
- Method of growth inhibition was determined as apoptotic due to critically shortened telomeres.

*Original Statement Task 2.* Prevent the spontaneous immortalization of Li-Fraumeni Syndrome Human Mammary Epithelial Cells using telomerase inhibitors, originally Months 12-24:

- The HMECs were treated with telomerase inhibitors and tamoxifen.
- The lifespan of the treated and untreated cells was monitored and aliquots of cells were either saved for DNA, RNA and protein analyses or stored frozen.
- Approximately ten population doublings before cells enter crisis, fluctuation analyses were performed.
- The frequency of spontaneous immortalization of Li-Fraumeni Syndrome HMECs was reduced with tamoxifen and telomerase inhibitors.

## Reportable Outcomes

Publications

**Herbert, B.-S.,** Wright, A.C., Passons, C.M., Kopelovich, L., Ali, I., Wright, W.E., and J.W. Shay. Effects of Chemopreventive and anti-telomerase agents on the spontaneous immortalization of breast epithelial cells. *J Natl Cancer Inst* 93:39-45, 2001.

**Herbert, B.-S.,** Pitts, A.E., Baker, S.I., Hamilton, S.E., Wright, W.E., Shay, J.W., and D.R. Corey. Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. *Proc Natl Acad Sci* 96:14276-14281, 1999.

Abstracts

**Herbert, B.-S.,** Kopelovich, L., Ali, I., Wright, W.E., and J.W. Shay. Telomerase: A Breast Cancer Chemopreventive and Therapeutic Target. Susan G. Komen "Reaching for the Cure...Making a Difference" Mission Conference. Sept 17-19, 2000.

Corey, D.R., **Herbert, B.S.,** Pitts, A.E., Baker, S.I., Wright, W.E., and J.W. Shay. Inhibition of Human Telomerase in Immortal Human Cells Leads to Progressive Telomere Shortening and Cell Death. Geron Symposium No. 3 entitled, "Telomerase and Telomere Dynamics in Cancer and Aging." June 24-28, 2000.

**Herbert, B.-S.,** Steinert, S., Wright, W.E., and J.W. Shay. Telomerase inhibitors induce apoptosis in immortal human cells and prevent the spontaneous immortalization of breast epithelial cells from individuals predisposed to breast cancer. Geron Symposium No. 3 entitled, "Telomerase and Telomere Dynamics in Cancer and Aging." June 24-28, 2000.

Pongracz, K., **Herbert, B.-S.,** Matray, T., Pruzan, R., Chin, A., Yamashita, Y., Harley, C., Shay, J.W., and S. Gryaznov. Inhibitors of telomerase- oligonucleotide N3'→P5'

phosphoramidates. Geron Symposium No. 3 entitled, "Telomerase and Telomere Dynamics in Cancer and Aging." June 24-28, 2000.

**Herbert, B.-S.**, Kopelovich, L., Ali, I., Wright, W.E., and J.W. Shay. Telomerase inhibitors prevent the spontaneous immortalization of breast epithelial cells from individuals predisposed to breast cancer and induce apoptosis in immortal cells. DoD Breast Cancer Research Program Era of Hope Meeting. June 8-12, 2000.

Pitts, A.E., Baker, S.I., **Herbert, B.-S.**, Shay, J.W., and D.R. Corey. 2'-O-Methyl RNA oligonucleotides directed against the human telomerase template potently and selectively inhibit telomerase activity, eroding telomeres and slowing growth. Cold Spring Harbor Meeting on Telomeres and Telomerase. March 25-28, p.124, 1999.

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# Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death

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**The correlation between telomerase activity and human tumors has led to the hypothesis that tumor growth requires reactivation of telomerase and that telomerase inhibitors represent a class of chemotherapeutic agents. Herein, we examine the effects of inhibition of telomerase inside human cells. Peptide nucleic acid and 2′-*O*-MeRNA oligomers inhibit telomerase, leading to progressive telomere shortening and causing immortal human breast epithelial cells to undergo apoptosis with increasing frequency until no cells remain. Telomere shortening is reversible: if inhibitor addition is terminated, telomeres regain their initial lengths. Our results validate telomerase as a target for the discovery of anticancer drugs and supply general insights into the properties that successful agents will require regardless of chemical type. Chemically similar oligonucleotides are in clinical trials and have well characterized pharmacokinetics, making the inhibitors we describe practical lead compounds for testing for an antitelomerase chemotherapeutic strategy.**

Human telomerase is a ribonucleoprotein that adds repeated units of TTAGGG to the ends of telomeres (1, 2). Telomerase activity has been found in almost all human tumors but not in adjacent normal cells (3, 4). This correlation has led to the hypotheses that reactivation of telomerase is necessary for sustained cell proliferation in many tumors and that telomerase is an exceptional target for a class of chemotherapeutic agents that act by an unidentified mechanism (5). Supporting these hypotheses is the observation that early-stage neuroblastomas have little or no telomerase activity, and this minimal activity generally correlates with a favorable outcome, whereas the late-stage disease has high telomerase activity and correlates with a poor outcome (6). A similar linkage between telomerase activity and poor clinical outcome has been reported for ordinary meningiomas (7), and other studies have suggested that telomerase activity is correlated with the pathologic stage (8–11) or tumor aggressiveness (11, 12).

Additional evidence for the importance of telomerase activity for sustained cell growth comes from studies of mice that lack the mouse telomerase RNA component (mTR). Depending on their genetic backgrounds, these mice survived for four to six generations with few detectable phenotypic changes (13, 14); however, by the seventh generation, highly proliferative organ systems like the testis, bone marrow, and spleen appeared abnormal, and the mice were no longer able to reproduce (15). In some genetic backgrounds, an increased incidence of neural tube defects limited viability after only four generations (14). Other studies of these mice revealed shortened life spans, an increased incidence of chromosomal abnormalities, and a slight increase in spontaneous malignancies (16). The long lag between loss of telomerase activity and a detrimental phenotype suggests that telomerase will be a difficult target for effective chemotherapy, but the mice used in these experiments possessed much longer telomeres than those found in cancerous human cells, and inhibition of telomerase in human cancer would be expected to produce effects more rapidly. Furthermore, recent reports of the effect of the mTR deletion in different genetic backgrounds (17, 18) suggest that the role of telomerase in cancer development could be highly context dependent (19).

Mutation of the RNA component of telomerase of *Tetrahymena* (20), *Kluyveromyces lactis* (21), *Saccharomyces cerevisiae* (22), and human cells (23) also leads to decreased cell proliferation, whereas expression of antisense RNA complementary to the human telomerase RNA component (hTR) caused decreased proliferation of HeLa cells after 23 to 26 doublings (24). Conversely, transfection of cells with the gene encoding the human telomerase reverse transcriptase component (hTERT) and subsequent expression of active telomerase have been shown to extend the life spans of normal human fibroblasts and epithelial cells (25–27). Most recently, expression of telomerase in conjunction with expression of simian virus 40 large T oncoprotein and an oncogenic allele of H-ras has been shown to promote tumorigenic conversion of normal human cells (28). Thus, the lack of telomerase expression seems to curb growth of rapidly proliferating cells eventually, whereas an increase in telomerase permits indefinite proliferation.

A potential concern for the telomerase–cancer connection is the observation that a few rare tumors (29) and some experimentally immortalized cell lines (30) lack detectable telomerase activity. Thus, although telomerase activity can confer extended life span to cells, other mechanisms may also exist. A pathway termed ALT (alternative lengthening of telomeres) has been proposed to account for this phenomenon. Evidence for the existence of this pathway is found in yeast in which the gene for est1, a protein involved in telomere-length maintenance, has been deleted (31). The cells divide normally until telomeres shorten sufficiently to affect proliferation. At this stage, most cells die, but some rare survivors continue to proliferate through a recombination mechanism to maintain telomere length.

Telomerase will be an unusually challenging target for drug development because of the long lag period expected before telomeres would shorten sufficiently to produce detrimental effects on cell growth and because of the possibility of alternate mechanisms for maintenance of telomeres. Because of this uncertainty, discovery of the potential of telomerase as a target for human therapy requires development of potent and selective synthetic inhibitors and their testing inside cells. To confirm action through a telomerase-dependent mechanism, inhibitors

Abbreviations: mTR and hTR, mouse and human telomerase RNA component; hTERT, human telomerase reverse transcriptase component; PNA, peptide nucleic acid; HME, human mammary epithelial; TRF, terminal restriction fragment.

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must meet the following criteria: (*i*) inhibitors should reduce telomerase activity but, initially, should not affect cell growth rates; (*ii*) addition of inhibitors should lead to progressive shortening of telomeres with each cell division; (*iii*) addition of inhibitors should cause cells to die or undergo growth arrest; (*iv*) the time necessary to observe decreased proliferation should vary depending on initial telomere length; and (*v*) chemically related molecules that do not inhibit telomerase activity should not cause decreased cell proliferation or telomere shortening.

Herein, we examine how inhibition of telomerase by exogenously added molecules affects cellular phenotypes. We observe that 2′-*O*-MeRNA and peptide nucleic acid (PNA) oligomers complementary to the template region of hTR inhibit telomerase and cause telomeres to shorten and that extended treatment with 2′-*O*-MeRNA promotes cell death through apoptosis. Inhibition of cell proliferation suggests that oligonucleotides are a viable approach to antitelomerase therapy, and our results should encourage and guide further testing of other approaches to telomerase inhibition such as nucleotide analogues (32), G quadruplex interactive agents (33, 34), or other small molecules.

## Materials and Methods

**Cell Lines.** The human mammary epithelial (HME) cells used for these experiments were spontaneously immortalized from an epithelial culture derived from normal breast tissue from a patient with Li-Fraumeni syndrome (35). Cells were grown in serum-free medium (MCDB from GIBCO) supplemented with 0.4% bovine pituitary extract (Hammond Cell Tech, Alameda, CA), 10 ng/ml epidermal growth factor (GIBCO), 5 μg/ml insulin (Sigma), 0.5 μg/ml hydrocortisone (Sigma), 5 μg/ml transferrin, and 50 μg/ml gentamicin (Sigma). The medium was changed every 2–3 days. Cells were used between population doublings 100 and 150. The prostate-tumor-derived DU145 cells were maintained in DMEM containing 10% (vol/vol) FCS, 500 units/ml penicillin (Sigma), and 0.1 μg/ml streptomycin (Sigma) and incubated at 5% $CO_2$ at 37°C. Detection and quantitation of apoptosis by flow cytometry were performed with the ApoAlert Annexin V Apoptosis kit (CLONTECH).

**Oligonucleotides and PNAs.** We purchased 2′-*O*-MeRNA oligonucleotides from Oligos Etc. and Oligo Therapeutics (Wilsonville, OR). The sequence of the match phosphorothioate modified 2′-*O*-MeRNA is 5′-CAGUUAGGGUUAG-3′; the mismatch sequence is 5′-CAGUUAGAAUUAG-3′, where the underlined nucleotides possess phosphorothioate linkages. Match and mismatch PNAs (36) of similar sequences and nontemplate directed PNA AGCGGGCCAGCAGCTG were synthesized automatically with a PerSeptive Biosystems (Framingham, MA) Expedite 8909 Synthesizer by using Fmoc protocols and reagents obtained from PE Biosystems. PNAs were purified by HPLC and characterized by matrix-assisted laser desorption time-of-flight mass spectrometry by using a Voyager-DE mass spectrometry workstation (PE Biosystems) as described (37). The sequence of the match PNA is Gly-CAGTTAGGGTTAG-Lys; the sequence of the mismatch is Gly-CAGTTAGAATTAG-Lys. DNA oligonucleotides for transfection of PNA/DNA complexes were obtained from Life Technologies (Gaithersburg, MD). The sequence of the DNA oligonucleotide complexed to the match PNA is (5′–3′) TCTAACCCTAA; the sequence of the DNA oligonucleotide complexed to the mismatch PNA is (5′–3′) TCTAATTCTAA.

**Uptake of 2′-*O*-MeRNA and PNA into Cells.** HME50-5E and HME50-hTERT cells were transfected with 2′-*O*-MeRNA and mismatch control oligomers after the FuGENE6 Transfection Reagent protocol (Roche Molecular Biochemicals). Briefly, $5 \times 10^4$ cells were allowed to adhere overnight in appropriate media. The next day, the oligomers were incubated for 15 min with the previously combined FuGENE6 Transfection Reagent and PBS. After changing to fresh medium, the oligomer mixture was added dropwise to the cells. Cells were harvested after 4 days, counted, and replated, and telomerase activity was determined.

DU145 cells were plated at 25,000 cells per well in a 24-well plate in DMEM supplemented with 10% (vol/vol) FCS, 500 units/ml penicillin, and 0.1 μg/ml streptomycin. For DNA/PNA transfections 100 μM PNA was hybridized with 109 μM appropriate DNA oligonucleotide in 0.5× PBS. After allowing the cells to adhere, they were transfected with 2.0 μl (7 μg/ml) of Lipofectamine (Life Technologies) and 0.5 μM 2′-*O*-MeRNA oligonucleotide (38) or 1 μM PNA/DNA complex (39) in 200 μl of total Opti-Mem (Life Technologies) according to the manufacturer's directions. After 6 h at 37°C, the transfecting mixture was removed, and medium without antibiotics and with 20% (vol/vol) serum was added. Cells were then harvested 12–15 h later after three washes with PBS and treatment with trypsin or allowed to grow for 3 days. Cells were harvested after 3 days, counted, replated, and assayed for telomerase activity.

**Measurement of Telomerase Activity and Telomere Length.** Telomerase activity was measured by telomere repeat amplification protocol by using the TRAPeze telomerase detection kit (Intergen, Purchase NY; ref. 40). After the extension of the substrate by telomerase, the products were amplified by PCR in the presence of an $^{32}$P-end-labeled TS primer, resolved on 10% polyacrylamide gels, and revealed by exposure to a PhosphorImager cassette (Molecular Dynamics). Telomerase activity was calculated as the ratio of the intensity of telomerase ladders to the intensity of the 36-bp internal standard. Percentage of inhibition was calculated by comparing telomerase activity of oligomer-treated cells with telomerase activity of cells treated with lipid alone. The levels of telomerase activity were within the linear range of the TRAP assay (40). Mean telomere length was evaluated by using telomere restriction fragment analysis, a variation of standard Southern analysis, and was quantitated as described (41). Digested samples were resolved on a 0.7% agarose gel and hybridized to a telomeric probe [[$^{32}$P](T-TAGGG)$_4$ oligonucleotide].

## Results

**Effect of 2′-*O*-MeRNA on Cell Proliferation.** We used cationic lipids to introduce 2′-*O*-MeRNA complementary to the template region of hTR into the immortalized human cell lines HME50-5E and DU145. We also introduced 2′-*O*-MeRNA into HME50-hTERT cells, a cell line generated by infecting preimmortal HME50 cells with the gene encoding hTERT. A 2′-*O*-MeRNA containing mismatched bases was employed as a control for the sequence-specificity of inhibitor action. Analysis of telomerase inhibition by TRAP indicated that the complementary 2′-*O*-MeRNA oligomer blocked more than 95% of telomerase activity 1 day after transfection, and more than 70% of activity was inhibited 3 days after transfection (Fig. 1). The 2′-*O*-MeRNA oligomers used in these studies are stable to degradation inside cells (38); thus, it is likely that the reduced inhibition is due to dilution of inhibitor as the cell population increases.

To determine whether inhibition of telomerase would ultimately limit proliferation, we transfected HME50-5E, DU145, and HME50-hTERT cells with 2′-*O*-MeRNA oligomers at 3- to 4-day intervals for 120 days. Because of the extreme length of these experiments, stringent precautions were taken to avoid contamination of cultured cells, and all experiments were completed successfully without interruption. After 15–25 days, proliferation of HME50-5E cells treated with the complementary oligomer began to slow, and after 110 days, no treated cells remained (Fig. 2*a*). The growth of cells treated with the control

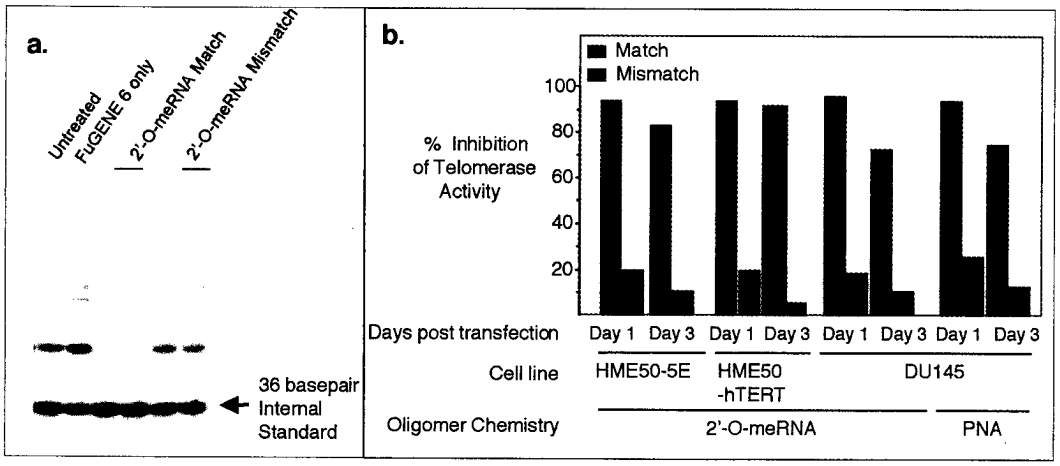


**Fig. 1.** (*a*) Inhibition of telomerase activity by 2′-*O*-MeRNA oligomers delivered into HME50-5E cells by using FuGENE6 lipid, measured 3 days after transfection. Similar results were observed on introduction of 2′-*O*-MeRNA oligomers into HME50-hTERT cells and on introduction of 2′-*O*-MeRNA or PNA oligomers into DU145 cells. (*b*) Inhibition of telomerase activity in HME50-5E and DU145 cells by 2′-*O*-MeRNA and PNA oligomers as detected by the TRAP assay. HME50-5E and DU145 cells were collected 1 and 3 days after transfection with 2′-*O*-MeRNA or PNA oligomers. Telomerase activity was quantitated as described (38).

oligomer containing mismatch bases was not affected. During the first 60 days after initial transfection, less than 5% of cells treated with the fully complementary 2′-*O*-MeRNA underwent apoptosis. By 80 days, however, 14% of cells treated with the match oligomer were observed to undergo apoptosis, and this percentage increased to 56% after 100 days with cell viability lost after 110 days (Fig. 3). By contrast, cells treated with lipid alone or the 2′-*O*-MeRNA oligomer containing mismatched bases underwent apoptosis at a rate of 2–3% throughout the entire experiment. To confirm that the effects of inhibitor addition were reproducible, treatment of HME50-5E cells with 2′-*O*-MeRNA was repeated, and a similar decrease in proliferation was observed.

Addition of complementary 2′-*O*-MeRNA to DU145 cells caused proliferation of cells to begin to slow after 60 days (Fig. 2*b*). By the end of the experiment (after 120 days), these cells had undergone 20 fewer population doublings than had cells treated with mismatch-containing oligomer. HME50-5E cells have a mean terminal restriction fragment (TRF) length of 2,000 bp, whereas DU145 cells possess a mean length of 3,600 bp. TRF length corresponds qualitatively to telomere length, and the finding that proliferation of treated HME50-5E cells decreases more dramatically than does cell proliferation of treated DU145 cells may be due to HME50-5E cells possessing shorter telomeres. Consistent with this hypothesis, HME50-hTERT cells have a much longer mean TRF length (7.6 kilobases) than either HME50-5E or DU145 cells, and we observed no significant reduction in their growth rates during the 120-day treatment period (Fig. 2*c*).

**Telomere Shortening On Addition of 2′-*O*-MeRNA.** To support the hypothesis that the decrease in cell proliferation is caused by the 2′-*O*-MeRNA oligomer through a telomerase-dependent mechanism rather than being caused by long-term toxicity that is

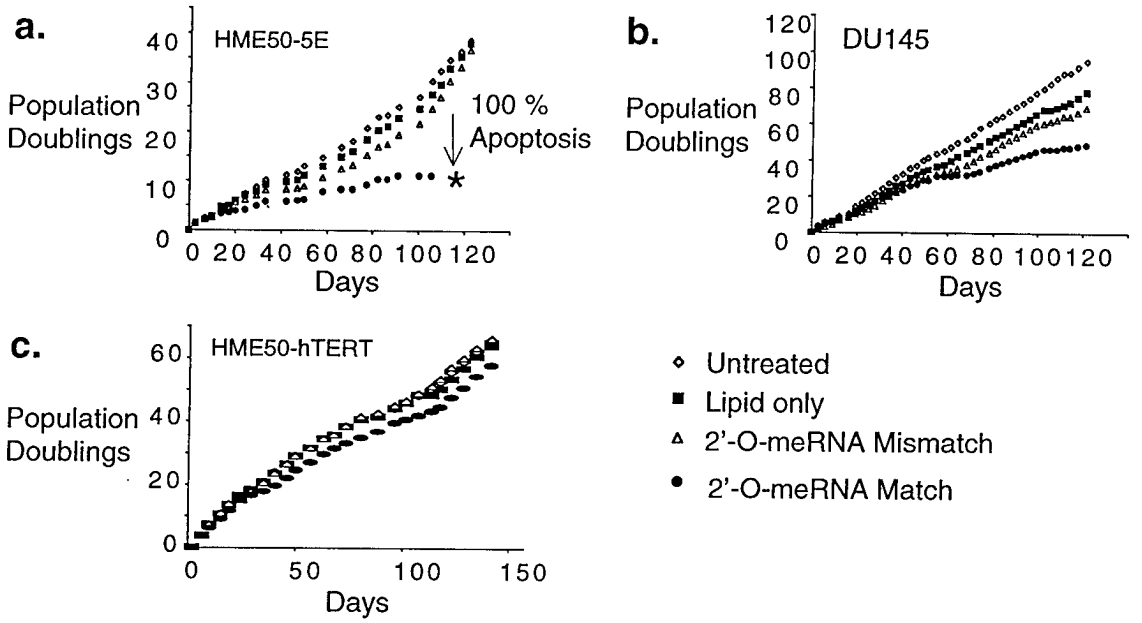


**Fig. 2.** Effects on cell growth after long-term transfection with match or mismatch 2′-*O*-MeRNA. (*a*) HME50-5E cells transfected with 2′-*O*-MeRNA oligomers. (*b*) DU145 cells transfected with 2′-*O*-MeRNA oligomers. (*c*) HME50-hTERT cells transfected with 2′-*O*-MeRNA oligomers.

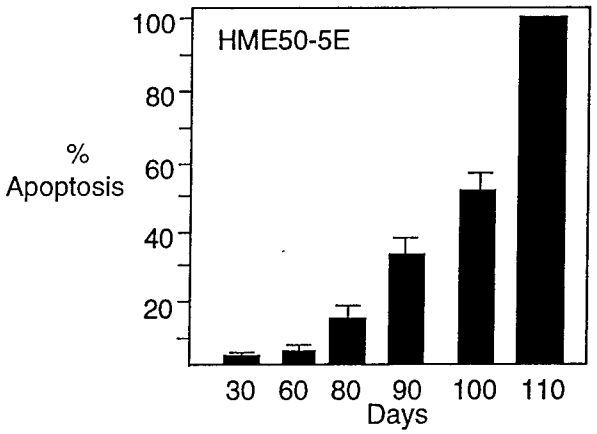


**Fig. 3.** Increase of apoptosis of HME50-5E cells after repeated transfection with fully complementary 2′-*O*-MeRNA. Levels of apoptosis were measured by staining with 4′6-diamidino-2-phenylindole followed by microscopy. Background apoptosis of cells that were untreated or that were treated with 2′-*O*-MeRNA containing mismatched bases was 2–3%. Levels of apoptosis were confirmed by flow cytometry with the ApoAlert Annexin V Apoptosis kit (CLONTECH).

independent of telomerase inhibition, we monitored the effect of inhibition on telomere length. Within 60 days of treatment, the mean telomere length of HME50-5E cells treated with 2′-*O*-MeRNA complementary to hTR was reduced from 2,000 to 1,700 bp (Fig. 4*a*). This decrease in measured TRF length may be an underestimate of the total loss of chromosomal DNA, because the TRF assay does not monitor erosion of subtelomeric regions and because relatively little telomeric DNA remained to hybridize with radiolabeled probe. The TRF length of cells treated with 2-*O*-MeRNA containing mismatches relative to hTR remained at 2,000 bp, and the signal intensity of probe hybridization was undiminished.

We also examined the mean TRF length of DU145 cells that had been treated with 2′-*O*-MeRNA oligomers for 76 days. Because DU145 cells possess longer telomeres, we were able to observe a dramatic erosion of telomere length, with the mean TRF length of cells treated with the 2′-*O*-MeRNA complementary to the template region of hTR decreasing from 3,600 bp to 2,200 bp (Fig. 4*b*). As observed with HME50-5E cells, the signal intensity of probe hybridization was greatly reduced because of the reduction in telomeric repeats. We also examined the effect of inhibitor treatment on HME50-hTERT cells and found that TRF length decreased from 7,600 to 6,800 bp after 75 days (results not shown).

The 2′-*O*-MeRNA inhibitor contained four phosphorothioate linkages, a chemical moiety noted for its propensity to produce misleading cellular effects that seem sequence-specific but are actually unrelated to binding to the intended target (42). Demonstrating that decreased cell proliferation derives from a telomerase-dependent mechanism involving binding of inhibitor to the RNA template is a critical prerequisite for embarking on lengthy studies in animals and humans. Therefore, to provide further evidence that the reduction of telomere length was due to Watson–Crick recognition of the template region of hTR, we tested the effects of addition of match and mismatch PNAs (36).

PNAs are ideal agents for confirming the specificity of oligonucleotides, because they possess a neutral amide backbone and have a much lower propensity for making unwanted interactions with macromolecules that bind the repetitive anionic linkages of DNA and RNA. PNAs cannot be introduced into cells by direct complexation with cationic lipid, because PNA backbone linkages are uncharged; however, PNAs can be hybridized to DNA oligonucleotides and introduced into cells as cargo on complexation of DNA with lipid (39). Addition of a PNA that was complementary to the hTR template to DU145 cells inhibited over 90% of telomerase activity after 1 day (Fig. 1*b*) and caused telomere shortening similar to that caused by addition of complementary 2′-*O*-MeRNA (Fig. 4*b*). A PNA directed to a nontemplate region of hTR inhibited only 50% of telomerase activity after 1 day and did not cause telomeres to shorten (results not shown).

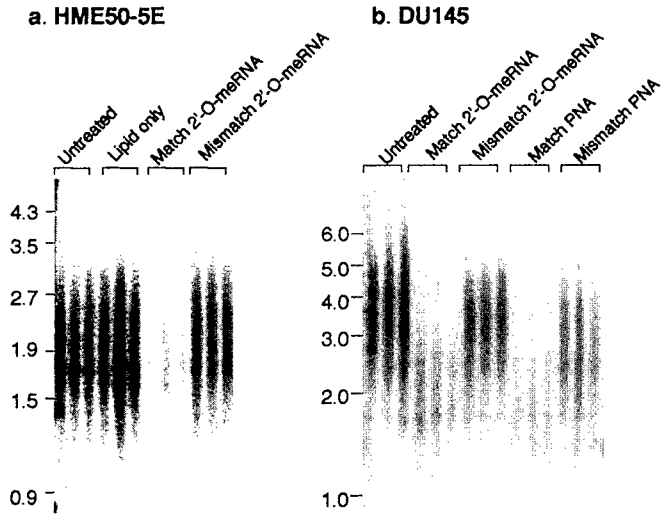


**Fig. 4.** Measurement of telomere restriction fragment length (TRF) in HME50-5E and DU145 cells treated with 2′-*O*-MeRNA and PNA oligomers. (*a*) HME50-5E cells that had been treated with 2′-*O*-MeRNA oligomers for 60 days, with the results of independent experiments shown in triplicate. (*b*) DU145 cells treated with 2′-*O*-MeRNA and PNA oligomers for 76 days, with the results of independent experiments shown in triplicate. In parts *a* and *b*, the signal intensity in the lanes showing the outcome of treatment with fully complementary oligomer is weak, because telomeres have eroded and few telomeric repeats remain to hybridize with radiolabeled probe. Equivalent amounts of chromosomal DNA were loaded in each lane. TRF lengths are expressed as kilobase pairs.

**Effects of Terminating Inhibitor Addition.** An inherent advantage of using synthetic inhibitors to reduce gene expression rather than genetic knockouts is that the phenotypic effects of regaining function can be evaluated by terminating inhibitor addition. After 76 days, we stopped adding inhibitor to DU145 cells that had been treated with fully complementary or mismatch-containing 2′-*O*-MeRNA or PNA oligomers. After 3 weeks, we noted that the eroded telomeres of cells treated with fully complementary oligomers had returned to approximately their initial lengths (Fig. 5). Telomeres remained near their initial lengths when measured a second time at 5 weeks. We also evaluated the effect of inhibitor withdrawal on proliferation of HME50-5E cells that had been treated with the fully complementary 2′-*O*-MeRNA until population growth was static. We observed that, within 3 weeks of terminating inhibitor treatment, previously static HME50-5E cells regained the ability to grow at the same rate as cells treated with mismatch-containing 2′-*O*-MeRNA.

## Discussion

**Telomerase as a Target for Chemotherapy.** Cancer remains a major cause of morbidity and mortality in spite of substantial progress toward understanding the molecular basis of the disease. The discovery of new drugs is urgent, and telomerase inhibitors have the potential to provide an additional option for chemotherapy. Telomerase inhibitors might not only limit growth of human

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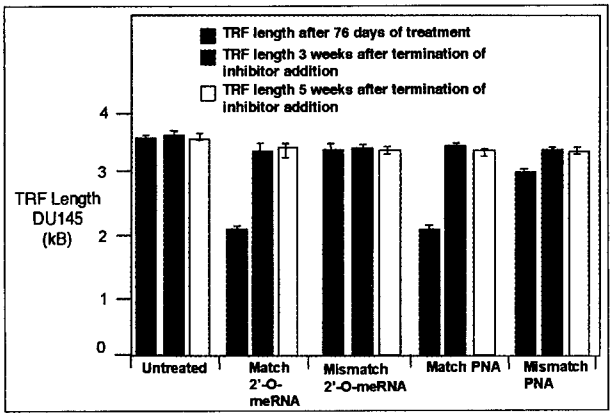


**Fig. 5.** The TRF length of DU145 as a function of inhibitor addition. TRF lengths were measured after 76 days of addition of fully complementary or mismatch-containing 2′-*O*-MeRNA or PNA oligomers. TRF lengths were then measured again 3 or 5 weeks after terminating oligomer addition. kB, kilobase.

tumors directly but might also act in a synergistic fashion with existing inhibitors and amplify their efficacy. For example, after initial chemotherapy or surgery, telomerase inhibitors might be used in an adjuvant setting to limit the recovery of residual cancer cells, making them more susceptible to attack by the immune system or killing by existing chemotherapeutic agents. The use of telomerase inhibitors is particularly attractive for situations of ongoing cell turnover, as occurs with tumor-static antiangiogenesis inhibitors. Systemic administration of telomerase inhibitors will also inhibit telomerase in normal stem cells. This inhibition may result in few side effects, because the relatively short telomeres of tumors may erode to a critical length before irreversible harm is done to other cells.

**Oligonucleotides as Telomerase Inhibitors.** Telomerase is an ideal target for oligonucleotides, because its RNA template is essential for activity and is intrinsically accessible to binding by nucleic acids. Oligonucleotides are being tested in 13 ongoing clinical trials (43), and recently, one oligonucleotide, Fomivirsen, has been approved for the treatment of cytomegalovirus retinitis (44). In addition, 2′-*O*-MeRNA and other 2′-*O*-alkyl-derivatized oligomers bind more tightly to complementary RNA sequences than do analogous DNA oligomers and have improved resistance to degradation by nucleases, reducing the need for phosphorothioate linkages and improving the selectivity of antisense effects (45, 46); 2′-*O*-alkyl RNA is currently being used in two phase II clinical trials including one trial directed against the R1-$\alpha$ subunit of protein kinase A, an application similar to the use of telomerase inhibitors to prevent tumor regrowth. Experience with 2′-*O*-alkyl oligomers in clinical trials, combined with our observation of both decreased cell proliferation and increased apoptosis, will encourage the testing of antitelomerase oligonucleotides in animals and humans.

**Phenotypic Effects Are Mediated by a Sequence-Specific, Telomerase-Dependent Mechanism.** Use of oligonucleotides to affect cellular phenotypes is often met with skepticism, because non-sequence-specific effects and misleading phenotypes have plagued their use. Tu *et al.* (47) have performed a comprehensive survey of the literature, leading Stein (48) to estimate that non-sequence-selective effects may be at least partially implicated in results reported in 94% of the 2,026 studies published through 1997. Indeed, we have previously observed that oligonucleotides that contain uniform phosphorothioate oligonucleotides inhibit telomerase in a non-sequence-specific fashion, presumably through binding to hTERT (38).

We offer multiple independent lines of evidence in the present study that the effects we observe are due to Watson–Crick recognition of telomerase. Treatment with match 2′-*O*-MeRNA and PNA oligomers cause much greater decreases in telomere length and cell proliferation than does treatment with oligomers that contain mismatched bases or treatment with a less efficient PNA inhibitor directed to a nontemplate region of hTR. The fact that inhibition of telomerase has a more rapid and pronounced effect on proliferation of HME50-5E cells, which have shorter telomeres than DU145 cells, supports a mechanism of decreased proliferation involving telomere shortening rather than nonselective toxicity. Similarly, even though the genetic background of HME50-hTERT cells is similar to that of HME50-5E cells, proliferation of treated HME50-hTERT cells did not decrease, presumably because their initial telomere length of HME50-hTERT cells is approximately 4,000 bp longer. PNA and 2′-*O*-MeRNA oligomers cause similar reductions in telomere length, suggesting that the effects are caused by Watson–Crick recognition rather than by non-base-pair-mediated contacts with backbone phosphodiester or phosphorothioate linkages. The use of PNAs to confirm the mechanism of oligonucleotide action has important general implications for the use of oligonucleotides within cells, because validating the mechanism of oligonucleotide action is a key problem for antisense research. If neutral and anionic oligomers produce the same effects, they likely derive from specific Watson–Crick base pairing rather than misleading non-sequence-specific interactions. Recent work with PNAs introduced into cells by electroporation has shown decreased cell viability, supporting our findings (49).

**General Implications for Development of Telomerase Inhibitors.** Typically, when a putative antiproliferative agent is applied to cells, an effect is observed within hours or days. Telomerase is an unusually challenging target for drug discovery, because a cellular response that depends on telomere shortening will require weeks to become apparent. Although this outcome should not have serious consequences for administration of proven drugs to patients accustomed to chronic treatment of residual disease, it greatly complicates the assays needed to develop and test such drugs. Our primary finding is that telomerase is a viable target for synthetic agents aimed at controlling proliferation of immortal human cells. No evidence for adoption of an ALT (alternative lengthening of telomeres) pathway for telomere maintenance was observed, suggesting that this pathway may not be readily adopted by some immortal cell types. The decreased proliferation we observe should encourage further development and testing of other promising classes of compounds such as nucleoside derivatives directed to hTERT (32) or small molecules designed to disrupt telomere synthesis by promoting G quadruplex formation (33, 34).

Our results have broad implications for how telomerase inhibitors must act within cells to be effective and on how protocols for animal and clinical testing must be designed. We observe telomere shortening and decreased cell proliferation, even though between 5% and 30% of telomerase activity remained after treatment with inhibitor. However, addition of a PNA directed to a nontemplate region of hTR, which is a less potent inhibitor, does not lead to telomere erosion, and telomeres rapidly regain their lengths once inhibitor addition is terminated. Taken together, these results suggest that, although obtaining a striking phenotypic effect does not require full inhibition of telomerase, potency remains an important design consideration. Inhibitors will be most effective against tumor cells with short telomeres, and inhibition of telomerase must be maintained at a sustained high level over time to prevent the rapid regrowth of telomeres. Regrowth to initial telomere length

on cessation of inhibitor addition suggests that there is a "set point" for telomere length in human cells and that the telomeres of stem cells that erode during therapy may recover once treatment is terminated. HME50-5E cells were derived from HME50 preimmortal cells that had one mutant p53 allele and lost the second allele during spontaneous immortalization, whereas DU145 cells have two mutant alleles, indicating that functional p53 is not necessary for the reduced proliferation of these cell lines on treatment with telomerase inhibitors.

**Conclusions.** The potential for telomerase to be a target for anticancer chemotherapy has engendered a debate that evokes both great enthusiasm and great skepticism. Our findings indicate that telomerase is a viable target for chemotherapeutic drugs and are important for two reasons. The first is that our data will encourage investment in the demanding long-term studies needed to discover and test other classes of inhibitors and guide the design of protocols that optimize the likelihood of obtaining definitive results in animals. The second is that oligonucleotides seem to be excellent candidates for antitelomerase drugs in their own right. The exposed RNA template of hTR makes telomerase an ideal target for oligonucleotides, and the presence of similar oligonucleotides in clinical trials suggests that it should be possible to test potent antitelomerase oligonucleotides *in vivo* in the near future.

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# Effects of Chemopreventive and Antitelomerase Agents on the Spontaneous Immortalization of Breast Epithelial Cells

*Brittney-Shea Herbert, Angelique C. Wright, Christina M. Passons, Woodring E. Wright, Iqbal Unnisa Ali, Levy Kopelovich, Jerry W. Shay*

***Background:*** **Activation of telomerase is an early event in the development of breast and other cancers that may lead to cell immortalization, a critical and rate-limiting step in cancer progression. Breast epithelial cells from women with Li–Fraumeni syndrome (LFS) immortalize spontaneously and reproducibly in culture. We, therefore, tested whether immortalization of these cells could be prevented by treating them with chemopreventive agents and by inhibiting telomerase activity.** ***Methods:*** **Noncancerous, preimmortal breast epithelial cells derived from a patient with LFS were treated for 3 months with nontoxic concentrations of the chemopreventive agents oltipraz, difluoromethylornithine, tamoxifen, and retinoic acid or with two different telomerase inhibitors. The frequency of spontaneous immortalization of LFS-derived cells was estimated by an approach based on fluctuation analyses. Statistical analyses were two-sided.** ***Results:*** **The frequency of spontaneous immortalization events of LFS-derived breast epithelial cells was reduced by long-term treatment with retinoic acid ($P<.001$) or tamoxifen ($P<.05$) compared with solvent-treated cells. The frequency of immortalization was also reduced by treating LFS-derived cells with an antitelomerase antisense oligonucleotide ($P<.001$) or by inducing the cells to express a dominant negative mutant of telomerase ($P<.025$) compared with cells treated with a control oligonucleotide or with empty vector, respectively.** ***Conclusions:*** **Treatment of preimmortal LFS breast epithelial cells with chemopreventive and antitelomerase agents decreased the frequency of spontaneous immortalization *in vitro*. These studies validate the application of a new cell culture model system to screen the effects of novel chemopreventive agents by use of cell immortalization as an end point. The results also suggest that the telomerase ribonucleoprotein complex may be an important molecular target for breast cancer prevention. [J Natl Cancer Inst 2001;93: 39–45]**

Li–Fraumeni syndrome (LFS) is an autosomal dominant trait that results in childhood sarcomas and early-onset breast cancer. LFS is frequently characterized by inherited mutations in the tumor suppressor gene, p53 (also known as TP53) *(1)*. Alterations in the p53 gene are thought to lead to genomic instability by allowing uncontrolled cellular proliferation, which perpetuates further instability *(2)*. Breast tumors are the most prevalent cancer among women in LFS families; 25% of the breast cancers are diagnosed in women under age 30 years and 89% in women under age 50 years *(1,3,4)*. The molecular mechanisms that lead to the specific increase in the incidence of breast cancer as opposed to other cancers in LFS families are not understood *(5,6)*.

The cell immortalization that leads to breast cancer in LFS patients presumably involves the activation of telomerase. Telomerase is a ribonucleoprotein complex that adds telomeric repeats to the ends of chromosomes *(7)*. In most normal human cells, with the exception of germ cells and stem cells, the chromosomes progressively shorten their telomeres with each cell division because telomerase is not active. Cellular senescence occurs when cells with critically shortened chromosomes undergo a permanent growth arrest.

Cell immortalization is thought to occur when the progressive telomere shortening that normally occurs in most cells is prevented by the reactivation or increased expression of telomerase, although other mechanisms that stabilize and maintain telomeres are also possible *(8)*. Indeed, telomerase activity was detected in nearly all (90%) cancers tested *(9,10)*. Immortal cells emerge by escaping crisis, which is a period of balanced cell growth and cell death followed by a decrease in the total number of surviving cells. If telomerase activation is necessary for cell immortalization, then telomerase may provide a target for cancer treatment and prevention. Previous reports *(11–13)* have demonstrated that treatment of immortal and cancerous cell lines with antitelomerase agents can inhibit their growth, leading to apoptotic cell death in a p53-independent manner.

To study the effects of chemopreventive and antitelomerase agents on immortalization, it is necessary to examine cells that become immortal *in vitro*. However, the spontaneous immortalization of human cells in culture is an extremely rare event: It requires mutations in several genes, such as p16, p53, and pRb, and their cellular pathways that are involved in cellular senescence *(8,14,15)*. Although normal human breast epithelial cells *in vitro* never immortalize spontaneously, breast epithelial cells derived from LFS patients do so at a detectable and reproducible frequency of $5 \times 10^{-7}$. These cells, which initially are heterozygous for wild-type p53 and lack telomerase activity, spontaneously inactivate p16 and the remaining wild-type p53 allele *in vitro*, resulting in immortalized cultures that express telomerase *(16)*.

To investigate the nature of the immortalization process, we surveyed a panel of clinically validated chemopreventive agents for their effects on the spontaneous immortalization of LFS-derived breast epithelial cells *in vitro*. Because immortalization usually involves the activation of telomerase, we also used this model system to examine the effects of antitelomerase agents on breast epithelial cell immortalization. Telomerase activation occurs early in breast cancer progression; hence, it is an attractive target for the treatment and prevention of breast cancer *(10,17–20)*. We examined two antitelom-

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*See* "Notes" following "References."

erase agents: One is an antisense oligonucleotide complementary to the template region of human telomerase RNA, and the other is a dominant-negative mutant of the catalytic subunit of human telomerase. These studies provide a new model to screen novel chemopreventive agents that target cellular immortalization, a critical step in cancer progression.

## Materials and Methods

### Cell Culture

The human mammary epithelial (HME) cells used for these experiments were derived from the noncancerous breast tissue of a 31-year-old female diagnosed with LFS described previously *(16)*. These cells contain a germline mutation (Met133Thr) in the p53 gene that affects the conformation of the wild-type p53 protein *(16)*. Immunohistochemical analysis confirmed that these cells do not express the estrogen receptor-α (data not shown). The cells were grown in MCDB 170 media (Life Technologies, Inc. [GIBCO BRL], Rockville, MD) supplemented with 0.4% bovine pituitary extract (Hammond Cell Tech, Alameda, CA), 10 ng/mL epidermal growth factor (Life Technologies, Inc.), and 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 5 μg/mL transferrin, and 50 μg/mL gentamicin (all from Sigma Chemical Co.; St. Louis, MO). The medium was changed every 2–3 days.

### Treatment With Telomerase Inhibitors

**Antitelomerase agents.** Phosphorothioate-modified 2′-*O*-methyl RNA oligomers were purchased from Oligos Etc. (Wilsonville, OR). The sequence of the antisense RNA complementary to the template region of human telomerase RNA is 5′-CAGUUAGGGUUAG-3′; the sequence of the mismatched RNA is 5′-CAGUUAG*AA*UUAG-3′, where the underlined nucleotides possess phosphorothioate linkages and mismatched nucleotides are indicated by italics. The antisense and mismatched oligomers were introduced into cells every 4 days during the course of the study by transfection with FuGENE6-transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN).

**Construction and expression of dominant-negative telomerase mutant.** The D869A-hTERT complementary DNA (cDNA) is a derivative of the modified hTERT cDNA that contains a point mutation within the highly conserved reverse transcriptase motif that changes the aspartic acid residue at amino acid 869 to an alanine *(21)*. The D869A-hTERT cDNA was inserted into the *Eco*RI site of the retroviral vector pBABEpuro to create pBABEpuro D869A-hTERT *(22)*. The pBABEpuro D869A-hTERT and pBABEpuro (empty vector) were introduced separately into the mouse ecotropic packaging cell line PE501 *(23)* by electroporation. The ecotropic viruses that were produced were supplemented with polybrene (Sigma Chemical Co.) to a final concentration of 4 μg/mL and used to infect the mouse amphotropic packaging cell line PA317 *(23)*. Infected cells were cultured in the presence of 4 μg/mL puromycin (Sigma Chemical Co.) for 1 week to select for puromycin-resistant cells that contained pBABEpuro D869A-hTERT or pBABEpuro (empty vector). The culture supernatants containing amphotropic viruses were then collected, supplemented with polybrene to a final concentration of 4 μg/mL, and used to infect HME cells. Infected cells were cultured in the presence of 150 ng/mL puromycin to select for puromycin-resistant cells that contained pBABEpuro D869A-hTERT or pBABEpuro (empty vector).

### Treatment With Chemopreventive Agents

HME cells were treated every 3 days for approximately 25 population doublings with the following clinically validated chemopreventive agents: oltipraz, difluoromethylornithine (DFMO), tamoxifen citrate, 9-*cis*-retinoic acid, and 13-*cis*-retinoic acid. Oltipraz, DFMO, tamoxifen citrate, and 9-*cis*-retinoic acid were obtained from the DCP Repository (McKesson BioServices, Rockville, MD). 13-*cis*-Retinoic acid was purchased from Sigma Chemical Co. Oltipraz, tamoxifen citrate, 9-*cis*-retinoic acid, and 13-*cis*-retinoic acid were dissolved in dimethyl sulfoxide and stored at −80 °C as 1000× stocks. DFMO was dissolved in HME culture media and stored at −80 °C as 1000× stocks. Chemopreventive agents were diluted to the appropriate working concentrations in HME culture media.

### Fluctuation Analysis to Determine the Frequency of Immortalization

The frequency of immortalization was estimated by use of a fluctuation analysis as described previously *(16,24,25)*. Each clone of cells that emerged from crisis was counted as an independent immortalization event. Frequency is defined as the probability of obtaining an immortal cell line on the basis of the total number of cells plated at each passage, not on the number of cell divisions, and is calculated by dividing the total number of independent immortalization events among all dishes by the total number of cells plated. In this study, untreated, mock-treated, and treated cells were expanded to 10 culture dishes approximately 10 population doublings before they would have normally reached crisis *(24,25)* and were maintained at a constant density of $10^6$ cells per dish. Once the cells reached crisis, they were harvested, replated, and re-treated at least once every 3 weeks until virtually no surviving cells remained or until the culture had a small focus of growing cells. Cells were considered to be immortal if they expressed telomerase and had undergone vigorous, postcrisis growth *(16)*. Each small focus of growing cells was counted as an immortal event; the total number of immortal events from each of the 10 dishes for each treatment was recorded. Frequency of immortalization was calculated by dividing the number of immortal events by the total number of cells plated ($10 \times 10^6$ cells). For each set of treatments, we performed and averaged three fluctuation analyses at three separate times by use of the same starting populations of cells recovered from freezer stocks.

### Measurement of Telomerase Activity

Cells were resuspended in lysis buffer (i.e., 10 m*M* Tris–HCl [pH 8.0], 1 m*M* $MgCl_2$, 1 m*M* EGTA, 1% Nonidet P-40, 0.25 m*M* sodium deoxycholate, 10% glycerol, 150 m*M* NaCl, and 5 m*M* β-mercaptoethanol) at a concentration of 1000 cells/μL, were incubated on ice for 30 minutes, and were then centrifuged at 14 000*g* for 20 minutes at 4 °C *(21,26)*. The resulting supernatants were used directly to detect telomerase activity (by use of 500 cell equivalents per assay) or were flash-frozen and stored at −80 °C. Telomerase activity was measured by use of the TRAP-eze Telomerase Detection kit (Intergen, Purchase, NY), with the telomerase substrate (TS) primer (5′-AATCCGTCGAGCAGAGTT-3′) as the substrate. After the extension of the substrate by telomerase (for 30 minutes at room temperature), the extension products were amplified by polymerase chain reaction by use of the TS primer end labeled with $^{32}P$, resolved on a 10% polyacrylamide gel, and revealed by exposure to a PhosphorImaging cassette (Molecular Dynamics, Sunnyvale, CA).

### Statistical Analysis

Each dish was counted as having an immortalization event occur or not having an immortalization event occur. Because the data were collected as three different experiments for the cells treated with chemopreventive agents (Table 1) and with antitelomerase agents (Table 2) and the proportion of dishes with an immortal event were small in some treatments, a two-tailed Fisher's exact test, using the number of dishes with an immortal event, was performed for each agent separately to examine the association between treatment and experiment to determine if the data across the three experiments for each treatment could be combined in further analyses. Because there was no statistically significant association between experiment and treatment for the chemopreventive agents ($P = .932$) or for the antitelomerase agents ($P = .915$), the data for each treatment across three experiments were combined. Comparisons of immortalization events for the seven chemopreventive treatments and the five antitelomerase treatments were performed separately by use of two-sided chi-square tests of independence and, when significant, were followed with Tukey-type *post hoc* multiple comparison tests for proportions *(27)* to examine which treatments were statistically significantly different. The alpha level for all statistical tests was set to 0.05 and the Fisher's exact and chi-square analyses were performed by use of the SAS program (version 6.12; SAS Institute, Cary, NC). The Tukey-type *post hoc* multiple comparisons tests for proportions were programmed in Microsoft Excel, and significance was determined by consulting "Critical Values of the q Distribution" by Harter [reprinted in *(27)*, Appendix p. 57–73], where exact *P* values are not available.

## Results

### Concentrations of Chemopreventive Agents Used for Long-Term Study

To determine any cytotoxic effects of the chemopreventive agents, we tested a range of concentrations for each of the chemopreventive agents. Fig. 1, A, shows the dose–response of LFS-derived breast epithelial cells treated with 1 n*M*–100 μ*M* of oltipraz, DFMO, tamoxifen, 9-*cis*-retinoic acid, and 13-*cis*-retinoic acid compared with that of control cells. The

**Table 1.** Effects of chemopreventive agents on the spontaneous immortalization of Li–Fraumeni syndrome-derived breast epithelial cells

| Treatment, concentration* | No. of dishes with an immortal event/No. of dishes †,‡ Experiment 1 | Experiment 2 | Experiment 3 | *P*§ | Average frequency of immortalization per $3 \times 10^7$ cells‖ |
|---|---|---|---|---|---|
| Untreated | 5/10 | 5/10 | 6/10 | | $5.33 \times 10^{-7}$ |
| Solvent | 4/10 | 6/10 | 5/10 | | $5.0 \times 10^{-7}$ |
| Oltipraz, 10 n*M* | 3/10 | 3/10 | 4/10 | >.05 | $3.33 \times 10^{-7}$ |
| Difluoromethylornithine, 10 n*M* | 3/10 | 3/10 | 1/10 | >.05 | $2.33 \times 10^{-7}$ |
| Tamoxifen citrate, 10 n*M* | 2/10 | 1/10 | 1/10 | <.05 | $1.33 \times 10^{-7}$ |
| 9-*cis*-Retinoic acid, 10 n*M* | 2/10 | 0/10 | 0/10 | <.005 | $0.67 \times 10^{-7}$ |
| 13-*cis*-Retinoic acid, 1 n*M* | 1/10 | 0/10 | 0/10 | <.001 | $0.33 \times 10^{-7}$ |

*Cells were treated for several months with nontoxic concentrations of chemopreventive agents (*see* the "Materials and Methods" section). Three separate experiments were conducted, and the numbers of immortalization events are shown for each.

†An immortalization event is expressed as a clone of cells emerging from crisis in a dish. Each dish was maintained at $10^6$ cells/dish.

‡The combined data had a chi-square of 37.738, 6 *df*, and a $P = .001$.

§*P* values from the Harter tables [reprinted in *(27)*] from Tukey-type multiple comparison tests for proportions comparing solvent-treated control cells. Untreated and solvent-treated controls were found to be not statistically significantly different. The solvent-treated control cells, having the lower proportion of immortalized cells, were compared with each of the other treated cells.

‖The frequency of spontaneous immortalization is expressed as the probability of obtaining an immortal cell line based on the total number of cells plated. For example, if one maintained 10 dishes at a minimum population size of $10^6$ cells per dish, for a total pool size of $10 \times 10$ (or $10^7$), and three immortalization events in three dishes were observed, this would yield a frequency of 3 divided by $10^7$, $3.0 \times 10^{-7}$ *(16)*. Statistical analyses were performed using the number of immortalization events, not frequency.

**Table 2.** Effects of anti-telomerase agents on the spontaneous immortalization of Li–Fraumeni syndrome-derived breast epithelial cells

| Treatment, concentration* | No. of dishes with an immortal event/No. of dishes †,‡ Experiment 1 | Experiment 2 | Experiment 3 | *P*§ | Average frequency of immortalization per $3 \times 10^7$ cells‖ |
|---|---|---|---|---|---|
| Untreated | 5/10 | 5/10 | 6/10 | | $5.33 \times 10^{-7}$ |
| Mismatch control, 500 n*M* | 6/10 | 6/10 | 5/10 | | $5.67 \times 10^{-7}$ |
| Antisense RNA, 500 n*M* | 2/10 | 0/10 | 0/10 | <.001 | $0.67 \times 10^{-7}$ |
| Vector-only control | 5/10 | 5/10 | 5/10 | | $5.0 \times 10^{-7}$ |
| Dominant-negative hTERT | 3/10 | 1/10 | 1/10 | <.025 | $1.67 \times 10^{-7}$ |

*Preimmortal cells were treated for several months with nontoxic concentrations of 2′-*O*-methyl RNA or mismatch oligonucleotides. A separate set of cells was also infected with D869A-hTERT, a dominant negative-acting mutant of hTERT several population doublings before crisis (*see* the "Materials and Methods" section). Three separate experiments were conducted, and the numbers of immortalization events are shown.

†An immortalization event is expressed as a clone of cells emerging from crisis in a dish. Each dish was maintained at $10^6$ cells/dish.

‡The combined data had a chi-square of 27.847, 4 *df*, and a $P = .001$.

§*P* values from the Harter tables [reprinted in *(27)*] from Tukey-type multiple-comparison tests for proportions comparing untreated control cells. Untreated and mismatch oligonucleotide/vector-only treated controls were found to be not statistically significantly different. The untreated control cells, having the lower proportion of immortalized cells, were compared with each of the other antitelomerase-treated cells.

‖The frequency of spontaneous immortalization is expressed as the probability of obtaining an immortal cell line based on the total number of cells plated. For example, if one maintained 10 dishes at a minimum population size of $10^6$ cells per dish, for a total pool size of $10 \times 10^6$ (or $10^7$), and three immortalization events in three dishes were observed, this would yield a frequency of 3 divided by $10^7$, $3.0 \times 10^{-7}$ *(16)*. Statistical analyses were performed using the number of immortalization events, not frequency.

final concentrations of the chemopreventive agents used in these studies did not affect the growth rate of these preimmortal, noncancerous cells (Fig. 1, B). After 3 days of treatment at concentrations of 1 n*M*–1 μ*M*, the chemopreventive agents were ineffective at inhibiting telomerase activity in telomerase-positive LFS-derived breast epithelial cells that had spontaneously immortalized (data not shown). Inhibition of telomerase activity at 100 μ*M* was associated with cell growth toxicity.

### Effect of Long-Term Treatment of LFS-Derived Breast Epithelial Cells With Chemopreventive Agents on the Frequency of Spontaneous Immortalization

To determine if clinically validated chemopreventive agents have any effect on cell immortalization, we treated preimmortal LFS breast epithelial cells before undergoing crisis with oltipraz, DFMO, retinoic acid, and tamoxifen. Before any treatment, all cells lacked telomerase activity. After the cells were grown for 25 population doublings in the presence or absence of the chemopreventive agents, they underwent a period of balanced growth and death (i.e., crisis) *(16)*. The small fraction of cells that survived crisis expressed telomerase activity (Fig. 2, A) and were considered to be spontaneously immortalized because they continued to grow vigorously after crisis *(16)*. Fluctuation analysis was used to estimate the frequency of immortalization for untreated and treated breast epithelial cells. As Table 1 shows, LFS-derived breast epithelial cells treated with tamoxifen, 9-*cis*-retinoic acid, and 13-*cis*-retinoic acid had fewer spontaneous immortalization events compared with untreated or solvent-treated cells ($P<.05$, $P<.005$, and $P<.001$ for tamoxifen, 9-*cis*-retinoic acid, and 13-*cis*-retinoic acid, respectively). Long-term treatment of the cells with oltipraz and DFMO also decreased the number of spontaneous immortalization events, but these effects were not statistically significant.

### Effect of Long-Term Treatment of LFS-Derived Breast Epithelial Cells With Antitelomerase Agents on the Frequency of Spontaneous Immortalization

We treated LFS-derived breast epithelial cells with two different antitelomerase agents to determine if specifically inhibiting telomerase activity affected the frequency of spontaneous immortalization in these cells. One set of cells was transfected every 4 days over a 3-month period with an antisense RNA that was directed against the template region of human telomerase RNA. Another set of cells was infected with a retrovirus containing a dominant negative mutant of the telomerase catalytic subunit (hTERT) and then

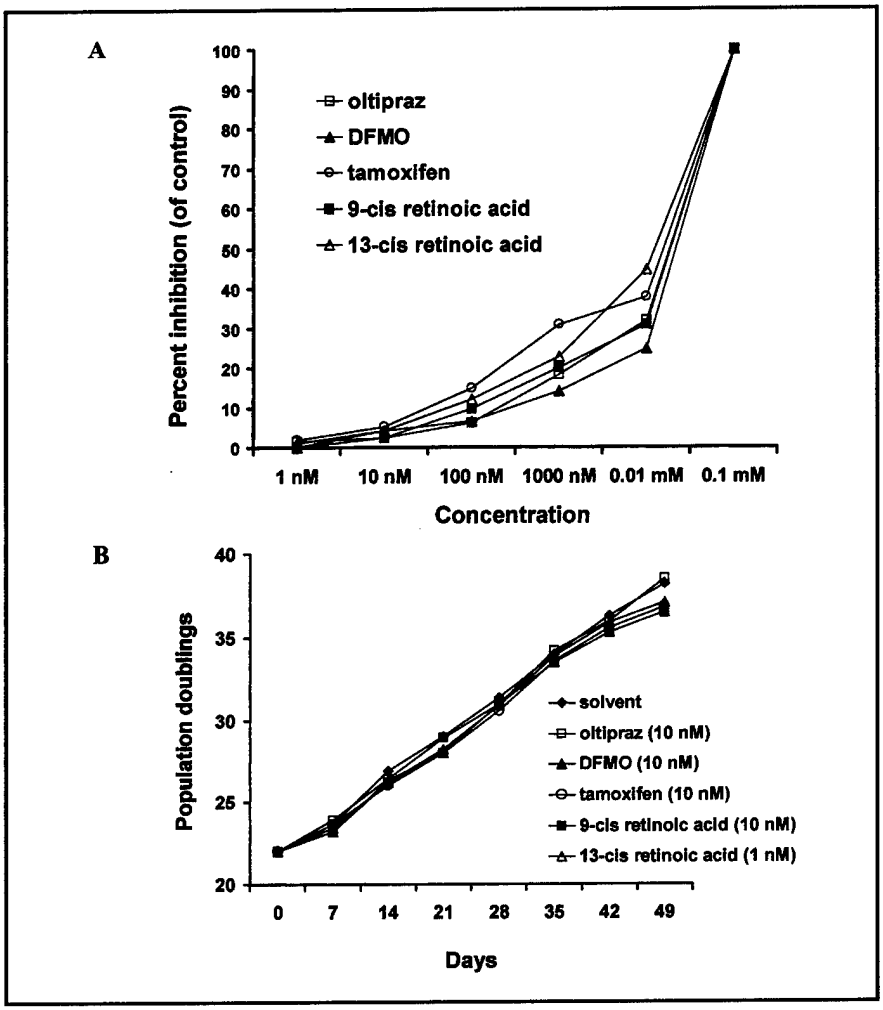


**Fig. 1.** Concentrations of chemopreventive agents used for long-term studies (1–10 n*M*) did not affect cell proliferation and were not toxic to the cells. **Panel A:** dose–response of growth inhibition by chemopreventive agents as shown by percent inhibition of Li–Fraumeni syndrome (LFS) breast epithelial cell number treated for 3 days with oltipraz, difluoromethylornithine (DFMO), tamoxifen, 9-*cis* retinoic acid, and 13-*cis* retinoic acid (1 n*M*–0.1 m*M*) as compared with control cells. Equal numbers of cells plated on multiple six-well dishes were treated with various doses of each chemopreventive agent or with solvent or media only (controls). After 3 days, cells were harvested and counted. The percent inhibition of cell number compared with controls was plotted. **Panel B:** growth of LFS-derived breast epithelial cells treated with nontoxic doses of the chemopreventive agents. LFS-derived breast epithelial cells were grown in culture for approximately 20 population doublings, after which they were grown in the presence of solvent or chemopreventive agents at the indicated concentrations for several weeks. Cells were split when necessary to maintain a constant cell density. Population doublings were determined by the following formula: population doublings = log(number of cells recovered at each subcultivation/number plated)/log 2. LFS-derived breast epithelial cells undergo crisis after approximately 50 population doublings in culture.

grown under conditions that selected for expression of the mutant protein. As summarized in Table 2, both antitelomerase agents decreased the absolute number of spontaneous immortalization events in the LFS-derived breast epithelial cells. The differences between untreated or mismatch- or vector-treated control cells and cells treated with the antisense oligonucleotide or the dominant-negative mutant hTERT were statistically significant ($P<.001$ and $P<.025$, respectively).

Because treatment with telomerase inhibitors did not completely prevent spontaneous immortalization of LFS-derived breast epithelial cells, we wanted to confirm whether telomerase was active in these cells after they had immortalized. The immortalized cultures derived from cells infected with the dominant-negative mutant of hTERT began to express telomerase activity, even though they were still resistant to the selectable drug resistance marker contained on the vector (data not shown). Western blot analysis detected no expression of the mutant hTERT in immortalized cells derived from cells infected with the dominant-negative mutant hTERT, confirming that the immortalized cells had lost the dominant-negative effect of the mutant hTERT because they no longer expressed the mutant protein (data not shown). The loss or decrease of expression of this mutant hTERT (and eventual reactivation of telomerase activity) within an infected cell population is not uncommon in tumor cell lines grown in continuous culture *(13)*. Therefore, the appearance of immortalized clones from cells infected with the dominant-negative mutant of hTERT was most likely due to loss of expression of the mutant hTERT and reactivation of telomerase.

To ensure that the antitelomerase antisense oligonucleotide was an effective inhibitor of telomerase activity, we infected HME cells with a retrovirus containing wild-type hTERT (HME plus hTERT cells) and then transfected them with an antisense RNA complementary to the template region of human telomerase RNA. Three days after transfection, cells were collected and telomerase activity was measured. As shown in Fig. 2, B, transfection of HME plus hTERT cells with the antitelomerase antisense RNA effectively inhibited telomerase activity.

## Discussion

This is the first demonstration that nontoxic concentrations of chemopreventive and antitelomerase agents inhibit the spontaneous immortalization of human breast epithelial cells derived from an LFS patient. Our results suggest that this approach may be a useful model system to screen for chemopreventive agents that inhibit the progression of breast cancer. These results also support the use of antitelomerase agents as an approach to prevent the activation of telomerase.

Breast epithelial cells derived from an LFS patient provide an efficient model for examining the effects of treatment on the probability of immortalization because they spontaneously immortalize at a measurable frequency of $5 \times 10^{-7}$ [this study and *(16)*]. In contrast, most normal human cells, including breast epithelial cells, do not spontaneously immortalize at measurable frequencies *in vitro (28,29)*.

Immortalization is thought to be a critical, rate-limiting step in cancer progression. One mechanism by which cells immortalize is by activating telomerase. The chemopreventive agents used in this study reduced the frequency of immortalization

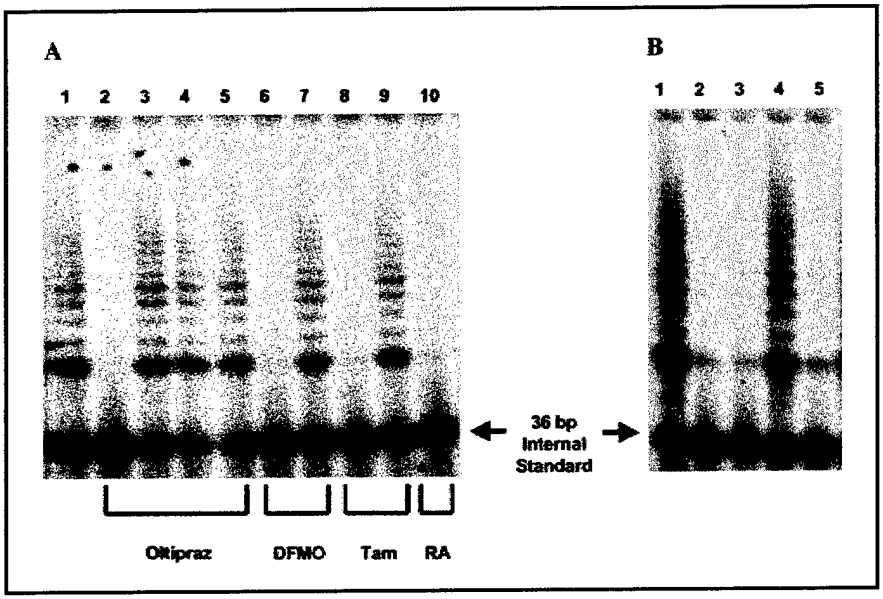


**Fig. 2.** Telomerase activity of treated and untreated human mammary epithelial (HME) cells. **Panel A:** cultures that immortalized after long-term treatment with chemopreventive agents expressed telomerase activity. Telomerase activity was measured for 500 HME cell equivalents per lane by use of a radiolabeled oligonucleotide primer and a polymerase chain reaction (PCR)-based assay (*see* the "Materials and Methods" section). Labeled PCR reaction products were resolved on polyacrylamide gels and visualized by PhosphorImaging. A ladder of bands represents the extension of the substrate primer by telomerase. **Lane 1** = untreated HME cells that have spontaneously immortalized; **lane 2** = preimmortal HME cells treated with oltipraz; **lanes 3–5** = three independent immortalization events of HME cells treated with oltipraz; **lane 6** = preimmortal HME cells treated with difluoromethylornithine (DFMO); **lane 7** = one immortalization event of HME cells treated with DFMO; **lane 8** = preimmortal HME cells treated with tamoxifen; **lane 9** = one immortalization event of HME cells treated with tamoxifen; and **lane 10** = preimmortal HME cells treated with retinoic acid. In this experiment, no immortalization events were observed in HME cells treated with retinoic acid. TAM = tamoxifen; RA = 9-*cis* retinoic acid. **Panel B:** Antisense RNA inhibits telomerase activity. Preimmortal HME cells derived from a patient with Li–Fraumeni syndrome were infected with a retrovirus containing wild-type hTERT to induce the cells to express telomerase activity. Cells expressing exogenous hTERT were then transfected with antisense RNA complementary to the template region of human telomerase RNA. Three days after transfection, cells were collected and telomerase activity was measured for 500-cell equivalents per lane by use of a radiolabeled oligonucleotide primer and a PCR-based assay (*see* the "Materials and Methods" section). Labeled PCR reaction products were resolved on polyacrylamide gels and visualized by PhosphorImaging. A ladder of bands represents the extension of the substrate primer by telomerase. **Lane 1** = 500 H1299 lung carcinoma cells served as a positive control for the telomerase assay; **lane 2** = lysis buffer served as a negative control; **lane 3** = preimmortal HME cells; **lane 4** = preimmortal HME cells infected with the hTERT-containing retrovirus; and **lane 5** = preimmortal HME cells infected with the hTERT-containing retrovirus and treated with an antisense RNA oligonucleotide complementary to the template region of human telomerase RNA.

and, by some mechanism, must have prevented the ability of cells to emerge from crisis. In fact, a decrease in the frequency of immortalization was also observed for cells treated with chemopreventive agents for shorter times (only approximately 10 population doublings) before crisis. Moreover, removal of the treatments before crisis results in an immortalization frequency similar to that of untreated or mock-treated cells. These results suggest that the agents are likely to be affecting cells when they are in crisis. Whether these chemopreventive agents affect immortalization through prevention of telomerase activation is a current area of interest in our laboratory.

Since 90% of cancers exhibit telomerase activity and activation of telomerase is an early event in breast cancer progression, the development of chemoprevention strategies that specifically target telomerase is of great interest. The recent cloning of the hTERT promoter has enabled researchers to dissect the molecular mechanisms regulating transcription of hTERT *(30,31)*. Kyo et al. *(32)* showed that estrogen increases hTERT messenger RNA and activates telomerase by inducing the binding of the estrogen receptor to estrogen-response elements on the hTERT promoter. Antiestrogen therapies may, therefore, directly or indirectly affect the regulation of telomerase expression and thus prevent a step that is necessary for the progression of most cancers. One such antiestrogen drug, tamoxifen, has recently been shown to affect telomerase activity and the proliferation of breast carcinoma cell lines *(33)*. Although our results show that tamoxifen is a potent agent in the prevention of spontaneous immortalization, this effect is unlikely to involve antiestrogen activity because the HME cells used in this study do not contain the normal estrogen receptor. Tamoxifen has been shown, in some cases, to act independently of the estrogen receptor *(34)*. Whether the observed effects of tamoxifen on spontaneous immortalization of the LFS cells were indirect (i.e., through an estrogen-like nuclear receptor) remains to be determined.

Retinoids also have been shown to reduce telomerase activity, as seen after the induction of differentiation by retinoic acid and a sharp decline in cell proliferation caused by the treatment of mammary tumors with the synthetic retinoid 4-(hydroxyphenyl) retinamide *(35–38)*. These findings confirm the need to understand the molecular mechanisms by which conventional chemopreventive agents act via their respective receptors and to investigate whether they act by preventing the activation of telomerase during the progression to cancer.

Oltipraz and DFMO also decreased the frequency of immortalization, but not as much as the other chemopreventive agents used in this study. Oltipraz is an anticarcinogen that inhibits HIV-1 replication and some reverse transcriptases *(39)*. The chemopreventive properties of oltipraz have been studied in animal models of colon and lung carcinogenesis *(40,41)*. DFMO is a potent and irreversible inhibitor of the enzyme ornithine decarboxylase, which is involved in polyamine synthesis. DFMO has been reported to have chemopreventive activity in colon, skin, and breast carcinogenesis *(42,43)*. Because polyamine synthesis is necessary for cellular proliferation, DFMO may derive its chemopreventive activity from its antiproliferative properties.

Although some of the chemopreventive agents may act indirectly on telomerase, other agents are being designed specifically to target telomerase directly *(11–13,18,44–46)*. Oligonucleotides complementary to the template region of the RNA component of telomerase have been shown to inhibit telomerase activity and cell growth after long-term treatment of immortal and cancerous cell lines

*(11,47,48).* The other telomerase inhibitor used in this study is a cDNA that contains a point mutation in one of the conserved reverse transcriptase motifs in the human telomerase catalytic subunit, hTERT. When assembled into the telomerase ribonucleoprotein complex, this mutant subunit acts in a dominant-negative fashion to inhibit telomerase activity in tumor cell lines, shortening telomeres and inducing cell death *(12,13,21).* Our study confirms the potential of antitelomerase agents to prevent the activation of telomerase and to inhibit spontaneous immortalization *in vitro.*

Our finding that treating cells before crisis with antitelomerase agents diminishes the spontaneous immortalization of LFS-derived breast epithelial cells *in vitro* indicates a potential approach for the development of rational chemoprevention strategies by use of both clinically and preclinically validated chemopreventive agents for women with a genetic predisposition to breast cancer. Moreover, the prevention of spontaneous immortalization described in this work offers a new intermediate end point for validating novel chemopreventive agents.

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## NOTES

W. E. Wright and J. W. Shay are on the scientific advisory board and own stock in the Geron Corporation, Menlo Park, CA.

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