GRANT NUMBER DAMD17-97-1-7032

TITLE: Novel Strategies Targeting Telomere Maintenance for the Treatment and Prevention of Breast Cancer

PRINCIPAL INVESTIGATOR: Elizabeth H. Blackburn, Ph.D.

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, California 94143-0962

REPORT DATE: July 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
The goal of this research is to test two new strategies to treat breast cancer, based upon altering telomerase action. The ribonucleoprotein telomerase synthesizes telomeric DNA by copying a template RNA sequence within the essential RNA of telomerase. Without telomerase, as cells divide their telomeres shrink and eventually the cells cease dividing. Telomerase activity is commonly found in human breast cancer cells but not in many normal cells. First, inhibitors of telomerase will be used to prevent telomerase from maintaining telomeres. We previously found that we can inhibit telomerase from breast cancer cell lines with reverse transcriptase inhibitors. The second novel (gene therapy) strategy will direct telomerase to synthesize "toxic" telomeric DNA sequences in breast cancer cells, thereby quickly causing them to cease dividing. In this strategy, the telomerase RNA template is altered. Progress to date: 1) we tested 139 compounds and found three new telomerase inhibitors. 2) We have introduced engineered telomerase RNA genes that direct synthesis of mutated telomeres into breast cancer cells in culture. Next we will determine which telomerase RNA gene sequences cause deleterious telomeric DNA sequences to be synthesized, thereby preventing cell division.
FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

________________________ 28/7/98
PI - Signature Date
INTRODUCTION

Telomerase, a cellular ribonucleoprotein enzyme which we discovered several years ago in my laboratory, (Greider and Blackburn, 1985) has the function of synthesizing one strand of telomeric DNA. It specifies the sequence of this essential chromosomal DNA by copying a short template sequence within the RNA moiety of telomerase into telomeric DNA. Telomerase adds this telomeric DNA to the ends of chromosomes to make up for the inability of other DNA polymerases in the cell to replicate these ends completely. The overall aim of the research funded by this grant is to manipulate telomerase activity in human breast cancer cells or pre-cancerous breast epithelial cells, in order to block cancer progression.

Previous results from our and other laboratories have shown that telomerase is often activated in immortalized human breast cancer cell lines, and in cells from breast tumors (Kim et al., 1994; Avilion et al., 1996; Strahl and Blackburn, unpublished work; Kim et al., 1995). It is much less active, or not active at all, in normal breast epithelium. Telomerase is thus a potential therapeutic target for breast cancer treatment. The core of this proposal is to explore two related approaches, one completely novel, for the treatment of breast cancer and/or prevention of breast cancer progression. Both approaches consist of interfering with telomere maintenance by telomerase, and each represents a new potential avenue for chemotheraphy of human breast and other cancers.

Toward the first approach, we had previously shown that we can shorten the telomeres of immortalized human lymphoid cells in culture using a telomerase inhibitor (Strahl and Blackburn, 1996). Inhibition of telomerase in cells would potentially block telomerase from maintaining telomeres, leading to telomere shortening, and hence potentially block or slow cancer cell growth. In the past funding year we have tested 139 compounds for their possible action as telomerase inhibitors. The ultimate long-term goal is to determine whether telomerase inhibitors will be useful clinically in the prevention and/or treatment of breast cancer. Such inhibitors have not been tested clinically or even in breast cancer model systems.

The second approach is a novel gene therapy approach. This exploits the activation of telomerase commonly seen in breast cancer cells. The strategy is to engineer the telomerase RNA in breast cancer cells, causing the synthesis of "toxic" telomeres and thereby preventing cancer cell multiplication. This second strategy is completely novel in concept and nature, because instead of attempting to inhibit telomerase action in cancer cells, it actually turns that action itself against cancer cells. The design of mutated template sequences was based on those we have used for the Tetrahymena, K. lactis and S. cerevisiae telomerase RNA templates (Yu et al., 1990; Gilley et al., 1995; McEachern and Blackburn, 1995; Kirk et al., 1997; Smith and Blackburn, 1998). Such mutations caused synthesis of telomeric DNA at the ends of the chromosomes that led to blocked cell division.

The long-term goal is to develop the information from these experiments into therapies for clinical use in the treatment and/or prevention of breast cancer.

Technical objectives
Specific aims for the 24-month funding period:

1) Determine the effects of inhibiting telomerase activity on growth of immortalized breast cancer cells in culture.

2) Determine telomerase activity levels in breast epithelial cancer cells grown in primary short-term cultures directly from patient tumors, and the effects of inhibiting telomerase activity on the growth of these cells.

3) Introduce mutated telomerase RNA genes, engineered so that they direct synthesis of mutated telomeres, into breast cancer cells in culture, to determine which telomerase RNA gene sequences cause deleterious telomeric DNA sequences to be synthesized, thereby
preventing cell division. The long-term goal is to determine whether the tumor growth of these telomerase template-engineered cells is altered after xenografting them into the nude mouse tumor model system.

BODY

A. Identification of novel telomerase inhibitors in vitro: Specific Aims 1) and 2).

Rationale
First, the goal is to test whether telomerase inhibitors can prevent telomerase from maintaining telomeres. Telomeric tracts are several thousand base pairs long in normal somatic cells, but are often shorter in cancerous cells. In addition, cancer cells divide more rapidly than typical normal somatic cells. Therefore, even if telomerase activity is needed over the long term for some normal cell types, inhibiting telomerase over the short term may be a way of targeting cancerous cells for chemotherapy without severe effects on normal cells. Toward that goal, we have first tested 139 different compounds for effects on telomerase activity in vitro.

The bases for choosing these compounds were:

(i) Nucleoside analogs that mimic the normal substrates, and thus may compete or bind to sites that affect activity. We have shown such effects for AZT and ddG with human lymphoid cell and breast cancer cell telomerase, which act (at least in part) by competing for the nucleoside triphosphate substrate binding site of telomerase (Strahl and Blackburn, 1996). In addition, high dGTP concentrations and G-rich primers stimulate telomerase by binding elsewhere on the telomerase ribonucleoprotein (Lee and Blackburn, 1993), and accordingly we tested a large number of purine (base) analogs made by combinatorial chemistry techniques for inhibitory effects that may be exerted through that mechanism.

(ii) A set of compounds derived from the compound calcomine orange, and that were designed to fit into the HIV reverse transcriptase active site.

(iii) Antisense PNA (peptide nucleic acid) oligonucleotides, shown by D. Corey and colleagues to inhibit telomerase polymerization activity in vitro, it is thought by binding to the telomerase template region.

Experimental methods
Effect of reverse transcriptase inhibitors on telomerase activity in vitro.
We tested additional nucleoside analog inhibitors besides dGTP and AZT-TP, including the inhibitors we have tested previously with Tetrahymena and human B- and T-cell telomerase. Initially, established breast cancer cell lines were used for direct comparison of conventional and TRAP in vitro telomerase assays, with which we have extensive experience (Strahl and Blackburn, 1996). The 1-tube TRAP in vitro telomerase assay was adopted as the best method for screening of inhibitors.

Toward the first anticancer approach, we have tested 139 compounds to determine whether they act as telomerase inhibitors in vitro. Telomerase was assayed using preparations from MCF-7 breast cancer cell lines and also, for comparative purposes, Jurkat cells (a T-cell leukemic line). The extracts were partially purified by a method we had previously developed and optimized (Strahl and Blackburn, 1996 and unpublished work). We defined the range of cell or protein amounts (determined by micro-Bradford assays) within which the assay showed linearity. The assays were tested to be in the linear range by titrating the amounts of extract used and using only those amounts that gave linear quantitative activity levels. We found that this is especially critical
for breast epithelial cells. In addition we identified artifactual positive signals in the TRAP assay, and devised controls to ensure signals are attributable to telomerase action itself.

Additional controls (besides those standardly used in telomerase activity assays - RNase controls, no extract controls) included adding the solvent DMSO that was used to dissolve a subgroup of the inhibitors, at the highest concentration used. To distinguish between effects caused by inhibition or effects on the PCR (Taq polymerase) step of the assay, each assay consisted of a pair of reactions, one (the experimental) in which the inhibitor was added at the beginning of the telomerase elongation step, and the other (the control) in which the inhibitor was added after the elongation step and before the PCR product amplification step. Each inhibitor was tested at three concentrations - 1, 5 and 10 micromolar. Products were analyzed by DNA sequencing gel electrophoresis. This type of gel produced the highest resolution of products.

Results and discussion
139 different compounds were screened for their ability to act as inhibitors of telomerase in vitro. The following results are divided into the three classes of compounds i, ii and iii described above:

(i) Purine analogs
Three compounds at 5 micromolar concentration inhibited telomerase, although they also inhibited the PCR step: 6-(4-methoxy) purine, Compound SJK-III-37 (see Figure 3) and Compound 5-2.

Another group of test compounds (5 guanine derivatives) appeared to stimulate telomerase activity.

(ii) Calcomine orange derivatives
3 out of 20 tested had an apparently specific inhibitory effect on telomerase, and not the PCR step, at 5 micromolar. These are therefore lead compounds for further investigation. Of the remaining 17, 10 inhibited the telomerase elongation step but also the PCR reaction at 5 micromolar, and 7 others had no effect on telomerase or the PCR step at 1 micromolar. Sample telomerase assay results are shown in Figure 2 and the structures of the three inhibitors are shown in Figure 3.

(iii) Antisense PNA oligonucleotides.
PNA oligonucleotides (11 residues) long) with sequences antisense to the template region and elsewhere on the human telomerase RNA, were prepared in-house. One was the same sequence as those found by Corey et al. to inhibit human telomerase in vitro at subnanomolar concentrations (Norton et al., 1996). In our hands they inhibited activity assayed by the TRAP method but only at apparent higher (micromolar) concentrations. However the purity of these PNAs was not established. Therefore these PNAs have been ordered from PerSeptives Biosystems Inc (Framingham MA). They will be assayed with the TRAP assay and the conventional assay.

Problems encountered
Attempts to assay telomerase activity using the conventional non-amplified method from Jurkat cells (and the goal is ultimately to use MCF-7 cells), have to date been hampered by technical problems in these cells, and are still underway. This method is necessary to determine which aspect of the telomerase reaction is affected by the agent.

Terminal deoxynucleotidyl transferase (TdT): a mimic of telomerase in the TRAP assay that potentially produces false positives for telomerase activity.
In collaboration with Thea Tlsty's group, Department of Pathology, University of California San Francisco, we tested whether TdT could give a false positive reaction for telomerase activity. Using a set of primers (TS and CS primers; Kim et al., 1995) we found that commercially available
purified TdT gave a 6 base ladder pattern consisting of bands with electrophoretic mobilities that were indistinguishable (even in high resolution sequencing gels) from those produced by authentic telomerase activity. At high RNase concentrations this reaction was inhibited. However these RNase concentrations were much higher than the concentration needed to inhibit telomerase activity completely. Therefore such inhibition by high concentrations of RNase was unspecific; such unspecific inhibition was also seen with the PCR amplification step. Controls for telomerase activity were therefore done using the low RNase concentrations that we have shown inhibit telomerase specifically.

**B. Synthesis of "toxic" telomeres: Specific Aim 3)**

**Rationale**

About 99% of cells in the adult human body are not proliferating, and are therefore unlikely to be affected by altering telomerase. Therefore, we initially have used an inducible promoter that is not tissue specific, in order to determine, first, whether "toxic" telomere effects can be found in cells in culture. The design of the mutated telomerase RNA templates was based on those that we have shown produce "toxic" telomeres in *Tetrahymena, K. lactis* and *S. cerevisiae*.

**Experimental methods**

During the funding period we subcloned a 598 bp segment containing the 451 base human telomerase RNA gene (hTER gene) plus ~147 bp of downstream region (Feng et al., 1995). The gene was cloned by PCR from the genome of a human cell line with highly active telomerase (the Jurkat T-cell lymphocyte cancer cell line) in order to have a telomerase RNA allele that was known to be functional. The cloning required optimization of the PCR conditions for this particular gene sequence. What initially appeared to be a problem was that, upon obtaining sequence, the sequence did not match that in the published database at several positions in the coding region of the hTER gene. However, that original database sequence was subsequently been shown to be in error at those positions. We found a sequence that did match the correct sequence that was published subsequently. To ensure that we had obtained the correct sequence, 6 independently obtained cloned PCR products were sequenced. Half (3/6) had a novel base change (at position 137 in the coding sequence of the RNA) that differed from that in the database and was not a PCR artifact. (One cloned sequence also had a PCR -generated error). Cloning was also accomplished from normal (non-cancer cell) human genomic DNA. 5 independent clones were sequenced and these results confirmed the sequencing results obtained with the Jurkat cell clones, and also confirmed the published corrected sequence and another allelic variant (a single base change outside the mature RNA sequence at position 514 (Bryan et al. reference).

We subcloned the wild-type hTER gene into a vector with a selectable marker. Mutated template sequences were made by standard methods. The sequences of the mutagenized RNAs were confirmed by DNA sequencing and are shown in Figure 1. Constructs were made with a vector containing a Tetracycline-inducible promoter (commercially available, Invitrogen) to control transcription of the telomerase RNA gene.

Two breast cancer cell lines were used: MDA231 (supplied by Dr. Chris Benz, UCSF) and MCF-7-Tet off (purchased from Clontech). MDA231 cells were transfected with the Tet-on Plasmid. In the future, quality control for inducibility with tetracycline will be performed.

MCF-7-Tet off cells were transfected with 5 different mutant telomerase RNAs, as well as the wild-type telomerase RNA gene as a control. The 5 mutant template sequences are listed in Table 1. Cell division rates were monitored after selection for the marker carried on the plasmid. Expression of the telomerase RNA has been monitored by RT-PCR for some of the cell lines, using oligonucleotide primers specific for the altered template region sequences (see Table 1). Mutant telomerase RNA expression will also be monitored by Northern blotting, using oligonucleotide probes specific for the altered template region sequences.
Results and discussion
We have successfully achieved the objective of expression of mutant telomerase RNAs in MCF7 breast cancer cells.

Cell growth properties are being monitored. This work is in progress.

Recommendations in relation to statement of work

Task 5: Months 2-4. For inhibitors available in triphosphate form, use TRAP assays as described previously (Strahl and Blackburn, 1996) to test that telomerase activity from each cell line is sensitive to inhibitor. Test non-nucleoside analogs (e.g., Foscarnet) directly.

We deemed it more cost effective to go directly to task 5. Task 5 has been completed. We now have a new set of compounds that can be tested on tissue culture cells themselves. This requires much larger amounts of the test compounds than were initially available for most of the compounds. Therefore tasks 1-4 will be done in the next year.

Task 15: Months 1-5. Perform site-directed mutagenesis of subcloned telomerase RNA template bases, as described by us previously (e.g., Gilley et al., 1995). Insert into suitable vector.

Task 16: Months 6-24. Transfect mutated (or wild-type control) telomerase RNA gene in vector construct into breast cancer cell lines. Identify transformant lines. Where appropriate, induce expression of mutated telomerase RNA gene construct.

This has been competed.

Cell growth and phenotypic properties of selected mutant RNAs will be monitored in months 14-24.

CONCLUSIONS

The 3 compounds identified in this work as inhibitors of telomerase in vitro are lead compounds for further investigation as possible drugs that prevent telomere maintenance in cells. Their effects on telomerase action and telomere length in cells need to be investigated.

We have successfully achieved the objective of expression of mutant telomerase RNAs in MCF7 breast cancer cells. These initial studies used an inducible promoter that can be experimentally turned on or off.

If we find that cell proliferation can be stopped by synthesis of "toxic" telomeres, then, one long-term goal will be to minimize potentially deleterious expression of mutant telomerase RNA in those normal cells with active telomerase. To that end, the Her2 gene promoter (reviewed in Hung et al., 1995), which is specific for breast tissue cells, will be used to target expression of the altered telomerase RNA gene to breast cancer cells.
REFERENCES


APPENDICES

TABLE 1

Transfection of breast cancer cell lines with telomerase RNA template mutants

<table>
<thead>
<tr>
<th>Mutant*</th>
<th>WT</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of stable transfectant lines obtained:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>34</td>
<td>42</td>
<td>31</td>
<td>28</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Number of cell lines induced and telomerase RNA prepared:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>21</td>
<td>28</td>
<td>9</td>
<td>12</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Number of cell lines tested by RT-PCR and shown to express the introduced telomerase RNA:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ND**</td>
<td>3</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1</td>
</tr>
</tbody>
</table>

*Mutant designations. Telomerase RNA base(s) mutated are indicated by the residue number in the telomerase RNA coding sequence, numbered from the 5' end of the RNA, and the base to which the residue was changed indicated by G (guanine) or A (adenine). +AA: two additional A residues were introduced. WT: wild-type. See Figure 1.

WT         wild-type
mutant 1    46, 47, 50, 51G
mutant 2    46, 47, 50-53G
mutant 3    47A, 50G
mutant 4    47A, 50G, 53A
mutant 5    49+AA, 49A

** ND, not done
Template sequence of the human telomerase RNA gene and constructed mutations

56 55 54 53 52 51 50 49 48 46 47

CAAUCCCACAUC

MUTATIONS
47A, 50G  
CAAUCCGGAAAC

47A, 50G, 53A  
CAAAACCGGAAAC

46, 47, 50, 51G  
CAAUCCGGAAAC

46, 47, 50 - 53G  
CAAGGGGAAGG

49 + AA  
CAATCCCACAUC

PREDICTED 
TELOMERIC REPEAT
TTTGGC

TTTGGC

TTCCCCC

TTCCCC

TTTTAGGG

Figure 1
The position of each nucleotide within the template is indicated above the wild type telomerase RNA sequence at the top of the page. The wild type sequences are in grey, mutated nucleotides are in black. The predicted telomeric repeat is shown for each mutant template sequence at the right.
Study of inhibitory effects of Calcomine Orange derivatives on MCF-7 telomerase activity

<table>
<thead>
<tr>
<th></th>
<th>319 DCJ</th>
<th>319 CCJ</th>
<th>293 ECJ</th>
<th>293 DCJ</th>
<th>270 A</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 uM</td>
<td>0.2 uM</td>
<td>0.2 uM</td>
<td>0.2 uM</td>
<td>0.2 uM</td>
<td>1 uM</td>
</tr>
<tr>
<td>1 uM</td>
<td>1 uM</td>
<td>1 uM</td>
<td>1 uM</td>
<td>1 uM</td>
<td>1 uM</td>
</tr>
<tr>
<td>5 uM</td>
<td>5 uM</td>
<td>5 uM</td>
<td>5 uM</td>
<td>5 uM</td>
<td>5 uM</td>
</tr>
</tbody>
</table>

Figure 2
TRAP assay on MCF-7 extract testing inhibitory effects of 5 Calcomine Orange derivatives at 3 concentrations: 0.2, 1, and 5 uM. All of these compounds show inhibition at 5 uM. The lane on the far right is a negative control run without extract.
Figure 3

Structures of compounds for further study -- putative telomerase inhibitors

Calcomine Orange Derivatives

![Chemical structure of KWM-3-19-DCJ]

![Chemical structure of KWM-2-96-BCD]

![Chemical structure of KWM-2-70-A]

Purine Analogs

![Chemical structure of SJK-III-37]

![Chemical structure of 6-(4-methoxy)purine]