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TITLE: Inhibition of Telomerase as a Therapeutic Target for the  
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| <b>13. ABSTRACT (Maximum 200 Words)</b><br>Malignant prostate cells must divide many times to form a tumor, metastasize or recur after therapy. Critical for this type of endless cell division is an enzyme named telomerase. This enzyme is usually dormant in most normal tissues, but is resurrected in prostate cancer cells by yet unknown genetic changes. Since the enzyme is required for survival of cancer cells but is not present in most normal cells, inhibition of telomerase may specifically target prostate cancer cells. We have shown that impeding the function of telomerase by expressing a mutated version of this protein kills prostate cancer cells in an experimental setting. We now wish to translate this finding into a more practical application by searching for smaller molecules capable of inhibiting telomerase activity. The approach we propose to take is one that capitalizes on reagents already in hand. First, we have mapped parts of telomerase that are extremely sensitive to even small disruptions. We now wish to find molecules that bind to these sensitive regions, as such regions represent the most attractive parts of the protein to target for inhibition. We will therefore screen millions upon millions of small molecules, termed peptides, for those few that adhere to these regions of the telomerase enzyme. Such peptides could serve as a blueprint to develop even more clinically relevant inhibitors of telomerase. |                                                                 |                                                                |                                                                             |
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## 1.0 Introduction

**Subject:** Inhibition of telomerase as a therapeutic target for the treatment of prostate cancer.

**Purpose and scope of research:** Telomerase is ribonuclear protein enzyme<sup>1</sup> that is activated in the vast majority of human cancers<sup>2-4</sup>, including prostate<sup>3,5-16</sup>, where it plays an essential role in allowing cells to continue to divide<sup>2,17,18</sup>. We found that inhibition of telomerase catalytic enzyme activity through the expression of a dominant-negative hTERT protein is lethal in prostate cancer cell lines<sup>19</sup>. It therefore stands to reason that other modes of inhibiting telomerase activity may be equally effective at killing these cancer cells. Since the enzyme requires the correct association of its RNA and protein subunits<sup>20,21</sup>, we propose to screen for peptides that bind to the RNA-binding domain of the protein subunit to sterically block RNA-protein interactions, which in turn would inhibit telomerase catalytic activity.

## 2.0 Body

**Task 1:** Map the C-terminal boundary of domain D. *a.* Create a series of tandem NAAIRS substitutions of hTERT 3' of the last substitution by site directed mutagenesis. Clone these mutants into a retroviral vector. *b.* Create retroviruses derived on the mutant hTERT-expressing retroviral constructs, infect telomerase-negative cells and select for stable cell lines. *c.* Assay cell lines for telomerase activity and cell viability.

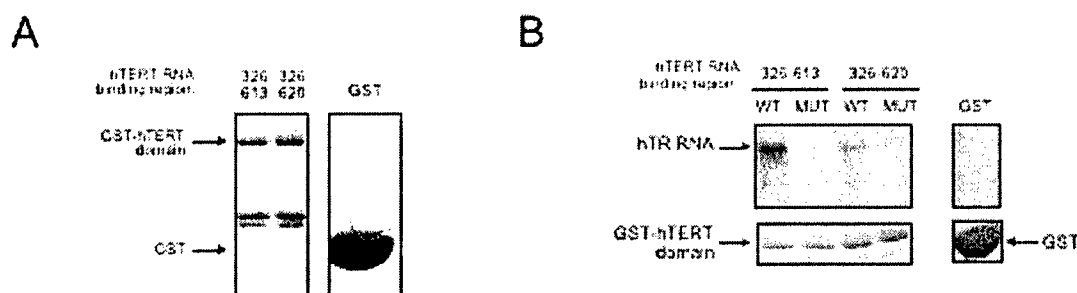
**Task 2:** Produce large quantities of recombinant RNA-binding domains of the hTERT protein. *a.* Create bacteria expression plasmids encoding HIS<sub>6</sub>-FLAG tagged domains C and D of hTERT. *b.* Purify recombinant proteins.

**Task 3:** Assay recombinant domain proteins for hTR RNA binding. *a.* Assay recombinant protein *in vitro* for RNA binding in solution. Optimize assay where required. *b.* Assay recombinant protein *in vitro* for RNA binding when immobilized on ELISA plates. Optimize assay where required.

**Task 4:** Screen for peptides that bind to the recombinant telomerase protein by phage display. *a.* Perform the first cycle of the phage display screen, optimizing where necessary. *b.* Rescreen candidate phage repeatedly to isolate phage expressing high affinity peptides. *c.* Determine the sequence of the peptides of candidate phage. *d.* Synthesize candidate peptides

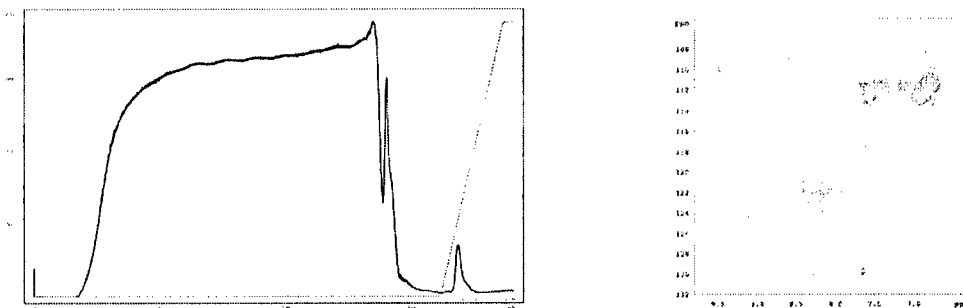
**Task 5:** Screen peptides for specific inhibition of telomerase function *in vitro*. *a.* Screen peptides for inhibition of hTERT-RNA interactions *in vitro*. Optimize where required. *b.* Begin to screen peptides for inhibitory activity by a real-time PCR-based telomerase assay, optimizing where necessary. *c.* Begin to assay specificity by comparing degree of telomerase inhibition of candidate peptides compared with control peptides, optimizing where necessary.

We were able to produce recombinant polypeptides from bacteria encompassing the hTR-binding region of hTERT that bound to the hTR *in vitro* (Figure 1). This represented completion of tasks 1,2 and most of task 3. While we made fast progress on these tasks, producing highly purified protein for a high throughput screen turned out to be a daunting task, and hence we engaged in a collaboration with Dr. Maurizio Pellecchia at the Burnham Institute to produce highly purified recombinant protein for phage display, and additionally, to determine the NMR structure of this polypeptide for the expressed use of identifying small molecules that would bind to the RNA-protein interface.



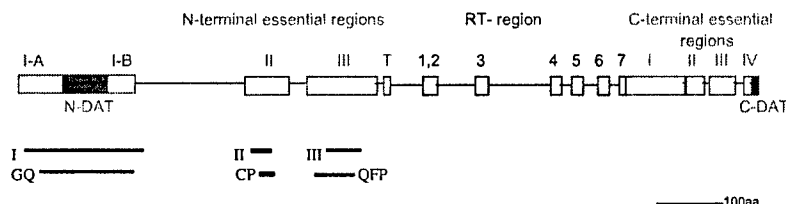
**Figure 1.** Recombinant hTR-binding domains of hTERT are functional. A) Two GST fusion polypeptide fragments comprised of the hTR-binding domains of hTERT (denoted by the amino acids they encompass) were produced in bacteria, immunoprecipitated with an anti-GST antibody and resolved by SDS-PAGE. The fragments differ only by the length of their C-terminus. B) The same two polypeptides (WT), or versions containing a mutation in hTR-binding domain (MUT), were incubated in the presence of  $^{32}\text{P}$ -labelled hTR, immunoprecipitated as before and resolved by SDS-PAGE. hTR association was visualized by exposure to film (top) whereas immunoprecipitated fusion proteins were visualized by Coomassie Blue staining (bottom). GST serves as a negative control in A and B.

The Pellecchia lab discovered that the hTR-binding domain peptide encompassing amino acids 326-613 described in figure 1 could not be reliably expressed in bacteria, despite trying five different strains of bacteria and a multitude of growth conditions (temperature, broth, induction times, etc). There was more success with the fragment encoding amino acids 326-620, which could be produced as a soluble protein in one strain of bacteria (BL21(DE3)). Moreover, the GST-tagged version of this peptide could be purified, although it later turned out to simply not be possible to get enough of this peptide to perform any analysis. In an effort to overcome these limitations a smaller version of the hTR-binding domain (amino acids 326-437) was generated as a HIS-tagged polypeptide. This polypeptide was expressed at high levels in bacteria (15mg/L of culture), although it unfortunately accumulated in inclusion bodies. Protein could be solubilized from inclusion bodies using 6M guanidine-HCl, but the solubilized protein loaded onto a HIS-Trap column charged with Ni could not be eluted unless washed with 6M guanidine-HCl, indicating that the protein was precipitating on the column (not shown). To overcome this problem, it was found through a large amount of effort that the peptide could be refolded in 50 mM PBS, pH 6.5; 1 mM EDTA; 500 mM arginine; 1 mM DTT; 250 mM NaCl and 440 mM sucrose. Hence, after purifying the peptide on the HIS-Trap column under denaturing conditions, the peptide was refolded by rapid dilution in the aforementioned conditions, although this resulted in a significant loss of material that limited the ability to generate NMR spectral data (Figure 2). Nevertheless, it is now possible to generate a peptide of the hTR-binding domain, and continued efforts on refolding may eventually allow good spectral analysis of this region.



**Figure 2.** His-tagged hTR-binding region 326-437 peptide purification and spectral analysis. LEFT: Blue line: Trace of HIS-tagged protein elution. Red line: gradient of an increase in imidazole up to 500 mM. RIGHT:  $[^1\text{H}-^{15}\text{N}]$  HSQC analysis of the aforementioned polypeptide after refolding.

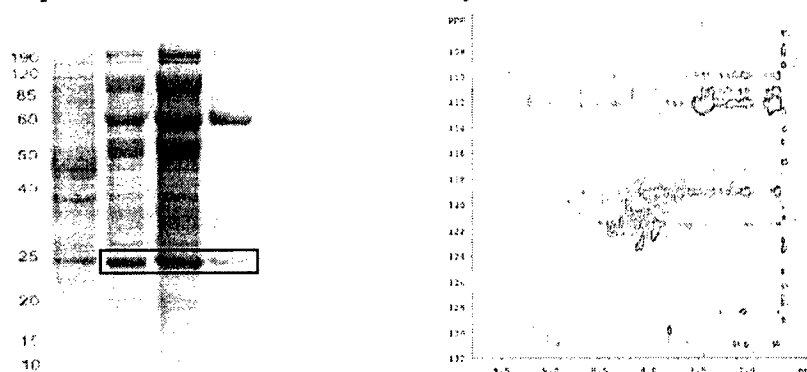
Due to the difficulties in producing enough recombinant hTR-binding domain polypeptide for these studies, we identified another target that has all the advantages of the hTR-binding domain, but may be more readily produced as a recombinant polypeptide in bacteria. Specifically, we have shown that small mutations in the N-terminal DAT domain abolish the ability of hTERT to elongate telomeres, indicating that this domain is essential for telomerase function, and hence a target to develop small molecular weight inhibitors of telomerase<sup>22</sup>. This domain is nestled within a short N-terminal region flanked at one end by the initiation Methionine (amino acid 1) and the other by a long stretch of random coil (amino acids 176 to 349) that is insensitive to mutation (Figure 3), arguing that a fragment encompassing this region (i.e. 1-200) could be produced as recombinantly as a functional polypeptide<sup>22</sup>. Indeed a similar fragment has been produced in rabbit reticulocyte lysates, and shown to restore the telomerase catalytic activity of an N-terminally deleted hTERT fragment<sup>23</sup>.



**Figure 3.** Pictorial diagram of the functional domains of hTERT. Black, reverse transcriptase (RT) region; red, essential domains identified by mutagenesis described in section C2; open boxes, essential regions or motifs; closed boxes, DAT domains; lines, linker or regions of low homology. Regions of homology defined by mutagenesis in yeast (I, II and III) or TERT alignments (GQ, CP and QPF) are shown below.

To this end, a HIS-tagged polypeptide encompassing amino acids 1 to 200 was expressed in bacteria and was easily induced by IPTG. Importantly, this polypeptide was soluble, validating our prediction that this region is an excellent target to purify as it is flanked by a random coil at one end and is the N-terminus of the protein at the other end. However, purification on the HIS-Trap column revealed that the eluted protein was highly contaminated with other proteins. Nevertheless, this could be cleaned up to a large extent by an ion-exchange column, although there still remains a band at ~63kDa that is either a contaminant or a dimer of the peptide. There was enough material to perform NMR analysis, although the data from such analysis clearly indicates that there

is further work to be done (Figure 4). This polypeptide can be much more easily purified, and hence represents the best bet for future study.



**Figure 4.** His-tagged DAT domain (amino acids 1 to 200) polypeptide purification and spectral analysis. LEFT: Protein purification as follows, lane 1- uninduced, lane 2- induced with IPTG, lane 3- elution of polypeptide from HIS-Trap column, lane 4- elution of polypeptide from ion-exchange column. Box denotes the His-tagged DAT-domain polypeptide. RIGHT:  $[^1\text{H}-^{15}\text{N}]$  HSQC analysis of the aforementioned polypeptide.

#### 4.0 Key accomplishments

- Purification and initial spectral analysis of a folded polypeptide encompassing hTR-binding domain of hTERT.
- Purification and initial spectral analysis of a folded polypeptide encompassing DAT domain of hTERT.

#### 5.0 Reportable outcomes

- Bacterially produced recombinant polypeptides of the hTR-binding domain and DAT domain of hTERT.

#### 6.0 Personnel supported by this grant

- Soma Banik, Kevin Crowell, Brian Freibaum, Chris Counter

#### 7.0 Conclusion

There is currently no effective long-term therapy for recurrence and metastasis of prostate cancer. Activation of telomerase plays a critical role in permitting cancer cells to divide sufficiently to undergo these processes<sup>18</sup>. Inhibitors of this enzyme may therefore have therapeutic value in the treatment of late stage prostate cancer. Indeed, we show that inhibition of the enzyme by experimental means can abolish the tumorigenic potential of prostate cancer cell lines isolated from metastatic disease<sup>19</sup>. In an effort to translate this preliminary work into a more clinically relevant approach we have identified and purified two domains of the telomerase catalytic subunit essential for enzyme activity that can be used to design small molecular weight inhibitors. This represents a huge technical feat given the difficulty in their purifications and refolding. This purification represents a rate-limiting step that has thwarted the field, and hence is a huge step in the right direction towards determining the structure of critical domains of telomerase for the purpose of modeling telomerase inhibitors. We are now poised to determine their structure by NMR spectroscopy, which will finally provided much needed structure data to begin the process of modeling telomerase inhibitors.

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