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# THE DIETARY ISOPRENOID PERILLYL ALCOHOL INHIBITS TELOMERASE ACTIVITY IN PROSTATE CANCER CELLS

by

Tabetha Sundin
B.S. May 2005, Old Dominion University

A Thesis Submitted to the Faculty of
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## ABSTRACT

THE DIETARY ISOPRENOID PERILLYL ALCOHOL INHIBITS TELOMERASE ACTIVITY IN PROSTATE CANCER CELLS

Tabetha Sundin
Old Dominion University, 2012
Co-Advisors: Dr. Patricia Hentosh
Dr. David Gauthier

This is the first evidence that a plant-derived compound–perillyl alcohol – regulates telomerase activity via the mammalian target of rapamycin (mTOR) pathway in prostate cancer cells. Telomerase–the enzyme responsible for immortalizing cells through telomeric repeats addition–is de-repressed early in an aspiring cancer cell. We hypothesized that perillyl alcohol regulates hTERT (human telomerase reverse transcriptase) at the translational and post-translational levels via its effects on the mTOR pathway. A rapid suppression of telomerase activity was detected in prostate cancer cell lines (PC-3 and DU145) in response to biologically-relevant concentrations and short incubations of perillyl alcohol or the mTOR inhibitor–rapamycin.

Western blot analysis revealed a decrease in hTERT protein levels in response to either agent that did not coincide wholly, with loss of telomerase activity suggesting a further level of regulation. Using immunoprecipitation we established the presence of a hTERT-mTOR-S6K (p70 S6 kinase)-Hsp90 (Heat shock protein 90)-Akt complex previously detected in activated NK cells in DU145 prostate cancer cells. Further, western blot analysis demonstrated that perillyl alcohol or rapamycin disrupted the binding interactions between RAPTOR and hTERT, mTOR, S6K, and Hsp90, establishing an additional mechanism by which these agents decrease telomerase activity.

Prostate cancer cells overexpress eIF4E (eukaryotic initiation factor 4E) the rate-limiting protein that mediates cap-dependent translation by way of

mTOR signaling. Immortalized Chinese hamster ovary (CHO) control cells (pMV7) and CHO cells with forced eIF4E-overexpression (rb4E) were used to elucidate the role of eIF4E in telomerase regulation by perillyl alcohol and rapamycin. Telomerase activity and TERT protein levels were dramatically attenuated in rb4E cells by perillyl alcohol or rapamycin, but the pMV7 cells were unresponsive to either agent. Through western blot analysis we determined eIF4E-overexpression activates Akt–an upstream regulator of mTOR–through a positive-feedback loop thereby increasing the phosphorylation of downstream targets of Akt. These findings demonstrate that eIF4E-overexpression in CHO cells alters protein synthetic processes and gene regulation, thus enabling the inhibitory effects of perillyl alcohol and rapamycin on telomerase activity and TERT protein levels. This study provides evidence for a unique link between perillyl alcohol- and rapamycin-mediated regulation of mTOR and hTERT.

This dissertation is dedicated to my husband, Christopher Brown. He has made countless sacrifices so that I could pursue my dream. He has truly been a blessing in my life and my children are lucky to have such an amazing father. To my children Alexis and Xavier Brown, who are my inspiration to push myself further than I thought possible. To my beautiful mother who nearly gave up her sanity to raise my sisters, brother and I; she truly is an amazing woman. To my father who I aspire to be like: smart, hard-working, strong, and yes, even good-looking. For all of my family, mentors, and friends who helped me along the way. And finally, to my mentor Dr. Hentosh, who believed in me even when I didn't believe in myself, I will always be grateful and feel I have made a lifelong friend. Without her I would never have completed the program.

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# CHAPTER I

# INTRODUCTION, SPECIFIC AIMS AND SIGNIFICANCE

## INTRODUCTION

### *Telomeres*

Human telomeres, specialized nucleoprotein structures found at the ends of chromosomes, consist of a repeated series of the hexameric DNA sequence $(TTAGGG)_n$, along with a 6-protein complex called shelterin (Fig. 1) [1-3]. Telomeres function to prevent chromosomal degradation and genomic instability and therefore the loss of genetic information. Although human telomeres are of heterogeneous lengths, human cells begin with approximately 12 kilobases (kb) of telomeric DNA; by the time adulthood is reach this number has been reduced to around 8 kb of telomeric DNA [4]. Therefore, telomeres function as molecular clocks that ultimately link cellular aging to cell division [5]. DNA polymerases require a double-strand/single strand interface in order to bind DNA and therefore replicate the strands. The interface is provided by an RNA primer laid down by an RNA primase. Although only used once at the beginning of replication for the leading strand, this RNA primer is used repeatedly for the lagging strand. After DNA polymerase α uses the primer to initiate replication, RNA sequences are removed, degraded and replaced by DNA. Space becomes limiting at the 3'-end of the lagging strand, and the RNA primase may no longer bind the strand to provide an interface. Therefore, DNA polymerase cannot fully replicate the lagging strand resulting in a single-stranded 3' overhang that will eventually be cleaved off leading to DNA loss.

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The journal format for this dissertation is modeled after Archives of Biochemistry and Biophysics

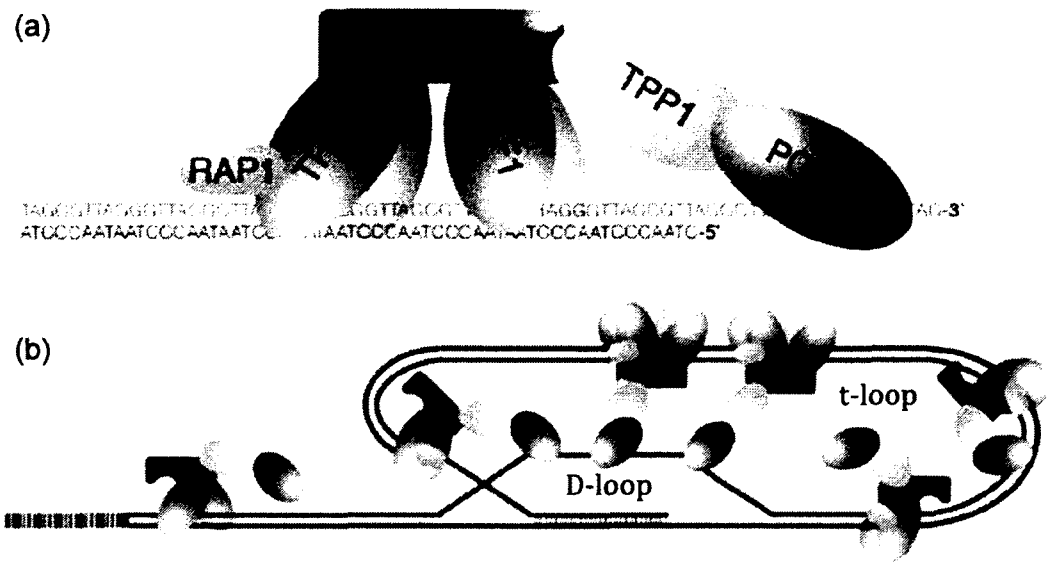


**Fig. 1**. The shelterin complex. **(a)** The six protein complex that constitutes shelterin. **(b)** Schematic of shelterin complex bound to a telomere. TRF1 and TRF2 bind both double-stranded TTAGGG repeats and TIN2. TIN2 also binds TPP1, which binds POT1. POT1 binds the single-stranded portion of the telomere end, creating the D-loop. Reprinted from DNA Repair, 8, Give me a break: How telomeres suppress the DNA damage response, page 1119, © 2009 Elsevier B.V., with permission from Elsevier. [1].

The inability of DNA polymerase to replicate DNA to the end of the chromosome is referred to as the 'end replication problem' [6, 7]. The telomeric repeat (TTAGGG) is non-coding, serving only as a substitute for the loss of chromosomal DNA that may otherwise occur during replication.

In humans, the protein complex shelterin protects the single-stranded 3'-end of the telomere by inducing secondary structure formation. The 3' single-stranded overhang is tucked back into the double-stranded telomeric DNA creating a t-loop and a D-loop (Fig. 1 (b)) [1, 8]. This secondary structure acts to

sequester the 3' single-stranded overhang from cellular repair proteins so that it will not be recognized as a single-stranded break [9]. Shelterin is composed of: telomeric repeat binding factors 1 and 2 (TRF1/2), TRF2- and TRF1-Interacting nuclear protein 2 (TIN2), protector of the telomere (POT1), the human ortholog of the yeast repressor/activator protein 1 (RAP)1, and the protein formerly known as TINT1, PTOP, or PIP1 (TPP1) (Fig. 1 (a)) [10]. Despite the efforts of shelterin, 50-200 bp of telomeric DNA are lost with each round of replication [11]. When telomeres reach a pre-determined critically short length (< 200 bp), the secondary structure provided by shelterin is disrupted [12]. Through a complex signaling cascade, the loss of secondary structure provided by shelterin signals the cells to go into an irreversible state termed senescence [12]. Although the cell is viable during senescence, it is unable to proliferate. Without further damage the cell can remain in the senescent state for long periods of time. Chromosomes that lack sufficient telomeric repeats are prone to chromosomal degradation, recombination and fusion events. In this manner, telomeres act as the protective cap at the end of the chromosome and have been likened to the plastic tips at the end of a shoelace [13].

*Telomerase*

The hexameric repeats $(TTAGGG)_n$ at the ends of telomeres are synthesized and maintained by an enzyme called telomerase [14]. Telomerase is a ribonucleoprotein, consisting of three major components: human telomerase reverse transcriptase (hTERT), human telomerase RNA component (hTERC), and the protein dyskerin, all of which are necessary to counteract telomeric shortening during replication [15-18]. Human TERT is a DNA polymerase that is also classified as a reverse transcriptase (RT) due to its ability to copy an RNA template into DNA [15]. Blackburn, Greider and Szostak shared the Nobel Prize in 2009 for their work with telomeres and their discovery of telomerase. Although both hTERT and hTERC are necessary for telomerase activation, hTERT is the catalytic portion of the enzyme and is considered the rate-limiting component [19, 20]. In fact, when the *TERT* gene is transfected into human cell lines under the

control of a constitutive promoter, the cells bypass senescence and become immortal [21]. Three structural components comprise hTERT: a long N-terminus with DNA and RNA-binding domains, a catalytic reverse-transcriptase domain, and a short C-terminus extension [22]. Similar to most other polymerases, there are notable fingers, palm and thumb DNA polymerase motifs found in the TERT protein [23, 24].

The hTERC portion of telomerase enzyme consists of an 11-nucleotide template core region that provides the RNA template for the enzyme to generate telomeric repeats [25]. In addition to a template region, hTERC has a conserved region 4 and 5 (CR4/CR5), a pseudoknot motif and the box H and ACA elements (H/ACA domain) that provide enzyme fidelity, processivity, and are responsible for the interaction between hTERT and hTERC [26]. Dyskerin, the protein portion of the telomerase holoenzyme, is necessary for enzymatic regulation. Current research favors the model of dyskerin along with two ATPases, pontin and reptin, serving to stabilize hTERC, while hTERC and hTERT assemble. Although pontin and reptin dissociate, dyskerin remains a part of the active enzyme [27]. Once all components are together in the complex, telomerase becomes active and synthesizes telomeric repeats.

Telomerase extends telomeres through a reaction involving cycles of primer recognition and binding, synthesis and translocation. Through this reaction, telomerase adds hundreds of nucleotides to the DNA strand. The first part of the reaction, primer recognition and binding, is carried out once telomerase recognizes the 3' single-stranded overhang of the telomere that serves as the DNA primer for this polymerase [24]. Telomerase recognizes the guanine-rich strand of the telomere as a primer *in vivo*; it also appears that any guanine-rich template can serve as a primer for telomerase *in vitro* [28]. During the synthetic portion of the cell cycle, telomerase processively adds deoxynucleoside triphosphates (dNTPs) to the end of the telomere, known as repeat addition processivity. During synthesis, the RNA-DNA hybrid is kept at a constant length of seven to eight base pairs, due to 5'-bonds melting and 3'-bonds being created at the same rate [29]. When the telomerase enzyme

reaches the 5'-end of the template, it translocates to reposition the DNA at the 3'-end of the template to repeat the cycle. The ability of telomerase to add repeats processively is unique. Most reverse transcriptases are only able to copy an RNA genome into a single DNA molecule. Telomerase is capable of repeat addition processivity due to DNA-binding 'anchor sites' that are present in hTERT [30]. In addition to anchor sites, it has also been shown (*in vitro*) that the telomere-binding protein heterodimer TPP1-POT1 stimulates telomerase activity and processivity, although the mechanism by which this occurs has not been elucidated [31].

*Telomerase and Cancer*

Generally, somatic cells and normal cells in culture lack hTERT expression, so their telomere length continues to shorten with each cell replication. Consequently, these telomerase-deficient cells have a limited number of cell divisions prior to senescence, or a non-replicative state [9]. Some sub-populations of normal human cells do express low levels of telomerase that are insufficient to achieve immortality. These include mainly stem cells in proliferating tissues, germ cells, and activated lymphocytes [32-34]. Telomerase activation or derepression is a critical event in a cell that is progressing towards a cancerous state [35]. Activating/de-repressing telomerase immortalizes ~90% of cancer cells [36]. The other ~10% of cancer cells activate a mechanism known as alternative lengthening of telomeres (ALT), in which recombination events lead to the extension of the telomere [37]. Telomerase expression does not make a cell cancerous, but allows a cell to live long enough to acquire mutations that increase its likelihood of becoming cancerous. Mouse models have shown that overexpression of *TERT* leads to increased tumor formation [38, 39]. This low level of telomerase is not sufficient to prevent the telomere from shortening, but it does slow the rate at which the process occurs [40]. Cancer cells have shorter telomeres than normal cells, sensitizing them to telomerase inhibition [41]. Therefore telomerase inhibition is an attractive target for cancer therapy. Telomerase inhibition in cancer cells has been shown to decrease telomere length and cause cellular senescence or apoptosis, while having little effect on

normal cells [41]. Telomerase inhibitors thus have a promising role as adjuvant therapeutics or as chemopreventives [42]. Telomerase inhibition is a key target for anticancer studies due to the specificity of telomerase expression and the correlation between telomerase presence and cell immortality.

*Telomerase Regulation*

The rate-limiting component of the telomerase holoenzyme, hTERT, is regulated at both the transcriptional and post-transcriptional level. *hTERT* mRNA levels are controlled through a series of transcriptional factor interactions with promoter regions. Posttranscriptional control mechanisms such as structural changes of the holoenzyme, localization of hTERT, hTERT phosphorylation, protein degradation and alternative splicing account for a significant degree of regulation [43]. A critical level of regulation is governed by transcriptional processes [44]. Somatic cells and normal cells in culture do not have detectable levels of *hTERT* mRNA, although the RNA component-hTERC-is transcribed and present at finely regulated levels [19, 45]. Eventually normal cultured cells with a finite number of growth divisions will enter into a stage of growth arrest, termed senescence. In time the telomeres of these cells will become so short that the ends of the chromosomes began to fuse and break. This cellular stage is termed crisis; all but a few of these cells will succumb to apoptotic death [46]. The limited number of cells that survive crisis become immortalized, a process characterized by a surge in *hTERT* mRNA levels [45]. Comparable transcriptional derepression of *hTERT* is observed in tumor cells relative to adjacent normal tissues [45]. The promoter region of the *hTERT* gene has multiple binding sites for a vast array of transcription factors (both activators and repressors), providing clues to the extent of regulatory complexity [47]. Specifically, two Myc/Max binding sites (E-boxes) have been identified in the *hTERT* promoter, and c-myc directly activates *hTERT* transcription [48-50]. Alternatively, Mad-1 can displace Myc and form a heterodimer with Max, thereby repressing transcription by blocking the E-box found in the *hTERT* promoter [51]. The human papillomavirus type 16 E6 oncoprotein (E6) also binds the E-box, thereby activating *hTERT* [52]. Myc is not the only transcription factor that can

alter *hTERT* expression. Estrogen has been shown to activate telomerase via effects on the *hTERT* promoter [53].

Adding to the complexity of *hTERT* transcriptional regulation, signal transducer and activator of transcription (STAT)3 and STAT5 bind the promoter of *hTERT* [54]. Telomerase is also upregulated by hypoxia-inducible factor-1 (HIF-1) and via portions of the Ras (rat sarcoma) pathway [55, 56]. Additionally, leptin, an adipose-secreted hormone, increases the expression of *hTERT* mRNA and protein, providing a mechanism of action to explain the increased cancer incidence in obese patients [57]. Telomerase expression is inhibited transcriptionally by the p53-mediated binding of the transcription factor, Sp1 [58]. Almost all cancer cells have aberrant Ras signaling and constitutively activated *c-myc*. Interestingly, the minimum genetic alterations to induce a fibroblast to become cancerous include Ras activation, *hTERT* expression and SV40 large antigen, which targets the master tumor suppressors, p53 and pRb [59]. Linking oncogenic pathways to telomerase activation provides a mechanism by which an aspiring cancer cell can bypass many hurdles simultaneously.

After *hTERT* transcription has been activated, molecular failsafes still remain to squelch the pro-cancer activities of a cancer cell apprentice. *hTERT* mRNA must be translated and the protein readied for its role. Reversible phosphorylation of hTERT protein regulates the protein's function, cellular localization and ultimately telomerase activity [60]. Human TERT is phosphorylated by more than one kinase at different sites in the protein. A well-described relationship between hTERT and a kinase is the association between hTERT and Akt (also known as protein kinase B). Akt is an important protein in the phosphatidylinositide-3 kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) signaling pathway that governs protein translation. Akt, a serine/threonine kinase, phosphorylates hTERT at Serine 824 (Ser 824) and Serine 227 (Ser 227) [61]; phosphorylation of either site upregulates telomerase activity. Protein kinase C (PKC) has also been to shown to induce hTERT expression and modulate its activities post-transcriptionally by phosphorylation [36, 62].

Likewise, hTERT phosphorylation is important in the nuclear localization of the protein where it may join hTERC and activate the telomerase holoenzyme [63]. Human TERT can only bind its nuclear translocator, nuclear factor (NF)-κβ, in its phosphorylated form [64, 65]. Upon localization in the nucleus, phosphorylated hTERT binds 14-3-3 signaling proteins that act to sequester hTERT in the nucleus where it may associate with the other components of the telomerase holoenzyme to perform its function to extend the telomeres [66]. Tumor cells with high levels of telomerase activity contain phosphorylated forms of hTERT that are found mainly in the cell nucleus [67, 68]. Conversely, protein phosphatase 2 A (PP2A) abrogates telomerase activity by dephosphorylating the protein. This maybe a direct dephosphorylation event or alternatively it could be a downstream effect of PP2A dephosphorylating Akt rendering it incapable of phosphorylating hTERT [69-71]. Heat shock protein 90 (Hsp90), a protein known to associate with both hTERT and Akt, also has an important role in telomerase activity. Hsp90 prevents PP2A from dephosphorylating Akt [69]. Further, Hsp90 is necessary for assembly of the telomerase holoenzyme, and is itself regulated through phosphorylation [72]. Reactive oxygen species (ROS) have been shown to induce phosphorylation of hTERT Tyrosine 707 (Tyr707) via Src (sarcoma) kinase [73]. Tyr707 phosphorylation has the opposite effect of that observed with Ser824 phosphorylation; this event is critical for hTERT nuclear export, translocation back into the cytoplasm and loss of telomerase activity. Thus, nuclear localization is another level in the multistep regulation of telomerase activity.

Human TERT protein levels are additionally regulated through the actions of the ubiquitin-protein ligase (E3 ligase) Makorin-1 (MKRN1) [74]. MKRN1 recognizes hTERT and targets it for degradation via the ubiquitin-26 S proteasome pathway (UPS). Hsp90 is thought to rescue hTERT from degradation by preventing the actions of MKRN1 [74]. While the mechanism is not understood currently, the association of Hsp90 with hTERT may prevent MKRN1 from recognizing hTERT as a substrate. MKRN1 levels dramatically rise when a cell enters the G1 state of the cell cycle, providing a possible link

between telomerase activity and the cell cycle [75]. Although MKRN1 is the only E3 ligase that has been identified currently, there may be other E3 ligases that target hTERT for degradation.

*Non-Telomeric Functions of Telomerase*

In addition to providing a cell with immortality, telomerase has a much larger role in cancer development. When hTERT is overexpressed in a variety of cells, the cells become resistant to apoptosis [76-79]. In fact hTERT blocks both the intrinsic and extrinsic apoptotic pathways [80-82], and is itself a target for caspase-6 and caspase-7 cleavage [83]. The mechanism by which hTERT blocks apoptosis is unclear; however it appears to inhibit an early step in the apoptotic pathway prior to caspase activation [84].

Besides the anti-apoptotic functions of hTERT, telomerase is involved in multiple levels of DNA repair. hTERT and hTERC both have a role in regulating the ATM (ataxia telangiectasia mutated)-ATR (ataxia telangiectasia and Rad3-related kinase) DNA damage pathway. hTERT upregulates ATM causing cell cycle arrest to allow DNA repair or apoptosis [85]; hTERC inhibits ATR, thereby preventing cell cycle arrest [86]. Cells that overexpress hTERT have 20-fold less spontaneous chromosome breaks and increased levels of ATP, possibly due to increased mitochondrial DNA protection [87]. Many of the DNA damage response factors are dependent on ATP hydrolysis, including chromatin decondensation responsible for activating ATM, which results from a double-strand break [88, 89]. Telomerase repairs these double-strand breaks by the *de novo* addition of telomeres in a process termed 'chromosome healing' [90]. This extends the life of a cell with a defective genome, thus increasing the possibility of that cell becoming cancerous.

Ectopic hTERT expression further revealed that hTERT is responsible for regulating nearly 300 genes that participate in functions such as cell cycle progression, apoptosis, metabolism and signaling [91]. Further, hTERT has been implicated in pRb hyperphosphorylation, causing unchecked cell cycle progression and providing a growth advantage for hTERT-overexpressing cells

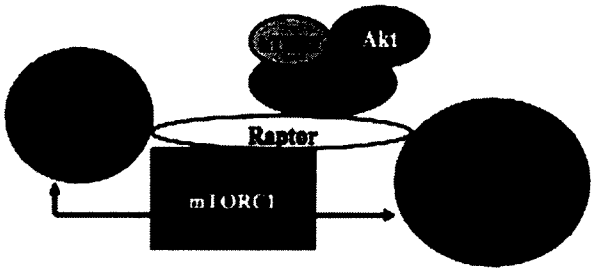


**Fig. 2.** Proposed schematic representation of the hTERT-mTOR-RAPTOR complex in DU145 prostate tumor cells. The presence of mTOR in a complex with TERT provides compelling evidence for the mTOR-mediated control of telomerase activity. The arrows represent phosphorylation of the substrates by mTORC1.

[92-94]. Additionally, hTERT is responsible for the transcriptional activation of cyclin D1 [95].

The cancerous phenotype associated with hTERT-overexpressing cells is exacerbated by the ability of hTERT to upregulate epidermal growth factor receptors (EGFRs) [96]. EGFRs are responsible for multiple oncogenic signaling pathways. Increased numbers of cell surface receptor sensitizes a cell to low levels of growth factors. Thus under conditions of limited growth factors, signaling pathways deceive the cell that nutrients are ample. Cancer cells are known for their ability to survive in low nutrient environments by usurping EGFR signaling pathways.

*mTOR*

Hsp90, Akt, hTERT, p70 S6 kinase (S6K), and mTOR form a physical complex with one another (Fig. 2) [70]. This complex provides compelling evidence mTOR-mediated control of telomerase activity. Through kinase cascades, mTOR regulates cell size, progression of the cell cycle, and cell

survival, and is considered a master regulator of protein synthesis [97]. mTOR, a serine/threonine kinase that is often dysregulated in cancer cells, is a member of the PI3K-related kinase (PIKK) family [98]. By nucleating two different functional multi-protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), mTOR responds to nutrient, energy and oxygen stresses on the cell [99, 100]. The best characterized of these is the the mTORC1 homodimer complex that consists of mTOR, accessory protein RAPTOR (regulatory-associated protein of mTOR), mammalian lethal with Sec13 protein 8 (mLST8) (also known as GβL), PRAS40 (proline-rich AKT substrate 40 kDa), and DEP-domain-containing mTOR-interacting protein (DEPTOR) (Fig. 3) [99]. mTOR can alternatively associate with the complex involving RICTOR (rapamycin-insensitive companion of mTOR), mLST8, DEPTOR, PROTOR (protein observed with RICTOR), and mSIN1 (mammalian stress-activated protein kinase interacting protein); this complex is termed mTORC2 (Fig. 3) [99]. In addition to

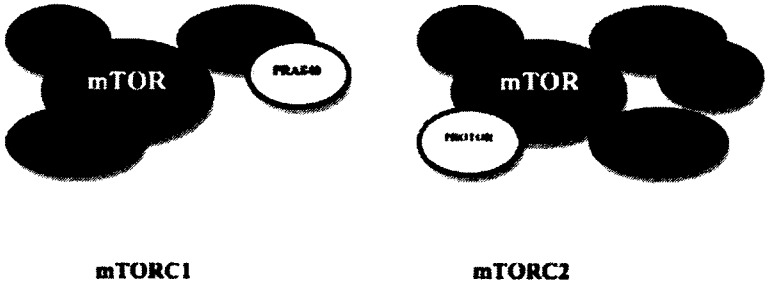


**Fig. 3.** Schematic representation of the mTORC1 and mTORC2 complexes. mTOR is known to nucleate two distinct protein complexes. The mTORC1 complex consists of mTOR, RAPTOR, PRAS40, DEPTOR, and mLST8. mTORC2 consists of mTOR, RICTOR, mSIN1, DEPTOR, PROTOR, and mLST8. Despite having a few proteins in common, these complexes are known to behave very differently from one another.

being necessary for the catalytic activities of mTOR, both RICTOR and RAPTOR help to recruit downstream targets to the complex [101]. mTORC1, touted for its nutrient sensing abilities, is associated with the predominant pathway by which mTOR controls cell growth and proliferation, the PIK3-Akt-mTOR pathway. It is now understood that mTORC2 has a role in this pathway by activating Akt through phosphorylation, creating a positive feedback loop [102]. mTORC2 is also known for its ability to direct actin remodeling [100]. The well-established functions of mTOR are due to mTORC1, as mTORC2 functions are just now beginning to be elucidated [103].

*Upstream Regulation of mTOR*

The PI3K-Akt-mTOR pathway is an important mechanism that allows communication between cellular and intracellular proteins responsible for growth and proliferation. When insulin binds its receptor on the cell membrane surface, a kinase cascade is initiated. The binding event signals insulin receptor substrate 1(IRS1) to the intracellular portion of the receptor [104]. IRS1 then activates the first kinase in this pathway, PI3K, which recruits Akt to the intracellular portion of the cell membrane [104-107]. Akt is then activated through both phosphoinositide-dependent protein kinase I (PDK1) and mTORC2. Akt in turn phosphorylates the GTPase activating protein (GAP)-tuberous sclerosis 2 (TSC2)-thereby deactivating the protein. In the absence of a growth factor such as insulin, the active TSC2 forms a complex with TSC1 that accelerates the exchange of Rheb (Ras homolog enriched in brain)-GTP (guanosine triphosphate) to Rheb-GDP (guanosine diphosphate). Rheb must be in the GTP form in order to directly stimulate mTORC1 [104, 105]. As a consequence of Akt phosphorylation, TSC2 is deactivated, thereby allowing mTORC1 activation through Rheb-GTP. Akt also phosphorylates PRAS40, an inhibitor of mTORC1, therefore deactivating it [108]. Although PRAS40 is a known binding partner of mTORC1, in the absence of insulin, PRAS40 functions to inhibit Rheb activation of mTORC1 [108].

**Translational Control: Regulation of eIF4 and p70 S6 Kinase**

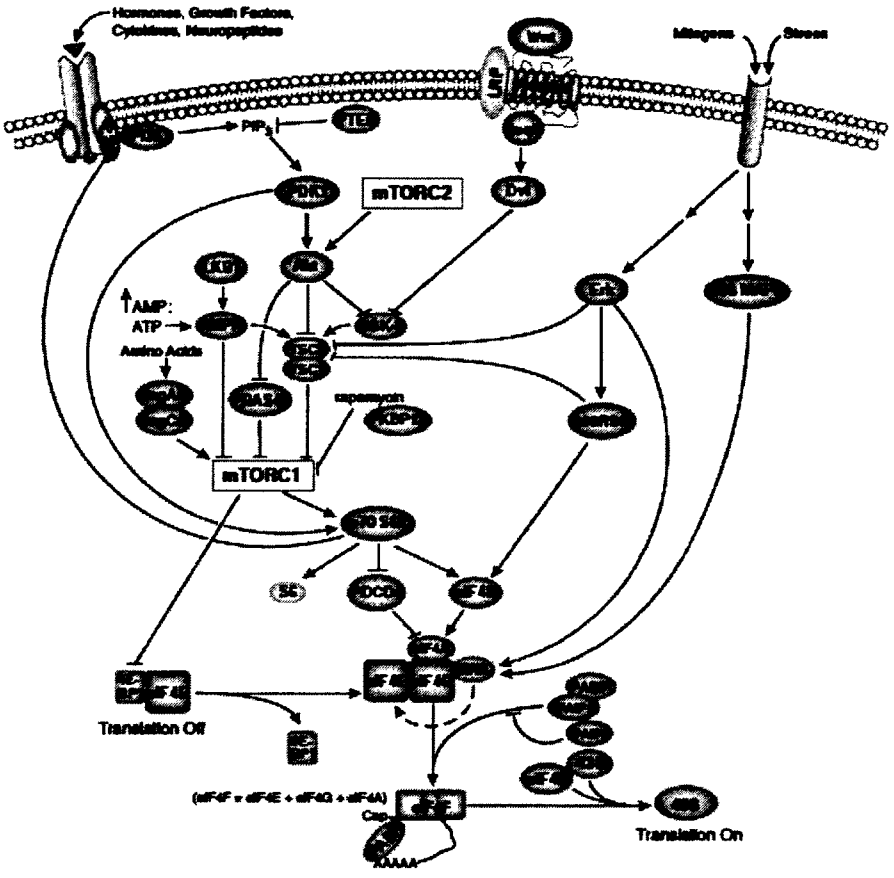


**Fig. 4.** The mTOR pathway. mTOR is considered a master regulator of protein translation. This pathway depicts the numerous proteins upstream and downstream of mTOR, which ultimately determines when cap-dependent translation is turned on or off. Illustration from Cell Signaling Technology, Inc., 2003-2010.

*Down-stream Targets of mTOR*

Mitogenic activation of mTORC1 increases cap-dependent translation initiation through phosphorylation of S6K and 4E-binding protein 1 (4E-BP1) (Fig. 4). mTORC1 activates S6K through the phosphorylation of specific sites on the

protein. Only the active or phosphorylated form of S6K can act as a kinase and phosphorylate S6, a ribosomal protein. S6 activation is necessary for translation of ribosomal proteins. Through a negative feedback loop, S6K also phosphorylates IRS1, therefore deactivating the protein necessary to initiate PI3K-Akt-mTOR pathway [109]. S6K also activates eukaryotic initiation factor (eIF) 4B, a protein responsible for activating eIF4A, a helicase necessary to unwind the 5' untranslated region (UTR). In addition, S6K inhibits programmed cell death protein 4 (PDCD4), a protein that inhibits eIF4A [110]. mTORC1 also acts as a kinase that directly phosphorylates 4E-BP1, an inhibitor of translation, resulting in dissociation of eIF4E. This allows the mRNA cap-binding protein to associate with the scaffold protein eIF4G [111]. The association of activated eIF4A with eIF4E and eIF4G completes formation of the 7-methylguanosine triphosphate (m7GpppX) cap-binding complex termed eIF4F [112]. Binding of eIF4F to the 5' cap of mRNA is followed by circularization of the mRNA, a process that occurs via interactions between eIF4G and the poly-A binding protein (PABP) [113]. The 43S complex, consisting of the 40S ribosomal subunit, eIF2/GTP/Met-tRNAi, and eIF3, binds to eIF4F via interactions between eIF3 and eIF4G forming the preinitiation complex [111]. This complex begins scanning the mRNA in a 5' to 3' direction from the mRNA cap. Once a translation initiation codon or AUG with the optimal consensus sequence is located, the pre-initiation complex is released and an initiating methionine is inserted into the aminoacyl site formed by association of the 60S ribosomal subunit with the 40S ribosomal subunit to form an 80S ribosomal complex. Subsequent elongation of the protein occurs though translocation of the nascent peptide to the peptidyl site of the 80S complex and formation of peptide bonds [111].

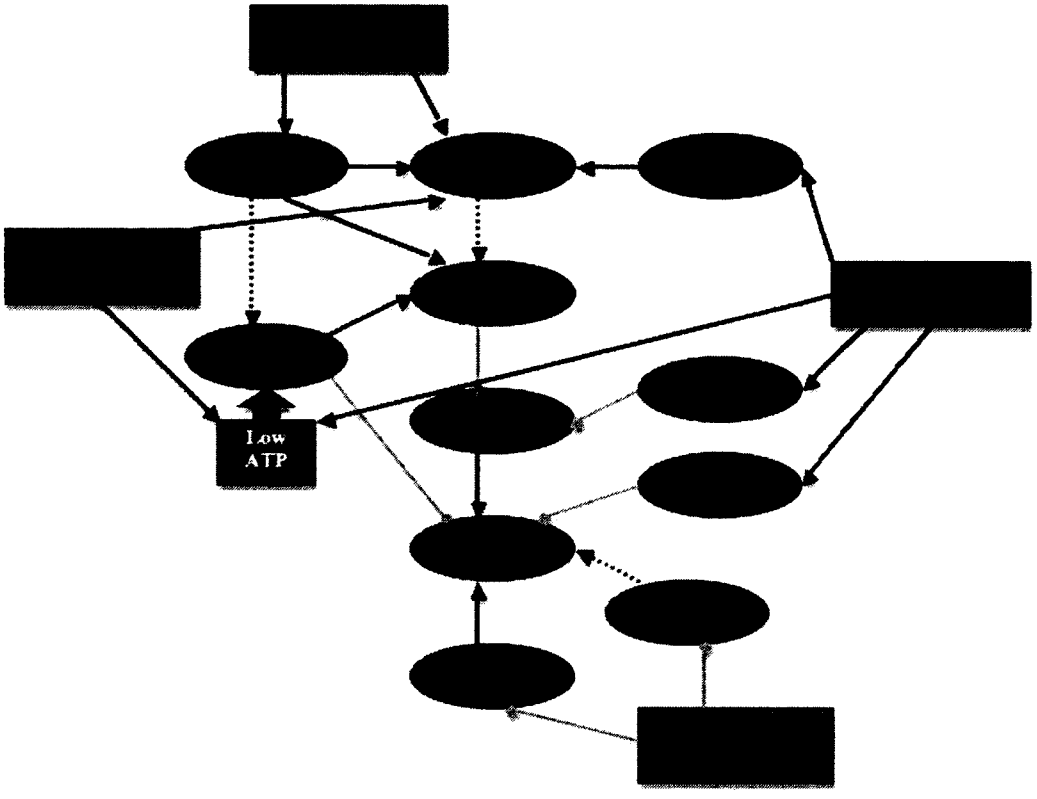


**Fig. 5.** How cellular stressors regulate mTOR activation. Activation of mTOR by growth factors is depicted as a black line. mTOR inhibition by a protein is depicted by a grey line. Pathways that have not been confirmed as a direct association between the protein and mTOR is shown as a dotted line.

Importantly, eIF4E is the rate-limiting component of the translation initiation complex [114]; its release from 4E-BP1 due to mTORC1 activation, controls protein translation. mRNAs with 5'-UTRs that are G-C rich have substantial secondary structure, and cannot compete for eIF4E as well as mRNAs without stable 5-UTR secondary structure [115-117]. Consequently, free eIF4E levels are tightly regulated through mitogenic activation of the mTOR signal transduction pathway [118]. Due to the ability of mTORC1 to inhibit an inhibitor

(4E-BP1) and activate a protein crucial to translation initiation (S6K), mTORC1 is a master regulator of protein synthesis [119].

In addition to its well-established function in modulating translation initiation, mTOR is responsible for cell growth regulation, transcriptional control, and autophagy repression [105]. mTORC1 mediates cell cycle regulation by increasing eIF4E levels sufficiently to initiate cap-dependent translation of cyclin D1 mRNA [120]. Conversely, in cells where mTORC1 is inactivated, translation initiation of cyclin D1 mRNA is suppressed sufficiently to arrest the cell in G1 [120]. The mTORC1 pathway also controls cell size and glucose homeostasis apparently through the activation of S6K, although the mechanisms are not yet clear [121]. S6K activation also increases the levels of survivin, an inhibitor of apoptosis [122]. mTOR-mediated autophagy repression is a result of a direct interaction of mTOR with the ULK (Unc-51-like kinase 1) complex necessary to initiate autophagy [105]. With assorted downstream targets of mTOR whose activation or repression can result in either survival or cell death, it is imperative to properly regulate the mTOR pathway to preserve normal cellular homeostasis.

*Cellular mTOR Inhibition*

Diverse cellular stressors inhibit the mTOR pathway through various mechanisms (Fig. 5) [97]. Protein translation occurs at a high adenosine triphosphate (ATP) cost for the cell. Therefore, shutting it down when the cell is stressed is imperative. DNA damage is one such cellular stress that activates both p53 and Redd1/RTP01 [123]. Through downstream signaling, both of these proteins activate TSC 1/2 and inhibit mTOR via Rheb [124]. Hypoxia is a well characterized stressor that signals activation of BCl2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), promyelocytic leukemia tumor suppressor (PML), and Redd1 via induction of HIF-1 [125]. BNIP3 inhibits Rheb independently of TSC 1/2; PML directly inhibits mTOR [125]. Hypoxia and glucose derivation lead to low ATP, a signal that activates 5'AMP-activated protein kinase (AMPK) [126]. AMPK inhibits mTOR directly and activates TSC 1/2 [126]. Glucose deprivation is third type of stress that activates Redd1, making Redd1 a critical activator of TSC 1/2. Interestingly, it has been shown

that cigarette smoke can also induce Redd1 [127]. Amino acid deprivation inhibits two activators of mTOR, GTPase Rag (Ras related GTPase) and Map4k3 (mitogen-activated protein kinase kinase kinase kinase 3) [128]. When active, Rag serves to bind RAPTOR and translocate mTORC1 to the surface of an endomembrane compartment, serving along with Rheb to activate mTORC1 [129]. The redundancy in pathways mediating mTOR inhibition highlights the importance of maintaining protein only under ideal cellular conditions.

*mTOR and Cancer*

The mTOR pathway is often constitutively active in cancer cells, leading to aberrant protein translation, cell growth, and proliferation. The tumor suppressor, phosphatase and tensin homolog (PTEN), is commonly deleted or truncated in endometrial, breast, prostate and ovarian cancers leading to overexpression of the PI3K-Akt-mTOR pathway [130]. A commonly observed mutation in *PIK3CA* in breast, colorectal, and endometrial cancers results in overexpression of Akt and increased transformation *in vitro* and *in vivo* [131]. The *AKT1* gene may also be amplified causing it to be overexpressed in some tumor cell types. *AKT1* mutations have been shown to constitutively activate the protein, as seen in some breast, colorectal and ovarian cancers [132-134]. Enhanced expression of the downstream target of mTORC1, *eIF4E*, by gene amplification is also regularly observed in tumor cells, leading to increased protein translation [135]. eIF4E-overexpression is an independent biomarker of cancer recurrence in head and neck cancers [136]. The array of mutations identified that lead to mTORC1 pathway upregulation in numerous diverse cancers support the role of mTOR as an attractive anti-cancer target.

*Rapamycin*

The classical mTOR inhibitor is the macrolide antibiotic, rapamycin (Fig. 6 (a)). The vast array of mTORC1 functions were revealed through the use of rapamycin. Rapamycin was discovered in *Streptomyces hygroscopicus* in the soil on Easter Island in the 1970s [137]; it was not until the 1990s that the cellular target of this drug, mTOR, was uncovered [99]. Rapamycin binds to the 12 kDa

immunophilin FK506-binding protein (FKBP12), forming a drug-receptor complex, which then binds to mTOR. This process weakens the association of mTOR with RAPTOR, thus inhibiting the kinase activity of mTOR [138-140]. Although mTORC2 is considered rapamycin-insensitive, it has been shown that prolonged treatment with rapamycin does inhibit mTORC2 assembly [141].

Cellular treatment with rapamycin causes decreased phosphorylation of 4E-BP1, therefore reducing the level of free eIF4E for translation initiation [142]. Rapamycin likewise attenuates the phosphorylation of S6K and S6 ribosomal protein [142]. It has been shown that rapamycin at 10 nM at 4 hr, a clinically relevant concentration [143], is capable of inhibiting the mTORC1 pathway, thus impeding cellular proliferation, growth and translation initiation [144, 145]. Analogs of rapamycin ('rapalogs') have been approved for use in several forms of metastatic cancers and continue to be evaluated in Phase III solid tumor studies.

*An mTOR and telomerase liaison*

An association between mTOR and telomerase activity was first proposed by Zhou et al. (2003) and later by Zhao et al. (2008) who noted that rapamycin,

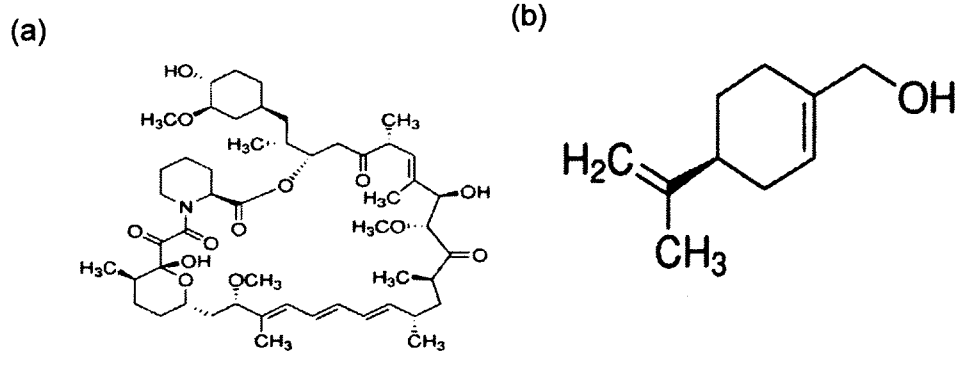


**Fig. 6.** Biochemical structures of rapamycin and perillyl alcohol. (a) Rapamycin; (b) Perillyl alcohol. www.SigmaAldrich.com

at high concentrations (100 - 1000 nM) and long treatment conditions (48 to 72 hr), inhibited both telomerase activity and *hTERT* mRNA levels [146, 147]. Effects on *hTERT* mRNA levels were somewhat surprising based on the known cellular target of mTOR—protein translation. It should be pointed out that the maximum tolerated dose of oral rapamycin administered to adult cancer patients on a daily basis has been reported at ~6 mg/d, which results in a maximal plasma concentration of ~22 nM [143]. hTERT co-immunoprecipitates with mTOR, S6K, Hsp90 and Akt, suggesting that these proteins form a physical and functional complex [70]. Bu et al. also proposed in 2007 [148] that mTOR signaling was 'coupled' to telomerase regulation. In contrast to the above two studies, Bu et al. showed that rapamycin (10 nM) had no effect on the *hTERT* promoter, but that hTERT protein levels were decreased; they concluded that hTERT regulation by rapamycin was post-transcriptional [148]. Researchers and clinicians may be able to take advantage of this relationship between mTOR and telomerase for effective combination therapy and/or cancer chemoprevention. Bu et al. (2007) also noted synergy in regards to down-regulation of telomerase activity between rapamycin and fluorouracil treatment of hepatocarcinoma cells in culture [148].

In addition, because telomerase activation/de-repression is an early event in cancer cell formation, the ideal chemopreventive would be present and active before cancer is detectable. Rapamycin itself has been touted as a new potent cancer chemopreventive agent [149, 150], but grave concerns about toxic side effects (immunosuppression), safety (feedback activation of Akt and enhanced tumorigenesis) and pharmacokinetic issues temper enthusiasm for this approach [151, 152]. The potential effectiveness of plant-derived dietary factors with relatively low acute toxicity [153] contrasts to that of rapamycin; consequently phytochemicals may represent a more feasible and effective method for cancer prevention. As described below, isoprenoids have been reported to modulate either mTOR signaling or telomerase activity. These data reported in this study collectively form the basis of our proposal that mTOR and telomerase regulation are coupled.

*Isoprenoids*

Isoprenoids (also designated as terpenes), found widely in fruits and vegetables, represent the largest group of natural products with ~25,000 structures reported [154, 155], and are recognized for their ability to suppress carcinogenic processes *in vivo* and *in vitro* [153, 156-158]. Isoprenoids are small, lipophilic products of the plant mevalonate biosynthetic pathway [153]. Isoprenoids are so-named because of their basic chemical structure consisting of multiples of an isoprene (5-carbon) subunit: monoterpenes consist of C10, sesquiterpenes, C15; diterpenes, C20 and others. In plants they function as repellents, attractants or toxins, and provide resistance to aphid infestations or fungal- and bacterial-caused diseases [154]. Isoprenoids are relatively non-toxic in human studies even at high doses [159].

Perillyl alcohol, a structurally simple monoterpene, is found predominantly in oils from cherries, cranberries, lavender, celery seed and spearmint (Fig. 6 (b)) [79, 160]. Perillyl alcohol and other related terpenes, such as farnesol and geraniol, exhibit chemopreventive and cytotoxic activity against a wide variety of cancer cell lines [153, 156, 158, 161]. Perillyl alcohol was found to act uniquely and mechanistically on protein translation through modulation of mTOR signaling pathway [145]. It specifically decreases 4E-BP1 phosphorylation and disrupts the eIF4F 5′- cap-binding complex [144, 145]. While perillyl alcohol has been shown to suppress the mTOR pathway, the mechanism appears to be distinct from that for rapamycin. In addition to the mTOR pathway, perillyl alcohol has the ability to inhibit other pathways associated with the cancer phenotype, such as cell growth, pRB phosphorylation and Cdk phosphorylation [162-165]. Perillyl alcohol can also counteract proliferation and initiates apoptosis and G1 arrest [165-170]. Although a small, natural compound, perillyl alcohol is becoming well-established as a cancer chemopreventive agent [153, 158].

*Rationale of Study and Specific Aims*

Prostate cancer is a leading cause of cancer death among men, second only to lung cancer. One in six males will be diagnosed with prostate cancer during their lifetime, with no effective cure available for those with advanced stages of the disease. hTERT, the limiting factor for telomerase activation, is de-repressed in prostate cancer cells and in part makes these cells immortal. It has been reported that hTERT forms a functional complex with mTOR, S6K, Hsp90 and Akt in activated natural killer cells (NK). mTOR is a master regulator of protein translation through its ability to release the rate limiting component of cap-dependent translation, eIF-4E, from 4E-BP1. The presence of mTOR in association with hTERT strongly signifies mTOR's involvement in regulating telomerase activity. The macrolide rapamycin effectively treats hormone-related cancers through its modulation of the mTOR pathway. A plant-derived isoprenoid perillyl alcohol also suppresses mTOR signaling; its mechanism of inhibition appears to be similar but distinct from that for rapamycin. Perillyl alcohol is potentially more effective at preventing prostate cancer than is rapamycin due to its small size, lipophilic nature and relatively low toxicity. Recent studies have shown that rapamycin at high concentrations and long incubations inhibits telomerase activity and *hTERT* mRNA levels. Currently, it is unknown if perillyl alcohol also modulates telomerase activity. Thus perillyl alcohol may be important clinically due to the documented immunosuppressive toxicity of rapamycin in cancer patients. Therefore, perillyl alcohol-mediated inhibition of hTERT represents a potential cancer chemopreventive or adjuvant for therapy.

Our long-term goal is to understand the mechanism by which isoprenoids are effective as chemopreventives. The objective of this research is to explore the relationship between perillyl alcohol, telomerase function and the mTOR pathway. *The central hypothesis is that perillyl alcohol regulates hTERT at the translational and post-translational levels via its effects on the mTOR pathway.* The hypothesis is supported by studies demonstrating similarity between the mechanisms of action of rapamycin and perillyl alcohol, and the known inhibitory

relationship between rapamycin and telomerase activity. The rationale supporting the proposed research is that elucidating novel mechanisms by which perillyl alcohol inhibits prostate cancer cell proliferation will enhance exploitation of this agent as a chemopreventive for prostate cancer. The central hypothesis will be tested and the objective of this proposal met through the following three specific aims:

**Specific Aim 1**. Establish the translational and post-translational effects of perillyl alcohol treatment on hTERT regulation in cultured human prostate cancer cells. Specifically, DU145 and PC3 cells will be treated with solvent, perillyl alcohol or rapamycin as a positive control, and:

A. Telomerase activity assessed using real-time polymerase chain reaction telomeric repeat amplification protocol (RTQ-TRAP) as well as non-denaturing polyacrylamide gel electrophoresis (PAGE);

B. SDS/PAGE and western blots performed to detect potential modulation of hTERT protein levels, phosphorylation status, cellular location and degradation; and

C. Co-immunoprecipitation with a RAPTOR or mTOR antibody, silver stained gel analysis, and western blots carried out to establish the presence of co-precipitating proteins in the hTERT-mTOR-RAPTOR protein complex in untreated cells and possible disruption of the complex by perillyl alcohol and/or rapamycin.

**Specific Aim 2**. Define the transcriptional effects, if any, of perillyl alcohol treatment on *hTERT* regulation in prostate cancer cells. DU145 and PC3 cells will be treated with solvent, perillyl alcohol or rapamycin as a positive control, and:

A. Real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR) utilized to measure isoprenoid effects on *hTERT* mRNA levels. *3-Hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase* mRNA will be included as a control; results will be interpreted using the $\Delta\Delta$ $C_T$ method.

**Specific Aim 3.** Examine telomerase activity, *TERT* mRNA and TERT protein levels in cells that over-express eukaryotic initiation factor 4E (eIF4E)–the rate-limiting factor for cap-dependent translation regulated by mTOR. Immortalized Chinese hamster ovary (CHO) cells transfected with an *eIF4E*-expressing vector or a control vector will be treated with solvent, perillyl alcohol or rapamycin as a positive control and:

A. SDS/PAGE and western blots performed to study the effect of elevated eIF4E on TERT, Akt, S6K, and 4E-BP1 protein levels and the phosphorylation status of Akt, S6K, and 4E-BP1;

B. Real-time RT-PCR conducted to assess the consequence of amplified eIF4E on *TERT* mRNA. *Actin* mRNA will be included as a control; and

C. RTQ-TRAP exploited to measure alterations in telomerase activity when eIF4E is over-expressed.

## SIGNIFICANCE

The proposed research is innovative, because a relationship between telomerase activity, hTERT and perillyl alcohol has not been explored or exploited previously. Our study is projected to first: identify and establish a translational and/or post-translational inhibitory relationship between perillyl alcohol and hTERT. This finding will allow us to determine the mechanism by which perillyl alcohol can be used as a cancer chemopreventive. Secondly, we expect to find that perillyl alcohol down-regulates hTERT through the mTOR pathway. *With this knowledge perillyl alcohol may be used as an adjuvant to current chemotherapy regiments that do not target the mTOR pathway.* By using a biologically relevant concentration of rapamycin for a reasonable period of time as a control, we will be able to determine the direct effects of mTOR inhibition on telomerase activity. The expected outcomes are foreseen to have an important

positive health impact because these findings will lead to novel cancer therapeutics/adjuvants and greatly advance the field of chemopreventives.

# CHAPTER II

# THE ISOPRENOID PERILLYL ALCOHOL INHIBITS TELOMERASE ACTIVITY IN PROSTATE CANCER CELLS

## INTRODUCTION

Nutritional epidemiologists emphasize that diet has a major role in the incidence and progression of prostate and other types of cancer [39, 153, 158, 171]. Isoprenoids (also referred to as terpenes), found widely in fruits and vegetables, represent the largest group of naturally occurring organic chemicals with ~25,000 structures reported [155], and are recognized for their ability to suppress carcinogenic processes *in vivo* and *in vitro* [172]. Perillyl alcohol is a small lipophilic isoprenoid found predominantly in oils from cherries, cranberries, lavender, celery seed and spearmint [79, 160]. Perillyl alcohol and other related terpenes, such as farnesol and geraniol, exhibit chemopreventive and cytotoxic activity against a wide variety of cancer cell lines [153, 156, 158, 161]. Additionally, perillyl alcohol alone or included as an adjuvant inhibited various tumor xenografts in mouse models including human pancreatic cancer cells [172-175]. Previous studies in our lab demonstrated that perillyl alcohol acts uniquely and mechanistically on protein translation through modulation of the mammalian target of rapamycin (mTOR) signaling pathway [145].

mTOR, a serine/threonine protein kinase, is a critical component of the phosphoinositide 3-kinase (PI3K)/Akt pathway. Through kinase cascades, mTOR regulates cell size, progression of the cell cycle and cell survival [119]. mTOR protein exists in two functional complexes: mTORC1 and mTORC2. mTORC1 regulates protein translation through phosphorylation and activation of p70 S6 kinase (S6K) and 4E-binding protein 1 (4E-BP1). Only the active form of S6K can subsequently phosphorylate S6, a ribosomal protein, which is necessary for ribosome assembly and protein translation. Phosphorylation of 4E-BP1, an inhibitor of translation, causes it to dissociate from eukaryotic initiation factor 4E (eIF4E) allowing translation to begin. Due to its ability to

inhibit an inhibitor (4E-BP1) and activate a protein crucial to translation initiation (S6K), mTORC1 is a master regulator of protein synthesis [119].

The classical mTOR inhibitor is the macrolide, rapamycin. Rapamycin treatment causes decreased phosphorylation of 4E-BP1. Unphosphorylated 4E-BP1 binds eIF4E efficiently and reduces cellular levels of eIF4E available for formation of the m7GpppX cap-binding complex–eIF4F. Rapamycin likewise reduces the phosphorylation of S6K and S6 ribosomal protein [142]. Perillyl alcohol displays similar properties to rapamycin, but is distinct, in that it suppresses 4E-BP1 phosphorylation in human prostate and colon tumor cells via mTORC1, with a similar or greater effect than that observed with rapamycin as detected by western blotting [144, 145]. Perillyl alcohol also disrupts the m7GpppX cap binding complex, eIF4F, by suppressing interaction of eIF4E with eIF4G [144, 145].

Recently, both mTOR and S6K were found to co-immunoprecipitate with human telomerase reverse transcriptase (hTERT), Hsp90 and Akt suggesting that these proteins form a physical and functional complex [70]. hTERT is one of three components of telomerase, a ribonucleoprotein complex responsible for adding 6 base pair (bp) repeats to the end of a chromosome to prevent loss of DNA during replication. This action is necessary due to the fundamental limitation of polymerization at chromosome ends. hTERT, the rate limiting enzymatic portion of telomerase, is a potential candidate for cancer therapy due to its absence in most normal somatic cells, but its re-activation in many tumor cells. Telomerase activation is an early and key event in the creation of tumor cells, and as such, is an important target in cancer prevention. The inclusion of mTOR and S6K in the hTERT complex is compelling evidence to support mTOR-mediated control of telomerase activity, and as such, we hypothesize that isoprenoids, such as perillyl alcohol, suppress telomerase activity. The effects of perillyl alcohol on telomerase activity are examined in this work.

## MATERIALS AND METHODS

*Cell lines and cell culture*

Human prostate cancer cell lines PC3 and DU145 (American Type Culture Collection, Manassas, VA) were maintained at 37°C in a humidified incubator with 5% $CO_2$ in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1X glutamine and 1X penicillin/streptomycin. Growth medium was changed every other day.

*Drug treatments*

Perillyl alcohol (Sigma-Aldrich, St. Louis, MO) was prepared in 100% ethanol; rapamycin (Cell Signaling Technology, Danvers, MA) was dissolved in 100% dimethyl sulfoxide (DMSO) [145]. PC3 and DU145 cells were plated at a concentration of $5x10^5$ cells per 60 mm plate in 3 mL medium and allowed to attach overnight. Cells were then treated with one of the following: 400 µM perillyl alcohol for 16 hr, 10 nM rapamycin for 4 hr, 0.1% DMSO for 4 hr or 0.1% ethanol for 16 hr as described by Peffley et al. [145]. In separate experiments, shorter incubations with either drug were also conducted to establish a time course of possible effects.

*Protein extraction for telomerase activity*

Cells were harvested using trypsin-EDTA (Gibco/Invitrogen, Carlsbad, CA) and then pelleted by centrifugation (500 x g) for 8 min. Cell pellets were washed with phosphate-buffered saline (PBS) and centrifuged again. Protein lysates were obtained by resuspending PC3 or DU145 cells in 200 µL of ice-cold 1X CHAPS lysis buffer per $1x10^6$ cells. The CHAPS lysis method was modified from that presented in Hou et al. and others [176-179]. CHAPS lysis buffer consisted of 0.5% 3-[(3-Cholamidopropyl)dimethylammonio]-l-propanesulfonic acid (CHAPS), 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA), and 10% glycerol. RNase inhibitor (New England Biolabs, Beverly, MA) (final concentration of 10 units/mL) and ß-mercaptoethanol

(BME) (final concentration of 5 mM) were added just prior to use. Cell lysates were incubated at 4°C for 30 min and then centrifuged at 12000 x g for 30 min at 4°C. The supernatant was collected, protein concentration was interpolated using a Bradford assay and extracts were stored at -86°C.

*RTQ-TRAP assay*

Telomerase activity was detected by performing real-time quantitative telomerase repeat amplification protocol (RTQ-TRAP) as derived from a protocol described by Hou et al. [178]. In detail, each 25 µL RTQ-TRAP reaction contained: 1X SYBR Green Master mix (50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM each dNTP, 3 mM $MgCl_2$, iTaq DNA polymerase at 0.05 units/µL) (Bio-Rad, Richmond, CA), 10 mM EGTA, 0.2 µg T4 gene protein (New England Biolabs), 0.35 µM TS primer (5'-AATCCGTCGAGCAGAGTT-3') (Tm 53.6°C) and 0.35 µM ACX primer [5'-GCGCGG$(CTTACC)_3$ CTAACC-3'] (Tm 66.4°C) [180], and 2.5 µg of protein extract. Primers were purchased from Integrated DNA Technology (IDT, Coralville, IA). All samples were analyzed in duplicate or triplicate in a 96-well plate on a Bio-Rad CFX96 thermal cycler, and 1X CHAPS buffer was included as a negative control. Reactions were incubated at 25°C for 20 min to allow for elongation of the TS primer by cellular telomerase. The PCR protocol began with a 95°C hot start to activate *Taq* polymerase, followed by 40 cycles at 95°C for 20 s, 50°C for 30 s, 72°C for 90 s [178], ending with a melt curve protocol. Telomerase activity was comparatively assessed based on threshold cycles ($C_T$) [178]. $C_T$ values greater than 35 were considered false positives due to primer dimers. Inhibition of telomerase activity was determined as follows:

$$(C_T \text{ treatment}) - (C_T \text{ control}) = \Delta C_T$$

$$(1 / 2^{\Delta C_T}) \times 100 = \%\ \text{ACTIVITY REMAINING}$$

*RTQ-TRAP product analysis by native Polyacrylamide Gel Electrophoresis (PAGE)*

TRAP products were also analyzed on a non-denaturing 10% polyacrylamide mini-gel (8.3 x 6.4 x 0.1 cm) (Bio-Rad) in 0.5X Tris-borate-EDTA (TBE) (50 mM Tris, pH 8.4, 44.5 mM boric acid, 0.5 mM EDTA) buffer. The protocol was derived from that presented by Dikmen et al. [181]. DNA 100-bp ladder markers (Bio-Rad) were included for size references. DNA products (~20 µL) were re-suspended 1X Nucleic Acid Sample Loading Buffer (Bio-Rad) and electrophoresed for 30 min at 200 volts. Gels were then stained with SYBR® Green I Nucleic Acid Stain (Invitrogen) per manufacturer's suggestions. In most cases gels were additionally stained with 0.01 mg/mL ethidium bromide for 10 min to obtain the clearest picture. Gels were photographed on a Kodak image station.

*RNA extraction and quantification*

Total cellular RNA was isolated using PureZOL™ (Bio-Rad) according to the manufacturer's protocol. RNase-free-DNase I digestion (50 units) (Roche Applied Science, Indianapolis, IN) was also performed. RNA quantification was determined by UV absorbance on a BioPhotometer Plus (Eppendorf®, Hamburg, Germany). RNA intactness was assessed by analyzing 28S and 18S subunits of ribosomal RNA by ethidium bromide 2% agarose gel analysis.

*hTERT mRNA levels*

Expression of *hTERT* mRNA was detected using real-time RT-PCR. One µg of purified cellular RNA from untreated and treated cells was reverse transcribed into cDNA using iScript (Bio-Rad) at 42°C for 30 min in the presence of random hexamers and oligo(dT), Moloney murine leukemia virus reverse transcriptase and RNasin. Analysis of the expression of *hTERT* mRNA was performed by real-time PCR amplification using the Bio-Rad CFX96 thermal cycler. PCR primer sets for *hTERT* cDNA (GenBank ID: AF015950) and *Homo sapiens 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase* cDNA (GenBank

ID: NM_000859) sequences were optimized using Beacon Designer software (PREMIER Biosoft International, Palo Alto, CA). PCR efficiencies for each primer set were determined in triplicate by a dilution series of the cDNA template. A master mix contained 1X SsoFast EvaGreen (Bio-Rad), 0.5 µM of each forward and reverse primer, and 1.5 µl of cDNA per reaction. A 140-bp *hTERT* fragment was amplified with the primer pair 5′-GAGTGTCTGGAGCAAGTTG-3′ (Tm 52.6°C, located in exon 3) and 5′-GGATGAAGCGGAGTCTGG-3′ (Tm 53.6°C, located in exon 4) (IDT). A 122-bp reductase amplicon was amplified as an internal control using the primer pair 5′-TGCAGAGCAATAGGTCTTGGTG-3′ (Tm 58.1°C, spans exons 13 and 14) and 5′-TCGAGCCAGGCTTTCACTTC-3′ (Tm 57.3°C, located in exon 14). The real-time PCR program used was 95°C for 30 s, followed by 35 cycles at 98°C for 30 s, 65°C for 30 s, ending with a melt curve analysis step where the temperature was reduced to 65°C, then increased to 95°C in 0.2°C increments every 5 s. The $\Delta\Delta C_T$ method was used to comparatively analyze the data as described in Livak and Schmittgen [182].

*Protein extraction and immunoblotting*

The protocol described by Peffley et al. [145] was used with minor changes. Briefly, cells were plated at $3x10^6$ per 100 mm culture dish and allowed to attach for 24 hr before treating with perillyl alcohol or rapamycin as above. Cells were lysed in 250 µL RIPA lysis buffer containing fresh 1X Complete protease inhibitor cocktail (Roche Applied Science), 1 mM $Na_3VO_4$ and 1 mM NaF, and protein concentrations were determined by a Bradford assay. Protein levels were assessed by western blotting according to [145] with some modifications. Thirty to 50 µg total protein per well were loaded on a 4-15% polyacrylamide TGX mini-gel (Bio-Rad) and resolved by electrophoresis in 1X Tris-glycine-SDS (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3) for 30 min at 200 volts. Electrophoretic transfer onto polyvinylidene fluoride (PVDF) membrane was performed in 1X Tris-glycine buffer. Membranes were blocked in protein-free bløk-CH™ buffer (Millipore, Temecula, CA) for 1 hr at room temperature and then incubated with gentle rocking in one of the following primary antibodies: mouse monoclonal anti-

eIF4E (1:250, clone P2, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal anti-hTERT (1:1000; clone Y182, Millipore), rabbit polyclonal anti-MKRN1 (Makorin-1) (1.25 µg/mL, Abcam, Inc., Cambridge, MA), mouse monoclonal anti-alpha-tubulin (1:250; clone 10D8, Santa Cruz Biotechnology, Inc.), or rabbit polyclonal anti-TERT (phospho S824) (1:1000, Abcam, Inc.).

Incubations were conducted overnight at 4°C and followed by extensive washes in 1X Tris-buffered saline (TBS)-Tween 20 (0.02M Tris, pH 7.4, 0.15 M NaCl, 0.05% Tween 20). HRP-conjugated secondary antibodies (goat anti-rabbit or goat anti-mouse [Santa Cruz Biotechnology, Inc.]) were diluted 1:5000 in bløk-CH™ buffer and incubated for 1 hr at room temperature along with 1 µL StrepTactin-HRP (Bio-Rad) per 10 mL solution to visualize Precision Plus protein standards (Bio-Rad). HeLa nuclear extract (25 µg, Millipore) was used a positive control for MKRN1 detection. Specificity of the hTERT antibody was confirmed by comparing band patterns from DU145 cell lysates (with re-activated TERT) to that of normal human XP30RO fibroblasts (TERT negative) (Coriell Institute, Camden, NJ). Anti-actin antibody (1:1000; clone H-300; Santa Cruz) was included to confirm equal protein amounts in both lanes. Subsequent membrane washes were as described [145] and then blots were incubated for 5 min in Immun-Star™ HRP detection system (Bio-Rad) before capturing images on a Kodak Image Station. Densitometric analysis of protein bands was conducted using Kodak Molecular Imaging Software version 4.0.4. Band intensities for proteins of interest were compared relative to the levels of eIF4E, which do not change in response to the above treatments and provide a control for loading and transfer onto PVDF membranes [145].

*Cytoplasmic and nuclear lysates*

DU145 cytoplasmic and nuclear lysates were obtained using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL) according to manufacturer's instructions. Briefly, $3x10^6$ DU145 cells were harvested using trypsin-EDTA, centrifuged at 500 x g for 5 min, rinsed with PBS and then centrifuged again. The supernatant was removed, and 300 µL of ice-

cold CER I solution was added. After the appropriate vortexing and incubation steps, 16.5 µL of CER II solution was added. Subsequent vortexing and centrifugation were performed following the manufacturer's protocol, resulting in a supernatant (cytoplasmic fraction) of DU145 cells and an insoluble pellet. The insoluble pellet was suspended in 100 µL of NER, vortexed and centrifuged, which produced a nuclear fraction in the supernatant.

*Statistical analysis*

All experiments were conducted three to five times with sub-sampling of each independent quantitative experiment. Data are expressed as a mean +/- standard deviation. Data were analyzed using Model I ANOVAs with SPSS version 19.0. The relationship of interest was perillyl alcohol versus control and rapamycin versus control; therefore, Dunnett's test was used as a follow-up to determine statistical significance of the results.

## RESULTS

*Telomerase activity is decreased by both perillyl alcohol and rapamycin*

Telomerase is necessary to maintain telomere lengths in over 90% of cancers, imparting them with immortality [36]. To examine effects of perillyl alcohol or rapamycin on prostate tumor cell telomerase activity, we performed RTQ-TRAP on protein extracts obtained from perillyl alcohol- or rapamycin-treated DU145 and PC3 cells. The drug treatment conditions used–400 µM perillyl alcohol for 16 hr or 10 nM rapamycin for 4 hr–were those shown previously to suppress 4E-BP1 phosphorylation in human prostate cancer cells and to inhibit cap-dependent translation via the mTOR pathway [144, 145]. Concentrations used also emulate plasma concentrations of the respective agents achieved in cancer patients [143, 159]. For reference, EtOH- or DMSO-treated control cell protein samples were also analyzed. RTQ-TRAP assay, used to assess telomerase activity, is preferred over previous TRAP methods due to its increased sensitivity and quantitative nature based on threshold cycle ($C_T$) values [178]. A one $C_T$ difference between two samples represents a twofold

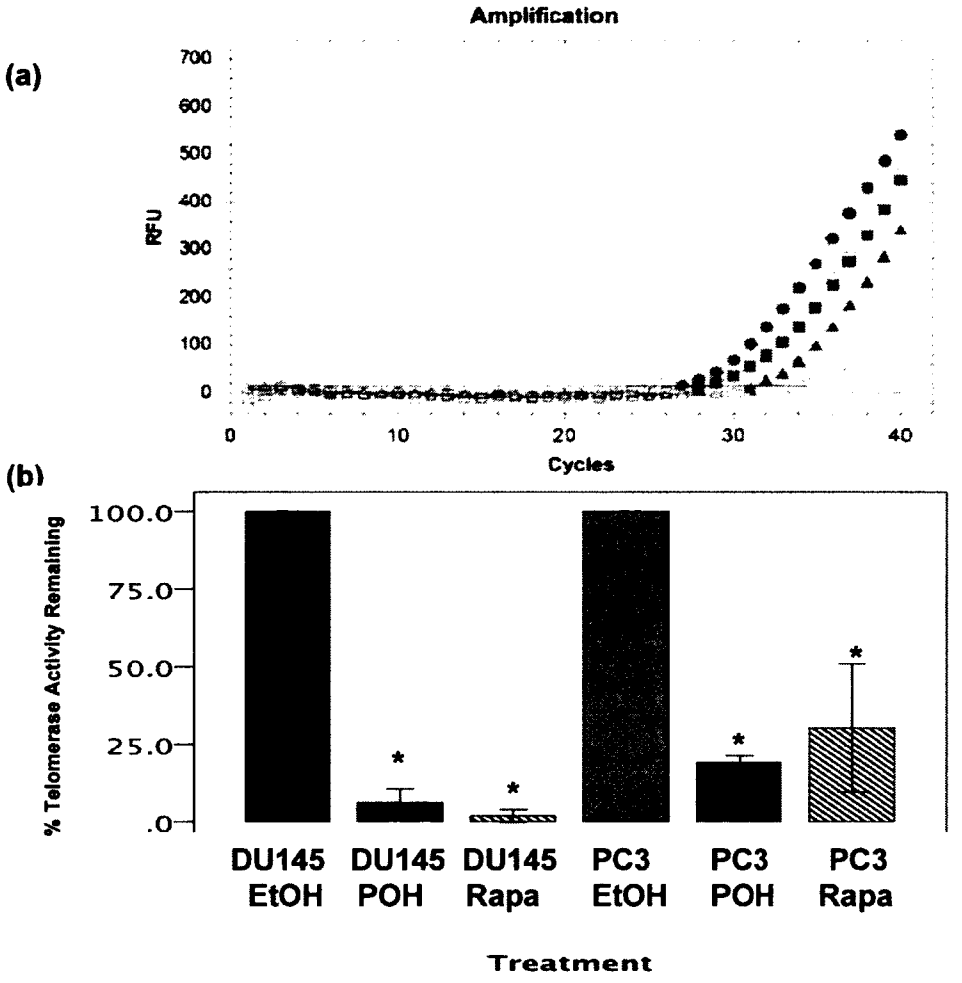


**Fig. 7.** Perillyl alcohol (POH) and rapamycin (Rapa) inhibit telomerase in DU145 and PC3 cells. **(a)** Relative levels of telomerase activity in prostate cancer cells with and without perillyl alcohol or rapamycin determined by RTQ-TRAP. Cells were treated with one of the following: 400 μM POH for 16 hr, 10 nM Rapa for 4 hr or 0.1% ethanol (EtOH) for 16 hr before lysis in CHAPS buffer. Open symbols: DU145 cells; solid symbols: PC3. ●,○: EtOH. ■,□: Rapa. ▲, Δ: POH. Representative results from DU145 and PC3 cell lines assessed by RTQ-TRAP. An increase in $C_T$ value indicates a decrease in telomerase activity. **(b)** RTQ-TRAP assays were run in triplicate and the resultant $C_T$ values for each treatment condition and cell line were averaged. Remaining telomerase activity was determined as described in Methods relative to EtOH-treated cells, which were set at 100%. Error bars shown represent the mean +/- standard deviation (SD) of three independent experiments; * represents significant differences as determined by ANOVA.

difference in starting material or in this case, a 50% reduction in telomerase activity; a $C_T$ value change of 3.3 represents a 10-fold difference or 90% reduction in activity. Fig. 7 (a) shows representative results from RTQ-TRAP experiments that were conducted on standardized protein concentrations at least three times. In the absence of reagents (EtOH only), DU145 cells (open circles) were found to have much greater telomerase activity than did PC3 cells (solid circles), i.e., the average $C_T$ value was lower for untreated DU145 for equivalent protein amounts than that found for untreated PC3 (24.7 and 27.4, respectively in the experiment depicted)–indicative of greater initial activity. Results from three separate experiments demonstrated that PC3 cells had ~17% of the telomerase activity found in DU145 cells. Disparate levels of telomerase activity have been noted previously for these two lines [183]. A large increase in $C_T$ values (due to loss of telomerase activity) was identified in perillyl alcohol (open triangles)- and

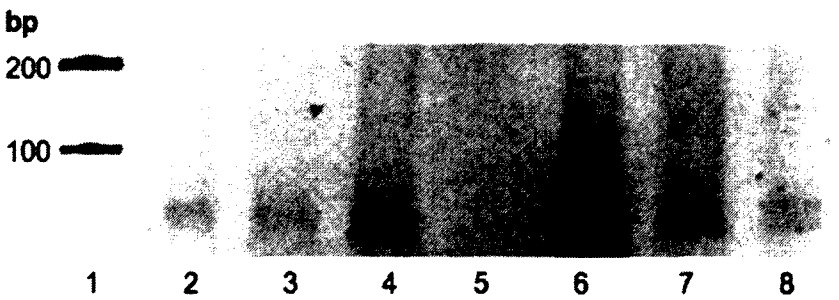


**Fig. 8.** Verification of telomerase activity in DU145 and PC3 cell extracts by PAGE. RTQ-TRAP generated amplicons were electrophoresed on a 10% non-denaturing polyacrylamide mini-gel that was subsequently stained in SYBR Green and ethidium bromide. Lane 1: 100-bp markers included as a size reference. Lane 2: Rapamycin-treated PC3. Lane 3: Perillyl alcohol-treated PC3. Lane 4: PC3 EtOH-treated control sample. Lane 5: No template control sample. Lane 6: DU145 EtOH-treated control sample. Lane 7: Perillyl alcohol-treated DU145 sample. Lane 8: Rapamycin-treated DU145 cells.

rapamycin (open squares)–treated DU145 samples relative to controls ($F= 1214.996$; $df= 2$; $p<0.0005$). Correspondingly, PC3 samples also showed attenuated telomerase upon perillyl alcohol (solid triangles) and rapamycin (solid squares) treatment compared to controls ($F=40.275$; $df=2$; $p<0.0005$). A Dunnett's test revealed the significant differences in both cell lines between perillyl alcohol and control treated sample (DU145 and PC3 $p<0.0005$), and between rapamycin and control treated samples (DU145 and PC3 $p<0.0005$). Resultant $C_T$ values for each treatment condition and cell line were averaged and remaining telomerase activity determined as described in Methods relative to EtOH-treated cells, which were set at 100% (Fig. 7, (b)). Perillyl alcohol and rapamycin inhibited telomerase activity in both cell lines. Treatment of PC3 cells with perillyl alcohol resulted in ~80% loss of telomerase activity; a 70% reduction was observed with rapamycin. Correspondingly in DU145 cells, both perillyl alcohol and rapamycin dramatically attenuated telomerase activity (~93% and 98%, respectively).

Telomerase activity results were confirmed by analyzing amplicons generated by RTQ-TRAP on non-denaturing 10% polyacrylamide gels as modified from Dikmen et al. [181] followed by SYBR Green/ethidium bromide staining (Fig. 8). The product from the no template control reaction was run on the gel to account for artifacts due to primer-dimer formation (Fig. 8, lane 5). RTQ-TRAP-generated amplicons produced a characteristic laddering pattern indicative of telomerase activity as seen in control lanes for PC3 and DU145 cellular extracts (Fig. 8, Lanes 4 and 6 respectively). Greater telomerase activity (i.e. number of bands) and band intensities were again apparent in the control DU145 lane relative to PC3. In perillyl alcohol-treated samples for PC3 and DU145 cells, there was a marked decrease in the intensity of the ladder (lanes 3 and 7, respectively), and an even further reduction in the intensity after rapamycin treatment (lanes 2 and 8 respectively), indicating decreased telomerase activity. Interestingly, we were able to detect the ladder using a mini-gel. This enables electrophoretic gel analysis in 30 min, versus the standard gel run of 2-4 hrs. The waning intensity of the ladders confirmed that low

**Table 1.**
**Time course of perillyl alcohol or rapamycin treatment effects on telomerase activity in DU145 cells.[a]**

| Condition | 1 hr | 2 hr | 4 hr | 8 hr | 16 hr |
|---|---|---|---|---|---|
| POH | ---------- | ---------- | 82.8 +/- 19.9 | 86.5 +/- 16.4 | 92.5 +/- 12.8 |
| Rapamycin | 88.2 +/- 4.5 | 92.2 +/- 5.4 | 98.3 +/- 1.8 | ---------- | ---------- |

[a]Percent reduction in telomerase activity relative to control untreated cells. Each value represents the mean of three independent replicates +/- standard deviation. POH = Perillyl Alcohol. Statistical significance of perillyl alcohol treatment vs. control or rapamycin treatment versus control was assessed using ANOVA with a Dunnett's posthoc test.

concentrations and short exposure times of perillyl alcohol or rapamycin were sufficient to decrease telomerase activity in DU145 and PC3 cells.

The remarkable loss of telomerase activity after relatively brief incubations with either perillyl alcohol or rapamycin led to examination of even shorter treatment times to gain insights into their respective mechanisms of modulating telomerase. In addition, because DU145 cells had significantly greater amounts of telomerase activity compared to PC3, all additional studies focused on DU145. Cells were treated with rapamycin for 1 or 2 hr, or perillyl alcohol for 4 or 8 hr, after which they were lysed and assayed with RTQ-TRAP. Rapamycin markedly arrested telomerase activity by ~88% +/- 4.5% (SD) within 1 hr (Table 1). Although much smaller and more simple structurally than rapamycin, perillyl alcohol likewise exerted substantial inhibitory effects at 4 hr and reduced telomerase by ~83% +/- 19.9% (SD); at 8 hr incubation, telomerase activity was attenuated by ~87% +/- 16.4% (SD). The rapid inhibition of telomerase activity by perillyl alcohol and rapamycin provides insights for their mode of action.

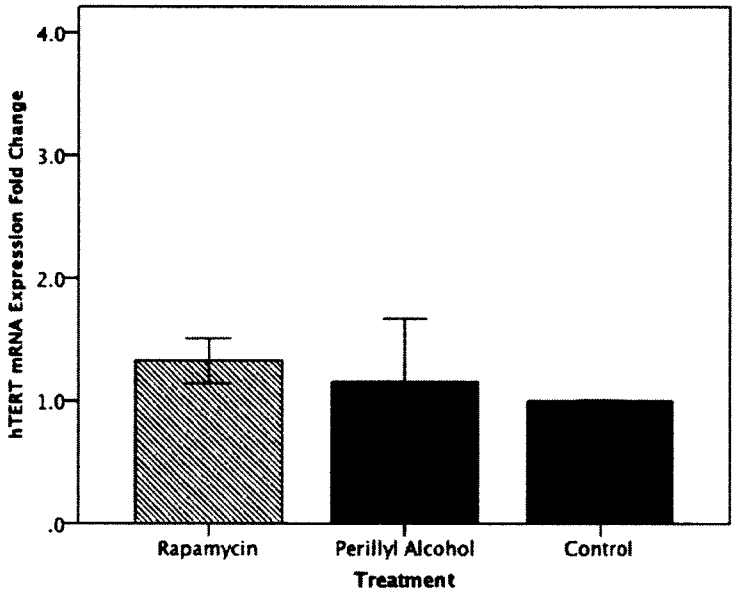


**Fig. 9.** Quantitative real-time RT-PCR analysis of *hTERT* mRNA levels in DU145 cells treated with perillyl alcohol or rapamycin. *hTERT* mRNA expression was determined based on $C_T$ values. Levels were normalized to the expression of *HMG CoA reductase* and expressed as fold change. No significant difference is detected when comparing treatment groups to control. Results are shown as a mean +/- SD of at least three experiments.

*Neither perillyl alcohol nor rapamycin alters hTERT mRNA levels*

Telomerase activity is regulated at both transcriptional and translational levels, although transciptional processes have been identified as the critcal governing factor [44]. mRNA levels of *hTERT*, the rate limiting component of telomerase, correlate to telomerase activity [184]. Rapamycin–at high concentrations and/or for long periods (48 to 72 hr)–has been reported to decrease *hTERT* mRNA levels [146, 147]. Likewise, 24 hr cellular treatment with the isoflavone genistein reduced *hTERT* mRNA expression [185]. Using real-time RT-PCR, we examine if telomerase activity was altered by perillyl alcohol due to changes in *hTERT* mRNA levels, and to verify the reported effects of

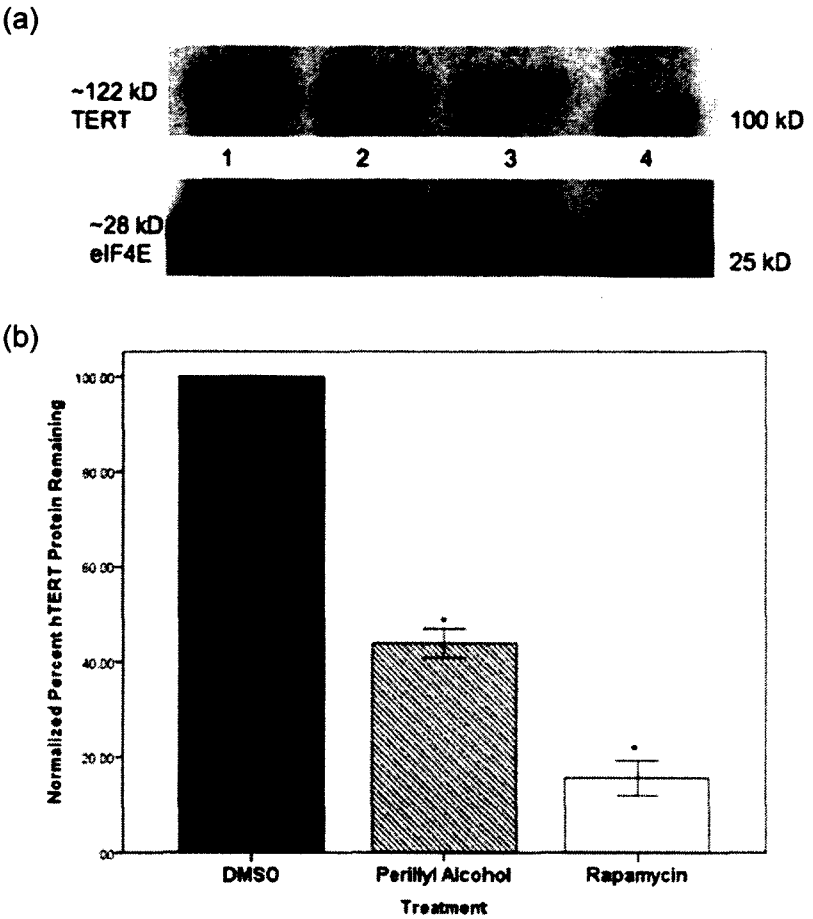


**Fig. 10.** Western blot analysis to detect hTERT protein levels in DU145 cells following perillyl alcohol or rapamycin treatments. **(a)** Lanes 1-3 were loaded with 50 µg of DU145 total protein. Lane 1: control DMSO-treated sample. Lane 2: 400 µM perillyl alcohol for 16 hr. Lane 3: 10 nM rapamycin for 4 hr. Lane 4: Precision Plus Protein™ WesternC™ Standards. Membrane in the top panel was probed with an anti-hTERT antibody. **(b)** Depiction of the western blot produced by probing with anti-eIF4E antibody as a loading control. B. Histogram representation of four independent experiments +/- 1 standard deviation.

rapamycin (Fig. 9). An internal control gene was included–*HMG-CoA reductase*–whose mRNA level does not change in response to perillyl alcohol and rapamycin [145]. Real-time RT-PCR analysis showed that at the pharmacologically relevant levels used during this study [143, 159], neither perillyl alcohol (0.4 mM, 16 hr) nor rapamycin (10 nM, 4 hr) produced a significant decrease in their respective average $C_T$ values in DU145 cells as compared to untreated controls when normalized to *HMG-CoA reductase* levels as described in Livak and Schmittegn ($F= 0.219$; $df=2$; $p= 0.808$) [182]. Thus, neither perillyl alcohol nor rapamycin–under the conditions used here–appear to regulate telomerase activity at the transcriptional level.

*hTERT protein levels diminish after perillyl alcohol or rapamycin incubation*

Additional levels of telomerase regulation due to post-translational processes such as hTERT protein phosphorylation, cellular localization and/or degradation are known [43]. It was hypothesized that a direct translational effect due to suppressed 4E-BP1 phosphorylation and therefore decreased hTERT protein synthesis by either compound was likely. Further, due to the rapid rate of reduction observed in the above studies, telomerase activity inhibition may be additionally modulated at a post-translational level. Western blot analysis was then conducted to assess possible hTERT protein level changes in response to treatments with perillyl alcohol or rapamycin. After transfer onto PVDF, membranes were incubated with either rabbit anti-hTERT (detects ~122 kDa protein) or mouse anti-eIF4E (~28 kDa protein) as a control for loading (Fig. 10 (a)). Densitometric analysis was performed on the western blot bands; results from four independent experiments are shown (Fig. 10 (b)). Bands resulting from the DU145 protein lysates treated with either perillyl alcohol or rapamycin were compared to the band intensity resulting from the control-treated sample (lane 1, Fig. 10 (a)). Perillyl alcohol treatment reduced hTERT protein levels by ~30% (lane 2), whereas rapamycin treatment (lane 3) caused a ~65% reduction in hTERT protein levels. Four independent experiments produced similar results with ~40% loss of hTERT protein after perillyl alcohol treatment, and ~76% loss

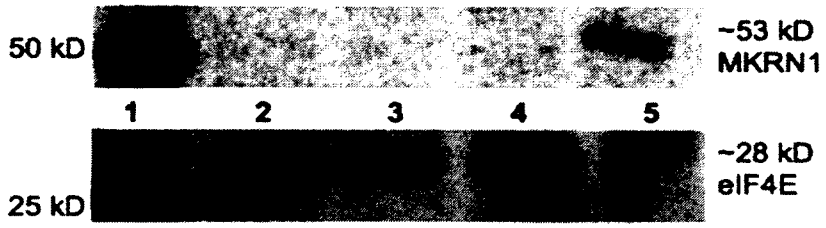


**Fig. 11.** Western blot analysis to detect MKRN1 expression after perillyl alcohol or rapamycin treatment. Lanes 2-4 were loaded with 50 µg of total protein from DU145 cells. Lane 1: Precision Plus Protein™ WesternC™ Standards. Lane 2: Rapamycin-treated sample. Lane 3: Perillyl alcohol - treated sample. Lane 4: Control (DMSO)-untreated sample. Lane 5: HeLa nuclear extract (25 µg) was run as a positive control. Membranes were probed with anti-MKRN1 (top); antibody against eIF4E was used as a loading control (bottom).

of protein after rapamycin ($F=63.893$; $df= 2$; $p<0.0005$). A Dunnett's test further revelead that loss of hTERT protein caused by either perillyl alcohol or rapamycin were both highly significant ($p<0.0005$). Thus, perillyl alcohol and rapamycin lowered hTERT protein levels with short exposure times and at biologically relevant concentrations. This significant attenuation of hTERT protein by either agent supports an effect on protein translation mediated by the mTOR pathway.

*Degradation of hTERT by Makorin-1 does not appear to contribute to loss of hTERT protein*

Degradation of hTERT is controlled by Makorin-1 (MKRN1)–an E3 ligase that mediates ubiquitination of hTERT for proteasome processing [74, 75]. Cancer cells typically contain low MKRN1 levels [75]. However, MKRN1 mRNA and protein levels rise dramatically in G1 arrest to signal the proteolytic breakdown of hTERT [75]. Perillyl alcohol and rapamycin treatment cause G1

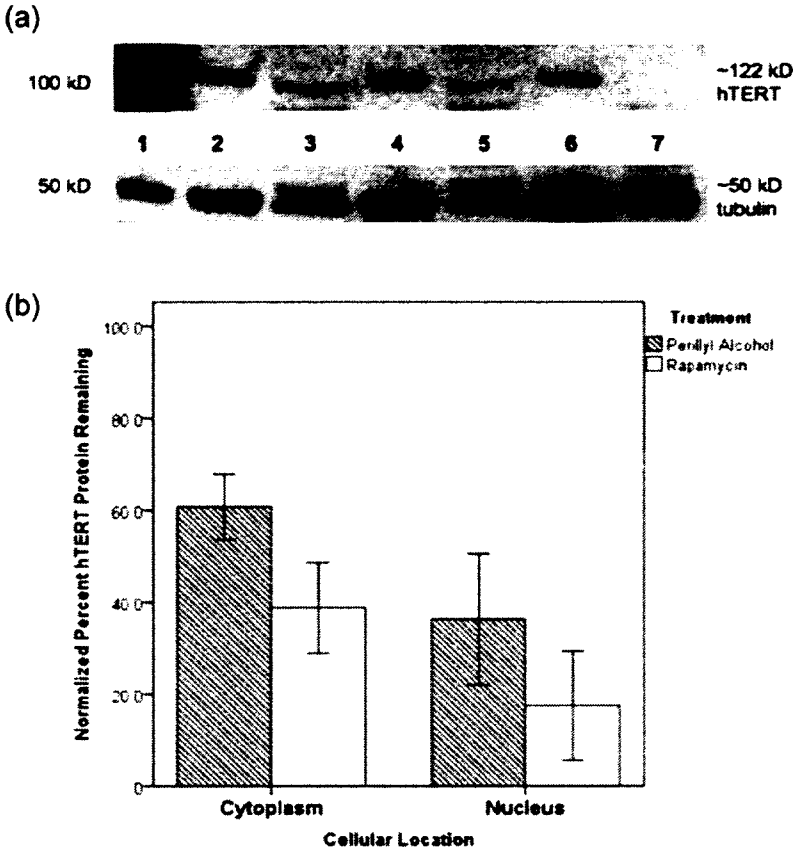


**Fig. 12.** Western blot analysis to detect cellular localization changes after perillyl alcohol or rapamycin treatment. **(a)** Lanes 2, 4, and 6 were loaded with 40 µg of cytoplasmic protein from DU145 cells. Lanes 3, 5, and 7 were loaded with 40 µg of nuclear protein from DU145 cells. Lane 1: Precision Plus Protein™ WesternC™ Standards. Lanes 2 and 3: Control (EtOH)-untreated samples. Lanes 4 and 5: Perillyl alcohol-treated samples. Lanes 6 and 7: Rapamycin-treated samples. Membranes were probed with anti-TERT antibody (Top); antibody against alpha-tubulin was used as a loading control (Bottom). **(b)** Histogram representation of four independent experiments +/- 1 standard deviation.

arrest in DU145 cells and a concomitant increase in $p21^{cip}$ levels [186]. Therefore western blotting was used to examine whether MKRN1 levels increased after either treatment, which would cause the ubiquitination and degradation of hTERT (Fig. 11). MKRN1 protein was not detected in control-treated DU145 cells (Fig. 11, lane 4). Furthermore, no increase was noted with perillyl alcohol or rapamycin treatment (lanes 3 and 2, respectively). MKRN1 protein was observed in the control lane (lane 5) with 25 µg nuclear extract from HeLa cells, which are reported to have low but measurable MKRN1 levels [75]. Longer exposures (20 min vs. 2 to 10 min) likewise did not reveal MKRN1 protein bands in DU145 cellular extracts.

*Cellular localization of hTERT and phosphorylation status*

Telomerase activity is also dependent on phosphorylation and nuclear localization of the hTERT protein [63]. Telomerase activity is thought to be modulated through phosphorylation of the hTERT subunit at Ser824 (and other sites such as Ser1125 and Ser227) by Akt protein kinase [61] and by various protein kinase C isoenzymes [67, 187, 188]. Conversely, decreased phosphorylation of Ser 824 with concomitant phosphorylation of Tyr707 is associated with hTERT export out of the nucleus into the cytoplasm [73, 189].

To compare the effects of perillyl alcohol and rapamycin on hTERT cellular distribution, nuclear and cytoplasmic fractions were separated and hTERT levels in both fractions analyzed by western blotting (Fig. 12 (a)). hTERT protein was found in both the nucleus and cytoplasm of untreated DU145 cells, with greater protein in the cytoplasm. In response to isoprenoid or rapamycin treatment, no appreciable redistribution of hTERT protein from the nucleus into the cytoplasm was detected. Instead, protein levels in both fractions decreased relative to their counterpart control amounts. With the caveat that western blots are only semi-quantitative, densitometric analyses of band intensities relative to alpha-tubulin (loading control) from four experiments revealed that in response to perillyl alcohol, nuclear hTERT protein levels decreased by ~52 %, and that in the

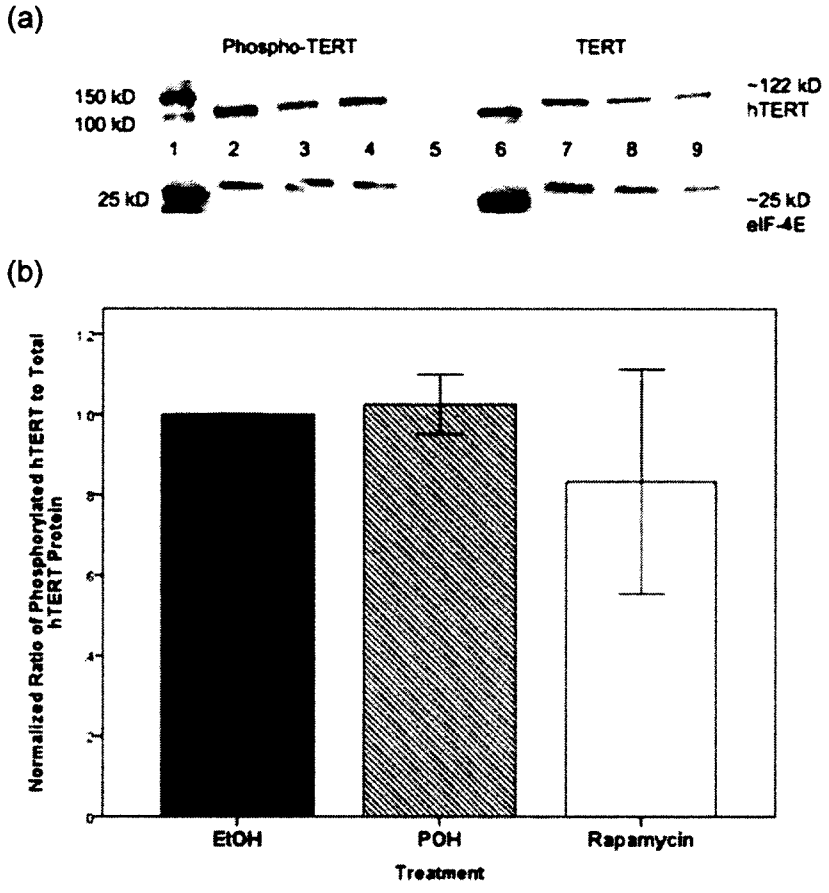


**Fig. 13.** Western blot analysis to detect phosphorylation changes after perillyl alcohol or rapamycin treatment. **(a)** Lanes 2-4 and 7-9 were loaded with 50 µg of total protein from DU145 cells. Lanes 1 and 6: Precision Plus Protein™ WesternC™ Standards. Lanes 2 and 7: Control (EtOH)-untreated samples. Lanes 3 and 8: perillyl alcohol-treated samples. Lanes 4 and 9: Rapamycin-treated samples. Lane 5 was loaded with running buffer. Membranes were probed with anti-phospho (Ser824)-TERT (Top left); anti-TERT (Top right); antibody against eIF4E was used as a loading control (bottom). **(b)** Histogram analysis of five independent experiments +/- 1 standard deviation.

cytoplasmic fraction decreased by ~40 % (F=0.493; df=1; p=0.505) (Fig. 12 (b)). Rapamycin treatment mimicked this trend. hTERT protein in nuclear fractions declined by ~77 % compared to that in the cytoplasm, which was reduced by ~61 % (F=0.151; df=1; p=0.709). In both situations, the respective reductions in nuclear and cytoplasmic hTERT protein levels were not significantly different than the total loss of protein observed after either treatment. The phosphorylation status of hTERT Ser824 in response to either treatment condition was also assessed (Fig. 13 (a) and (b)). The dephosphorylation pattern followed the reduction in hTERT protein levels. Results from four experiments showed that perillyl alcohol treatment caused a ~41 % decrease in hTERT protein and a ~56 % loss of hTERT (Ser824) phosphorylation (F=1.048; df=1; p=0.336) (Fig. 13 (b)). Rapamycin caused a ~60 % reduction in hTERT protein and ~46 % loss in Ser824 phosphorylation (F=2.315; df=1; p=0.167)(Fig. 13 (b)). There is no significant difference in the the decrease in hTERT Ser824 phosphorylation with either perillyl alcohol or rapamycin treatment versus the loss of hTERT protein observed. Hence the loss of telomerase activity is not due to a change in Ser824 phosphorylation or due to export of the protein from the nucleus.

## DISCUSSION

The rate-limiting component of the telomerase holoenzyme, hTERT, is regulated by both transcriptional and post-transcriptional factors, such as mRNA levels, cellular localization of hTERT protein, hTERT phosphorylation and protein degradation [43]. Somatic cells and normal cells in culture do not have detectable levels of *hTERT* mRNA, although the RNA component–*hTERC*–is present [45]. Eventually normal cultured cells enter into crisis; all but a few will succumb to apoptotic death [46]. The few cells that survive crisis become immortalized as detected by a surge in *hTERT* mRNA levels [45]. Comparable transcriptional derepression of *hTERT* is observed in patients' tumor cells relative to adjacent normal tissues [45]. The promoter region of the *hTERT* gene has multiple binding sites for a vast array of transcription factors, providing clues to

the extent of regulatory complexity [47]. Specifically, two Myc/Max binding sites (E-boxes) have been identified in the *hTERT* promoter, and c-myc directly activates *hTERT* transcription [48-50]. It is well known that c-myc activation is an early event in nearly all cancers as is telomerase activation. However, after *hTERT* transcription has been de-repressed, molecular failsafes remain to squelch the pro-cancer activities of a cancer cell apprentice. Posttranscriptional control of hTERT becomes the cell's last resort to forestall telomerase activation and therefore immortalization.

Results of this study demonstrate that both perillyl alcohol and rapamycin attenuate telomerase activity without altering *hTERT* mRNA levels. The ability of perillyl alcohol–a structurally simple plant monoterpene–to inhibit telomerase activity has not been previously reported. These findings indicate that effects on *hTERT* core promoter by either perillyl alcohol or rapamycin are not involved in repression of telomerase activity. In addition, *hTERT* mRNA stability is an unlikely target because mRNA levels did not change within the time-frame studied. Thus perillyl alcohol and rapamycin appear to act as sentinels to counter increased *hTERT* mRNA levels. These results with perillyl alcohol contrast to those reported for genistein–a soybean-based natural product–and other isoflavones or plant products that appear to regulate telomerase transcriptionally [65, 161, 185, 190]. Likewise, epicatechins found in green tea have been reported to down-regulate *hTERT* mRNA levels in carcinoma cells [191]. Zhao (2008) and Zhou (2003) also found decreased *hTERT* mRNA levels with high concentrations (100 - 1000 nM) and long incubation times (48 to 72 hr) of rapamycin [146, 147]. Others have established that genistein depletes telomerase via an epigenetic mechanism–through site specific hypo-methylation at an E2F-1 binding site in the *hTERT* promoter [60, 188].

In the absence of *hTERT* mRNA inhibition, we proposed that perillyl alcohol- and rapamycin-mediated loss of telomerase activity were likely due to translational (hTERT protein synthesis) or a combination of translational and post-translational mechanisms such as phosphorylation, nuclear translocation or degradation of the hTERT protein. Our experiments clearly demonstrated that

both agents instigated a significant loss of hTERT protein. The half life of hTERT has been reported at ~ 6 to 12 hr depending on the cell type studied [178, 192]. Protein loss was observed at 4 hr treatments, therefore it could be due in part to inhibition of translation initiation and protein turnover attributed to effects of either compound on S6 kinase and/or 4E-BP1. Within this work, protein turnover cannot be ruled out or distinguished from degradation. Importantly the findings that rapamycin, the classical mTOR inhibitor, or perillyl alcohol, an isoprenoid with known anti-mTOR effects, both decrease hTERT protein provides compelling evidence that perillyl alcohol or rapamycin-mediated control of telomerase is due to translational mechanisms mediated by the mTOR pathway.

Likewise our findings are noteworthy in that biologically relevant concentrations and brief exposure times for perillyl alcohol or rapamycin were used: 0.4 mM for 16 hr, and 10 nM for 4 hr, respectively. Under these conditions, suppressed phosphorylation of a downstream target of mTOR (4E-BP1), disruption of the m7GpppX cap binding complex eIF2F and/or inhibition of cap-dependent translation have been observed [144, 145, 193]. Shorter incubation times with either agent likewise resulted in attenuated telomerase activity. Thus the cellular effects are very rapid. In contrast, virtually all of the studies cited above-in which *hTERT* mRNA effects were observed–were performed for 24, 48, or 72 hrs. We believe the transcriptional effects on hTERT may be due to the secondary effects of shutting down cap-dependent translation through the mTOR pathway for a prolonged period of time. It should be emphasized that the maximum tolerated dose of oral rapamycin administered to adult cancer patients on a daily basis has been reported at ~6 mg/d, which results in a maximal plasma concentration of ~22 nM [143]. Correspondingly, the perillyl alcohol concentration used in this study emulates plasma concentrations of perillic acid--the main metabolite of perillyl alcohol detected in patients [159, 194]. The suprapharmacological rapamycin concentrations used in the above two *in vitro* studies and/or prolonged incubations are unwarranted, and effects observed likely reflect accumulating damage to cellular components, secondary metabolic effects and possibly autophagy [195]. In addition, drug studies conducted with

unrealistic concentrations may disguise the true cellular pathways modified or provide artifactual results [196, 197].

Moreover, western blot results revealed that MKRN1– the E3 ligase known to target hTERT for degradation- was not up-regulated in perillyl alcohol– and rapamycin-treated cells, a result somewhat surprising based on several earlier reports linking hTERT degradation to MKRN1 [74, 75, 192]. However, the findings should have been anticipated to some extent. Although previous studies found that MKRN1 mRNA and protein levels rose dramatically (~6 fold) after either cell cycle arrest was induced [75] or a chaperone inhibitor geldanamycin was used [74], the enhanced MKRN1 protein levels occurred over a period of 6 to 12 hrs [75]. Effects observed with both perillyl alcohol and rapamycin were much faster in the present experiments, in which diminished telomerase activity was measured 1 or 2 hr post-rapamycin incubation or after a 4 hr perillyl alcohol treatment. Due to the rapid decrease in protein observed in the current experiments, ubiquitination and proteasome breakdown of hTERT may indeed still have a role, but it is unlikely to involve MKRN1. Lee and Chung (2010) and Kim et al. (2005) have proposed that hTERT degradation may be mediated additionally by Hsp90/Hsp70-associated U-box ubiquitin ligase CHIP or other unidentified E3 ligases [74, 192].

The rapidity at which a decline in telomerase activity was detected with rapamycin (1 hr) or with perillyl alcohol (4 hr) incubation led us to explore supplementary telomerase activity regulation at a post-translational level. Intracellular reshuffling of hTERT contributes to post-translational regulation of telomerase activity [63]. hTERT redistribution between the nucleus and cytoplasm with nuclear localization is thought to be essential for telomerase activity [63, 66]. However, our findings show that neither perillyl alcohol nor rapamycin causes cellular redistribution of hTERT. Interestingly, the average of the cytoplasmic and nuclear loss of hTERT is approximately equivalent to the total loss of hTERT seen in the previous studies (data not shown).

As a corollary to the cellular localization studies, hTERT phosphorylation status was examined to confirm the absence of hTERT redistribution.

Phosphorylation events have been linked to hTERT exit from or entrance into the nucleus. In accordance with the above results, we did not detect a change in phosphorylation of hTERT (Ser824), which has been shown to be targeted by Akt [61]. Furthermore, our findings are consistent with previous observations that neither perillyl alcohol nor rapamycin suppressed Akt (Ser473) phosphorylation in prostate cancer cell lines [145]. Conversely, agents that do affect Akt kinase activity, such as a PI3-kinase inhibitor wortmannin, inhibit telomerase activity and downregulate hTERT protein phosphorylation [61]. Jagedeesh et al. (2006) also reported that 50 μM genistein treatment of DU145 cells for three days decreased phosphorylation of Akt (Ser473) concomitant with a reduction of hTERT protein phosphorylation (non-specific serines) [65]. Likewise, hTERT translocation into the nucleus was prevented. Clearly a change in phosphorylation status did not contribute to the rapid loss of telomerase activity that we observed with our short, biologically relevant treatment parameters.

Rapamycin has been touted as a new potent cancer chemopreventive agent [149, 150], but concerns about toxic side effects (immunosuppression), safety (feedback activation of Akt and enhanced tumorigenesis) and pharmacokinetic issues temper enthusiasm for this approach [151, 152]. The potential efficacy of naturally occurring dietary factors with relatively low acute toxicity [153] contrasts to that of rapamycin; consequently, isoprenoids represent a more cogent and effective method for cancer prevention. Perillyl alcohol exhibits clear and definitive effects on a distinct signaling pathway–mTOR–that has a strong, critical clinical role as well as a role in cell proliferation. Dietary compounds such as isoprenoids likely contribute to chemoprevention by inhibiting a slight growth advantage (or hyperproliferation) in an early stage or pre-malignant cell, and there is evidence that metastasis and angiogenesis are also targeted [198]. In this study we have uncovered an additional target–telomerase activity–that may be paramount in the chemopreventive capacity of perillyl alcohol. All cancers are excellent candidates for chemopreventive measures and approaches, but prostate cancer is particularly appropriate due to its relatively long latency, late age of onset, slow growth, and high incidence [171, 199, 200]. Our findings also

emphasize that isoprenoids may be important clinically not as single agents but rather as chemotherapeutic adjuvants or sensitizing agents to diminish telomerase activity in tumor cells.

# CHAPTER III

## DISRUPTION OF A HTERT-MTOR-RAPTOR PROTEIN COMPLEX BY THE PHYTOCHEMICAL PERILLYL ALCOHOL AND RAPAMYCIN

### INTRODUCTION

Gene regulation is a complex process mediated by numerous cellular pathways. At the forefront of current gene regulatory mechanism research are post-translational modifications that alter the ability of a protein to perform its functions. Traditional mechanisms of post-translational regulation include protein folding, phosphorylation changes, and cellular localization. An equally important post-translational process is the assembly of proteins into complexes that allow a cell to carry out diverse functions that the individual proteins found in the complex could not perform on their own. Determining which proteins are in the complex of interest discloses clues about regulation of the cellular process imparted by the complex. This molecular puzzle of protein interactions in a complex ultimately reveals regulation.

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is the central player in numerous protein complexes. One of its primary functions, regulation of cap-dependent translation, is mediated through phosphorylation of 4E-BP1. mTOR's association with various protein complexes imparts unique regulatory functions, a process that emphasizes the importance of protein-protein interactions and associated complex formation in expanding the regulatory role of individual proteins. For example, through multiple protein interactions mTOR not only regulates protein translation, but also cell growth and cell size, as well as autophagy and ribosome biogenesis.

mTOR associates with RAPTOR in a complex termed mTORC1 that governs cap-dependent translation [109]. Further, the downstream targets of mTORC1–S6K and 4E-binding protein 1 (4E-BP1)–have been shown to physically associate with RAPTOR in a larger complex [201, 202]. mTORC1 phosphorylates S6K and 4E-BP1, and complex formation apparently facilitates

the efficiency at which down-stream signaling may occur. Hsp90 likewise forms a physical complex with RAPTOR and is required for certain mTORC1 functions in the complex [203, 204]. Furthermore, inhibition of Hsp90 decreases RAPTOR expression, suggesting that Hsp90 may stabilize this complex [204].

Hsp90 is also an integral part of the telomerase complex. Hsp90 and a co-chaperone p23 are required for efficient telomerase activity [205]. Telomerase provides immortality to most cancer cells by extending telomeric DNA sequences with a hexameric repeat, thus allowing cancer cells to escape senescence. Human telomerase enzyme is minimally composed of hTERT–the catalytic subunit and reverse transcriptase–and hTERC, the RNA component [2, 16, 40, 60]. Other proteins such as dyskerin have also been found to be supporting members in the telomerase complex. Kawauchi et al. further described that upon IL-2 stimulation of natural killer (NK) cells, telomerase activity was de-repressed, and hTERT protein formed a physical and functional complex with mTOR, S6K, Hsp90 and Akt [70]. The inclusion of mTOR and S6K in the hTERT complex is persuasive evidence to support mTOR-mediated control of telomerase activity.

Using a plant-derived isoprenoid–perillyl alcohol–and the classical mTOR inhibitor–rapamycin–we recently demonstrated that both agents rapidly and effectively attenuate telomerase activity and hTERT protein levels in human prostate cancer cells. These decreases in telomerase activity and hTERT protein levels occur in the absence of any effect on *hTERT* mRNA (Chapter 2). Both compounds were previously observed in our laboratory to suppress 4E-BP1 phosphorylation and cap-dependent translation in cancer cells [144, 145]. In addition, the known mechanism of action for rapamycin involves dissociation of the endogenous mTOR-RAPTOR complex with a concomitant suppression of mTOR kinase activity [139, 206]. The ability of perillyl alcohol to likewise disrupt the mTOR-RAPTOR complex as a mechanism to down-regulate protein synthesis has not been explored.

Based upon the known important protein interactions described above, we hypothesized that an hTERT-mTOR-RAPTOR complex exists in prostate cancer cells. As demonstrated in this study, perillyl alcohol and rapamycin-mediated

dissociation of this complex has a unique role in post-translational inhibition of telomerase activity.

## MATERIALS AND METHODS

*Cell lines and cell culture*

Human prostate cancer cell line DU145 (American Type Culture Collection, Manassas, VA) was maintained at 37°C in a humidified incubator with 5% $CO_2$ in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1X glutamine and 1X penicillin/streptomycin. Growth medium was changed every other day.

*Drug treatments*

Perillyl alcohol (Sigma-Aldrich, St. Louis, MO) was prepared in 100% ethanol; rapamycin (Cell Signaling Technology, Danvers, MA) was dissolved in 100% DMSO [145]. DU145 cells were plated at a concentration of $3x10^6$ cells per 100 mm plate in 8 mL medium and allowed to attach overnight. Cells were then treated with one of the following: 400 μM perillyl alcohol for 16 hr, 10 nM rapamycin for 4 hr, or 0.1% ethanol for 16 hr as described by Peffley et al. [145]. Concentrations used emulate plasma concentrations of the respective agents reported in clinical studies on cancer patients [143, 159].

*Protein extraction and immunoprecipitation*

Cells were extracted using the Pierce® Classic IP kit according to the manufacturer's instructions. The IP lysis/wash buffer was supplemented with 1X Complete protease inhibitor cocktail (Roche Applied Science), 1 mM $Na_3VO_4$ and 1 mM NaF. Protein concentration was interpolated using a Bradford assay and extracts were either analyzed immediately or stored at -86°C.

The complex of interest was captured using the Pierce® Classic IP kit according to the manufacturer's instructions. Briefly, 5 μg of rabbit polyclonal anti-RAPTOR antibody (Millipore) or 5 μg of anti-IgG (clone Sc-2027; Santa Cruz) was incubated overnight with 1 mg of protein extract in the IP lysis/wash

buffer with end-over-end mixing. Protein A-Sepharose from *Staphylococcus aureus* (Sigma-Aldrich®) was resuspended (1:1) in PBS. Thirty µL of protein A-Sepharose slurry was applied to the Pierce spin column. Washes were carried out according to the manufacturer's protocol. The protein-antibody solution was applied to the spin column containing protein A-Sepharose and incubated for 1 hr at 4°C with end-over-end mixing. Subsequent washes were carried out according to the manufacturer's protocol. The complex was eluted from the column/beads following the sample-buffer elution protocol provided in a 2X SDS loading dye. Eluted protein was subsequently used in the western blot protocol.

*SDS-PAGE and western blot*

One half the volume of captured protein was loaded per well on a 4-15% polyacrylamide TGX mini-gel (Bio-Rad) and resolved by electrophoresis in 1X Tris-glycine-SDS (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3) for 30 min at 200 volts. Odyssey® two-color protein molecular weight markers were included to determine protein sizes; all subsequent steps were performed with minimal light exposure. Electrophoretic transfer onto polyvinylidene fluoride (PVDF) membrane was performed in 1X Tris-glycine buffer. Membranes were blocked in protein-free bløk-CH™ buffer (Millipore) for 1 hr at room temperature and then incubated with gentle rocking in one of the following primary antibodies: rabbit polyclonal anti-RAPTOR (1:1,000; Millipore) (capture/loading control), rabbit polyclonal anti-hTERT (1:1000; clone Y182, Millipore), rabbit polyclonal anti-mTOR (1:1,000; Cell Signaling Technology®), rabbit polyclonal anti-Hsp90 (1:1,000; clone C45G5, Cell Signaling Technology®), or rabbit polyclonal anti-p70 S6 kinase (1:1000; Cell Signaling Technology®).

Incubations were conducted overnight at 4°C and followed by extensive washes in 1X Tris-buffered saline (TBS)-Tween 20 (0.02M Tris, pH 7.4, 0.15 M NaCl, 0.05% Tween 20). IR-conjugated secondary antibodies (goat anti-rabbit [IRDye 680LT] or goat anti-mouse [IRDye 800 CW]) were diluted 1:5000 in bløk-CH™ buffer and incubated for 1 hr at room temperature. Subsequent membrane washes were as described [145], and then blots were washed for 5 min in PBS

before capturing images on an Odyssey Infrared Imaging System (Licor®). Band intensities were assessed subjectively.

*SDS-polyacrylamide gel silver staining*

Immunocaptured proteins separated by SDS-polyacrylamide gel electrophoresis were detected by silver staining using the Pierce® Silver Stain Kit according to the manufacturer's suggestions. Experiment was repeated twice with similar results.

## RESULTS

*Perillyl alcohol or rapamycin treatment causes changes in the protein complex associated with RAPTOR*

We previously demonstrated in DU145 prostate cancer cells that perillyl alcohol and rapamycin dramatically and rapidly attenuate telomerase activity without altering hTERT mRNA levels (Chapter 2). With these two agents, suppressed phosphorylation of a downstream target of mTOR (4E-BP1), disruption of the m7GpppX cap binding complex eIF2F and/or inhibition of cap-dependent translation have been observed in our laboratory [144, 145]. Importantly, hTERT protein levels were diminished by both agents, supporting an effect on 4E-BP1 and/or S6K phosphorylation and reduced initiation of protein translation. However, we also noted that the reduction in hTERT protein did not coincide wholly with loss of telomerase enzymatic activity, suggesting a further level of regulation. Rapamycin in complex with FKBP12 (FK506- binding protein of 12 kDa) causes the dissociation of RAPTOR from mTOR, disrupts coupling of mTOR with its substrates and interferes with its kinase ability [206]. We therefore reasoned that the rapid modulation of telomerase activity in DU145 cells by perillyl alcohol or rapamycin was attributable in part to destabilization of an hTERT-mTOR-RAPTOR complex.

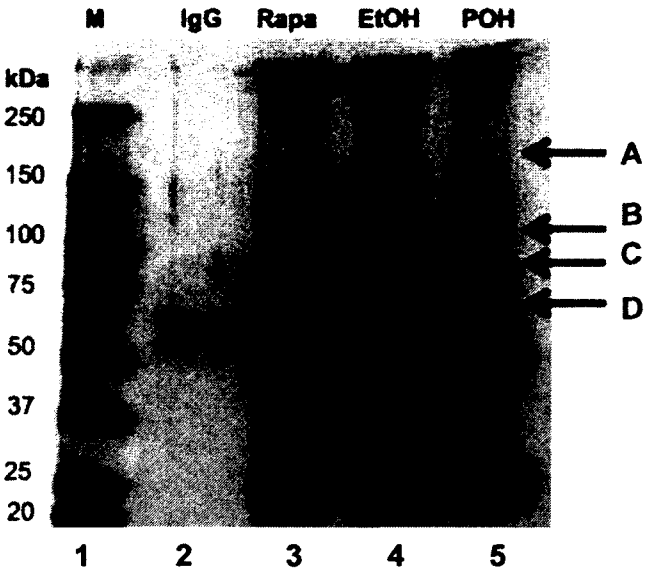


**Fig. 14.** Perillyl alcohol and rapamycin induce changes in proteins immunoprecipitated with RAPTOR in DU145 prostate cancer cells. Cells were treated with 400 μM perillyl alcohol (POH) for 16 hr, rapamycin (Rapa) at 10 nM for 4 hr, or 0.1% ethanol (EtOH) for 16 hr. Cellular extracts (500 μg) were incubated with anti-RAPTOR or anti-IgG antibodies and captured on Protein A-Sepharose. Proteins were eluted from the column/beads, separated by SDS-PAGE and detected by silver staining. Lane 1: Odyssey® two-color protein molecular weight markers (M). Lanes 2-5: 25 μl of eluted protein from the column/beads treated as indicated. Lettered arrows point out bands of interest.

Thus we used co-immunoprecipitation with a RAPTOR antibody and protein gel silver staining to detect RAPTOR-associated proteins in DU145 cells and to elucidate potential changes to the captured protein complex caused by either 400 μM perillyl alcohol for 16 hr, or rapamycin at 10 nM for 4 hr. Treatment with either perillyl alcohol or rapamycin modified the protein complex captured with RAPTOR antibody compared to untreated cells; moreover distinct differences were found between the two agents (Fig. 14). The pronounced bands at ~25 kDa and ~50 kDa are due to the light and heavy IgG chains,

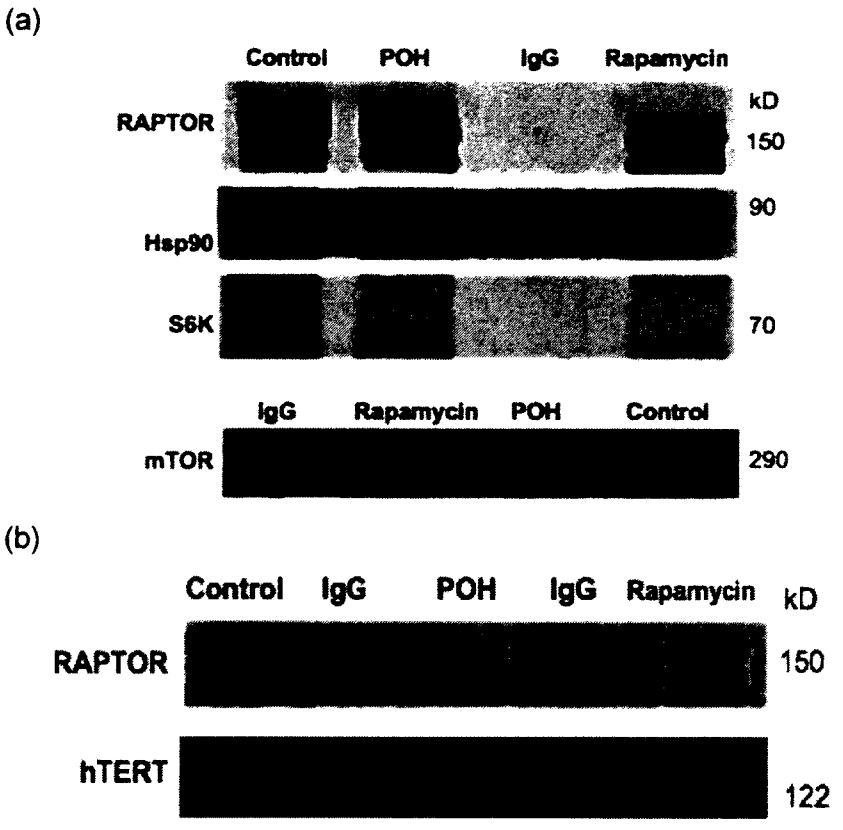


**Fig. 15.** Perillyl alcohol and rapamycin destabilize the hTERT-mTOR-RAPTOR complex found in DU145 prostate cancer cells. Representative western blots of RAPTOR-immunoprecipitated proteins are shown. Cells were treated and immunoprecipitated as described in Figure 14. After capture with anti-RAPTOR antibody and separation on SDS-PAGE, proteins were transferred to a PVDF membrane, and blocked prior to incubation with primary antibodies. **(a)** Blots were subsequently probed with anti-RAPTOR, anti-Hsp90, anti-S6K, or anti-mTOR antibodies as indicated. **(b)** Blots were probed with anti-RAPTOR or anti-hTERT antibodies. IgG: Negative control anti-IgG captured protein from EtOH-treated DU145 cells. All experiments were replicated at least five times with similar results.

respectively. Perillyl alcohol and rapamycin both suppressed proteins of approximately 170-180 kDa (A) and ~75 kDa (C) molecular mass (Fig. 14). The decrease in the 75 kDa (C) protein appeared to be greater with rapamycin than with perillyl alcohol (Fig. 14). Interestingly, rapamycin caused the loss of a ~100 kDa (B) protein, whereas perillyl alcohol increased the level of a protein at the same position on the gel (Fig. 14). Most noteworthy was the addition of a ~60 kDa (D) protein to the complex by both perillyl alcohol and rapamycin treatments (Fig. 14).

*Perillyl alcohol or rapamycin causes hTERT, S6K, Hsp90, and mTOR to dissociate from RAPTOR*

Cellular functions are often revealed through identification of protein complexes. Therefore, we assessed by western blotting for the presence or absence of protein partners described originally by Kawauchi et al. in an immunoprecipitated complex [70]. In control EtOH-treated cell extracts, immunoprecipitation with RAPTOR captured mTOR, S6K, Hsp90, and hTERT (Fig. 15 (a) and (b)) affirming the presence of a similar complex in DU145 cells. The above proteins were not detected when cellular extracts were immunoprecipitated with normal rabbit IgG. Although binding of 4E-BP1, a known RAPTOR-interacting protein, to the complex is of interest, it has a molecular weight of ~15-20 kDa; therefore its presence is masked by the IgG light chain. Further, the interaction between RAPTOR and 4E-BP1 has been previously confirmed [201, 207].

Similarly, Akt, has a molecular weight of 60 kDa, consequently the heavy chain IgG band conceals the protein band. However, immunoprecipitation with anti-Akt antibodies revealed the presence of hTERT, Hsp90, and mTOR (data not shown). The Hsp90 band was the most intense confirming a direct physical interaction with Akt. Additionally, the amount of these proteins that immunoprecipitated with Akt decreased in response to perillyl alcohol or rapamycin treatment. S6K did not co-immunoprecipitate with Akt, suggesting

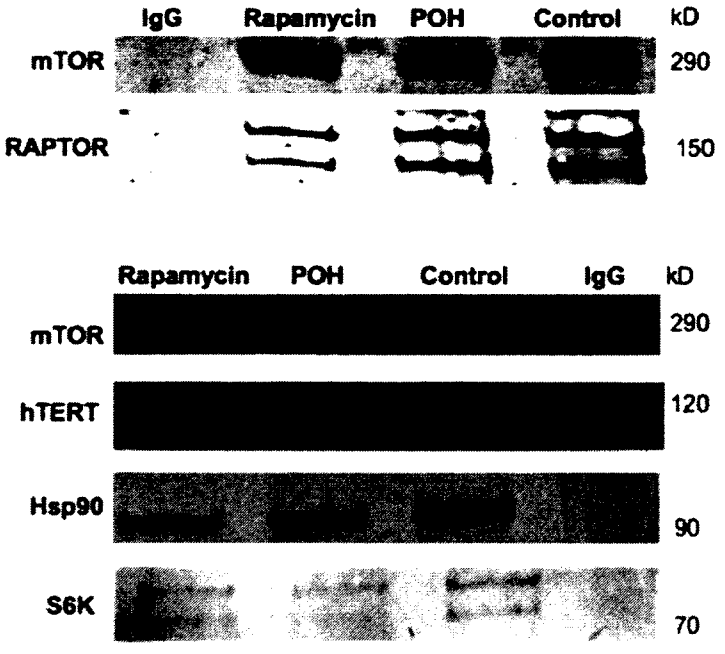


**Fig. 16.** Anti-mTOR immunoprecipitation confirms perillyl alcohol or rapamycin-mediated disruption of the hTERT-mTOR-RAPTOR complex. Cells were treated as described in Figure 14. Immunoprecipitations were conducted with either anti-mTOR or anti-IgG antibodies as a control. Blots were subsequently probed with anti-mTOR, anti-hTERT, anti-RAPTOR, anti-Hsp90 or anti-S6K antibodies as indicated. IgG: Negative control anti-IgG captured protein from EtOH-treated DU145 cells. POH, perillyl alcohol. All experiments were replicated at least five times with similar results.

they are not physically interacting with one another, but rather they are in the complex through common associations with another protein (data not shown).

Upon treatment with either perillyl alcohol or rapamycin, S6K completely dissociated from RAPTOR (Fig. 15 (a)). Additionally, a reduction in the amount of Hsp90 bound to RAPTOR was observed after either treatment. As expected based on previous literature reports, mTOR also separated from RAPTOR in cells treated with rapamycin [139]. A decrease in mTOR binding to RAPTOR

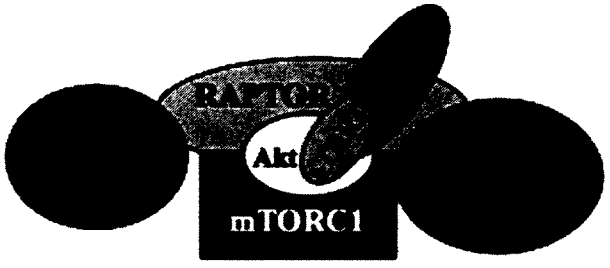


**Fig. 17.** Amended schematic model of the hTERT-mTOR-RAPTOR complex in DU145 cells. This schematic represents a more accurate model of the protein-protein interactions involved in the hTERT-mTOR-RAPTOR complex based upon our immunoprecipitation results. Treatment with either perillyl alcohol or rapamycin impairs the structural integrity of the complex.

was similarly noted in perillyl alcohol-treated samples, although the reduction was not as great as that detected with rapamycin (Fig. 15 (a)). Most interesting for our studies was the loss of hTERT binding to the RAPTOR-captured complex after treatment with either agent (Fig. 15 (b)). Importantly, perillyl alcohol caused a larger loss of hTERT protein than rapamycin did. This finding coupled with our previous reported results on the ability of these agents to attenuate telomerase activity and protein levels further supports our hypothesis of mTOR-mediated translational and post-translational regulation of hTERT.

*Perillyl alcohol or rapamycin causes hTERT, S6K, Hsp90, and RAPTOR to dissociate from mTOR*

As a corollary experiment, an antibody directed against mTOR was also used to co-immunoprecipitate the hTERT-mTOR-RAPTOR complex. The ability

of perillyl alcohol or rapamycin to compromise the structural integrity of the complex was also examined. Capturing protein binding partners in the complex with a different antibody could also potentially reveal specific physical interactions. Western blot analysis demonstrated that the mTOR antibody immunoprecipitated RAPTOR, Hsp90, S6K and hTERT (Fig. 16). Similarly, interactions among all four proteins with mTOR were disrupted by perillyl alcohol and rapamycin. Rapamycin and perillyl alcohol treatment decreased binding of RAPTOR and Hsp90 to mTOR, with rapamycin being more effective than perillyl alcohol. Although we detected a decrease in the hTERT and S6K bands upon treatment with either agent, the basal protein levels that immunopreciptated with mTOR were low. Our results are consistent with the postulate that the interaction between these two proteins with mTOR is via RAPTOR.

**DISCUSSION**

Protein complexes are the capstone of numerous cellular processes. Protein-protein interactions within these complexes add unique functions that individual proteins lack. Although it was surprising to find hTERT associated with a protein complex that included mTOR, it does provide a critical link between telomerase activity and a major signal transduction pathway that regulates cell cycle progression [70]. Here we have found that a complex similar to that described by Kawauchi et al. in NK cells also exists in DU145 prostate cancer cells [70]. To the best of our knowledge, this is the first report of an hTERT-mTOR-RAPTOR complex in cancer cells implicating the involvement of mTOR in controlling telomerase activity.

We previously demonstrated that perillyl alcohol or rapamycin individually inhibited telomerase activity and decreased hTERT protein levels (Chapter 2). We did, however, note discordance between the almost complete abrogation of telomerase activity and the moderate decrease in protein levels. Under our standard treatment conditions, both compounds down-regulated telomerase activity by greater than 90% (Chapter 2). Our previous results also noted that rapamycin decreased hTERT protein levels more effectively than did perillyl

alcohol: a 76% reduction versus a 40% loss, respectively. Such findings led us to explore alternative post-translational regulation, i.e., the destabilization of a multi-protein complex.

In the current study we demonstrate that treatment with either perillyl alcohol or rapamycin at short incubation times and biologically relevant concentrations disrupts this complex. Perillyl alcohol was as effective as rapamycin at dislodging hTERT from the hTERT-mTOR-RAPTOR complex. The smaller structure and lipophilic nature of perillyl alcohol might facilitate its diffusion into the complex and cause disruption of critical hydrophobic interactions. Based on our results, we propose that the ability of perillyl alcohol or rapamycin to perturb the functional hTERT-mTOR-RAPTOR complex helps resolve the variance noted between telomerase activity and hTERT protein loss in our previous study (Chapter 2).

Surprisingly, both losses and gains in RAPTOR-associated proteins were detected by silver stained gel analysis after perillyl alcohol or rapamycin treatment. We anticipated that either agent would destabilize the complex; therefore decreases in ~160 kDa (A) and 75 kDa (C) proteins after treatment supported our hypothesis (Fig. 14). However, analysis of the silver-stained gel also revealed the addition of a ~60 kDa (D) protein after treatment with either agent (Fig. 14). Further, perillyl alcohol caused the association of ~100 kDa (B) protein (Fig. 14). Although future studies are needed to identify these proteins by mass spectrometry, we believe the proteins are likely telomerase inhibitors that associate with RAPTOR post-treatment.

Others have reported drug-mediated disruption of protein-protein interactions between proteins in the hTERT-mTOR-RAPTOR complex; however we are the first to indicate complete dissociation of the multi-protein complex by either a simple phytochemical–perillyl alcohol–or rapamycin. In separate studies, rapamycin and curcumin were found to interrupt the mTOR-RAPTOR interaction [139, 208]. Geldanamycin–a Hsp90 inhibitor–not only displaced Hsp90 from RAPTOR [204], but additionally dislodged hTERT from Hsp90 leading to hTERT degradation [74]. Rapamycin and geldanamycin are both polyketide macrocyclic

antibiotics from *S. hygroscopicus* known to compromise protein-protein interactions [209]. Importantly, rapamycin inhibits Hsp90 complexes through modulation of the immunophilins FKBP52/54 [209]. These immunophilins are in the same family as the binding partner of rapamycin, FKBP12. The rapamycin-FKBP12 complex dissociates mTOR from RAPTOR by binding a hydrophobic pocket on the mTOR surface [206, 210]. Perillyl alcohol–a lipophilic compound–likely utilizes a similar mechanism to destabilize the hTERT-mTOR-RAPTOR complex.

Based on the RAPTOR and mTOR co-immunoprecipitation results reported in this study, as well as previous reports of known protein interactions, we developed a schematic representation of the hTERT-mTOR-RAPTOR complex (Fig. 17) [70, 139, 201-205, 211]. In the diagram, RAPTOR nucleates a complex among 4E-BP1, S6K, mTOR, hTERT, Akt, and Hsp90. Although Yip et al. report mTORC1 as an obligate dimer necessary for mTOR enzymatic activity, we have represented the hTERT-mTOR-RAPTOR complex as monomeric for clarity [109, 206]. RAPTOR serves as the scaffold that allows mTOR to phosphorylate its downstream targets–S6K and 4E-BP1–by physically binding them through a TOS motif [207], thus bringing them in close proximity to mTOR [201, 202, 204]. Other studies report that Hsp90 forms a physical complex with RAPTOR and is necessary for the mTORC1 functions of the complex, serving as another stabilizing scaffold [203, 204]. Additionally, Akt, an upstream effector of mTOR, was found in a physical complex with mTOR, supporting the idea that kinases must physically interact with their substrates [212]. Similarly, Akt phosphorylates TERT, and therefore, their presence together in the complex is not surprising [61]. TERT requires interaction with both Hsp90 and Akt–which also interact with each other–for efficient telomerase activity [70, 211, 213]. Hsp90 promotes and maintains large protein complexes, such as the proposed hTERT-mTOR-RAPTOR complex [214]. Thus placement of each protein within the diagram was based upon our results and previous reports of protein-protein interactions (Fig. 17) [70, 139, 201-205, 211]. As discussed earlier, rapamycin–and by inference perillyl alcohol–destabilizes both of the scaffolds–Hsp90 and RAPTOR– present

in the complex. Within this context, our results clearly show that rapamycin and perillyl alcohol cause dissociation of the hTERT-mTOR-RAPTOR complex, possibly providing a mechanism of action by which these agents decrease telomerase activity in prostate cancer cells.

In addition to enabling protein kinase activities, we venture that this large multiprotein complex has still more purposes. We further hypothesize that the physical interaction between mTOR and TERT is necessary for cancer cell survival [215]. By usurping the mTOR pathway, the cell may evade two impediments to long term survival. Constitutively active mTOR in the hTERT-mTOR-RAPTOR complex may stimulate TERT to ensure cancer cell immortality. An alternative potential role for the hTERT-mTOR-RAPTOR complex is to enable additional hTERT cellular functions, primarily DNA repair [84, 85]. Evidence supporting this premise is the discovery by Yip et al. that the dimeric mTORC1 complex has a central cavity large enough to accommodate double-stranded DNA [206]. Furthermore, mTOR is a member of the PIKK family, several of which mediate DNA repair [216].

Understanding the elaborate interplay among proteins that form this complex has established further regulation of the telomerase enzyme by the mTOR pathway. Agents that disrupt protein-protein interactions represent a novel class of telomerase inhibitors and potential therapeutics [217]. We propose that perillyl alcohol and rapamycin inhibit telomerase activity through unique synergistic decreases of hTERT protein translation and disruption of the hTERT-mTOR-RAPTOR protein complex. These findings strengthen the argument for use of perillyl alcohol as a potent cancer chemopreventive.

# CHAPTER IV

## EIF4E OVER-EXPRESSION IMPARTS PERILLYL ALCOHOL AND RAPAMYCIN-MEDIATED REGULATION OF TERT

### INTRODUCTION

Gene expression is tightly regulated by both transcriptional and translational processes. Although transcriptional control has long been touted as the principle regulator of gene expression, it is now clear that cells depend on translational regulation for rapid changes in protein levels. In eukaryotes, translational gene control is mediated mainly at the rate-limiting step of initiation [218]. Translation initiation is governed by a protein complex known collectively as eIF4F (eukaryotic initiation factor 4F) that includes eIF4A–a known RNA helicase, eIF4G–a scaffold protein, and eIF4E–the rate-limiting cap-binding protein [219]. Together, the proteins that comprise eIF4F ensure that secondary structure in the 5'-UTR (untranslated region) of mRNA is unwound to allow initiation of protein translation [112].

Not all mRNA sequences are able to compete equally for available eIF4E. mRNAs with 5'-UTRs that are G-C rich have substantial secondary structure that attenuates initiation of translation. Consequently, greater eIF4E levels are required to form the cap-binding complex, eIF4F, which unfolds secondary structure and permits efficient cap-dependent translation. mRNAs with extensive secondary structure are generally not translated in the presence of low cellular eIF4E levels, but instead are activated with mitogens through the mTOR pathway [115-117]. Additionally, elevated eIF4E levels associated with tumorigenesis have been shown to increase the translation of many oncogenic proteins. Free eIF4E levels are therefore tightly regulated through mitogenic activation of the mTOR signal transduction pathway [118].

Regulation of the rate of cap-dependent translation is mediated through the PI3K/Akt/mTOR pathway [99]. Under quiescent conditions, eIF4E is sequestered by 4E-BP1 in a configuration that prevents eIF4E from partaking in translation

initiation. Upon growth factor stimulation, a kinase cascade is activated that results in mTOR phosphorylating 4E-BP1, which causes eIF4E to dissociate [220]. Conversely, when cells encounter stressors that inhibit mTOR signaling, this cellular pathway ensures that proteins typically synthesized by cap-dependent translation are not generated. Not unlike many important pathways in the cell, mTOR signaling and therefore cap-dependent translation is frequently aberrant in cancer cells and is a leading target for anti-cancer therapies [218]. Treatment of cancer cells with the canonical mTOR inhibitor–rapamycin–causes decreased 4E-BP1 phosphorylation, therefore reducing the level of free eIF4E for translation initiation [142]. Additionally, our laboratory found that plant-derived compounds such as perillyl alcohol or genistein affect protein translation in cultured prostate cancer cells by modulating mTOR signaling [145], specifically both compounds decrease 4E-BP1 phosphorylation. Perillyl alcohol also disrupts the eIF4F 5'- cap-binding complex by suppressing interaction of eIF4E with eIF4G.

eIF4E is overexpressed in almost all cancers including carcinomas of the prostate, breast, lung, bladder, cervical, and head and neck [118]. eIF4E overexpression not only leads to many of the phenotypic changes associated with cancer cells including rapid proliferation, decreased apoptosis, and malignant transformation, it is also associated with a poor prognosis in human cancer patients [118, 221]. Most cancer cells are also characterized by a de-repression of telomerase, a ribonucleoprotein responsible for elongating telomeres by the addition of hexameric repeats that cause cellular immortalization [36]. Similar to eIF4E, *hTERT*, the rate limiting enzymatic portion of telomerase, is re-activated as an early and critical event in tumor cells.

Importantly, we previously observed that perillyl alcohol or rapamycin treatment of prostate cancer cells was associated with a significant and rapid loss of telomerase activity concomitant with a decrease in hTERT protein levels (Chapter 2). This finding strongly suggests that TERT protein levels and telomerase activity are mediated in part by the mTOR pathway, the master regulator of eIF4E. The above telomerase-mTOR regulatory phenomenon was

observed in tumor cells that endogenously over-express eIF4E. Here we examined the mTOR contribution to telomerase activity in an immortalized non-tumorigenic mammalian cell line with forced expression of eIF4E. The distinction between a cancer cell with elevated eIF4E and a normal immortalized cell with forced eIF4E expression will allow us to tease apart the eIF4E effects on telomerase activity versus those that may be mediated by other oncogenic pathways.

## MATERIALS AND METHODS

### *Cell Lines and Cell Culture*

Chinese hamster ovary (CHO)-derived cell lines (rb4E and pMV7) were maintained at 37°C in a humidified incubator with 5% $CO_2$ in MEM medium supplemented with 10% fetal bovine serum, 1X glutamine and 1X penicillin/streptomycin. Growth medium was changed weekly.

### *Geneticin Selection*

CHO cells permanently transfected with the vector pMV7-eIF4E containing the cDNA sequence for murine eIF4E under the regulation of a thymidine kinase promoter and a neomycin (neo) resistance gene or transfected with an empty vector (pMV7-neo) were created previously by Buechler and Peffley [144] and designated rb4E and pMV7, respectively. Vectors were originally provided by Nahum Sonenberg. Prior to initiating experiments, both cell lines were selected in geneticin (G-418) (Gibco/Invitrogen, Carlsbad, CA) according to manufacturer's suggestions to verify the presence of vectors. Briefly, a stock solution of geneticin was made by dissolving it in PBS at a concentration of 50 mg/mL and then sterile filtering. pMV7, rb4E, and control non-transfected cells were plated at 1 X $10^6$ in a 25 $cm^2$ flask with 10 mL of MEM media supplemented with 10% FBS. After an overnight incubation, geneticin was added to a final concentration of 0.4 mg/mL. Media was replaced at least weekly and supplemented with

geneticin for an additional 3 weeks to ensure only transfected cells would survive.

*Drug treatments*

Perillyl alcohol (96% stock, Sigma-Aldrich, St. Louis, MO) was prepared in 100% ethanol; rapamycin (stock 100 µM, Cell Signaling Technology, Danvers, MA) was dissolved in 100% DMSO [145]. pMV7 and rb4E cells were plated at a concentration of $5x10^5$ cells per 60 mm plate in 3 mL medium and allowed to attach overnight. Cells were then treated with one of the following: 400 µM perillyl alcohol for 16 hr, 10 nM rapamycin for 4 hr, or 0.1% ethanol for 16 hr as described by Peffley et al. [145]. Concentrations used of either compound are those that can be achieved in cancer patient plasma [143, 159].

*Protein Extraction for Telomerase Activity*

Cells were plated at a concentration of $1x10^6$ cells per 60 mm plate in 3 mL medium. Cells were harvested using trypsin-EDTA (ethylenediaminetetraacetic acid) and then pelleted by centrifugation (500 x g) for 8 min. Cell pellets were washed with ice cold phosphate-buffered saline (PBS) and subsequently centrifuged again. Protein lysates were obtained by resuspending pMV7 or rb4E cells in 200 µL of ice cold 1X CHAPS lysis buffer per 1 x $10^6$ cells. The CHAPS lysis method was modified from that presented in Hou et al. and others [176-179]. CHAPS lysis buffer consisted of 0.5% 3-[(3-Cholamidopropyl)dimethylammonio]-l-propanesulfonic acid (CHAPS), 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA), and 10% glycerol. RNase inhibitor (New England Biolabs, Beverly, MA) (final concentration of 10 units/mL) and ß-mercaptoethanol (BME) (Sigma-Aldrich, final concentration of 5 mM) were added just prior to use. Cell lysates were incubated at 4°C for 30 min and then centrifuged at 12000 x g for 30 min at 4°C. The supernatant was collected, protein concentration was interpolated using a Bradford assay and extracts were stored at -86°C.

*RTQ-TRAP Assay*

Telomerase activity was detected by performing RTQ-TRAP as derived from a protocol described by Hou et al. [178]. In detail, each 25 µL RTQ-TRAP reaction contained: 1X SYBR Green Master mix (50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM each dNTP, 3 mM $MgCl_2$, iTaq DNA polymerase at 0.05 units/µL) (Bio-Rad, Richmond, CA), 10 mM EGTA, 0.2 µg T4 gene protein (New England Biolabs), 0.35 µM TS primer (5'-AATCCGTCGAGCAGAGTT-3') (Tm 53.6°C) and 0.35 µM ACX primer [5'-GCGCGG(CTTACC)3 CTAACC-3'] (Tm 66.4°C) [180], and 2.5 µg of protein extract. Primers were purchased from Integrated DNA Technology (IDT, Coralville, IA). All samples were analyzed in duplicate in a 96-well plate on a Bio-Rad CFX96 thermal cycler, and 1X CHAPS buffer was included as a negative control. Reactions were incubated at 25°C for 20 min to allow for elongation of the TS primer by cellular telomerase. The PCR protocol began with a 95°C hot start to activate Taq polymerase, followed by 40 cycles at 95°C for 20 s, 50°C for 30 s, 72°C for 90 s [178], ending with a melt curve protocol. Telomerase activity was comparatively assessed based on threshold cycles ($C_T$) [178]. $C_T$ values greater than 35 were considered false positives due to primer dimers. Experiments were conducted at least three times. Inhibition of telomerase activity was determined as follows:

$$(C_T \text{ treatment}) - (C_T \text{ control}) = \Delta C_T$$

$$(1 / 2^{\Delta CT}) \times 100 = \% \text{ ACTIVITY REMAINING}$$

*RNA extraction and quantification*

Total cellular RNA was isolated using PureZOL™ (Bio-Rad) according to the manufacturer's protocol. RNase-free-DNase I digestion (50 units) (Roche Applied Science, Indianapolis, IN) was also performed. RNA quantification was

determined by UV absorbance on a BioPhotometer Plus (Eppendorf®, Hamburg, Germany).

*TERT mRNA Levels*

Expression of *TERT* mRNA was detected using real-time RT-PCR. One µg of purified cellular RNA from untreated and treated cells was reverse transcribed into cDNA using iScript (Bio-Rad) at 42°C for 30 min in the presence of random hexamers and oligo(dT), Moloney murine leukemia virus reverse transcriptase and RNasin. Additionally, minus reverse transcriptase and a minus RNA template reactions were included as negative controls. Analysis of *TERT* mRNA expression was performed by real-time PCR amplification using a Bio-Rad CFX96 thermal cycler. The complete Chinese hamster (*Cricetulus griseus*) *TERT* sequence is not defined, therefore the Chinese hamster shotgun sequence (AFTD01128649.1) was aligned with the known Golden hamster (*Mesocricetus auratus*) telomerase catalytic subunit (accession number AF149012). PCR primer sets for Chinese hamster *TERT* and an internal control gene β-actin sequence (*Cricetulus griseus* beta actin [ACTB], accession number U20114) were optimized using Beacon Designer software (PREMIER Biosoft International, Palo Alto, CA). PCR efficiencies for each primer set were determined in triplicate by a dilution series of the cDNA template. A master mix contained 1X SsoFast EvaGreen (Bio-Rad), 0.5 µM of each forward and reverse primer, and 1.5 µl of cDNA per reaction.

A 129-bp hamster *TERT* amplicon was generated using 5'-AGCATCATCTCCAACATAGC-3' (Tm 52.3°C) and 5'-TCGGTAGCAGACCAAC-3' (Tm 52.2°C). A 185-bp hamster β-actin gene amplicon was generated using 5'-GCACCACACCTTCTACAAC-3' (Tm 52.9°C) and 5'-TACGACCAGAGGCATACAG-3' (Tm 52.7°C). The real-time PCR program used was 95°C for 30 s, followed by 35 cycles at 98°C for 30 s, 65°C for 30 s, ending with a melt curve analysis step where the temperature was reduced to 65°C, then

increased to 95°C in 0.2°C increments every 5 s. The $\Delta\Delta C_T$ method was used to comparatively analyze the data as described in Livak and Schmittgen [182].

*Protein extraction and immunoblotting*

The protocol described by Peffley et al. [145] was used with minor changes. Briefly, cells were plated at $3x10^6$ per 100 mm culture dish and allowed to attach for 24 hr before treating with perillyl alcohol or rapamycin as above. Cells were lysed in 250 µL RIPA lysis buffer containing fresh 1X Complete protease inhibitor cocktail (Roche Applied Science), 1 mM $Na_3VO_4$ and 1 mM NaF, and protein concentrations were determined by a Bradford assay. Protein levels were assessed by western blotting according to [145] with some modifications. Fifty µg total protein per well were loaded on a 4-15% polyacrylamide TGX mini-gel (Bio-Rad) and resolved by electrophoresis in 1X Tris-glycine-SDS (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3) for 30 min at 200 volts. For western blots, Precision Plus protein standards (Bio-Rad) (chemiluminescence) or Odyssey® two-color protein molecular weight markers (infrared) were included to determine protein sizes. Electrophoretic transfer onto polyvinylidene fluoride (PVDF) membrane was performed in 1X Tris-glycine buffer.

Membranes were stained with Ponceau red to verify protein transfer and loading. Membranes were blocked in protein-free bløk-CH™ buffer (Millipore, Temecula, CA) for 1 hr at room temperature and then incubated with gentle rocking in one or more of the following primary antibodies: mouse monoclonal anti-eIF4E (1:250, clone P2, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal eIF4E (1:4,000, Cell Signaling, Danvers, MA), rabbit polyclonal anti-actin (1:500, clone H-300, Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-p70 S6 Kinase (1:1,000, Cell Signaling), rabbit monoclonal anti-phospho-p70 S6 Kinase (Thr389) (1:1,000, clone 108D2, Cell Signaling), rabbit polyclonal anti-Akt (1:1,000, Cell Signaling), rabbit monoclonal anti-phospho-Akt (Ser473) (1:1,000, clone 193H12, Cell Signaling), rabbit polyclonal anti-4E-BP1 (1:1,000,

Cell Signaling), rabbit polyclonal anti-phospho-4E-BP1(Thr37/46) (1:1,000, clone 236B4, Cell Signaling) and/or rabbit monoclonal anti-hTERT (1:1000; clone Y182, Millipore) that also recognizes hamster TERT. All antibodies recognize the rodent form of their respective protein and were diluted in bløk-CH™.

Incubations were conducted overnight at 4°C and followed by extensive washes in 1X Tris-buffered saline (TBS)-Tween 20 (0.02M Tris, pH 7.4, 0.15 M NaCl, 0.05% Tween 20). Membranes were incubated with HRP-conjugated secondary antibodies (goat anti-rabbit or goat anti-mouse [Santa Cruz Biotechnology, Inc.]) were diluted 1:5000 in bløk-CH™ buffer for 1 hr at room temperature along with 1 µL StrepTactin-HRP (Bio-Rad) per 10 mL solution to visualize Precision Plus protein standards (Bio-Rad). Subsequent membrane washes were as described [145] and then blots were incubated for 5 min in Immun-Star™ HRP detection system (Bio-Rad) before capturing images on a Kodak Image Station. Densitometric analysis of protein bands was conducted using Kodak Molecular Imaging Software version 4.0.4. Alternatively, membranes were incubated with IR-conjugated secondary antibodies (goat anti-rabbit [IRDye 680LT] or goat anti-mouse [IRDye 800 CW] diluted 1:10000 in bløk-CH™ buffer) and incubated for 1 hr at room temperature. Subsequent membrane washes were as described [145] and then blots were washed for 5 min in PBS before capturing images on an Odyssey Infrared Imaging System (Licor®). Band intensities for proteins of interest were compared relative to the levels of actin or eIF4E, which do not change in response to the above treatments and provide a control for loading and transfer onto PVDF membranes.

*Statistical analysis*

All experiments were conducted three to five times with sub-sampling of each independent quantitative experiment. Data are expressed as a mean +/- standard deviation. Data were analyzed using Model I ANOVAs with SPSS version 19.0. The relationship of interest was perillyl alcohol versus control and rapamycin versus control; therefore, Dunnett's test was used as a follow-up to determine statistical significance of the results.

## RESULTS

*Telomerase inhibition by perillyl alcohol or rapamycin is dependent on eIF4E overexpression in immortalized CHO cells*

Telomerase expression is necessary to extend telomeres found at chromosome ends to compensate for the loss of telomeric DNA that would lead to cellular senescence. In general, somatic cells and normal cells in culture do not have active telomerase. In contrast, most cancer cells and cultured cells that survive 'crisis' and become immortalized, such as CHO, have de-repressed the enzyme by either transcriptional or post-transcriptional mechanisms [13, 36, 222]. We previously established that perillyl alcohol and rapamycin at pharmacologically relevant concentrations (400 μM perillyl alcohol for 16 hr, 10 nM rapamycin for 4 hr), attenuated telomerase activity by over 90% in DU145 prostate cancer cells, signifying a link between mTOR signaling and telomerase regulation (Chapter 2). Here we performed RTQ-TRAP on protein extracts obtained from rb4E and pMV7 cells first, to assess the effect of eIF4E-overexpression on telomerase activity, and secondly, to identify if eIF4E-overexpression modulates the regulation of telomerase activity in response to either perillyl alcohol or rapamycin under the above standard treatment conditions.

Basal levels of telomerase activity based upon real time PCR $C_T$ values resulted in $C_T$ values of 24.13 +/- 0.794 SD for rb4E and 24.34 +/- 1.518 SD for pMV7 cells. Statistical analysis demonstrated no significant differences between rb4E and pMV7 cells (df=2; F=0.044; p=0.845). Surprisingly however, we found that in the absence of eIF4E overexpression, telomerase activity in pMV7 cells was unaffected by either perillyl alcohol or rapamycin (df=2; F=1.705; p=0.259) (Fig. 18). A Dunnett's test further revealed that the slight changes in telomerase activity in perillyl alcohol-treated (p=0.749) and rapamycin-treated samples (p=0.446) were insignificant as compared to the control. In contrast, in eIF4E-overexpressing rb4E cells, telomerase activity was dramatically attenuated by perillyl alcohol or rapamycin treatment, with 67% and 89% reductions,

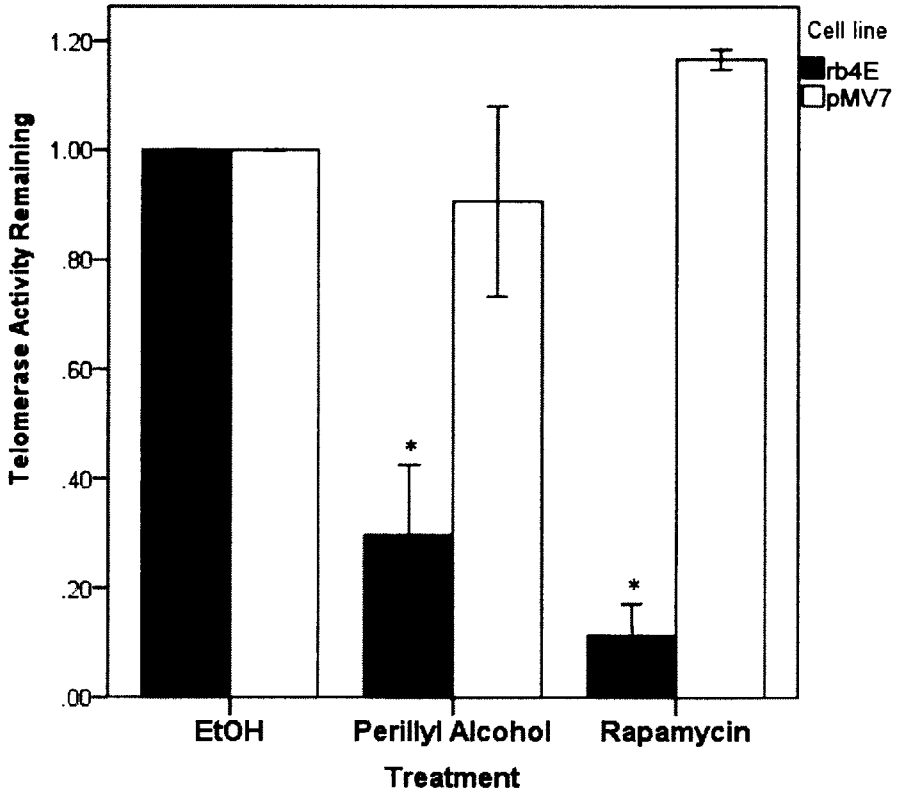


**Fig. 18.** Telomerase activity is reduced in rb4E cells in response to perillyl alcohol or rapamycin, however pMV7 cells are unaffected. RTQ-TRAP was used to assess the effects of perillyl alcohol or rapamycin on telomerase activity in both rb4E and pMV7. Cells were treated with one of the following: 400 μM perillyl alcohol for 16 hr, 10 nM rapamycin for 4 hr or 0.1% ethanol for 16 hr before lysis in CHAPS buffer. rb4E cellular extracts are represented by black bars; pMV7 cell lysates are represented by white bars. All experiments were conducted five times. Telomerase activity remaining was determined as described in Methods relative to ethanol (EtOH)-treated cells, which was set at 1 ($C_T$ value for rb4E = 24.13 +/- 0.794 SD ; $C_T$ value for pMV7 = 24.34 +/- 1.518 SD). Error bars represent +/- 1 standard deviation. Asterisks depict significant differences from control as determined by ANOVA.

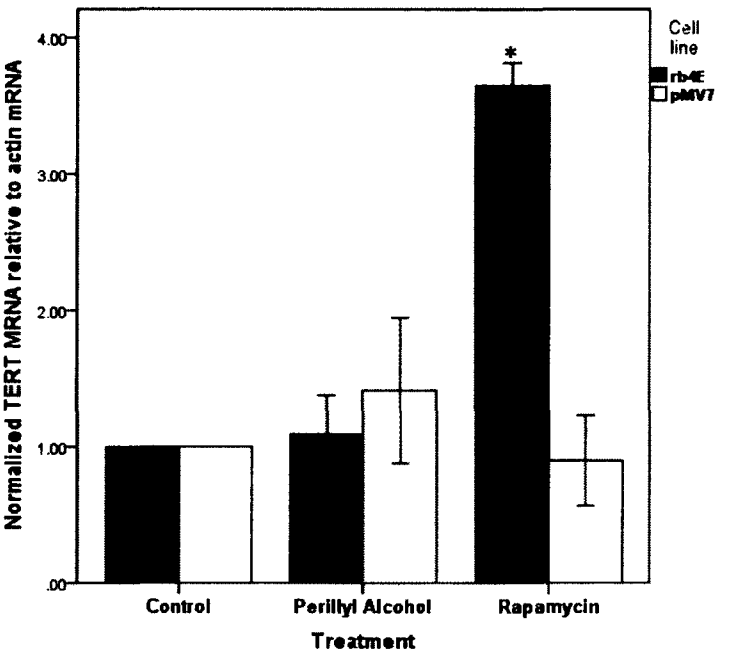


**Fig. 19.** Effects of perillyl alcohol or rapamycin on *TERT* mRNA levels in rb4E and pMV7 cell lines. Treatment conditions were as described in Figure 18. *TERT* mRNA expression was determined based on $C_T$ values derived from quantitative RT-PCR analysis. *TERT* mRNA levels were normalized to actin expression using the delta-delta $C_T$ method and expressed as a fold-change. Black bars represent rb4E *TERT* mRNA levels; white bars represent pMV7 *TERT* mRNA levels. Experiments were conducted in triplicate; error bars represent +/- 1 standard deviation. Asterisk depicts a significant difference from control as determined by ANOVA.

respectively (df=2; F= 34.073; p=0.001) (Fig. 18). A Dunnett's test revealed highly significant modulation of telomerase by both perillyl alcohol (p=0.001) and rapamycin (p<0.0005). The observed down-regulation was similar to that identified previously in our studies with human prostate cancer cells (Chapter 2).

*Neither perillyl alcohol nor rapamycin mitigates TERT mRNA levels regardless of eIF4E overexpression*

Although perillyl alcohol and rapamycin-mediated effects on telomerase activity in rb4E cells were relatively rapid suggesting translational or post-translational events, mRNA levels were assessed to verify that *TERT* transcription was not being altered. Analysis of the delta $C_T$ values of basal *TERT* mRNA levels found no significant differences between pMV7 and rb4E cells as assessed by quantitative RT-PCR ($df=2$; $F=0.178$; $p=0.688$) (not shown). Further, we found no inhibitory effect on *TERT* mRNA levels in response to perillyl alcohol or rapamycin treatment in either pMV7 or rb4E cell extracts (Fig. 19). Specifically in pMV7 cells, perillyl alcohol or rapamycin had little or no effect on *TERT* mRNA levels ($df=2$; $F=1.697$; $p=0.261$). In rb4E cells, an ANOVA on treatment effects demonstrated a substantial increase in *TERT* mRNA ($df= 2$; $F=186.48$; $p<0.0005$); however, a Dunnett's test revealed perillyl alcohol did not contribute to the upregulation of *TERT* mRNA ($p=0.782$). Rapamycin, in contrast, significantly enhanced *TERT* mRNA levels 3.7-fold in rb4E cells ($p<0.0005$). However, telomerase activity was significantly down-regulated under these conditions as observed in Figure 18. These results clearly show that *TERT* transcription was not being hindered; therefore translational processes must be at play in attenuating telomerase activity in rb4E cells (Fig. 19).

*TERT protein levels are diminished by perillyl alcohol or rapamycin only in the presence of eIF4E overexpression*

We previously reported that in DU145 prostate cancer cells, hTERT protein levels decreased in response to perillyl alcohol or rapamycin treatment (Chapter 2). Due to the apparent eIF4E-dependent inhibition of telomerase activity, we assessed the effect of eIF4E-overexpression on TERT protein modulation by perillyl alcohol or rapamycin treatment. Despite an ~5-fold increase in eIF4E levels in rb4E cell extracts compared to that found in pMV7 extracts ($df=2$; $F=36.941$; $p=0.004$), the basal levels of TERT protein in both cell lines were

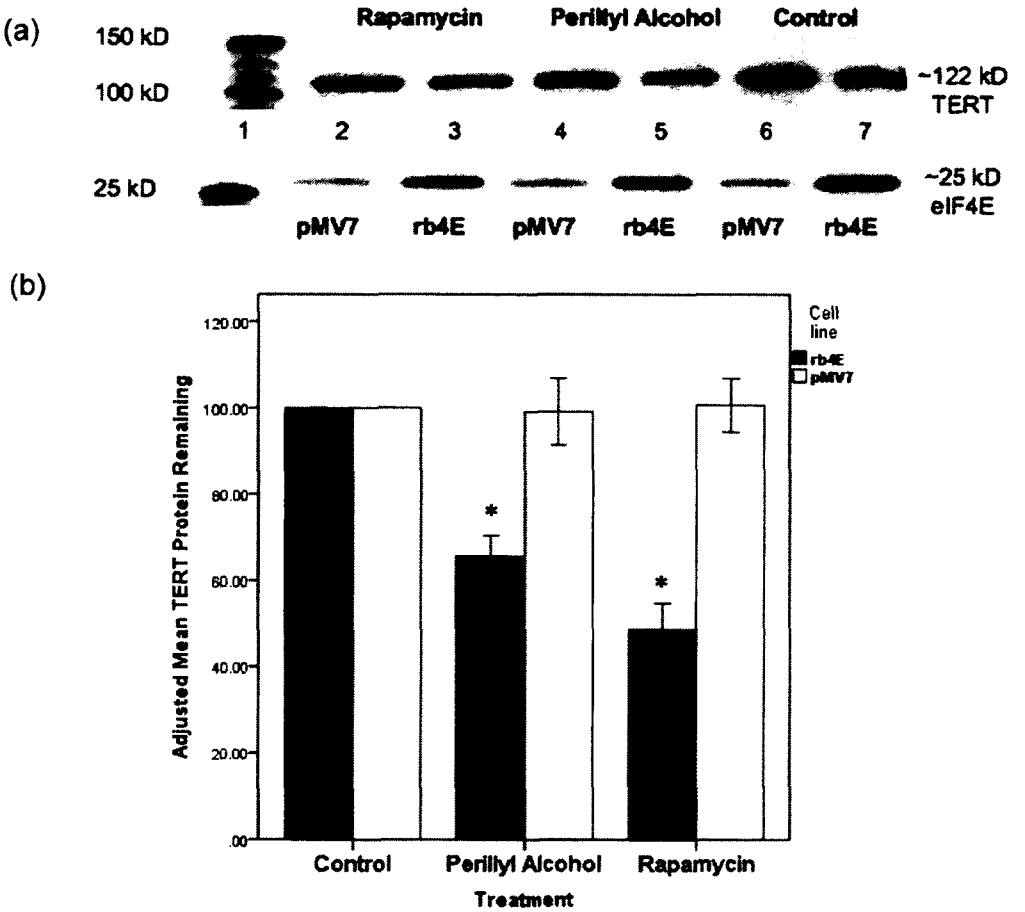


**Fig. 20.** TERT protein levels decrease in response to perillyl alcohol or rapamycin in rb4E cells but not in pMV7 cells. **(a)** Representative western blot analysis of whole cell lysates (50 µg of protein) resolved on a 4-15% polyacrylamide gel in presence of SDS and transferred to PVDF membrane. Membranes were probed with antibodies against TERT (top panel) or eIF4E (loading control, bottom panel). Lane 1: Precision Plus Protein™ WesternC™ Standards. Cells were treated as described in Figure 18. **(b)** Densitometric analysis of the effects of perillyl alcohol or rapamycin on TERT protein levels (three independent experiments). Error bars represent +/- 1 standard deviation. Asterisks depict significant differences from control as determined by ANOVA.

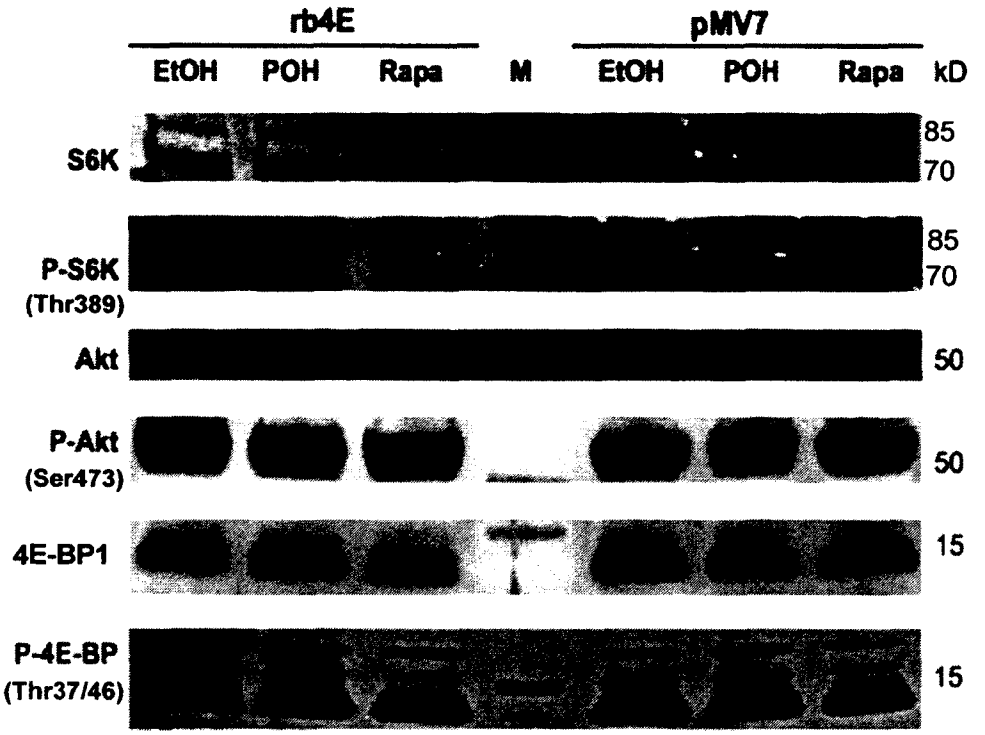


**Fig. 21.** eIF4E-overexpression alters phosphorylation of S6K, Akt, and 4E-BP1 and controls the response to perillyl alcohol or rapamycin. Representative examples of three independent experiments are shown. Rb4E or pMV7 cells were treated with biologically relevant concentrations of either rapamycin or perillyl alcohol as described in Fig. 18. Subsequently protein was extracted and 50 μg was resolved on a 4-15% polyacrylamide gel in presence of SDS and transferred to PVDF membrane. Membrane was immunoblotted with antibodies as indicated. POH, perillyl alcohol; Rapa, rapamycin, EtOH, ethanol; M, protein molecular weight markers.

virtually equivalent, therefore control bars were set to 100 (df=1; F=0.196; p= 0.681). Upon perillyl alcohol or rapamycin treatment of rb4E cells, however, a 34% and 51% decrease in TERT protein levels, respectively were found (df=2;

F=103.485; p <0.0005) (Fig. 20 (a) and (b)). A Dunnett's test further showed that the TERT protein responses to perillyl alcohol and rapamycin were highly significant in rb4E cells (p<0.0005). Rapamycin significantly decreased TERT protein levels despite the increase in mRNA levels. Multiple studies have shown mRNA levels do not often correlate to protein levels, suggesting additional regulatory mechanisms [223, 224]. In contrast, TERT protein levels were completely unaffected in pMV7 cells treated with perillyl alcohol or rapamycin (df=2; F=0.057; p=0.945) (Fig. 20 (a) and (b)). TERT modulation by perillyl alcohol or rapamycin is thus dependent on eIF4E-overexpression.

*eIF4E-overexpression alters phosphorylation of S6K, Akt, and 4E-BP1 and controls the response to perillyl alcohol or rapamycin*

eIF4E-overexpression activates Akt via increased phosphorylation of Ser473 and likewise increases phosphorylation of S6 ribosomal protein in mouse fibroblasts [116]. This suggested that greater eIF4E levels in rb4E may alter the cellular response to the mTOR pathway through a positive-feedback loop. Thus we examined the result of eIF4E-overexpression on the levels and phosphorylation status of Akt (Ser473), S6K (Thr389), and 4E-BP1 (Thr37/46). Furthermore, we addressed the effect of perillyl alcohol or rapamycin treatment on the phosphorylation of these proteins. Although rb4E cells have less Akt protein, the protein is more highly phosphorylated than that detected in pMV7 cells (Fig. 21). A major change in the level of Akt protein or its phosphorylation was not detected with either perillyl alcohol or rapamycin in rb4E or pMV7 cells. A comparison between pMV7 cells and rb4E cells revealed that rb4E cells had a more highly phosphorylated p85 isoform of S6K. S6K-phosphorylation (both isoforms) was almost completely abrogated by rapamycin in rb4E cells, despite lower levels of S6K protein compared to pMV7 cells (Fig 21). Perillyl alcohol had a similar, but more modest effect on S6K phosphorylation in rb4E cells. Strikingly, in pMV7 cells that do not overexpress eIF4E, diminished S6K phosphorylation was not observed with rapamycin or perillyl alcohol treatment (Fig. 21).

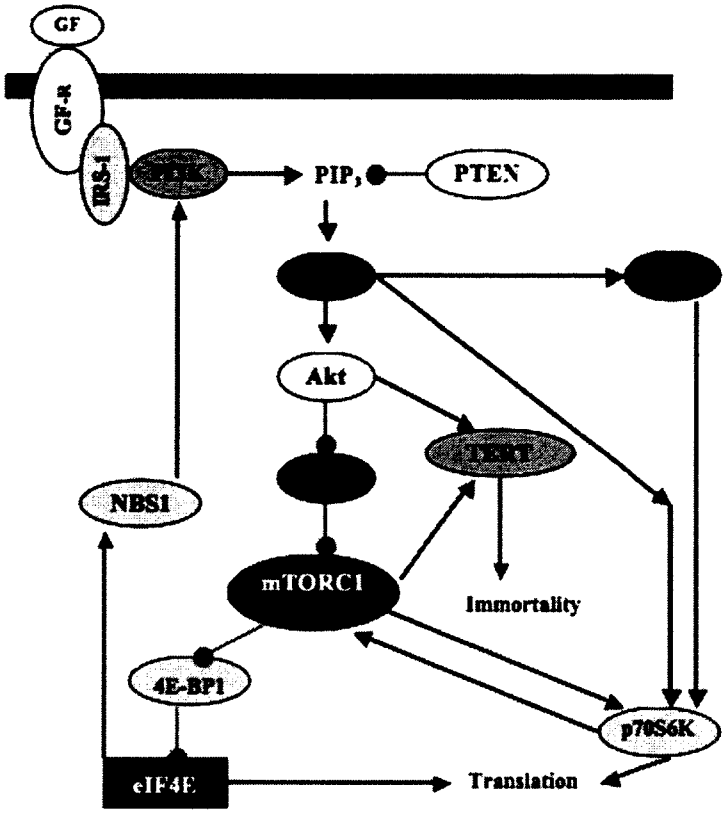


**Fig. 22.** A schematic representation of the positive feedback loop of eIF4E on the mTOR pathway via NBS1. eIF4E upregulation drives a positive feedback loop on the mTOR pathway resulting in increased phosphorylation of PDK1, Akt and p70 S6K. Nijmegen breakage syndrome (NBS1).

Both rb4E and pMV7 cells had similar levels of 4E-BP1, however rb4E cells had a higher level of phosphorylated 4E-BP1 (center and top bands). Perillyl alcohol did not affect the phosphorylation of 4E-BP1 in either cell line. Likewise, pMV7 cells exhibited no altered 4E-BP1 phosphorylation upon rapamycin treatment. Conversely, reduced phosphorylation of all isoforms of 4E-BP1 was observed with rapamycin treatment in rb4E cells, with the 4E-BP1 isoform represented by the center band being the most affected. These findings support

the presence of a positive feedback loop where elevated eIF4E in rb4E cells upregulates the PI3K/Akt/mTOR pathway as depicted in Figure 22 [116].

## DISCUSSION

Peffley et al. (2003) reported the first evidence that perillyl alcohol, limonene and other isoprenoids specifically affected gene expression at the translational level [193]. Furthermore they found that isoprenoids exerted chemopreventive and anti-proliferative effects, in part, by suppressing cap-dependent translation via mTOR/4E-BP1/eIF4E cascade, specifically through decreased 4E-BP1 phosphorylation and disruption of the eIF4F cap-binding complex [145, 193]. We recently observed that treatment of prostate cancer cells with perillyl alcohol and rapamycin individually was associated with a rapid and significant loss of telomerase activity and hTERT protein levels (Chapter 2). Of importance to this study was a previous finding from our laboratory that prostate tumor cells express levels of eIF4E that are approximately five-fold greater than their normal epithelial cell counterpart [186].

Although this is the first study to show perillyl alcohol or rapamycin-mediated telomerase inhibition is dependent on eIF4E, it was previously demonstrated that tumor cells are more responsive to perillyl alcohol-mediated growth inhibition than are non-cancer cells [170, 175]. Perillyl alcohol is relatively non-toxic and readily available through dietary sources thus making this compound a candidate for chemoprevention [153, 158]. Although perillyl alcohol's mechanism of action is still not fully resolved, the current study brings us closer to understanding how this small molecule may be useful in cancer prevention. In the context of chemoprevention, elevated eIF4E expression is a prerequisite for both perillyl alcohol-and rapamycin-dependent telomerase inhibition as described above (Fig. 18). eIF4E is overexpressed in most cancer cells compared to their normal counterparts [120, 225]. Additionally, eIF4E overexpression and telomerase re-expression both occur relatively early in the carcinogenesis process [35, 120]. Consequently, only cells that overexpress

eIF4E will be responsive to telomerase modulation, validating the use of perillyl alcohol for chemoprevention [170, 175].

The above results also indicate that TERT protein and telomerase activity are not likely regulated via enhanced eIF4E-driven cap-dependent translation because TERT protein levels were not elevated in rb4E cells (Fig. 20). This finding contrasts to our previous studies that examined HMG-CoA reductase protein in rb4E, in which reductase expression was increased by 400 to 500 percent compared to control cells [144, 145]. This eIF4E effect on reductase mRNA was specific and associated with only an overall 15 percent increase in total cellular protein synthesis. HMG-CoA reductase mRNA has extensive secondary structure in its 5'-UTR known to be responsive to eIF4E levels. In contrast, the *TERT* 5'-UTR has minimal secondary structure (Sundin and Hentosh, unpublished result), suggesting that translation of this mRNA does not require high eIF4E levels. Additionally, basal *TERT* mRNA levels were not upregulated in rb4E (Fig. 19).

The increased *TERT* mRNA levels associated with rapamycin treatment of rb4E cells were unexpected (Fig. 19). This phenomenon may be due to stabilization of *TERT* mRNA through inhibition of protein translation by rapamycin. Other translation inhibitors such as cycloheximide are often accompanied by heightened mRNA levels attributable to mRNA stabilization and protection imparted by ribosomal binding, as reported for autophagy–related proteins and others [226-228]. Alternatively, eIF4E has ancillary cellular roles related to protein translation including mRNA transport and turnover involving cytoplasmic processing-bodies (P-bodies) [219]. Mammalian P-bodies are cellular structures enriched in 5'-3' mRNA degrading enzymes [229, 230]. We propose that rapamycin treatment interferes with *TERT* mRNA association with, or transport to, the P-bodies, therefore enhancing *TERT* mRNA levels.

Support for this conjecture is the presence of an eIF4E-binding protein–4E-transporter (4E-T) found in P-bodies that interacts with eIF4E and represses translation [230]. eIF4E itself also localizes to P-bodies [230]. The 4E-T/eIF4E interaction serves as a prerequisite for targeting mRNAs to P-bodies. In

rapamycin-treated rb4E cells, 4E-BP1 was dephosphorylated (Fig. 21), which causes eIF4E to be sequestered. Perillyl alcohol treatment did not alter the phosphorylation status of 4E-BP1, allowing eIF4E to remain free to interact with 4E-T. We further contend that 4E-BP1/eIF4E binding supersedes the binding associations between 4E-T and eIF4E [219]. *TERT* mRNA levels were not altered in perillyl alcohol-treated cells likely due to the integrity of the eIF4E/4E-T complex (Fig. 19). In contrast, under rapamycin treatment, little or no *TERT* mRNA would be transported to the P-bodies; its mRNA would not be targeted for degradation triggering the elevated *TERT* mRNA levels observed in rb4E cells (Fig. 19). Despite high *TERT* mRNA levels, translation would likewise be negated due to eIF4E binding to 4E-BP1.

Our findings of altered TERT regulation and responses in rb4E must also be considered in light of recent studies that describe an eIF4E feedback loop in the mTOR pathway [116] (Fig. 22). As depicted, eIF4E-overexpression activates pro-survival Akt protein via increased expression of Nijmegen breakage syndrome 1 (NBS1) [116]. The ability of eIF4E to up-regulate NBS1 is independent of the translation initiation functions of eIF4E [116]. Once initiated by NBS1, the PI3K-Akt-PDK1 pathway instigates the downstream activation of mTOR, therefore S6K and 4E-BP1 become phosphorylated [231-233]. Upon activation, PDK1 in turn phosphorylates S6K directly [234, 235] and PKC– another upstream activator of S6K [236, 237]. Thus eIF4E activation of PI3K causes three distinct phosphorylation events of S6K, thereby amplifying the signaling pathway.

The redundant activation of S6K by three different proteins renders rb4E cells especially dependent on S6K activation rather than on 4E-BP1 activation. Consistent with this premise, our results show that the basal levels of S6K phosphorylation are greater in rb4E cells than those in pMV7 cells (Fig. 21). Additionally, S6K phosphorylation is dramatically curtailed by rapamycin, and reduced by perillyl alcohol only in the presence of eIF4E-overexpression (rb4E cells) (Fig. 21). In contrast, minimal amplification of 4E-BP1 activation was observed because it is phosphorylated via direct mTOR signaling. Although

diminished 4E-BP1 phosphorylation was detected in response to rapamycin in rb4E cells, the reduction is not as great as we would expect considering the S6K data. The reliance of the cell on S6K activation may make rb4E cells–and by default TERT protein levels and telomerase activity–more sensitive to perillyl alcohol or rapamycin-mediated S6K inhibition.

As cells transition from a normal condition to a cancerous state, eIF4E-overexpression may 're-wire' the mTOR pathway in such a way that a cell becomes more reliant on this pathway for survival and proliferation [238-241]. Although addiction of the EGFR oncogenic signal transduction pathways (upstream of the PI3K-Akt-mTOR pathway) has been previously described [241], the eIF4E-mTOR-TERT oncogenic dependence has not been demonstrated prior to this work. Weinstein and Joe emphasize that in cancer cells, a specific oncogene (i.e., eIF4E) may have a more vital and altered role in a given pathway compared with its function in normal cells [239]. eIF4E-overexpressing CHO cells are non-tumorigenic [242], however in our study on rb4E cells, eIF4E-overexpression mimics the behavior seen in cancerous cells and has assumed a role in TERT translation via S6K. If a cell such as rb4E becomes dependent on this pathway because of constitutive activation through elevated eIF4E expression, mTOR inhibitors such as perillyl alcohol and rapamycin would have a much greater effect on these cells due to a phenomenon now known as oncogenic shock [238]. Our results support an oncogenic shock hypothesis in that perillyl alcohol and rapamycin have no effect on phosphorylation or levels of mTOR-associated proteins–as well as TERT–in normal pMV7 cells with low eIF4E levels (Fig. 21). Conversely, cells over-expressing eIF4E become sensitive to their effects. Linking eIF4E-overexpression to the up-regulation of NBS1 may be one of the mechanisms by which eIF4E causes the suppression of apoptosis and enhancement of survival, a phenotype associated with cancer cells.

Elucidating the mechanism by which perillyl alcohol specifically manifests its effects against the mTOR pathway in cancer cells provides additional support for its efficacy as a chemopreventive agent. Similar–phytochemicals such as

curcumin, found in the plant *Curcuma longa* [218], has also been shown to have differential effects on protein translation in cancer cells versus normal cells [218]. Specifically, curcumin modulated cap-dependent translation more efficiently in cancer cells than in normal cells. This sensitivity may also be related to an activated mTOR pathway in tumor cells and account for the chemopreventive effects of curcumin [218]. Additional studies are necessary to further define the requirement of mTOR activation in prevention of cancer by natural products such as perillyl alcohol and related compounds. Likewise, our findings enhance the current understanding of perillyl alcohol's mechanism of action and highlight the importance of this simple isoprenoid for chemoprevention.

# CHAPTER V

# CONCLUSION

Defining the mechanism of action of perillyl alcohol in mTOR-mediated regulation of telomerase activity is essential to establish the value of perillyl alcohol as a chemopreventive. The collective findings presented in this work are compelling as they provide the first evidence that perillyl alcohol modulates telomerase activity via the mTOR pathway in prostate cancer cells. Our hypothesis that perillyl alcohol modulates telomerase expression through translational and/or post-translational mechanisms was supported by our findings. We determined that telomerase activity was inhibited by perillyl alcohol through a reduction in hTERT protein as well as a destabilization of the hTERT-mTOR-RAPTOR complex. Further, we revealed that perillyl alcohol or rapamycin-mediated inhibition of telomerase activity is dependent on eIF4E-overexpression.

We have uncovered many novel insights into telomerase regulation. Surprisingly, we found that overexpression of one mTOR-regulated protein (eIF4E) in a normal background, could in fact 're-wire' a cellular signaling pathway, thereby dramatically altering the way a cell responds to a drug (rapamycin or perillyl alcohol). Understanding these alterations in signaling pathways as a cell becomes cancerous will help establish new anti-cancer targets or improve drugs for the known targets.

These results will also have clinical relevance for chemoprevention through dietary intervention. eIF4E-overexpression and telomerase activation both occur relatively early as a cell shifts from a state of normalcy to a cancerous state, leaving a window of opportunity to target these cells before they multiply into a fully aggressive tumor mass. If the general population increases its consumption of fruits and vegetables, cancer rates may be decreased due to the ability of perillyl alcohol and other isoprenoids to dramatically curtail telomerase activity.

Although our study has revealed a mechanism by which perillyl alcohol inhibits telomerase activity, perplexing yet fascinating findings were encountered that present new avenues for discovery. This self-renewal pathway that we see repeatedly in science is our own sort of job security. The more that is discovered about a particular pathway/protein, the more questions arise to be answered about it. After this work, we are left questioning the function(s) of the hTERT-mTOR-RAPTOR complex. We speculate that DNA repair processes may be controlled by the complex, although further research will be necessary to confirm this hypothesis. hTERT has been implicated in dsDNA repair. Furthermore, the mTORC1 homodimer has a central cavity large enough to accommodate dsDNA. Additionally, mTOR is in a family of kinases (PIKKK) known for their DNA repair abilities. Interestingly, DU145 cells, which are radio-resistant, have very high levels of telomerase activity which would enable them to overcome radiation induced dsDNA breaks [243]. Whatever the reason RAPTOR, 4E-BP1, S6K, Akt, Hsp90, mTOR, and TERT were linked with one another evolutionarily, we now know the cell has made a compromise between efficiency (i.e., pairing kinases with their substrates) and vulnerability to being hijacked. Both EGFR-overexpression (upstream of the PI3K-Akt-mTOR pathway) and TERT-overexpression can independently cause the cell to display nearly every hallmark of cancer (i.e., evasion of apoptosis, unchecked cell cycle progression, sustained cell proliferation, resistance to growth inhibition, activation of invasion and metastasis, and induction of angiogenesis [244]). We now know how intimate these two pathways are. An aspiring cancer cell need only usurp one pathway to then control the cancer phenotype in a multifaceted manner.

Collectively these findings provide evidence for perillyl alcohol or rapamycin regulation of hTERT via the mTOR pathway in the presence of eIF4E-overexpression. Further, this work promotes the continued investigation of isoprenoids, such as perillyl alcohol, for use as chemopreventives. Together these results underscore the complexity of cellular responses that mediate anti-tumorigenic effects. Consequently, additional studies must be directed towards establishing the efficacy of these agents in a clinical setting.

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# APPENDIX A

## ABBREVIATIONS

*Abbreviations*

| | |
|---|---|
| 4E-BP1 | 4E-binding protein 1 |
| 4E-T | 4E-transporter |
| $m^7GpppX$ | 7-methylguanosine triphosphate |
| ALT | alternative lengthening of telomeres |
| AMPK | 5'AMP-activated protein kinase |
| ATM | ataxia telangiectasia mutated |
| ATP | adenosine triphosphate |
| ATR | ataxia telangiectasia and Rad3-related kinase |
| BME | ß-mercaptoethanol |
| BNIP3 | BCl2/adenovirus E1B 19 kDa protein-interacting protein 3 |
| bp | base pairs |
| BSA | bovine serum albumin |
| CHAPS | 3-[(3-Cholamidopropyl)dimethylammonio]-l-propanesulfonic acid |
| CHO | Chinese hamster ovary |
| $CO_2$ | carbon dioxide |
| CR4/CR5 | conserved region 4 and 5 |
| DEPTOR | DEP-domain-containing mTOR-interacting protein |
| DMSO | dimethyl sulfoxide |
| dNTP | deoxynucleoside triphosphate |
| E-box | Myc/Max binding site |
| E6 | human papillomavirus type 16 E6 oncoprotein |
| EDTA | ethylenediaminetetraacetic acid |
| EGFR | epidermal growth factor receptor |
| EGTA | ethylene glycol tetraacetic acid |
| eIF | eukaryotic initiation factor |
| EtOH | ethanol |
| FKBP12 | 12 kDa immunophilin FK506-binding protein |

*Abbreviations*

| | |
|---|---|
| GAP | GTPase activating protein |
| GDP | guanosine diphosphate |
| GTP | guanosine triphosphate |
| H/ACA | box H and ACA elements |
| HCl | hydrogen chloride |
| HIF-1 | hypoxia-inducible factor-1 |
| HMG-CoA | Homo sapiens 3-hydroxy-3-methylglutaryl-CoA |
| HRP | horseradish peroxidase |
| Hsp90 | heat shock protein 90 |
| hTERC | human telomerase RNA component |
| hTERT | human telomerase reverse transcriptase |
| IRS1 | insulin receptor substrate 1 |
| kb | kilobases |
| kDa | kilodaltons |
| KCl | potassium chloride |
| Map4k3 | mitogen-activated protein kinase kinase kinase kinase 3 |
| $MgCl_2$ | magnesium chloride |
| MKRN1 | makorin-1 |
| mLST8 | mammalian lethal with Sec13 protein 8; also known as GβL |
| mSIN1 | mammalian stress-activated protein kinase interacting protein |
| mTOR | mammalian target of rapamycin |
| mTORC1 | mTOR complex 1 |
| mTORC2 | mTOR complex 2 |
| $Na_3VO_4$ | sodium orthovanadate |
| NaCl | sodium chloride |
| NaF | sodium fluoride |
| neo | neomycin |
| NF | nuclear factor |
| NK | natural killer |
| P-bodies | processing bodies |

*Abbreviations*

| | |
|---|---|
| PABP | poly-A binding protein |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate-buffered saline |
| PDCD4 | programmed cell death protein 4 |
| PDK1 | phosphoinositide-dependent protein kinase I |
| PI3K | phosphatidylinositide-3 kinase |
| PIKK | PI3K-related kinase |
| PKC | protein kinase C |
| PML | promyelocytic leukemia tumor suppressor |
| POT1 | protector of the telomere |
| PP2A | protein phosphatase 2 A |
| PRAS40 | proline-rich AKT substrate 40 kDa |
| PROTOR | protein observed with RICTOR |
| PTEN | phosphatase and tensin homolog |
| PVDF | polyvinylidene fluoride |
| Rag | Ras related GTPase |
| RAP1 | the human ortholog of the yeast repressor/activator protein 1 |
| RAPTOR | regulatory-associated protein of mTOR |
| Ras | rat sarcoma |
| Rheb | Ras homolog enriched in brain |
| RICTOR | rapamycin-insensitive companion of mTOR |
| ROS | reactive oxygen species |
| rpm | revolutions per minute |
| RT | reverse transcriptase |
| RT-PCR | reverse transcription-polymerase chain reaction |
| RTQ-TRAP | real-time quantitative telomerase repeat amplification protocol |
| S6K | p70 S6 kinase |
| Ser | serine |
| SDS | sodium dodecyl sulfate |
| Src | sarcoma |

*Abbreviations*

| | |
|---|---|
| STAT | signal transducer and activator of transcription |
| TBE | tris-borate-EDTA |
| TBS | tris-buffered saline |
| TIN2 | TRF2- and TRF1-Interacting nuclear protein 2 |
| TPP1 | formerly known as TINT1, PTOP, or PIP1 |
| TRF 1/2 | telomeric repeat binding factor 1 and 2 |
| TSC | tuberous sclerosis |
| Tyr | tyrosine |
| ULK | Unc-51-like kinase 1 |
| UPS | ubiquitin-26 S proteasome pathway |
| UTR | untranslated region |

## APPENDIX B

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TERMS AND CONDITIONS

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**Abstracts**
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