Probing the Mechanistic and Therapeutic Intervention for the Drug Induced Age Reverse Procedure

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Fall 20-MSBI-NUST00000327214

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A thesis submitted in partial fulfillment of the requirement for the degree of Master's in Bioinformatics

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CERTIFICATE OF ORIGINALITY

I hereby declare that the research work presented in this thesis has been generated by me as a result of my own research work. Moreover, none of its contents are plagiarized or submitted for any kind of assessment or higher degree. I have acknowledged and referenced all the main sources of help in this work.

Marvah Mehmood Rana

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Dedication

This study is wholeheartedly dedicated to my grandparents

Sufi M. Anwar and *Irshad Beghum*

and my parents

Mehmood Iqbal and *Humaira Mehmood Rana*

who have been the source of inspiration and gave me the strength when I was about to give up, who continuously provided the emotional, spiritual, and financial support.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

hTERT	Human telomerase reverse transcriptase
hTERC	Human telomerase RNA component
BRN	Biological regulatory network
MDS	Molecular dynamic simulation
ROS	Reactive oxygen species
ALE-B	Average life expectancy at birth
TEN	Telomerase N-terminal domains
RT	Reverse transcriptase
CTE	C-terminal extension
TRBD	Telomerase RNA binding domain
DDR	DNA damage response
TEP	Telomerase protein component ALP
hALP	Human N-acetyle transferase like protein
TEIF	Transcription element interacting factor
STAT3	Signal transducer and
HIF1α	Hypoxia inducible factor 1 alpha
USF	Upstream stimulatory factor
KEGG	Kyoto encyclopedia
PDB	Protein data bank
RMSD	Root mean square deviation
FFT	Fast fourier transform

Abstract

Telomeres protect chromosomal ends during cell replication. Cell division shortens telomeres, causing senescence, differentiation, or necrosis and eventually aging. Aging caused by telomeres shortening is linked to diabetes, hypertension, Alzheimer's, and cancer. Therefore, activating hTERT (human telomerase reverse transcriptase), which lengthens telomeres, or boosting telomerase component expression can repair degeneration and postpone or reverse aging but the hyper activated hTERT promotes cell proliferation or cancer. hTERT mediated cell proliferation and p53 mediated cell apoptosis is critical in determining the cell fate. In order to reverse the aging effects of telomere shortening and keep cell proliferation under control, activities of hTERT and p53 must be kept within a normal range. A biological regulatory network (BRN) has been constructed and describe the dual role of Sp1 that activates both hTERT and p53. Moreover, the normal, moderately active and hyperactive states of Sp1 was related to the normal, moderately active and hyperactive concentration gradients of hTERT and p53 for the maintenance of normal cell proliferation and apoptosis. Furthermore, Protein-Protein docking helped in elucidating the binding patterns of hTERT activators like c-Myc and STAT3. Molecular dynamic simulations validated the stability of docked complex binding patterns by RMSD, RMSF, and Radius of gyration. Additionally, the binding site residues of hTERT Arg-224, Arg-293 and Arg-535 showed hydrogen bonding in c-Myc-hTERT complex before and after MD simulation, whereas the interacting residues Gly-35, Arg-63, Asp-147, Trp-203, Ser-206, Ser-227 and Ala-228 showed the stable hydrogen bonding before and after MD simulation in STAT3-hTERT complex, Arg-208, Arg-224 and Glu-533 formed the salt bridges in c-Myc-hTERT complex and Arg-63 and Glu-209 showed salt bridges in STAT3-hTERT complex. Thus, the interaction profiles of c-Myc-hTERT complex and STAT3-hTERT complex is identified as important for the future design of artificial activators of hTERT, or to classify the peptides or monoclonal antibodies of hTERT as activator or not-activator of hTERT by different machine learning models on the basis of evaluation by these binding patterns.

CHAPTER 1
INTRODUCTION

Chapter 1

1.   Introduction

1.1 The aging process

The beginning of aging occurred some 3.5 billion years ago, at the time that life on Earth first emerged. Because of aging's function as a risk factor in public health, the prevalence of several human diseases, including Alzheimer's disease, various forms of cancer, and metabolic disease/type II diabetes, has risen dramatically among the elderly. In order to meet the rising healthcare needs of an aging population, it is crucial to gain insight into the cellular and molecular mechanisms that decline with age and lead to increased disease susceptibility and frailty. The average life expectancy at birth in industrialized nations is quickly approaching plateau values as the aging changes associated with disease and the environment reach irreversible levels. In industrialized nations, where the average birth age is higher, the inborn aging process is the primary risk factor for disease and mortality beyond the age of roughly 28. As an approximate measure of the healthy, productive life-span, i.e. the functional life-span, these nations can only hope to increase their average life expectancy in the future by slowing the rate at which their populations are aging as a whole. [1]. Increased vulnerability to illness and mortality is one of the consequences of aging, which is the gradual accumulation over time of changes associated with or responsible for aging. There has been a lot of guesswork done on what exactly causes aging [2]. At now, there is great curiosity over the complex cause of the aging process in humans and animals, which has spawned various ideas. Although its origins are murky, aging has become the most pressing issue for doctors worldwide. The "old-age boom" predicted for the early 21st century has doctors extremely worried about age-related physiologic decline and age-associated illnesses. The successes of biomedical science in the 20th century have allowed more people to get close to the maximum human lifetime [3].

The buildup of variations that cause the sequence of changes that occur with increasing age and the concomitant, cumulative rises in the risk of illness and demise is what we call aging. The causes of aging have been the subject of several explanations. As research moves toward an agreement on the cause(s) of the aging process, it is imperative

that aging theories be studied for practical methods of enhancing the functional lifespan. While efforts to extend healthy life expectancy through traditional means have essentially reached an impasse, research into antiaging therapies has only begun [1]. Damage to cells and tissues from aging accumulates over time, reducing their ability to perform their functions and increasing the risk of mortality. Changes in old age can be traced back to a wide variety of sources, including development, genetic flaws, the environment, disease, and the aging process itself. Bettering people's standard of life reduces their risk of premature mortality [4].

1.2 Causes of aging

There are several causes of aging as shown in the **figure1.1**, like oxidative stress [5], telomere shortening [6], side reactions, glycation [7], aggregation of proteins [8], mutations [9] etc. However, telomere shortening is one of the primary causes of aging.

Figure 1.1. Causes of Aging are depicted in the figure that are stem cell exhaustion, genomic in stability, mitochondrial dysfunction, cellular senescence, altered intracellular communication, telomeres attrition, epigenetic alterations, deregulated nutrient sensing and loss of proteostasis.

In the 3D spatial structure of the nuclear genome, telomeres (the TTAGGG repeating DNA at the ends of linear chromosomes) play a role. In addition, certain patterns of controlled gene expression are set up via long-range 3D chromatin interactions. Dynamic nucleoprotein complexes called telomeres cap off each chromosome in a linear chromosome. They protect chromosome ends from being degraded, initiating a DNA damage response, fusing with other chromosomes, and being unstable [10]. Shelterin is a multi-protein complex found in telomeres that is accountable for protecting the G-rich nucleotide (TTAGGG) repeats at the end of chromosomes [11]. Interactions between telomere shelterin proteins and interstitial telomeric sequences (ITS) are a relatively recent area of research into the role that 3D telomere looping plays in cellular aging and genome stability.

1.4 Shelterin proteins

Six proteins work together to form shelterin: protection of telomere protein 1 (POT1), TRF1-interacting protein 2 (TIN2), repressor/activator protein 1 (RAP1), TIN2- and POT1-interacting protein (TPP1), telomeric repeat binding factors 1 and 2 (TRF1 and TRF2) [12]. POT1 binds to the telomeric DNA that is single-stranded [12] as shown in **figure 1.2**. Whereas TRF1 and TRF2 are linked to the telomeric DNA with double strands. The telomere replication factor 1 (TRF 1) is responsible for preventing telomere fusions and promoting telomeric replication [13]. TRF2 is essential for telomere maintenance and formation of higher telomeric structures such T-loops [14]. The shelterin protein TIN2 is essential to the shelterin complex because it binds both TRF1 and TRF2 at once and maintains the complex's structural integrity [15]. Telomere single-stranded tract protection is a joint effort between the protein POT1, which has a direct interaction with TPP1 protein, and TPP1 [16]. TRF2-interacting protein (RAP1) is a stabilizing protein that binds to and regulates the activity of TRF2 [17]. The shelterin complex has two crucial roles, one of which is to prevent the DNA damage machinery from recognizing chromosome ends, and the other is to recruit telomerase [11].
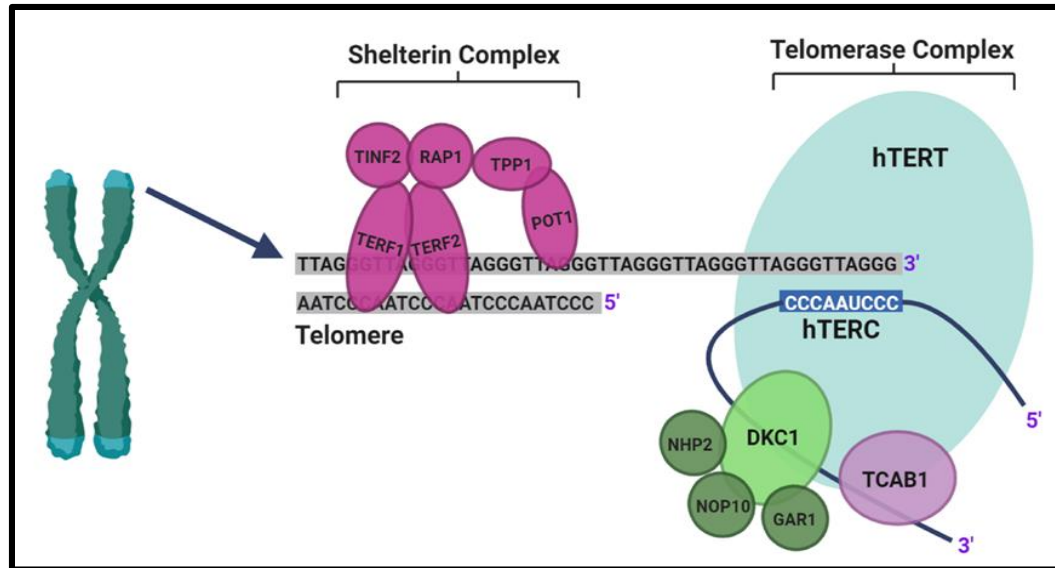
Figure 1.2. Six proteins making a shelterin complex along with the telomerase complex.

1.5 Telomerase

Telomerase, also known as telomere terminal transferase [5], is an enzyme that replicates the TTAGGG sequence at the end of chromosomes from an RNA template. The evidence for this is extensive [10]. A telomerase reverse transcriptase (TERT) protein and a telomerase RNA component (TERC) are the two main parts of telomerase. It wasn't until 1997 [16] that TERT, the catalytic component of the enzyme, was found. This was despite the fact that telomerase activity in extracts from the ciliate Tetrahymena had been reported by Greider and Blackburn in 1985 [18]. For telomerase biogenesis and localization activity, other proteins are needed, such as dyskerins, TCAB1, NHP2, NOP10, and GAR1 [19]. Telomere length reduces as we age. Telomere shortening causes apoptosis, senescence or neoplastic transformation of somatic cells, compromising an individual's lifespan and health. Shorter telomeres have been connected with higher prevalence of illnesses and poor survival [6]. In order to prevent telomere shortening caused by cell division, telomerase adds a G-rich nucleotide sequence (TTAGGG) to telomeres [20]. There are many steps involved in telomere replication, including binding, polymerization, and translocation. First, telomerase attaches to the 3′ overhang of the telomere by an interaction between TPP1 and telomerase RNA, or TERT. In the second stage of telomere replication, polymerization or telomere stretching occurs all the way to

the 5′ terminus of the DNA template. Once the telomere repeat has been fully elongated, telomerase can be repositioned to begin synthesis of a new telomere repeat sequence [21]. In adults, the telomerase gene is only expressed in highly dividing cells including germ cells, hematopoietic stem cells, and progenitor/stem cells [22]. To prevent excessive telomere shortening and promote ESC (embryonic stem cells) self-renewal, telomerase activity is also rather strong in ESC [23]. Immortalized cells and many tumor cells also have elevated levels of telomerase [24]. The fact that telomeres get shorter as we get older is widely recognized [23], since the quantity of telomerase activity is insufficient to counteract the constant regeneration in adult stem cells [25]. Telomerase and its activity can be controlled in cells by transcriptional regulation of TERT, alternative splicing variants of TERT, sex and growth hormone-like proteins found in telomeres and associated with the telomerase complex, androgen and estrogen, and other factors involved in phosphorylating and assembling telomerase or transporting its subunits [26]. TERT regulates telomerase activity in a number of ways, including alternative splicing, indirect and direct regulation of gene expression, posttranslational modification and protein tertiary folding [27]. Important elements in telomerase function include alternative splicing variations; for example, many cells contain distinct TERT variants from one another, which permits a wide range of cellular ages [28]. Phosphorylation of tyrosine or threonine/serine amino acids in a defined area of the catalytic subunit of TERT [27] is the mechanism by which telomerase is altered post-translationally [29]. Increased telomerase activity is linked to the interaction relationship between TPP1 and the TEN (Telomerase N-terminal) domain of TERT [28], further supporting the hypothesis that shelterin proteins play a significant role in telomerase action [30]. The p23 and hsp90 chaperone proteins, which are linked to efficient telomerase holoenzyme assembly, are also essential for telomerase activity [31]. Intracellular trafficking to the nuclear Cajal bodies, where the telomerase holoenzyme is assembled and/or matured, is another way by which telomerase activity is regulated [32]. The short sequence motif of TERC called the CAB box must be associated with TCAB1 in order for TERC to accumulate in Cajal bodies [33]. Cajal bodies and telomeres have been shown to coexist throughout the S phase of the cell cycle once the telomerase enzyme becomes active [34]. Unlike hTERT, which is only expressed in certain tissues, hTERC is expressed

everywhere. It serves as a template for the production of telomeric DNA. Some researchers have thus argued that it is not a limiting element in telomerase activity but hTERT is the main factor regarding telomeres lengthening [35, 36].

1.6 Telomeres shortening

Interstitial telomere loops (ITL) are generated by telomeres, and these loops interact with the internal transcribed spacer (ITS) to help control gene expression in a wide variety of genomic locations. In humans, laminopathies and telomeropathies frequently have short telomeres as a defining characteristic. It is possible that ITLs can vary and play functional roles in both normal and pathological processes. This is due to the fact that telomeres shorten in dividing somatic cells as a result of higher turnover and chronological age. Following reproduction, telomeres undergo a process of shortening and contribute less to the integrity of the nuclear genome. This can result in age-related disorders [37].

1.7 The hayflick limit

The deficiency of telomerase activity in human somatic cells causes telomere shortening with every cell division, leading to telomere malfunction and aging [38]. When telomeres shorten to an irreversible degree, cells have a biological reaction that prevents further cell division, leading to either or programmed cell death or senescence. The Hayflick limit was discovered to be the threshold at which cell proliferation stops being efficient [39]. In 1961, researchers Leonard Hayflick and Paul Sidney Moorhead found that human fetal fibroblasts grown in a lab had a limited ability to divide [40]. Hayflick proposed in 1965 that there were three stages in a cell's existence. His research shows that normal cell division starts in first stage, but subsequently slows down in the second stage. Stage 3 is the final stage before senescence sets in and cell division ceases [39]. Investigating the Hayflick limit aids in the study of cellular aging and the development of strategies to delay or halt the senescence of individual cells. DNA polymerase, which replicates linear DNA, was discovered to cause the loss of chromosomal terminal sequences (telomeres) in the early 1970s. Because of a delay in strand synthesis, DNA polymerase is unable to replicate the 3' end of linear DNA, a phenomenon known as the end-replication issue [41]. Telomerase and the shelterin

complex of the telomere help with this end-replication issue [42]. Eventually, as a result of the end-replication issue, telomeres shrink in human cells as we age [43].

1.8 Telomeres shortening and aging

Age-related disorders can be triggered by telomere shortening alone [42, 43]. These diseases include cardiovascular disease, liver cirrhosis, atherosclerosis [44], diabetes [45], infectious diseases, and Alzheimer's disease [46]. In addition to being linked to conditions like premature aging and bone marrow failure, telomere attrition has also been linked to human chronic disorders like hypertension [47]. Telomere shortening has been connected to aging and cellular senescence [43] and Human somatic cells' telomeres get more short as we age because telomerase activity declines, according to research. Studies in mice lacking telomerase (mTERC/) show that cell cycle halt and apoptosis result from telomere shortening that occurs between generations [48]. Hematopoietic cells, germ cells, embryonic stem cells and epithelial cells including liver, spleen and skin with extremely reformative/regenerative properties all express and contain telomerase at high levels [49-51]. To lower the danger of cancer, telomerase is inhibited in many somatic cells [52], which provides the evidence of its functions regarding cell proliferation. Human cells require telomerase activity for cellular immortalization, and it may be necessary for normal cells to be converted into cancer cells [24]. Cancer cells undergo transformation through telomerase activity and the concomitant deactivation of tumor suppressor genes or activation of oncogenes [53]. Inhibiting telomerase activity in cancer cells slows tumor development and promotes apoptosis [54]. To put it simply, oxidative stress brought on by reactive oxygen species (ROS) is to blame for both the initiation and the upkeep of senescence in cells. Multiple studies have shown that reactive oxygen species (ROS) may hasten telomere shortening in vitro and in vivo and can also directly harm DNA, particularly the structure of telomere [55-57].

1.9 Antiaging or age reversing

A growing body of evidence suggests that treating the aging process itself, rather than treating particular illnesses or symptoms of aging, could be one of the ways to go. This is inspiring research with the ultimate goal of bringing to market treatments that delay the aging process itself. There are several treatment techniques that can be brought to market much more quickly since they do not need FDA clearance. These include direct-to-consumer nutraceuticals and scientific diets that have undergone clinical trials. Nontraditional medicines, including blood-based therapy, are also being tested in an effort to delay the aging process. Using big data methods, researchers are attempting to create models of successful aging. Methods are increasingly being derived from findings on aging in model organisms. Death and medical expenses are primarily attributable to age-related illnesses. Slowing down the aging process would have significant health and economic implications. Companies specializing in antiaging treatments have sprung up in response to the discovery that several genes and pathways control aging in model organisms. Methods might include everything from drug development initiatives to big data approaches to DTC initiatives. Reliance on data from short-lived model species, a lack of biological knowledge of aging, and the difficulty of conducting clinical trials for aging all pose problems for commercialization. There are many possible therapies and targets related to aging, but only a tiny proportion can be studied for therapeutic applications because to the lengthy validation requirements. In any case, if even a single business succeeds, the results will be far-reaching [58].

As people age, the incidence of age-related disorders increases. Conditions associated with aging are now among the top triggers of mortality and disability everywhere in the globe. For this reason, it is of the utmost importance to discover effective therapies that can slow down the aging process and delay or prevent the onset of devastating age-related disorders. Enhancing autophagy, getting rid of senescent cells, transfusing plasma from young blood, intermittent fasting, boosting adult neurogenesis, working out, eating right, taking antioxidant supplements, and using stem cells are just few of the numerous antiaging methods now under investigation. To maintain normal health during aging and to delay age-related neurodegenerative illnesses like Alzheimer's, several pre-clinical research show that injection of autophagy enhancers, senolytic

medicines, plasma from young blood, medications that boost neurogenesis, and BDNF are potential options. The brains of the elderly and those with Alzheimer's disease have also showed promise for the potential of stem cell treatment to promote regeneration and restore lost function. The long-term effectiveness and safety of some of these methods is still up for debate and will be determined through clinical trials. However, non-invasive methods that appears to have low adverse effects, including as intermittent fasting, physical activity, and the use of antioxidants like resveratrol and curcumin, have showed tremendous promise for increasing function in aging. To conclude, various strategies are on the cutting edge of becoming standard therapy for slowing the aging process and delaying the onset of age-related disorders in the near future [59].

1.10 Telomerase (hTERT) activation and antiaging

The length of telomeres is a biomarker of cellular age and declines with age, making it a risk factor for age-related disorders. The pace of telomere shortening, which can be restored by telomerase, is influenced by environmental variables including nutrition and lifestyle. Natural molecule stimulation of telomerase has been proposed as a potential anti-aging modulator useful in the treatment of age-related disorders [60]. Because of its capacity to lengthen telomeres, the natural chromosomal endings that would else experience increasing attrition and finally threaten cell sustainability, telomerase endows most human cells with an infinite proliferative potential [61]. In contrast to its activation during development and neoplasia, telomerase is inhibited in normal human somatic cells. Telomerase is a specialized RNA-directed DNA polymerase that extends the telomeres of eukaryotic chromosomes. Repression of telomerase allows for the sequential shortening of telomeres in a chromosomal replication-dependent manner, whereas activation is implicated in the immortalization of neoplastic cells. For cells, the unidirectional catabolism of telomeres during the cell cycle serves as a means of keeping track of the amount of DNA they've lost and the number of times they've divided; when telomeres reach a critical shortness, cells cease replicating their chromosomes and enter cellular senescence. Forced production of the enzyme in senescent human cells increases their life span to one characteristic of youthful cells, whereas inhibition of telomerase has been found to limit the proliferation of cancer cells in vitro. Mechanisms by which telomerase is repressed in normal cells and activated in

neoplastic cells, as well as the signaling pathways between telomeres and these two cell types, remain to be determined. Thus, the ability to activate or deactivate telomerase may have significant therapeutic applications in the fight against aging and cancer both. Extensive research has revealed that post-translational protein-protein interactions, telomerase gene expression and protein phosphorylation all play important roles in mammalian cell telomerase control [62].

The increased probability of contracting a disease or passing away is a consequence of the time-dependent anatomical and physiological changes associated with the aging process [63]. Individuals and their cells begin to age soon upon birth and speed up as time goes on. After the embryonic stage, most human somatic cells have an inactive version of the telomerase gene, and telomere shortening is thought to occur naturally with age [64]. Since telomerase-expressing cells may keep their youthful state and continue to proliferate indefinitely [72], they have been considered a possible target for "antiaging" therapies [65]. Immortalized cells were once assumed to be a key component in the repair and replacement of worn out or diseased organs and tissues associated with aging. This therapy entails isolating cells from a patient with short telomeres and then increasing telomere length by expressing and activating TERT in those cells. Cells would be cultured, multiplied, and transplanted in place of failing or old organs and tissues [66]. Telomerase gene therapy may be useful in cases of decreased organ regeneration due to telomerase shortening, as shown in experiments with telomerase-deficient animals [67]. Increased telomerase expression achieved by telomerase gene therapy has been shown to reverse aplastic anemia characteristics in a mouse model without increasing cancer risk [68]. Using cell treatment to delay cell senescence is made clearer by the immortalization of cells via TERT concept. Traditional gene therapy uses TERT-modified cells for optimization of stem cell transplantation in vitro, the treatment of malignancies such as hepatocellular carcinoma, tissue engineering (including the formation of new blood vessels), and the treatment of chronic disorders such as atherosclerosis [69-71]. TERT is used to immortalize and increase the life span of several different types of human cells, including retinal-pigmented epithelial cells, dermal fibroblasts, keratinocytes, muscle cells, bone marrow stromal cells, endothelium cells, osteoblasts, odontoblasts,

and corneal epithelial cells [72-76]. Researchers have shown that transfecting telomerase-negative normal human cells with vectors encoding to TERT causes them to produce telomerase and shows a decrease in replicative senescence [65]. This includes retinal pigment epithelium cells. Several drugs, including histone deacetylase inhibitors and estrogen receptor agonists, can counteract the transcriptional downregulation of telomerase that occurs without transfection of the TERT gene [77]. In addition, telomerase, which locates in the nucleus and cytoplasm of cells, can be kept in those places by a protective mechanism by intracellular localization of telomerase and this can be considered as an age reversing therapy [78]. Because androgens can stimulate transcription of TERT , androgen therapy is utilized to treat aplastic anemia [79]. Danazol, a synthetic androgen, has been demonstrated to lengthen telomeres in leukocytes when used to treat telomerepathic individuals [80]. It has been shown that telomerase's classical role in elongating telomeres is substantially responsible for its antiaging activity. This avoids the buildup of dangerously short telomeres and loss of tissue homeostasis. This is comparable to the scenario in the so-called 'human telomere syndromes, in which telomere shortening in the setting of adult stem cell compartments causes significant impairment of stem cell mobilization and, ultimately, a deficiency in the ability to regenerate tissues. Therefore, cellular senescence or apoptosis refereed by the p53 pathway is triggered when short and/or unprotected chromosomal ends are detected as persistent and/or non-repairable DNA breaks [81].

Telomerase-deficient (Terc/) mice provide more evidence for telomerase's significance in tissue regeneration and organismal life span [82]. Increasing generations of telomerase-deficient mice interbred together shortens their lifespan [83], an impact that is evident in the first generation of mice without Terc, in which both the median and maximum life span are lowered [84]. Lastly, overexpressing telomerase is enough to increase the longevity of most cultured human cells [65]. Cumulatively, the data lends credence to the idea that mammalian life duration is limited by telomerase activity and telomere length, and to the idea that short telomeres actively contribute to aging by reducing the pace at which new tissues may form with age. One of the key predictions of this model is that reducing the rate of telomere shortening will extend lifespan. However,

telomere biology in cancer must be considered if this hypothesis is to be tested experimentally [61].

Joan S. Sonneborn's work shows that tissue deterioration may be reversed in elderly mice, providing the evidence for the potential for therapeutic TERT intervention in aging [85]. Examples of telomerase advantages arise in immunology [86], cardiology [87], chemotherapy-induced damage [88] and neurology [89, 90]. Cancer telomerase activity can be regulated by epigenetics [91] and TERT RNA splice variants [92]. Recently found telomerase cancer cell targeting permits eradication of cancer cells without downregulation of telomerase's beneficial effects in age-related disorders [93, 94]. Multiple disorders may benefit from the use of the TERT peptide GV1001 as an intervention medication, according to recent research [95].

1.11 Problem statement

Insufficient hTERT activity causes telomere shortening and cellular aging. Shortening of telomeres has negative effects on health and longevity by triggering apoptosis, cellular senescence and neoplastic transformation in somatic cells. That's why an enhanced but normal boost in hTERT and p53 activity is necessary to slow down or even reverse the aging process in cells.

1.12 Objectives

Objectives of this research are following:

1. To perform dynamic simulation of hTERT and p53 pathway to determine the concentration gradient of hTERT and p53 for the modulation of telomeres and for the regulation of normal proliferation/apoptosis in normal human cell.
2. To elucidate the interaction profile of positive regulators of hTERT and p53 for the design of new biological/chemical entities against age reversal process.
3. Co-targeting of hTERT and p53 by drug like entities for the telomeres mediated   age   reversing   process.

CHAPTER 2
LITERATURE REVIEW

Chapter 2

2.   Literature Review

2.1 Influence of aging

The mortality rate of a population at a specific age is a reasonable proxy for two things: (1) the average number of ageing changes and (2) physiologic age, or "actual age" ALE-B is based on a population's mortality rates. ALE-B estimates functional lifespan [4].

2.2 Structure and function of hTERT

In biology, ribonucleoprotein (RNP) complexes like telomerase are very important. The RNA part of telomerase, called hTR, acts as a template for making telomeric DNA and is at the centre of the enzyme's catalytic core [96]. Also, hTR binds to dyskerin to make sure holoenzyme is stable and to TCAB1 to help telomerase mature and move to Cajal bodies [34, 97]. **Figure 2.1** shows how the 1132 amino acids that make up the hTERT gene are put together. The hTERT protein has four domains: the telomerase N-terminal (TEN) domain, the telomerase RNA-binding (TRBD) domain, the reverse transcriptase (RT) domain, and the C-terminal extension (CTE). hTERT uses its TRBD- and RT-domains to bind hTR and put the hTR template in the RT-domain active site. This is how telomeric repeats are made [96]. The TEN-domain is involved in the catalysis of telomeric repeat addition [98]. The TEN-domain and CTE both have DAT (dissociates activity of telomerase) areas, with the former containing N-DAT and the latter C-DAT [99, 100]. Mutations in either DAT-region of telomerase stop immortalization but don't change the enzyme's ability to do its job [100]. Based on research with N-DAT mutations, it is thought that the TEN-domain brings telomerase to telomeres [101].
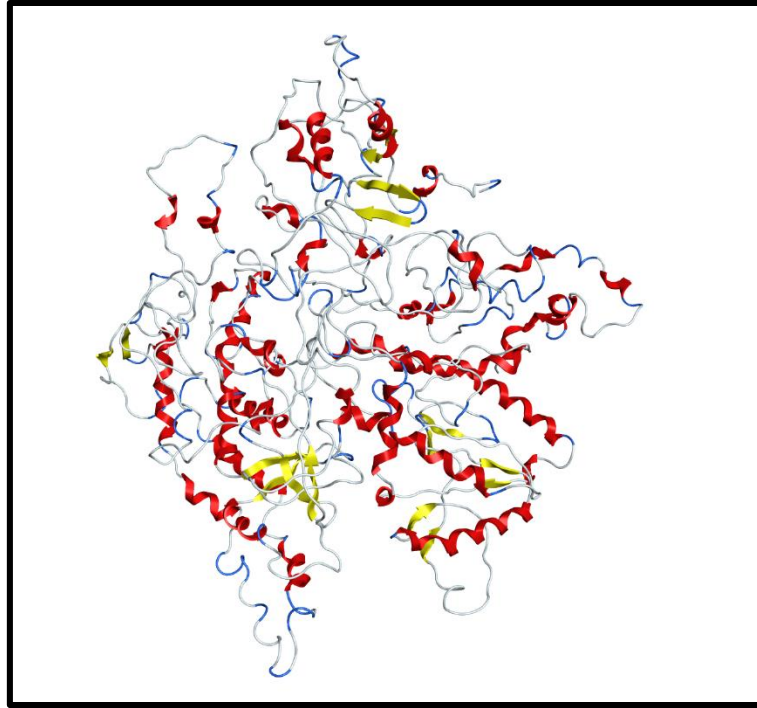
Figure 2.1. 3D Structure of hTERT comprised of 1132 residues and four domains visualized by MOE.

Telomeres are nucleo-protein complexes that safeguard the ends of chromosomes in eukaryotic cells. Muller and Meier determined the formation of telomeres in 1938. The DNA located at the ends of chromosomes is referred to as telomeric DNA. It consists of a 5 to 20 kb-long repeating pattern of the six nucleotides TTAGGG [10, 102, 103]. A telomeric loop (T-loop) that offers 3'end protection is formed by a series of telomeric DNA repeats and a terminal 3'G-rich single-stranded overhang [104, 105]. The shelterin protein complex and telomeric DNA act in tandem to preserve the integrity of the genome and chromosomes by preventing unneeded recombination, nucleolytic degradation and inter-chromosomal fusions[104, 106, 107]. Using shelterin protective caps, DNA damage repair (DDR) machinery can distinguish between genomic DNA damage and telomeric DNA [14, 108]. The "end-replication problem," age, oxidative damage and lifestyle (including stress, smoking, diet, work environment) shorten telomeric DNA after each replicative cycle throughout the lifetime of the cell [6, 109, 110]. When the telomeres of a cell get too short, the cell enters a dormant condition. This stage (M1) marks the beginning of DNA damage signaling and cellular senescence.

Together, they provide a crucial defensive mechanism that prevents a cell from becoming cancerous [107, 111]. However, there are situations where cells go beyond this senescence stage, entering a crisis state (M2) [111], bypassing crucial cell cycle checkpoints supplied by TP53, p16INK4a and Rb. Short telomeres and chromosome end fusion cause genomic instability, cell apoptosis, and ultimately cell death [111]. However, malignant transformation can be facilitated when cells gain the ability to divide indefinitely. Telomerase is responsible for sustaining or lengthening telomeres, a mechanism that promotes cellular immortalization [111-113]. Telomerase, an enzyme that may lengthen telomeric repeat sequences, was initially identified in 1985 [18, 114]. In 1989, telomerase activity was first demonstrated. It took almost a decade following telomerase's discovery for the protein component to be isolated and described for its function [115]. To progressively synthesize telomeric DNA repeats, this enzyme uses a huge ribonucleoprotein complex. The DNA polymerase telomerase is composed of two subunits: the human telomerase reverse transcriptase (hTERT), which is a catalytic protein subunit encoded by the TERT gene on chromosome 5p15.33, and the human telomerase RNA component (hTERC or hTR), which is an RNA component encoded by the TERC gene on chromosome 3q26 [116-118]. Additional proteins, including as Reptin, Nhp2, Gar1, Potin,, and Tcab1 were shown to be linked with the telomerase core complex and necessary for effective telomerase assemblage and employment to chromosomes [119, 120]. When combined with telomerase protein component (TEP1), dyskerin plays a crucial function in upholding the reliability of the telomerase complex [19, 121]. The telomerase enzymatic complex also includes the protein subunits (Ku heterodimer) Es1p and Es3p, which are important in assembly and maturation [122]. Human telomerase's three-dimensional structure has not been fully elucidated despite intensive study [123]. And, critically, only hTERC and hTERT are required for the restoration of telomerase activity [124-126]. Telomerase activity is strongly correlated with the amount of hTERT mRNA expressed, suggesting that hTERT is the major determinant of telomerase activity. According to the available data, the expression of hTERT is strictly controlled at the transcriptional level, making it the limiting factor in telomerase activity [65, 127-129]. Strong correlation between telomerase activity and hTERT expression has been shown in experimental settings [123].

Some model animals, including as chicken [130], zebrafish [131], dog [132] and pig [133], are used to investigate the role of telomerase and telomeres in the body. In maize, telomeres were found to be responsible for maintaining chromosomal integrity and genomic stability [134]. However, because mice are so comparable to humans in terms of telomere metabolism [135, 136], research conducted with mouse models have shown the most potential. Numerous analyses have shown that the biological responses to telomere disruption are essentially similar in both species. Somatic cell telomere length is thought to be closely related to telomerase control, which in turn may play a critical role in regulating normal metabolism, ageing, and cancer. Interesting, telomeres in mice are far longer than those in humans (25–40 vs. 10–15 kb, respectively). No visible changes are made to the fact that our lifetime is around a third that of humans. Without a doubt, the telomerase-deficient mouse model is invaluable for studying the effects of telomere dysfunction, such as aging, genomic instability, and cancer in mammals. Especially since telomere failure triggers the same molecular consequences in both species [135], including the p53 pathway being activated.

Human fibroblasts and lymphocytes undergo telomere shortening at a rate of about 50-100 base pairs (bp) per cell division in in vitro investigations [43, 137], which is quite close to a mouse model. The telomeric sequence is also the same, i.e., TTAGGG (n). Mouse Tert (mTert, mouse telomerase reverse transcriptase) telomerase activity and gene expression appear to be less strictly controlled in murine somatic cells compared to human somatic cells [135]. Since the expression of these subunits corresponds favorably with the distribution of telomerase activity in different cells [135, 138], it can be inferred that hTERT and mTert expression are the major determinants of telomerase activity.

2.3 Structure and function of p53

Depending on the type of cell and its environment, p53 is a very effective growth inhibitor that can stop the cell cycle or cause apoptotic cell death [139]. The p53 protein is a stress-inducible transcription factor that controls several genes involved in cell cycle arrest, apoptosis, differentiation, and even angiogenesis prevention. Approximately 50% of adult cancers have inactivated p53 (through mutation or deletion), whereas the remaining 50% have decreased wild type p53 activity. Thus, activation of the p53 pathway is viewed as a potentially interesting therapeutic method in the treatment of human tumors [140].

Normal cell division and tumor suppression need p53 activity regulation. p53 must be suppressed to allow normal growth and development while being activated in response to tumorigenesis-related stress. Several ways control p53. Although control of p53 transcription and translation has been identified, the key mechanisms influencing p53 function include control of p53 protein localization, regulation of p53 protein levels, and modulation of p53 activity, notably as a sequence-specific transcription factor. Post-translational modifications alter p53 [141] and cooperates with a variety of other cell proteins [142].

Trans activation domain, oligomerization domain, DNA-binding domain, proline-rich domain, and basic regulatory region are structural domains of transcription factors found in the 393-amino-acid p53 protein [143, 144] as shown in the **figure 2.2.** The N-terminal trans-activating domain (residues 1–62) is the part of the protein that interacts with other proteins. These proteins include basal transcription factors and regulatory proteins like MDM2, as well as co-activators acetyltransferases p300 and CBP [145-148]. The protein's N-terminal region also has a proline-rich domain, which spans residues 63 to 94 and contains five SH3-domain binding motifs. This domain may be found in the N-terminal region. This domain is very necessary for the p53-induced apoptosis that takes place when there is DNA damage [149]. The C-terminal section contains residues 325–356 that make up the oligomerization domain. This domain allows four monomers of p53 to form a homo-tetramer, which is necessary for the transcriptional activity of p53 [150, 151]. It is speculated that the essential regulatory region at the C-terminus, which spans

residues 357 to 393, is responsible for p53's sequence-specific binding [152, 153]. Finally, the core or central DNA-binding domain (residues 94-292) helps with the binding to sequence-specific double-stranded DNA and has a DNA binding surface that is formed of a central-sheet and two large loops (L2 and L3) that are stabilized by a zinc ion. This domain is located in the middle of the protein [154, 155]. The stability of the p53 protein is controlled by its central domain, and there is evidence that p53 has evolved to be minimally stable at physiological temperatures. This is supported by the fact that p53 has a melting point of 44 degrees Celsius and a half-life of nine minutes, both of which are lower than those of its paralogs p63 and p73 [156, 157].
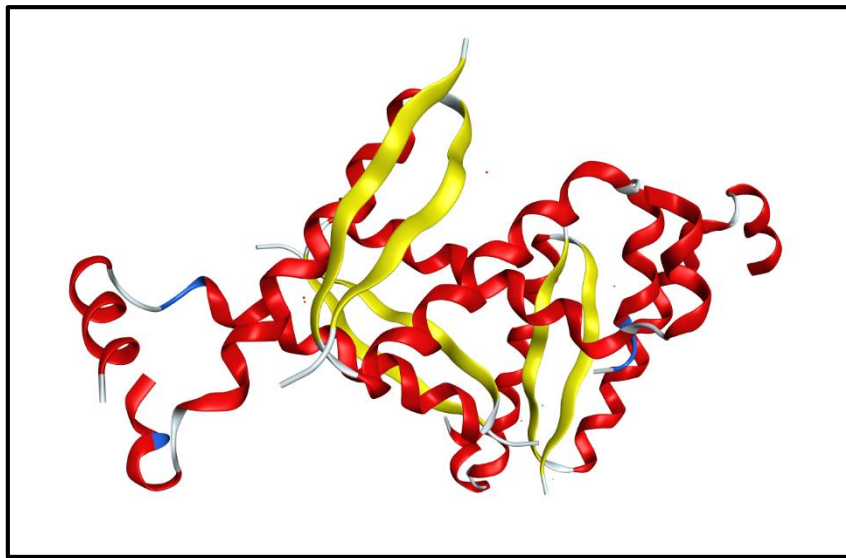
Figure 2.2. General 3D structure of p53 having 393 amino acid residues and contains transcription factor structural domain.

2.3 hTERT regulation and activation:

Telomerase activity is strongly correlated with the amount of hTERT mRNA expressed, suggesting that hTERT is the major determinant of telomerase activity. According to the available data, the expression of hTERT is strictly controlled at the transcriptional level, making it the limiting factor in telomerase activity [65, 127-129]. Strong correlation between telomerase activity and hTERT expression has been shown in experimental settings [123].

Both posttranscriptional and transcriptional processes are involved in hTERT regulation, which is a complex process that has yet to be fully elucidated [35]. Among them is hTERT pre-mRNA alternative splicing, which has been linked to diagnostic, prognostic, and clinical cancer characteristics [92, 158, 159] and is implicated in the control of telomerase activity. Germline, hematopoietic, stem, and rapidly renewing cells all have telomerase turned on permanently [49, 160]. In contrast, somatic cells have negligible or no telomerase activity mostly because of strict hTERT control [161]. Telomerase activity was found in the endometrial tissue (during the menstrual cycle), proliferative basal skin layer, hair follicles and the proliferative zone of intestinal crypts, but not in normal human blood cells [49, 160, 162, 163].

Although telomeres shorten gradually as cells approach replicative senescence [164], it is only during a crisis that they reach lengths at which they can no longer protect chromosomal ends [165]. Cells that are able to make it through a crisis are usually aneuploidy, suggesting that karyotype instability and fusions are more common at this time [166]. Although it has been hypothesized that telomere shortening causes both replicative senescence and crisis [167], it has also been recommended that replicative senescence is triggered by alterations in telomere status rather than telomere length [168, 169]. Given that most of this single-stranded overhang is lost in senescent human cells, the shape of the telomeric 3′ overhang reflects this changed condition [170, 171]. This means that although it is evident that telomere length, telomere shape, and telomerase all impact replicative lifetime in human cells, the interactions between these factors remain mostly correlative.

Changes in telomerase activity are temporally related with the expression of hTERT mRNA that occurs during cellular differentiation [172-174] and neoplasia [175], recent studies have shown that ectopic expression of hTERT is sufficient to restore telomerase activity in telomerase-negative cell lines, such as foreskin fibroblasts, mammary epithelial cells, retinal pigment epithelial cells, and umbilical endothelial cells, which supports the essential role that hTERT plays [65, 125, 176-178]. Other types of cell lines that do not have telomerase include retinal pigment epithelial cells. Although deactivation of the RB/p16 pathway is also required for hTERT-mediated

immortalization of keratinocytes and mammary epithelial cells, the correlation between hTERT expression and cellular immortalization has been well investigated and documented. This is the case despite the fact that the RB/p16 pathway must be deactivated. The combination of these theories and findings establishes the expression of hTERT as the bottleneck of telomerase activity, which propels the investigation of hTERT gene expression to the forefront of the research on telomerase regulation [179]. Conversely, there is a positive association between the length of the telomeres and lifespan [180]. Therefore, techniques that aim to increase telomerase activity in order to lengthen telomeres are considered crucial for fighting aging [61]. Multiple investigations in mice and rats shown that telomerase activity might reverse the aging process [136]. Mice with overexpressed TERT in many organs lived an additional 10% of their normal lifetime compared to wild-type mice in a single study, This resulted from the telomerase activation that occurred as a result of this treatment [181]. Increased expression of TERT in mice that were resistant to cancer was associated with a 40% increase in lifespan [61]. Adenovirus-mediated reactivation of telomerase can result in a 24% increase in lifespan [136]. In telomerase-deficient animals, reactivating telomerase causes telomere extension, reversal of degenerative phenotypes in gut, newborn neurons, testes, neural progenitors, spleen, and oligodendrocytes and decreased DNA damage signaling and associated cellular checkpoint responses [85]. TERT expression improves wound healing and keratinocyte proliferation in the bovine keratin 5 mouse model, compared to wild-type mice [182]. Protecting cells from apoptosis, telomerase encourages cell survival. By increasing hypertrophy and prolonging the life of myocytes in heart, TERT expression can delay the exit of cell cycle [56]. On the other hand, the expression of TERT is shown to inhibit the shortening of telomeres in neural cells [183]. TERT has also shown promising results when used to the central nervous system as a therapeutic target [183].

Positive transcriptional regulators can also enhance TERT expression via direct and indirect interactions with the promoter region. Direct-acting positive regulators include human N-acetyltransferase-like protein (hALP), transcriptional elements interacting factor (TEIF), signal transducer and activator of transcription 3 (STAT3), onco-proteins Ews-ETS, hypoxia inducible factor 1 alpha (HIF1α), and c-Jun [184]. Examples of indirect activators include the c-Myc oncogene, the apoptosis inhibitor

survivin, and the viral oncogene E6/NFX1. Survivin operates via SP1/c-Myc binding, and the oncogene c-Myc stimulates TERT expression by building a complex with max (c-Myc/Max) and binding to the E-box region. While p53 is degraded, c-Myc is activated, and upstream stimulatory factor (USF) based repression is broken by the viral oncogene E6/NFX1 [185-187], TERT expression is induced [188].

2.4 Signaling pathway of hTERT and aging

2.4.1 c-Myc

There was a correlation between c-Myc oncogene expression and telomerase activity prior to the identification of the hTERT promoter region, as shown in **figure 2.3** [189] was utilized to boost telomerase expression in human mammary epithelial cells, which are normally telomerase negative, to levels comparable to breast cancer cell lines. This was accomplished by introducing a c-Myc oncoprotein into the cells. Following transfection with the c-Myc vector, the amount of hTERT mRNA found in these cells was shown to have grown by a factor of 50 as determined by Northern blotting [189]. After characterizing the hTERT promoter region, c-Myc binding sites were discovered, enabling for additional investigation into the putative processes at work in this regulation [116, 190-192]. This c-Myc/Max dimer can recognise and bind to the 5′-CACGTG-3′ E-box consensus sequence [193, 194]. Because a similar sequence, 5′-CA(C/T)GCG-3′, also binds c-Myc/Max heterodimers, there is additional potential for regulation via binding at these non-canonical positions [193]. There are 29 putative c-Myc complex binding sites in the hTERT gene region, 18 of which are canonical [193]. Two E-boxes (5′-CACGTG-3′) in the midst of the hTERT minimal promoter have received special attention [190, 191, 195]. Gene reporter assays utilizing the luciferase gene and mobility shift tests indicate that c-Myc directly activates hTERT at these E-boxes [189-191, 193, 195]. Studies showing that c-Myc-induced hTERT elevation occurs without extra protein synthesis lend support to the concept that c-Myc directly activates hTERT [195].

Whether or not both of these E-boxes are required for the hTERT promoter to operate correctly has been the subject of considerable controversy. When the proximal E-box (at position -34) was deleted, the promoter activity was lowered by a factor of ten, attaining the same expression levels as promoter-free constructs [195]. Together, these results and the observation that the distal E-box is not preserved in the mouse TERT

promoter point to the proximal E-box [195] as the primary mediator of c-Myc's capacity to up regulate hTERT. Results from luciferase tests showed, however, that activity was drastically reduced in all cell types when the distal E-box (at position 242) was deleted or abolished [190, 191, 194]. These contradictory findings might point to a synergistic system in which the presence of c-Myc in both the distal and proximal E-boxes is necessary for full hTERT promoter activity.

2.4.2 Sp1

It has been observed that Sp1 can cooperate with c-Myc [194]. A two to seven fold increase in hTERT transcription is induced by co-transfection of a c-Myc or c-Myc/Max expression vector with a Sp1 expression vector [194]. Further investigation without Sp1 showed only partial activation, indicating that c-Myc requires Sp1 for complete activity [194]. Sp1 is usually believed to be a constitutively-expressed factor; yet, reports indicate that its expression can vary by as much as 100-fold amongst tissues [196]. These results raise the possibility that different amounts of endogenous Sp1 are responsible for c-Myc/cell Max's type-specific stimulation of hTERT expression [194]. Whether or not both of these E-boxes are required for the hTERT promoter to operate correctly has been the subject of considerable controversy. When the proximal E-box (at position -34) was deleted, the promoter activity was lowered by a factor of ten, attaining the same expression levels as promoter-free constructs [194]. The results for telomerase activity and hTERT expression were comparable. In addition, the levels of Sp1, Myc, and hTERT rise in a coordinated manner during SV-40-induced transformation of normal fibroblasts. Sp1 expression is substantially increased during cellular transformation, in contrast to Max protein expression, which is rather steady [194]. Sp1's involvement as a cooperative factor with c-Myc is extensively recognized, although the specific method by which it contributes to hTERT transcription remains elusive [190]. Furthermore, another research investigating estrogen's role as an activator of hTERT transcription have suggested that Sp1 may facilitate estrogen's activities in ovarian epithelial cells [197].

2.4.3 p53

The possible function of p53 in transcriptional control of hTERT was initially hypothesized based on data showing a negative correlation between p53 levels and telomerase activity, and it has now been confirmed. To determine if the absence of p53-mediated hTERT repression has any effect [198] contributes to the activation of telomerase seen during transformation, the effects of p53 overexpression in SiHa cervical cancer cells were explored. Two p53 binding sites, at 1954 and 1317 bp upstream of the ATG, were shown to be significantly involved in the reported inhibition of hTERT transcription [198]. Of note, the effects of exogenous p53 expression on transcriptional repression occurred before the suppression of cell growth typically associated with p53 activity [198]. These results provide further evidence that p53 may suppress hTERT gene expression directly to regulate cell growth. Sp1, a transcription factor, is considered to perform a cooperative role in p53-based control of hTERT, similar to what has been reported for the regulation of hTERT by c-Myc and estrogen [198]. When their binding sites to Sp1 were altered, the repressive effects of p53 were completely abolished, revealing a total dependence on Sp1 [194]. Sp1's dual function in hTERT activity raises the prospect of a more complex scenario involving protein-protein interactions between Sp1 and other transcription factors [194, 198, 199]. To learn how widespread p53-mediated regulation of hTERT is and to identify potential modes of action needing cooperation from Sp1, more research is needed.

2.4.4 ROS

The catalytic subunit of telomerase is human telomerase reverse transcriptase (hTERT), which is encoded by the tetrameric telomerase holoenzyme. Accumulating data suggests that hTERT is engaged in mechanisms outside telomere preservation and immortalization. hTERT overexpression reduces baseline ROS levels in cells and limits endogenous ROS generation in response to intracellular ROS triggers. In stressed cells, siRNA-mediated hTERT reduction boosted ROS production. ROS regulate hTERT in several ways. Changes to its expression, activity, location, and functions are among these [200-202]. It has also been shown that elevated ROS production encourages nuclear pore opening and subsequent hTERT export into the cytosol [203, 204].

Several other reports have also indicated that mitochondrial hTERT can considerably reduce levels of mitochondrial (and cellular) ROS [200, 205-209]. It has been shown that hTERT has the ability to reduce cellular redox potential and affect ROS-dependent signaling [210]. Since cells expressing WT hTERT had lower levels of mtROS, this finding suggests that autophagy activation might be mitigated by oxidative stress [211].
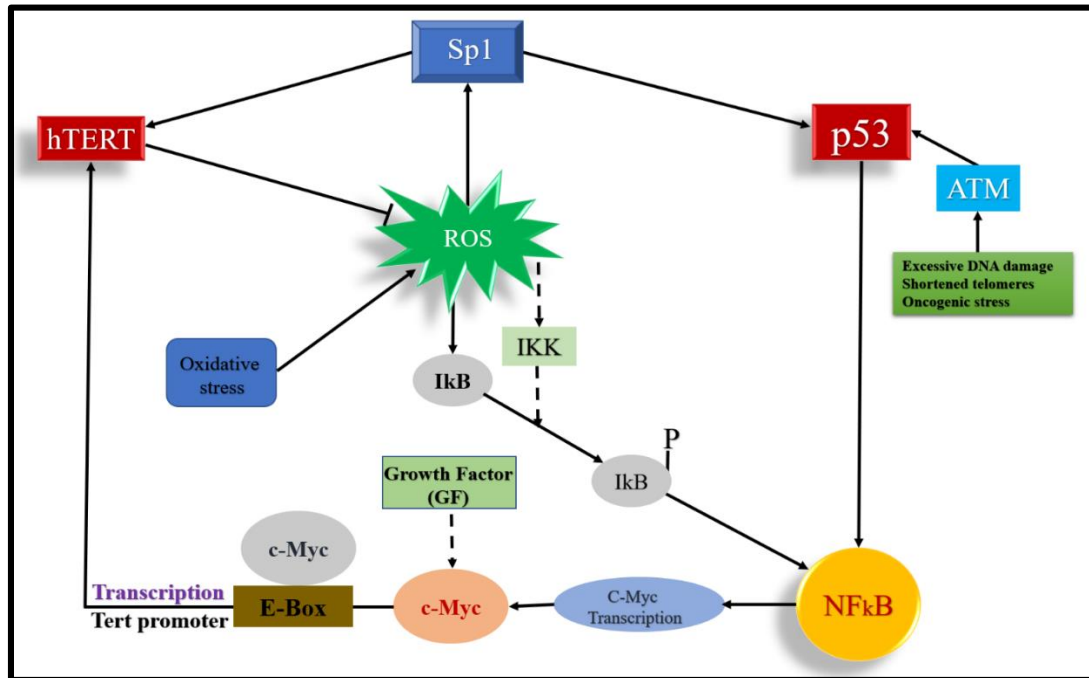
Figure 2.3. A knowledge driven biological regulatory network including different genes and proteins interacting positively or negatively with each other.

2.4.5 NFκB

A mechanism was explored that is mediated by ROS by which hTERT expression offers a survival advantage on cancer cells by attenuating apoptotic signals. As a result, cancer cells with naturally high hTERT levels would be resistant to chemotherapy treatments that employ ROS-mediated death pathways. In these instances, inhibiting hTERT or antioxidant defenses may render cancer cells more susceptible to treatment. NFκB, a crucial cellular transcription factor that interacts with ROS in a unique manner, is a plausible candidate for the mechanism through which hTERT regulates these antioxidant defenses. Multiple antioxidant components, such as MnSOD and -glutamyl-cysteine synthetase, the rate-limiting enzyme for GSH production, have been shown to be

controlled by cellular redox status. [212-214] ; NFκB has been shown to transcribe these factors and enhance the AP1's downstream target genes transcription that also includes sulfiredoxin [215, 216]. hTERT and NFκB have been shown to interact physically in a number of studies. Experiments were done to see if an active NFκB and p65 complex may function as a chaperone to help transport hTERT from the nucleus to the cytoplasm [217, 218]. Ectopic expression of hTERT enhances NFκB expression by more than 6-fold, according to research by Sharma et al. [219]. These researchers also demonstrated that this took place in a way that did not include direct interaction with the promoter regions of NFκB [219]. The level of nuclear p65 was likewise much higher in HT1 cells than in Neo cells. Furthermore, MnSOD, a transcriptional target of NFκB, is expressed at much higher levels in HT1 cells. It's tempting to speculate that hTERT's tight nuclear association with the active NFκB complex influences NFκB's DNA binding affinity and, by extension, the transcription of NFκB -driven antioxidant genes. Based on this and other recent research, it is clear that novel therapeutic methods are required to address the specific functional consequence of increased hTERT expression. Although expression of hTERT is essential for telomere maintenance, it has far-reaching biological consequences [208]. Multiple transcriptional regulatory elements are present in the human telomerase reverse transcriptase gene (hTERT). Increased hTERT transcription has been shown to be facilitated by a number of transcription factors [220-222] including c-Myc, Sp1, NFκB, and upstream stimulatory factors. Multiple cellular regulatory activities, including nuclear factor kappa B and hTERT, have been shown to govern cell proliferation [160, 223].

2.5 Existing strategies for antiaging

Antiaging science has immense potential because of the tight connection between aging and age-related disorders. Telomeres, which are protein-bound assemblies at the trimmings of chromosomes, have been shown to shrink during cell division and, in some tissues, with aging [224]. According to research, because human telomerase reverse transcriptase (hTERT) is not produced in human somatic cells, telomeres continually shorten and finally deplete with age. Activating telomerase may prove beneficial in the battle against age-related illnesses and as an antiaging strategy. hTERT, an antiaging supplement based on chemical telomerase activators, represents a new era in nutritional

antiaging research [225]. However, genetic modifications of telomerase in mice have yielded inconsistent findings [224, 226]. Overexpression of telomerase in adult mice increased median lifespan by 24% without increasing cancer incidence, according to one study. There is, however, a correlation between increased telomerase expression in mice and humans and a greater risk of developing cancer in both species [227]. Despite BioViva's self-experimentation with telomerase activation as an antiaging treatment, this therapy was not yet suitable for human usage because it was unknown how it may have worked. There were also security worries. Although some researchers anticipated that preserving telomeres might cause cancer, it was discovered that the mice used in the study were cancer-resistant [228]. Damage to essential biomolecules and aberrant gene expression, especially those related with aging, have been linked to chronic ROS overproduction. As a result, many aging-related clinical disorders attribute their beginning and course to this risk factor. To put it another way, aging-related disease situations can lead to unusually high ROS concentrations, which in turn can lead to permanently altered gene expression and signal transduction patterns [229]. Telomere shortening with age can be exacerbated by long-term exposure to chronic oxidative stress. Telomeres reduce with every division of a cell in the lack of telomerase activity, which is a crucial factor in aging processes [230]. In contrast, another research suggests that telomere shortening is influenced by both the frequency of cell divisions and the level of oxidative stress in the body [231]. These findings showed that oxidative stress may play a substantial role in the aging process and the diseases that accompany it, and that reducing or eliminating oxidative stress may be a useful strategy for delaying or slowing the aging process. As a result, many academics and medical professionals believe that taking antioxidant supplements can help delay or even prevent the onset of age-related diseases. However, the research conclusions on this subject are highly disputed. Randomized controlled trials of antioxidant supplements in humans are not linked to the improvement or prevention of cardiovascular disease or other age-related pathological disorders. Many studies show a link between vitamin supplements and an increased risk of death in well-nourished people, particularly in those who take vitamin A and vitamin E on a daily basis [232]. A study was performed to enhance the activity of telomerase by discovering potent activators and Telomerase activity increased 2 to 9

times more in in vitro model than in untreated cells. Importantly, the most powerful activator among commercially available supplements was the 08AGTL formulation, which contained *Centella asiatica* extract and increased telomerase activity by about nine-fold. Even more impressive was the potency of 08AGTL, which reached 17.3% of the telomerase activation of the positive control, a much greater percentage than the rest of the compounds examined, in terms of enhancing telomerase activity. They wanted to identify natural chemicals that could promote telomerase activity, which could lead to prolonged life expectancy and good aging. This natural product, 08AGTLF as shown in **figure 2.4**, which contains *Centella asiatica* extract, appears to have an impact on telomerase activity, but further study based on independent, randomized controlled studies investigating the underlying mechanisms is needed to confirm this. Telomere length, aging, and human health should be examined in future human intervention studies [60]. For the most part, only embryonic stem cells display telomerase activity. After birth, telomerase activity is dramatically reduced in all organs except for those that are capable of proliferation, such as the skin, gut, bone marrow, lymphocytes that divide, ovaries, and testes. Telomere shortening occurs as a result of telomerase deficiency in most human somatic cells. Cell immortalization is possible as a result of enhanced cellular telomerase activity and overexpression of TERT [233]. But there are evidences present where telomerase activity was enhanced along with the activation of tumor suppressor genes to prevent the danger of cell proliferation[61]. TERT, a component of telomerase, was found to be constitutively expressed in mice that were bred to be cancer resistant by increasing the expression of the tumor suppressors p53, p16, and p19ARF, according to the findings of the study. Activation of TERT increases the fitness of epithelial barriers, notably those of the skin and the intestine, and results in a systemic delay in aging, as well as an extension of the median life span in this environment. These findings reveal that constitutive expression of hTERT has antiaging action in the domain of mammalian organisms, which is consistent with previous findings[61].
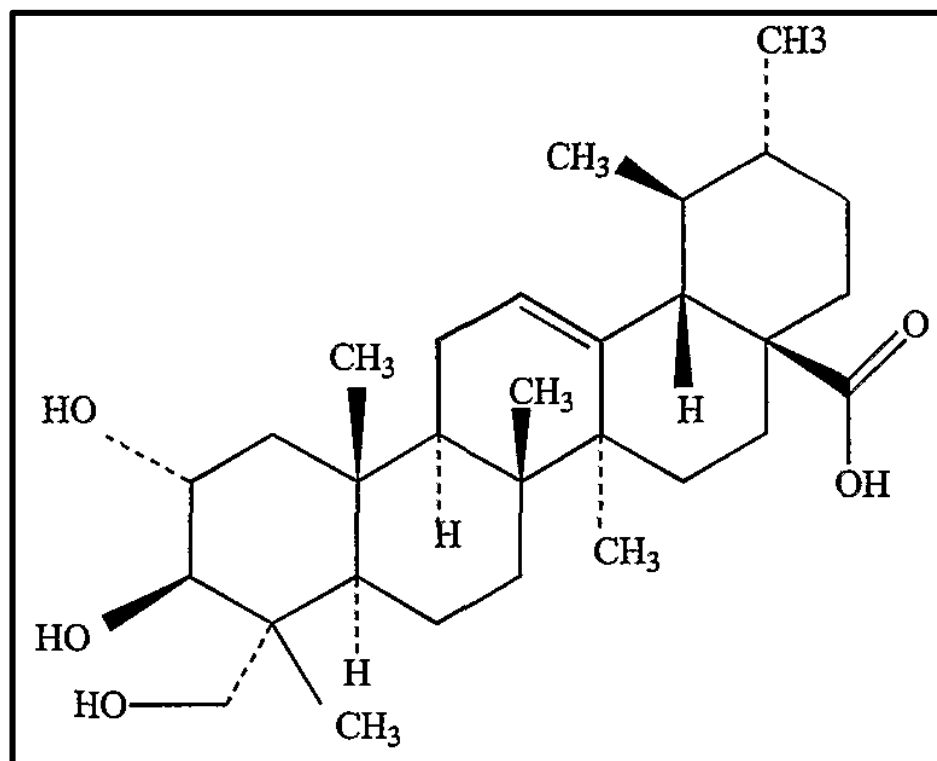
Figure 2.4. 2D structure of *Centella asiatica* extract formulation 08AGTLF that showed a positive impact on telomerase activity.

CHAPTER 3

METHODOLOGY

Chapter 3

3.   Methodology

3.1 Biological Regulatory Network (BRN) construction and its dynamic simulation

From a comprehensive literature search [193, 194, 208, 234] and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [235], To build a knowledge-driven biological regulatory network, researchers compiled a list of genes and proteins that interact (in either an activating or inhibitory fashion) with hTERT and p53 (BRN). Only modulatory proteins that have been shown to interact with hTERT or p53 in some way were chosen for use in the BRN's assembly. Different forms of protein interactions (activation and inhibition) were each represented as a directed edge linking two nodes in BRN. The BRN graph was created with the help of the user-friendly graphical software yEd v3.18.1.1 [236]. Initially, a model of a discrete biological regulatory network was built by assigning values to each item in the network: 0.0 for inactivity, 0.5 for moderate activity, and 1.0 for hyperactivity. The role of hTERT and p53 in cell proliferation and apoptosis was examined using continuous modelling and dynamic simulation of the network under three separate conditions: (a) normal hTERT activation, (b) moderate hTERT activation, and (c) hyper hTERT activation. The Java Genetic Regulatory Network Simulation Framework (Jimena) was used [237] to extrapolate the activation and inhibition states of various nodes described by the Boolean method [238]. As a whole, built BRN was simulated using continuous modelling, where nodes represent values in the interval [0, 1], and the network's behavior is determined by real-valued ODEs. The network's dynamic behavior was investigated by simulating it with the SQUAD simulation technique [239]. Briefly, SQUAD is an algorithm based on binary decision diagrams. By first viewing the network as a discrete dynamical system, the SQUAD approach generates a continuous dynamical biological regulatory network [214] by localizing the network's stable states [239]. With Wittmann et al., [240] explanation in mind, we designed a BRN where the node values were normalized-Hill functions, with SQUAD decay set to 1.0 and SQUAD steepness set to 10 [239-241]. With a step size (dt) of 0.05, the network was simulated for 100 simulation time seconds. To investigate the
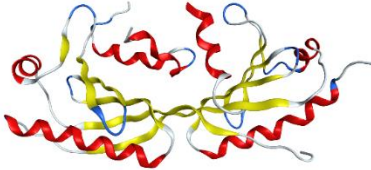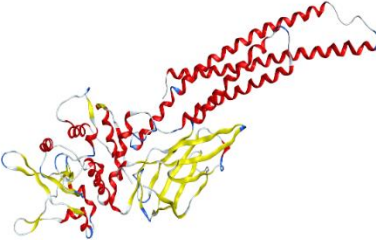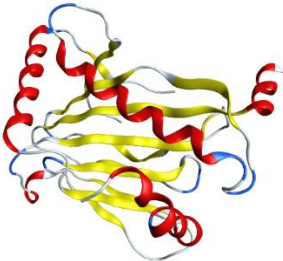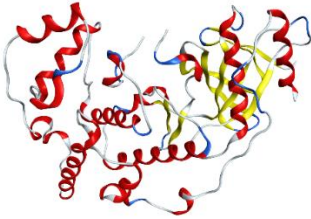
dynamical behavior of the chosen entities in the hTERT and p53 regulatory network, we imported the data acquired from simulations into the open-source interactive graphical program plotly (https://plot.ly/) [242].

3.2 Activators data collection

The data of positive transcriptional regulators of hTERT was available publicly. The structures of c-Myc, signal transducer and activation of transcription 3 (STAT3), hypoxia inducible factor 1 (HIF1α) and the transcription factor c-Jun [184] were retrieved from RCSB Protein Databank and these activators were selected through literature search as shown in the **table 3.1**. The structures were selected on the basis of their resolutions and recency. [243-246].

Table 3.1. The PDB IDs, resolutions and structures of positive regulators of hTERT.

Activator	PDB ID	Resolution	Structure	References
c-Myc	6e16	2.40 Å		[247]
STAT3	6tlc	2.90 Å		[248]
HIF1α	5la9	2.81 Å		[249]
c-Jun	6eq9	1.83 Å		[250]

3.3 Molecular docking

To investigate the molecular interactions between hTERT and its activators, the rigid protein docking was performed using ClusPro 2.0. Within this server three computational steps have been implemented that include: 1) rigid body docking using the FFT (Fast Fourier Transform) correlation approach, 2) RMSD (Root Mean Square Deviation) based clustering of the structures generated to find the largest cluster that will represent the likely models of the complex, and 3) the refinement of selected structures [251]. By default, the server settings of ClusPro 2.0 simultaneously generate four types of models using the scoring algorithms designated as balanced, electrostatic-favored, hydrophobic-favored, and van der Waals + electrostatic. We selected the top ranked (relative low energies) docking structures for all the activator complexes scored by the server. The molecular docking studies of p53 and its activators was not performed yet due to time constraints, but it is planned to perform in near future.

3.4 Analysis and visualization of docked complexes

Final docked output files were analyzed to check the interactions between the residues of both proteins within the complex. Information about the interface area and residues across all docked complexes was determined using PDBsum online server [252]. This server summarizes the information about bonded, non-bonded contacts and the interacting residues between the two proteins of a docked complex. Visualization of all the interacting residues was done by MOE [253].

3.5 Molecular dynamic simulation of the docked complexes

In order to assess the stability of the docked complexes, molecular dynamic simulation was employed on c-Myc-hTERT complex and STAT3-hTERT complex because of the early availability and better interactions of c-Myc and STAT3 with hTERT. The molecular dynamic simulation software GROMACS 2019.6 was used to assess the reliability of the docked complexes' binding residues and hydrogen bonds [254]. The structure of the docked protein-protein complex was constructed using the all-atom force field Charmm27. Solvation was carried out in a periodic box using the SPC216 water model, and the results were neutralized with Na+ and Cl- addition.

Initially energy minimization was completed using steepest descent minimization algorithm for 50000 steps and tolerance of 1000J/Å to eliminate the steric clashes of the complexes. Under conditions of constant temperature and pressure, equilibrium of minimum energy was reached for a pressure of 1000ps (default value). The Berendsen thermostat and barostat were used to maintain 300K temperatures and 1 atm pressure for the duration of the Molecular dynamic simulation sessions. Direct electrostatic interactions with a cut-off value of 1 nm were calculated using the fast smooth Particle-Mesh Ewald (PME) summation. Various MD runs up to 100ns duration were investigated.
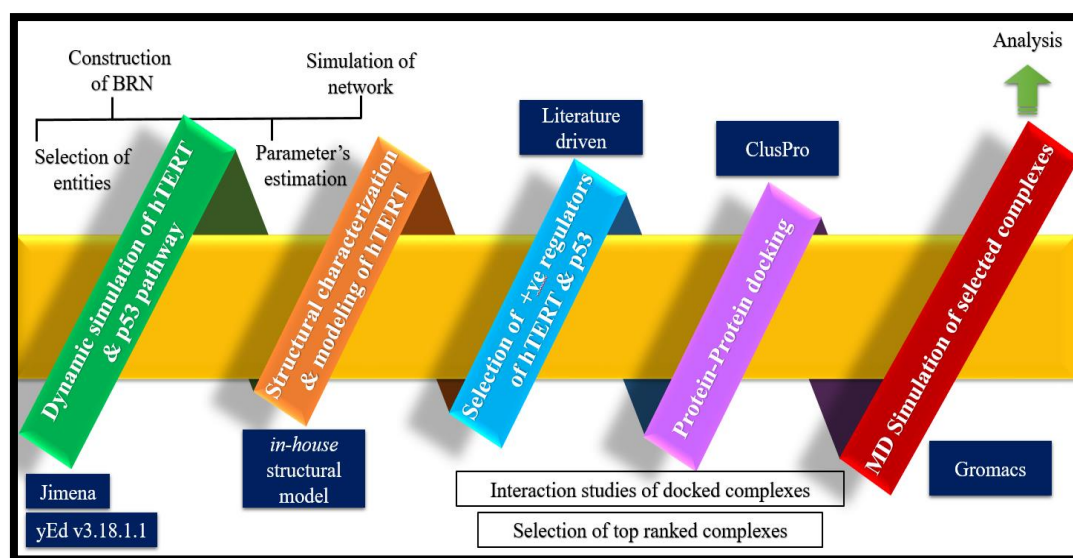
Figure 3.1 Schematic diagram representing the methodology used in this study

CHAPTER 4

RESULTS

Chapter 4

4.   Results

4.1 Biological Regulatory Network (BRN) simulations of hTERT and p53

Based on this information, the activators and inhibitors that interact with hTERT and p53 were chosen to form the theoretical BRN depicted in **figure 4.1**. Apoptosis and cell division were chosen as potential outcomes in the simulations [255, 256]. IκB was considered as the downstream signaling target of ROS that phosphorylates itself and activates NFκB [257]. NFκB was also included in BRN due to its importance in the regulation of the c-Myc transcription, that has a major role in cell cycle and proliferation [193, 258]. c-Myc further activates hTERT which is considered the most crucial factor in the regulation of cell proliferation. hTERT negatively regulates ROS because the mitochondrial production of ROS as a byproduct of oxidative respiration is enhanced by the shortening of telomeres, aging process and by exposure to carcinogens, radiation and other environmental toxins that produce elevated levels of intracellular ROS [259, 260]. Additionally, according to earlier research, hTERT overexpression lowers the basal levels of intracellular ROS and blocks the creation of endogenous ROS in response to stimuli that cause cells to produce ROS, hence reducing ROS-mediated cell apoptosis or programmed cell death [20, 21].   Moreover, p53 was considered important in the regulation of apoptosis which leads to cell death. p53 gets the activation signals from ROS and it further activates NFκB [261]. hTERT and p53 were considered the key contenders in the network, responsible for the responses of cell proliferation and apoptosis, respectively. Sp1 activated by ROS, was considered as the mainstream activating factor that positively regulates both the key contenders hTERT and p53 [194, 234].

In the network, it is essential to assume that Sp1 node is in a stable condition, where each node has a concentration parameter value of zero. In addition, Sp1 was regarded as the network's initiator, followed by dynamic simulations of continuous modeling. Initial parameters for scaled concentration levels in the BRN were defined as 0.0 for the inactive state, 0.1-0.4 for low activation states, 0.5-0.9 for the active state, and

1.0 for the hyperactive state. In order to accurately quantify the activation and inhibition relationship between the extracted nodes, the BRN was simulated until all states reached a stable state.
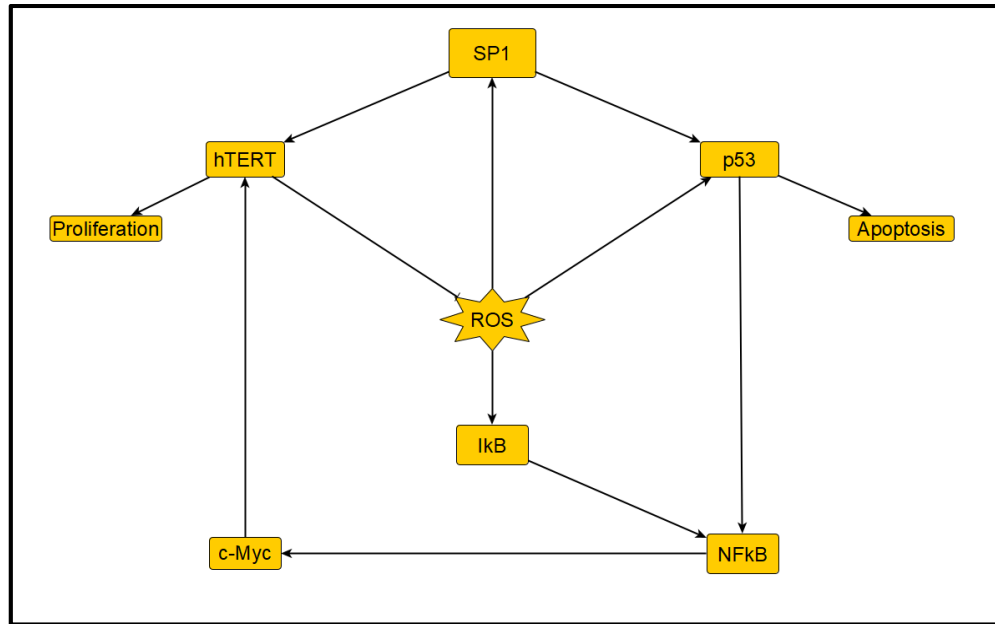
Figure 4.1. Biological regulatory network (BRN) of hTERT and p53 in a normal human cell. The hTERT mediated signals play an important role in cell proliferation and p53 plays a crucial role in cell apoptosis.

Under the dynamic simulations of a normal cell, the initial scaled concentrations of all the entities including the main activator Sp1 were kept in low activation range (0). To check the slightly more but normal activation of hTERT, the concentration of Sp1 was increased to moderately active state (0.5). And in the next round, the concentration of Sp1 was increased to hyperactive state (1.0) to check the over activation of hTERT, as shown in **table 4.1**.

Table 4.1. Parameter values of entities interacting with hTERT and p53 before and after simulations generated by Jimena.

Nodes	Sp1 value 0 (Normal Activation of hTERT)		Sp1 value 0.5 (Moderate activation of hTERT)		Sp1 value 1 (Hyperactivation of hTERT)	
	Before simulation	After simulation	Before simulation	After simulation	Before simulation	After simulation
Sp1	0	0.625	0.5	0.420	1	0.149
hTERT	0	0.676	0	0.825	0	0.916
p53	0	0.756	0	0.772	0	0.394
ROS	0	0.318	0	0.167	0	0.054
IκB	0	0.623	0	0.418	0	0.148
NFκB	0	0.727	0	0.852	0	0.815
C-Myc	0	0.600	0	0.795	0	0.933
Proliferation	0	0.682	0	0.833	0	0.946
Apoptosis	0	0.670	0	0.829	0	0.853

4.2 Dynamic model simulations:

During the simulation, the hTERT and p53 Sp1a's participation in the regulation of cell growth, development, and, intriguingly, apoptosis led to the initiation of efflux[262]. The Sp1 mediated effluxes of all the entities are shown in the form of graphs in **figure 4.2**. At each time step, a progressive increase in hTERT and p53 was detected, which eventually reached the moderately active expression level (0.676 and 0.756 respectively). At this level, the values for hTERT varied from 0.22-0.93, and the values for p53 ranged from 0.02-0.8, as shown in **figure 4.2 (a).** hTERT directly initiated the proliferation and p53 helped the cell to undergo apoptosis.
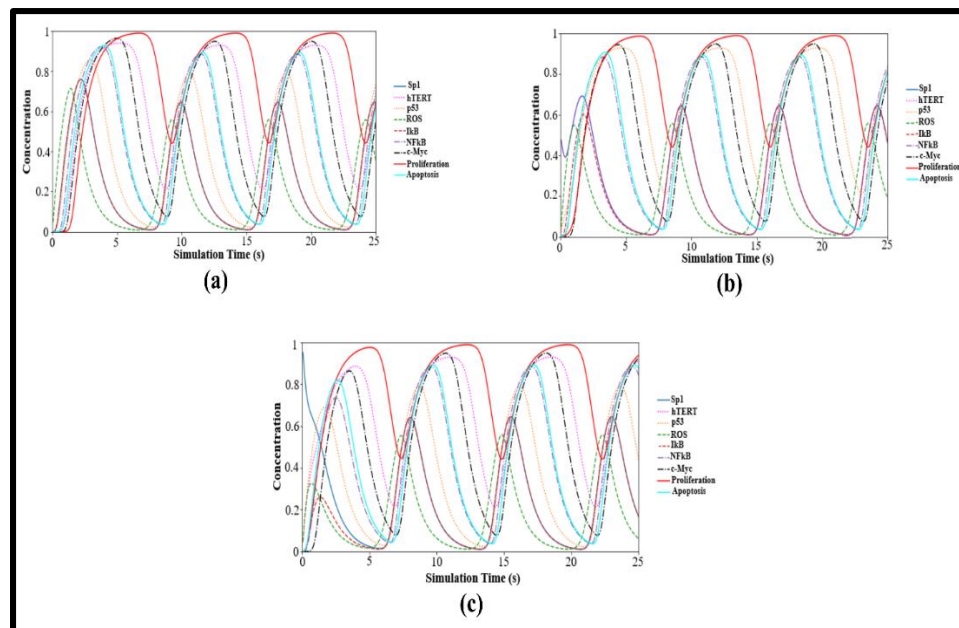
Figure 4.2. Jimena's dynamic simulations of the biological regulatory network of hTERT and p53 reveal the effect of activator-mediated effluxes on the regulation of cell proliferation and death by hTERT and p53. The x-axis indicates time in seconds and the y-axis indicates the relative concentration of activators and inhibitors. (a) The results of the simulation with Sp1 in its inactive initial state. b) The simulation outcomes when Sp1 is initially in the active state. (c) The simulated consequences when Sp1 is originally maintained in its hyperactive state.

hTERT alleviates the expression of ROS, so the slight increase in ROS expression (0.318) and it oscillated between 0.01-0.54 can be seen in figure 4.2 (a). By subsequent expression of ROS as well as assuming the prior activation of Sp1 (due to ubiquitous activation at promoter site within the human genome) in the BRN, the signal peak of Sp1 gradually reached at moderately active expression level (0.625) that ranged from 0.01-0.62 which in turn initiates both hTERT and p53 signaling. ROS further gives the positive signals to IκB which also gradually increases to moderately active level (0.623) that oscillated between 0.01-0.62. Activation of NFκB is controlled by sequential phosphorylation and ubiquitination of IκB. p53 also passes the positive activation signal to NFκB (0.727) which ranged between 0.03-0.89 throughout the simulation, that further activates c-Myc which then reaches its moderate level (0.600) which first oscillated between 0.1-0.84. c-Myc drives multiple synthetic functions necessary for rapid growth of cell division and activates hTERT for cell proliferation. Figure 4.2 (a) represents that the biological expression of hTERT and p53 is responsible for cell proliferation and apoptosis.

Next, the BRN was tested for Sp1, the mainstream activator's initial value 0.5. Due to this both p53 that swayed between 0.01-067 and hTERT that oscillated between 0.21-0.9, increased gradually towards slightly more active state, (0.825 and 0.77 respectively). This increment led the proliferation to gradually reach its more activated state (0.833) that first swayed from 0.45-0.97. Similarly, apoptosis also increased and reached its active state (0.829) that ranged 0.04-0.89 as shown in **figure 4.2 (b).** BRN was also tested by applying 0.5 as the initial values for both the activators, Sp1 and c-Myc as shown in the **figure 4.3 (a)** and the respective values of all entities are shown in **table 4.2**.
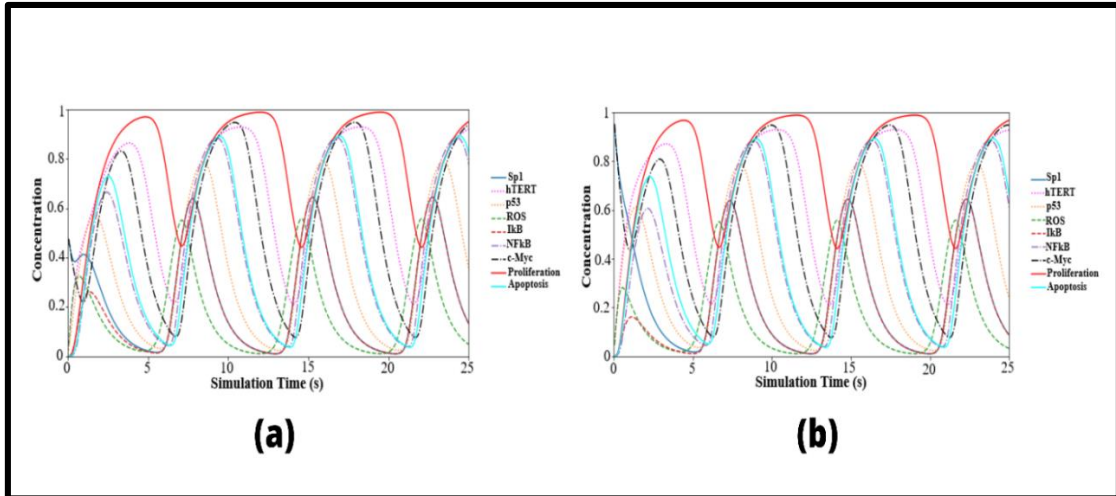
Figure 4.3. Jimena's dynamic simulations of the biological regulatory network of hTERT and p53 reveal the effect of two activator-mediated effluxes (Sp1 and c-Myc) on the regulation of cell proliferation and death by hTERT and p53. The x-axis indicates time in seconds and the y-axis indicates the relative concentration of activators and inhibitors. (a) The results of the simulation with Sp1 and c-Myc in their active beginning configuration. (b) The simulation outcomes when Sp1 and c-Myc are initially in hyperactive states.

Table 4.2. Parameter values of entities interacting with hTERT and p53 before and after simulations generated by Jimena.

Nodes	With Sp1 and c-Myc value 0.5		With Sp1 and c-Myc value 1	
	Before simulation	**After simulation**	**Before simulation**	**After simulation**
Sp1	0.5	0.118	1	0.078
hTERT	0	0.923	0	0.929
p53	0	0.318	0	0.214
ROS	0	0.043	0	0.028
IkB	0	0.117	0	0.077
NFκB	0	0.737	0	0.560
C-Myc	0.5	0.944	1	0.944
Proliferation	0	0.957	0	0.972
Apoptosis	0	0.785	0	0.605

Furthermore, the initial value of the mainstream activator Sp1 was set as hyperactive value 1 to check the changes in BRN. The expression level of hTERT first oscillated between 0.2 -0.95 and then reached its maximum near the hyperactive state (0.916). The expression level of p53 oscillated between 0.02-0.8 and finally decreased to slightly active state (0.394) and the responses, including proliferation (ranged between 0.48-0.99) and apoptosis (oscillated between 0.06-0.89) also increased to 0.946 and 0.853 respectively, as shown in **figure 4.2 (c).**

This BRN was again tested by setting the initial values of both activators Sp1 and c-Myc as hyperactive 1 and the values of the responses gradually increased to more active states because of the influence of two positive regulators, which can be seen in **figure 4.3 (b).**

To observe, if the expression level of important nodes and responses of BRN get back to the normal active states, an inhibitor (PLK1) was applied in the network and by doing so, both the main entities (hTERT and p53) gained the negative feedback from the inhibitor. So, the responses of BRN i.e., proliferation and apoptosis got back to the normal active state values (0.552 and 0.515 respectively).

4.3 Molecular docking

To analyze the interactions of hTERT protein with different naturally occurring activator proteins (c-Myc, STAT3, HIF1α and c-Jun), docking with in-house structural model of hTERT using ClusPro protein–protein docking program was performed. From the docking results of all the complexes, the top ranked cluster according to the highest number of poses and the least cluster center score, was selected. For each complex, the number of generated conformations and the cluster center score is shown in the **table 4.3**.

The complex with the highest number of poses generated and the lowest cluster center score depict the best binding complex. **Table 4.3** shows c-Myc-hTERT complex and HIF1α-hTERT complex as the better complexes as compared to others due to their higher number of generated poses and lower cluster center scores. But according to the literature, c-Myc and STAT3 are the activators that show better and early availability to hTERT [193, 263]. Therefore, depending on all these parameters, c-Myc-hTERT complex and STAT3-hTERT complex were selected for further analysis.

Table 4.3. The number of conformations generated and the cluster center score of the top ranked pose from all docked complexes.

Docked complex	No. of poses generated	Cluster center score
c-Myc-hTERT	64	-841.9 Kcal/mol
STAT3-hTERT	47	-826.8 Kcal/mol
HIF1α-hTERT	48	-1105.6 Kcal/mol
c-Jun-hTERT	36	-860.3 Kcal/mol

4.4 Interactions between the molecules of docked complexes

For c-Myc-hTERT complex, 30 residues of hTERT and 29 residues of c-Myc showed 207 different interactions with each other out of which there were 22 hydrogen bond interactions. Residues that are involved in H-bonds are His151, Arg155, Arg208, Ala210, Val212, Arg224, Ser227, Glu339, Glu533 and Arg535 as shown in **figure 4.4 (a).** Maximum residues of hTERT that interacted with c-Myc after docking were from the binding site of hTERT that is the RNA binding domain of hTERT. All the involved residues were from residue number 61-299 and then from residue no 339-535. From the STAT3-hTERT complex, maximum interactions were observed on the binding site of the receptor protein hTERT [264]. 193 different interactions were observed between 23 residues of hTERT and 33 residues of STAT3, out of which 21 were hydrogen bonds. Residues that showed hydrogen bonding are Gly35, Arg63, Asp147, His151, Trp203, Asn204, Arg208, Glu209, Arg224, Ser227 and Ala228 as shown in **figure 4.4 (b).** The residues that showed interactions are from residue no 34-299. From the HIF1α-hTERT complex, 31 residues of hTERT and 27 residues of HIF1α were involved in 196 linkages. There were 15 hydrogen bonds between the receptor protein and the ligand protein and involved resides are Arg3, Arg6, Cys7, Arg11, Arg15, Thr26, Arg48, Ala52 and Trp203 as shown in **figure 4.4 (c).** Interacting residues were from residue number 3-204 and all were from the binding site of hTERT. Similarly, from the c-Jun-hTERT complex, there were 289 interactions seen between 35 residues of hTERT and 35 residues of c-Jun and

most of the interactions were on the binding site of hTERT. 30 hydrogen bonds were formed by Arg185, Pro188, Arg208, Gly220, Ala221, Arg222, Arg223, Arg224, Ser227, Arg466, Arg485, Arg486, Arg489, Asn490, Lys493, Met512, Ser513, Arg535 and Arg537 as shown in **figure 4.4 (d).** Interacting residues were from residue no 185-227 and 466-537. Some residues from 1016 to 1027 were also involved in interactions.
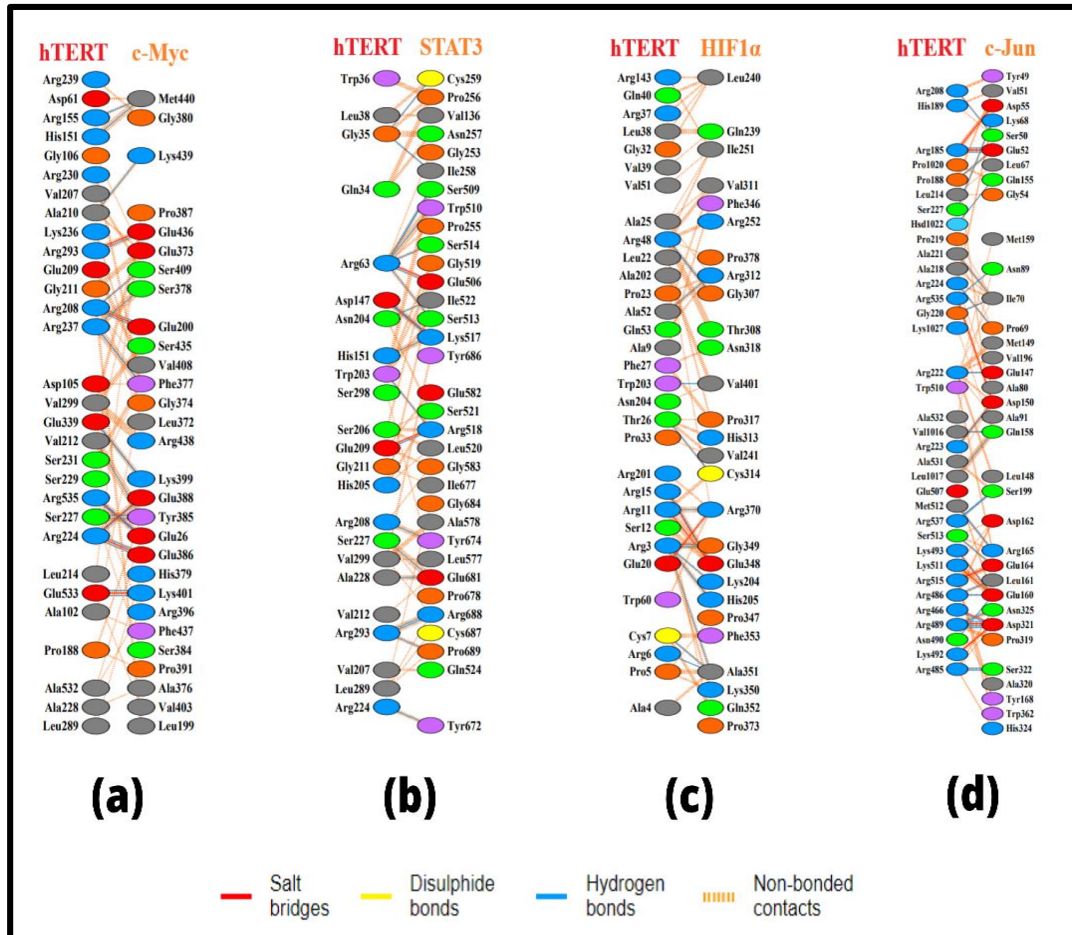
Figure 4.4. Interacting residues of all the activators (c-Myc, STAT3, HIF1α and c-Jun) with hTERT within a docked complex, given by pdbsum.

4.5 Molecular dynamic simulations of docked complexes

The molecular dynamic simulation of the selected activators complexes with hTERT helped in achieving the stability at human body temperature and pressure. The c-Myc-hTERT complex RMSD plot as shown in **figure 4.5**, represents that the complex was a little fluctuating till 20ns with approximately 0.4nm (4 Å) RMSD and the complex stabilized itself at around 0.5 to 0.6nm (5 to 6 Å) on 20 to 50 ns simulation time.
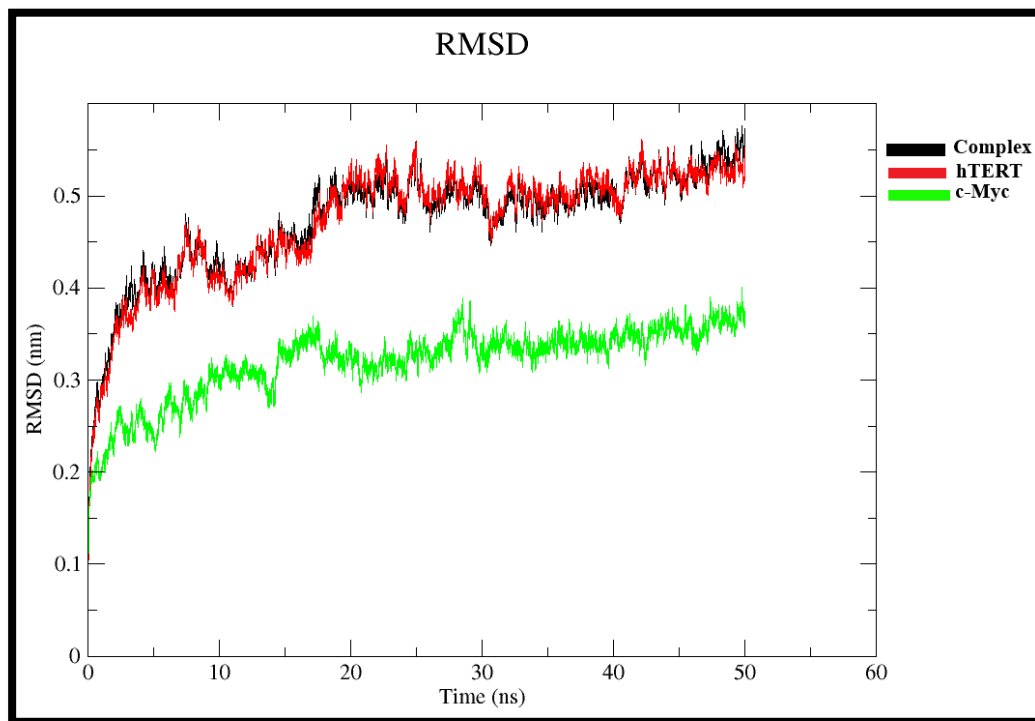
Figure 4.5. Molecular dynamic simulation of c-Myc-hTERT complex for 50 ns, which depicts lowest RMSD of complex at 0 ns and 0.5-0.6 at 50 ns.

The binding site of hTERT was not changed during the MD simulation of 50ns for the c-Myc-hTERT complex and the structure before and after MD was almost same as shown in **figure 4.6**. **Table 4.4** shows the binding site residues of hTERT before and after MD simulation and it can be identified that c-Myc-hTERT complex has hydrogen bonds formed by Arg-224 and Arg-535 were found common in the before and after MD structures. This indicates that the interactions identified after docking protocol were highly stable.

Before MD simulation   After MD simulation
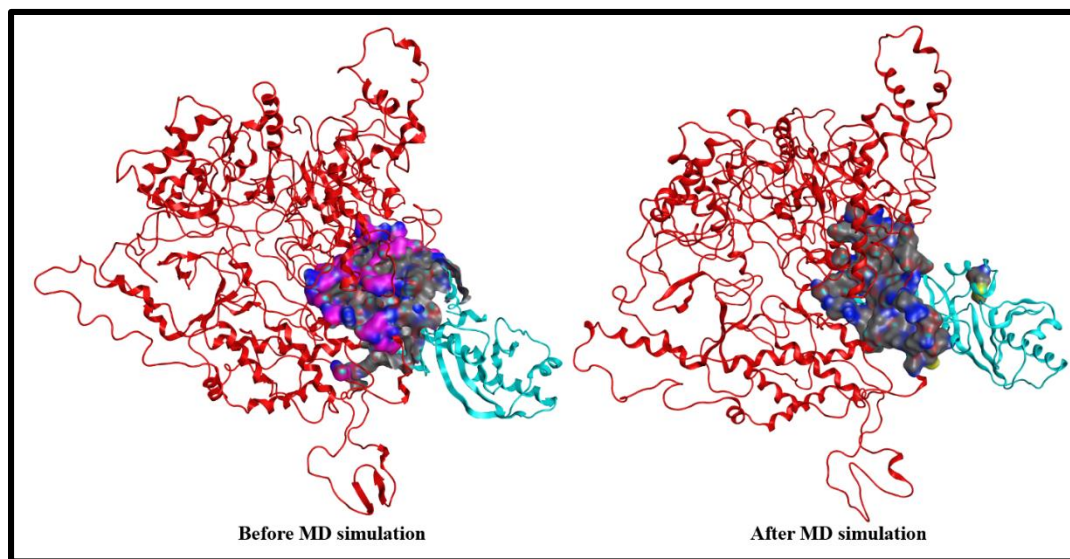
Figure 4.6. The interaction sites of binding residues before and after Molecular dynamic simulation in c-Myc-hTERT complex.

Table 4.4. c-Myc-hTERT complex hydrogen bonding residues and binding interactions at 0 ns and 50 ns.

	Before simulation				After Simulation		
Residues	Receptor protein atom	Ligand protein atom	H-bond Distance (Å)	Residues	Receptor protein atom	Ligand protein atom	H-bond Distance (Å)
His-151	ND1	O	2.81	Arg-108	NH1	OE1	2.60
Arg-155	NH1	O	2.68	Arg-224	NH2	OE1	2.66
Arg-208	NH1	OE1	2.70	Gly-225	N	OE2	2.85
Ala-210	O	NZ	2.51	Gly-226	N	OE2	2.72
Val-212	O	OH	2.75	Arg-239	NH1	OG1	2.80
Arg-224	NH2	OE1	2.72	Arg-293	NH2	OE2	2.60
Ser-227	OG	O	2.66	Ser-295	OG	OE2	2.69
Arg-237	NH2	O	2.73	Val-299	O	OG	2.66
Arg-293	NE	OE2	2.85	Gly-527	O	NZ	3.00
Glu-339	OE2	NZ	2.50	Ala-531	N	O	3.00
Glu-533	OE2	NZ	2.53	His-534	NH2	OE1	2.65
Arg-535	NH2	OE2	2.72	Arg-535	NH2	OE2	2.57

Moreover, the RMSF plot as shown in **figure 4.7** depicted the residues that are the most fluctuating from the complex. Leu-199, Asp197, His-303, Ser434 and Leu1103 are the residues that are fluctuating from the mean structure the most. But all of these residues were located in the loop, and this can be a reason of their fluctuation and these residues are also not involved in binding so it will not affect the binding pattern as well.
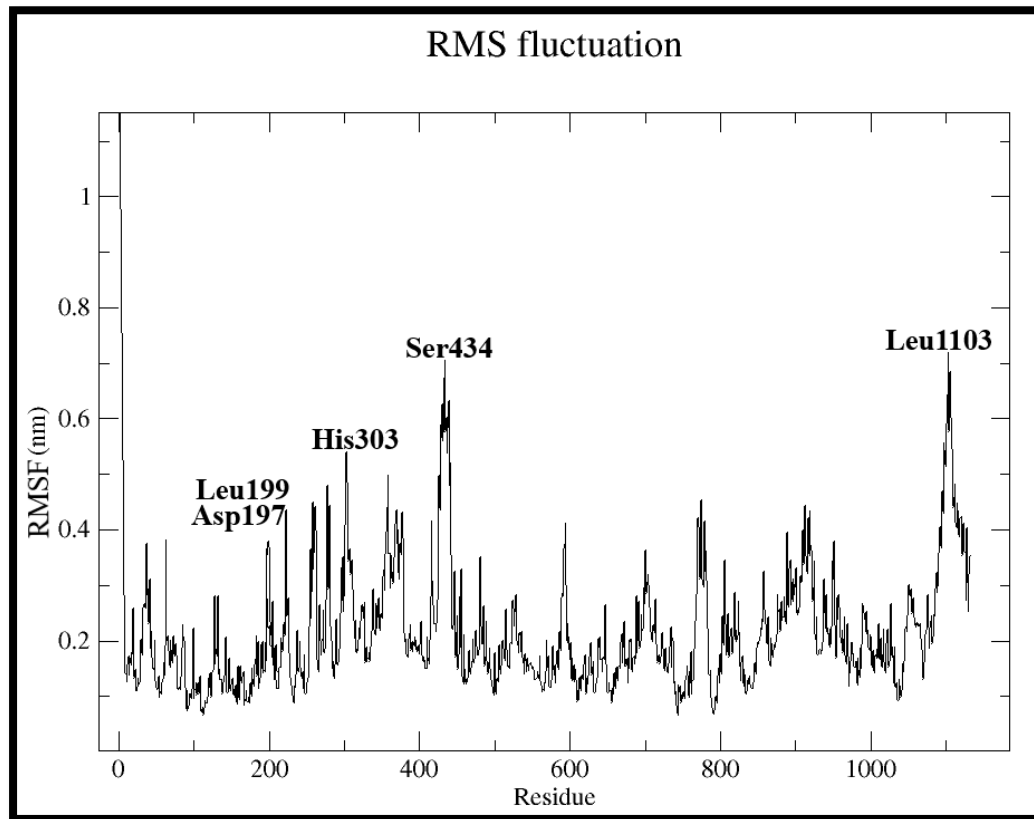
Figure 4.7. Serine, Histidine, Leucine and Aspartic acid are the most fluctuating residues in c-Myc-hTERT complex.

Furthermore, the radius of gyration for c-Myc-hTERT complex was stable at 3.54nm throughout the simulation time as shown in **figure 4.8**, which indicates the compactness of the complex.
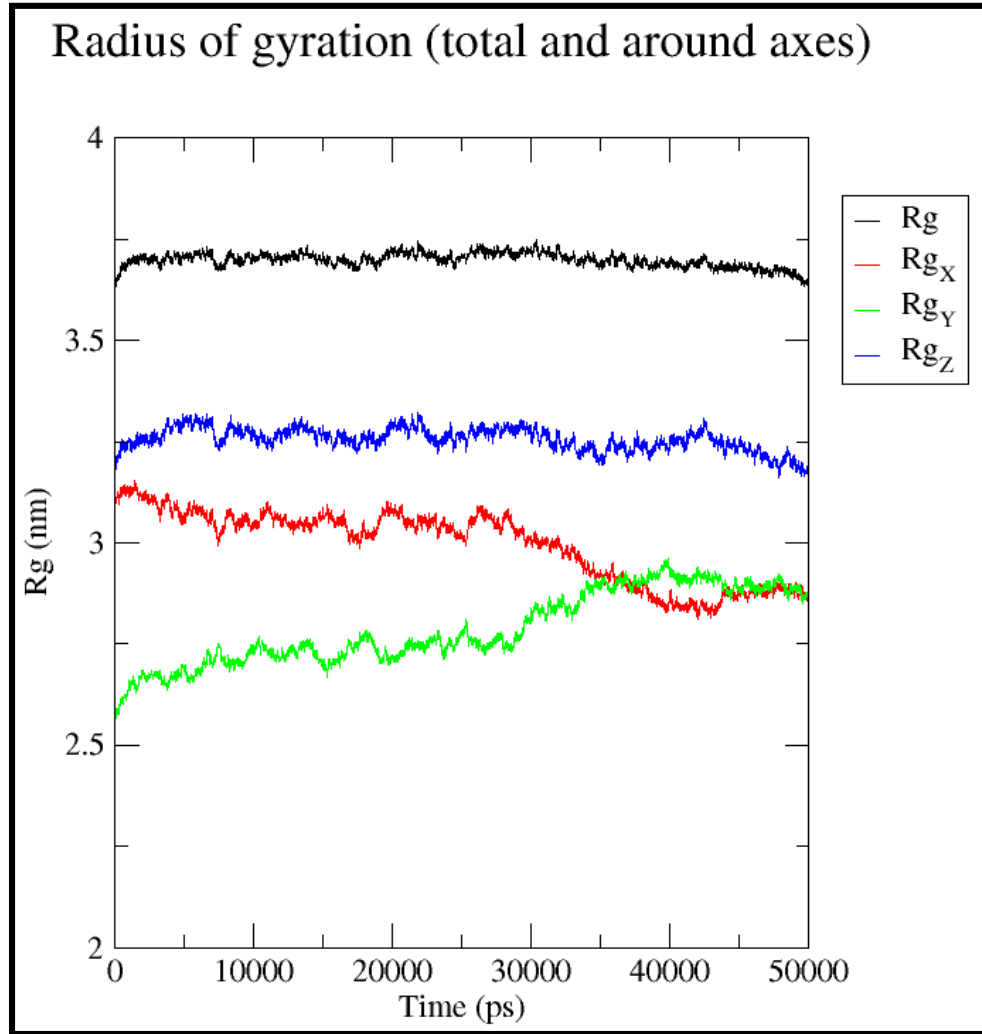
Figure 4.8. Radius of gyration at 3.7 nm shows that c-Myc-hTERT complex is significantly compacted.

In case of STAT3-hTERT complex, it was found that the complex showed little fluctuations in the start of the simulation having RMSD around 0.5nm (5 Å) as shown in the **figure 4.9**, but after 10ns, stability was gained with RMSD around 0.6nm (6 Å) till the end of the simulation.
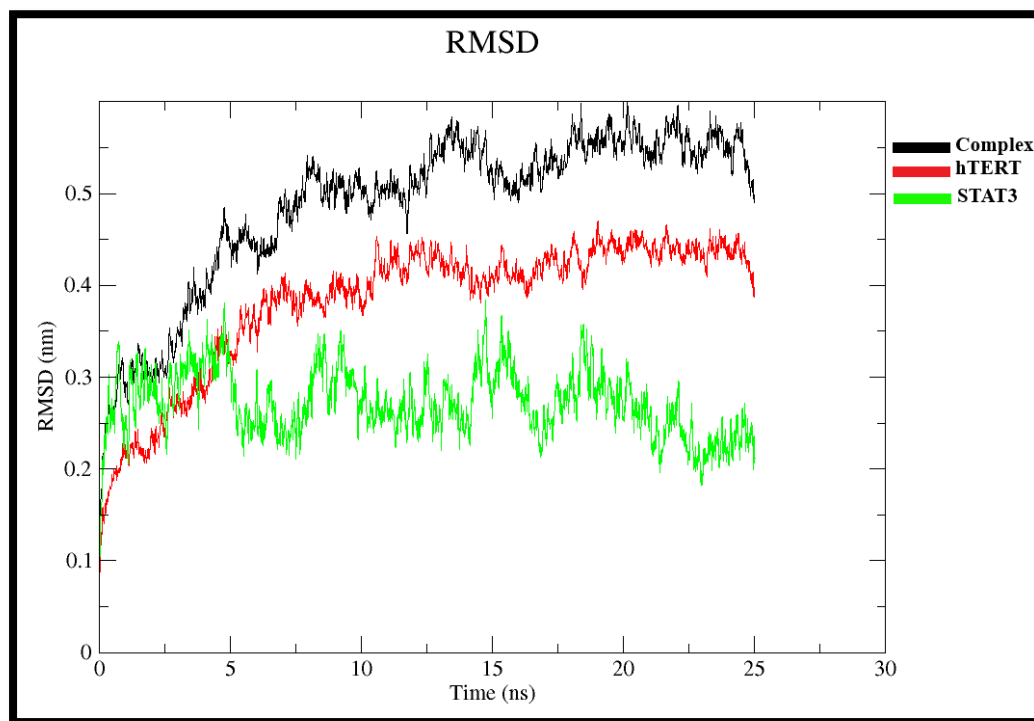
Figure 4.9. Molecular Dynamic Simulation of STAT3-hTERT complex for 25 ns, which depicts lowest RMSD of complex at 0 ns and 0.5-0.6 at 25 ns.

The binding site of STAT3-hTERT complex was not much changed during the 25ns simulation and the structure before and after MD was almost same as shown in **figure 4.10**. The binding site residues of hTERT in STAT3-hTERT complex are shown in **table 4.5**, and it can be observed that the hydrogen bonding residues Gly-35, Arg-63, Asp-147, Trp-203, Ser-206, Ser-227and Ala-228 were found common in both the before and after MD structures as shown in **figure 4.11.** This validates that the docking interactions of this complex were highly stable.
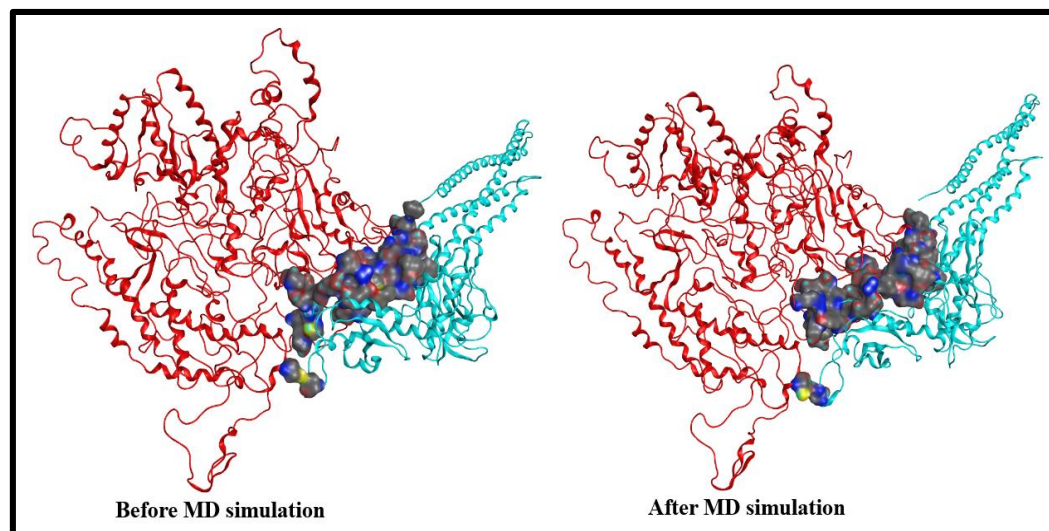
Figure 4.10. The interaction sites of binding residues before and after Molecular dynamic simulation in STAT3-hTERT complex.

The RMSF plot was also analyzed showing Arg-358, Ser-368, Glu-432 and Arg-774 are the highly fluctuating residues from their mean structure. But these residues are not involved in interactions, so they don't have any effects on the interaction profile of STAT3-hTERT.
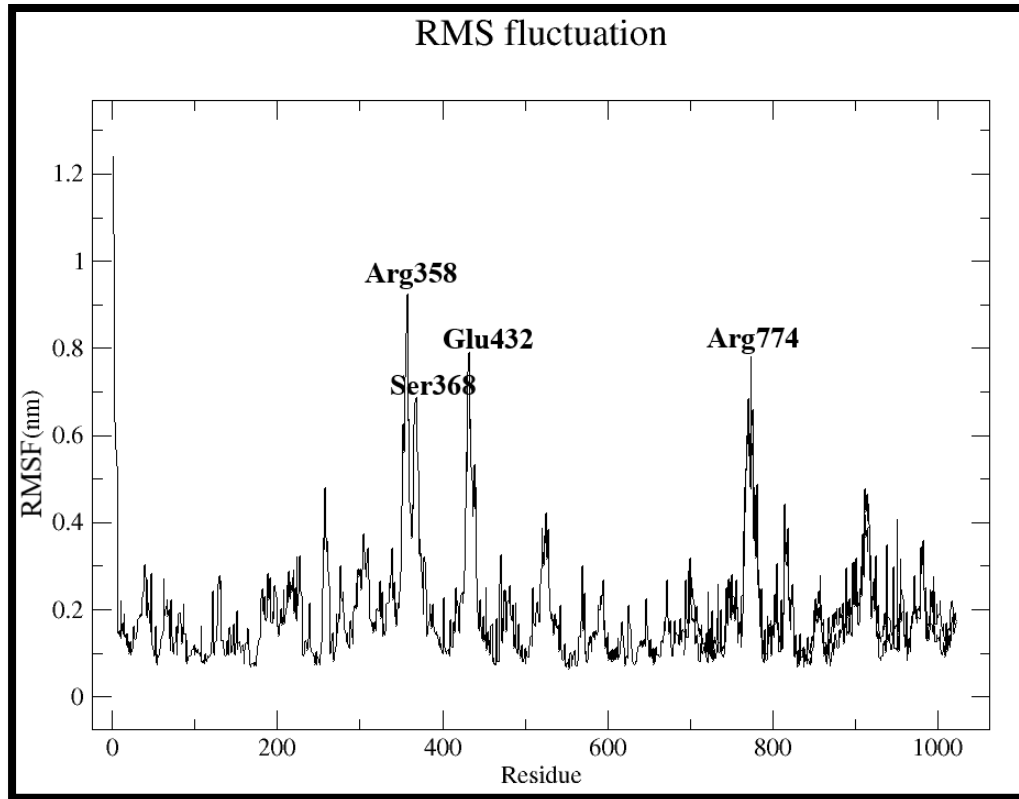
Figure 4.11. Arginine, Glutamic acid, Serine are the most fluctuating residues in STAT3-hTERT complex.

Additionally, the STAT3-hTERT complex was found highly compacted by the radius of gyration shown in **figure 4.12**, that was stable throughout the simulation at 3.3nm.
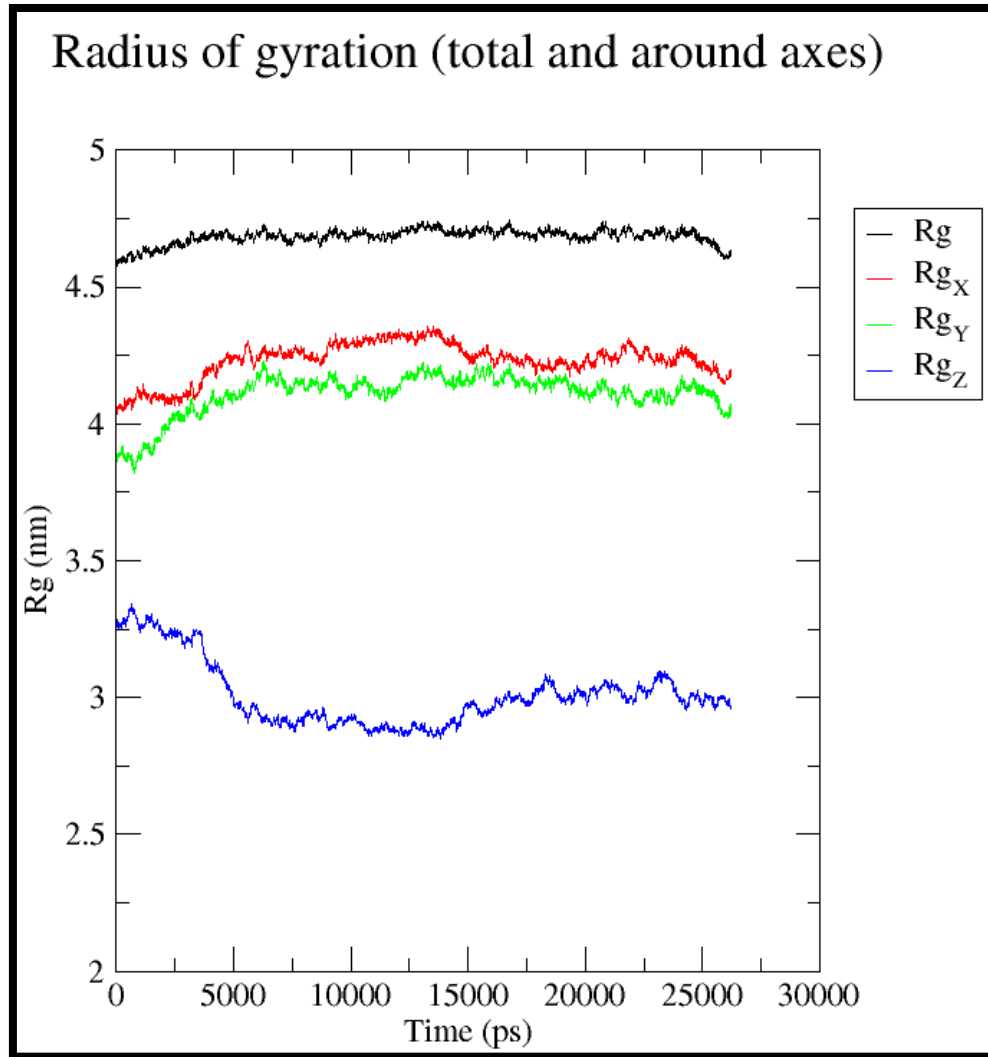
Figure 4.12. Radius of gyration at 3.3 nm shows that STAT3-hTERT complex is significantly compacted.

Table 4.5. STAT3-hTERT complex hydrogen bonding residues and binding interactions at 0 ns and 25 ns.

Before Simulation				After Simulation			
Residues	Receptor protein atom	Ligand protein atom	H-bond Distance (Å)	Residues	Receptor protein atom	Ligand protein atom	H-bond Distance (Å)
Gly-35	O	N	3.18	Gly-35	O	N	3.26
Asp-61	OD2	NH2	2.62	Arg-63	NH1	OG	2.68
Arg-63	NH1	OG	2.93	Asp-147	OD2	NZ	2.86
Asp-147	OD1	NZ	2.97	Trp-203	NE1	O	2.79
His-189	ND1	OD1	2.82	Ser-206	O	NH1	2.78
Trp-203	NE1	O	3.26	Ser-227	OG	OE2	2.58
Asn-204	OD1	N	3.20	Ala-228	N	OE1	3.04
Ser-206	N	OG	2.78				
Ser-227	OG	OE2	2.78				
Ala-228	N	OE1	3.34				

On the basis of these interaction profiles of the activators of hTERT, new artificial activators of hTERT can be designed in future to reverse the aging process. Furthermore, in this thesis work, we have just considered hTERT and its activators. However, in future, p53 and its activators can be explored along with the activators of hTERT. And later on different machine learning models can be built where these interactions or interaction sites (considered as attributes) can be evaluated with the interaction profiles of unknown peptides of monoclonal antibodies of hTERT to classify them as activator or non-activator of hTERT.

Chapter 5
Discussion

Chapter 5

5.   Discussion

hTERT plays a vital role in cell proliferation and delaying the aging process. And p53 plays an important role in cell apoptosis and tumor suppression [265, 266]. As both of these functions are significant for increasing the cell replication process but to a normal limit, this makes hTERT and p53 very significant and effective target for the age reversal process. The aim of this study was to activate hTERT to a normal range by co targeting p53 and to elucidate the interaction profile of hTERT and its activators. Herein, a knowledge-driven biological regulatory network (BRN) has been constructed to highlight the role of oncogenes and tumor suppressor genes interaction with hTERT and p53 in cell cycle progression, proliferation, and apoptosis. We have simulated the BRN and observed that the Sp1 node has a central role in the network. Where the changes in the concentration of Sp1 node (by setting its value 0.0, 0.5, 1.0 throughout the entire simulation) results in the changes in concentrations of hTERT and p53 and eventually effecting the cell proliferation and apoptosis by generating signal peaks.

From the constructed network, it can be observed that Sp1 is the main node that is activating both of our target's hTERT and p53 but it could not be considered as the main target of this study because Sp1 is not only present in this pathway rather it is involved in many other pathways as well. Moreover, Sp1 is not directly linked to the cell proliferation and apoptosis that are the main responses of this BRN. However, hTERT and p53 are directly linked to proliferation and apoptosis, respectively. Our simulation results demonstrate that Sp1 activates both hTERT and p53, hTERT further inhibits ROS but p53 further activates NFκB. ROS also activates NFκB via phosphorylated IκB. NFκB is responsible for the initiation of c-Myc transcription where c-Myc binds to the E-box on the TERT promotor region and triggers transcription process. That eventually leads to the activation of hTERT. These signaling nodes, pathways and the results of this simulation are also supported by the previous studies where hTERT mediated the cell proliferation and p53 lead to the tumor suppression by increasing apoptosis [193, 194, 208, 234, 267].

The simulation where Sp1 was set as 0.5 (moderately active) showed the most promising results where hTERT and p53 were activated till a balanced and normal concentration gradient. Furthermore, the responses (cell proliferation and apoptosis) were also lying in the moderately active range. Therefore, these results helped us achieving the objectives of this study that were to perform dynamic simulation of hTERT pathway to determine the concentration gradient of hTERT for the modulation of telomeres in normal human cells and to study the modulation of p53 along with hTERT for the regulation of normal cell proliferation/apoptosis by dynamic modeling of p53 along with hTERT pathway.

Another aim of this study was to elucidate the interaction pattern of hTERT with its activators, and it was not possible with the 3D structural model of hTERT and its naturally occurring biological activators. For that, an *in-house* homology model of hTERT was used, and the structures of activators were retrieved from PDB. The activators c-Myc, STAT3, HIF1α and c-Jun were docked individually with hTERT. This protein-protein docking showed several interactions between the hTERT and activators which showed the strong binding pattern between them. Furthermore, these activators were ranked on the basis of availability and interactions with hTERT and the top two docked complexes c-MYC-hTERT and STAT3-hTERT were selected for further analysis.

MD simulation of selected complexes c-Myc-hTERT and STAT3-hTERT was performed, and it was found that both the complexes achieved a stable RMSD between 5 and 6 Å. The MD simulation is the process that helps in the identification of the actively meaningful interaction for a specific period under the human body temperature and pressure [268]. The continuous force exerted on the molecules of complex only sustains the most stable interaction while breaking the less stable bonds. The stabilization of these complexes in a short time is due to the reason that the hTERT structure was also stabilized by the MD simulation before docking. The stability of the complex depends on the stability of the receptor protein that's why it led to the more stable complexes. It was also observed that there was no change in the binding cavity rather binding residues within the same cavity change. This change in binding interaction is due to the energy

minimization during MD production which makes new bonds and breaks old bonds to find the most stable conformation. Glu-209, Ser-227, Ala-228, Arg293 and Val-299 were found commonly interacting in both c-Myc-hTERT complex and STAT3-hTERT-complex, before and after MD simulations which represents that these are highly significant residues of hTERT. Moreover, the hydrogen bond interactions were also common in both the complexes which represents that this interaction profile can be further used for the future design of artificial activators of hTERT.

Conclusion

The average lifespan has increased dramatically during the past century as scientists have been looking for ways to increase the average human health span and lifespan. Telomeres are chromosomal DNA segments that play a critical role in the cell proliferation for antiaging. The loss of telomere length is an age-related cognitive decline that further leads to more age-related diseases like diabetes, chronic liver disease, hypertension, cardiovascular disease and neurological diseases. A key step in the development of new antiaging treatments is identifying the pathways that regulate longevity and this study demonstrates that hTERT and p53 mediated pathway can play an important role regarding this. Our findings highlight the therapeutic importance of hTERT mediated cell proliferation and p53 mediated cell apoptosis in normal human cells to reverse the aging process. In this study, a knowledge-driven pathway is being simulated in order to understand the effects of positive regulators of hTERT and p53 in the cell proliferation and apoptosis for reversing the age but also control the risk of cancerous cells by maintaining a balance between apoptosis and proliferation. Herein, different naturally occurring biological molecules like c-Myc, STAT3, HIF 1α and c-Jun have been studied to this end, as they can influence telomere length via stimulation of hTERT expression. The use of telomerase activators as a potential antiaging treatment seems encouraging. Docking protocol elucidated the interactions between hTERT and its activators and the Molecular dynamic simulation protocol helped validating these interactions. Glu-209, Ser-227, Ala-228, Arg-293 and Val-299 are the residues that are found interacting in docking as well as after MD simulations of both the c-Myc-hTERT and STAT3-hTERT complexes therefore, these are surely of significant importance in the hTERT interaction profile. Moreover, the interaction analysis will help in designing of artificial positive regulators of hTERT. By expansion of this study area and potential research in the future by activating hTERT and p53, we may be able to learn more about the substances and mechanisms that specifically target telomeres and so extend human life without keeping the risk of cancer.

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