

The Quest for Immortality: Introducing Metadichol® a Novel Telomerase Activator

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Abstract

Humans are keenly aware of their mortality. Given a limited time what we do with our life is a reflection of knowledge of our mortality. In 2009 the Nobel prize in medicine to Jack W Szostak, Elizabeth Blackburn, Carol W Greider for their work on Telomerase and scientific research exploded in this area. Telomere protect chromosome ends the Telomerase enzyme maintains Telomere length. This activity of Telomerase is essential in aging and stem cells and achieving longer life spans.

Telomerase is expressed in 85% of human cancer cell lines, but its enzymatic activity is not detectable in most human somatic cells which constitute the vast majority of the cells in the human body. There is a need for increased telomerase activity in stem cells for use in the treatment of therapies where there is an active role for telomerase. Umbilical Cord Blood (UCB) provides an attractive source of stem cells for research and therapeutic uses. Work shown here characterizes the gene expression changes from Umbilical cord cells differentiate toward telomerase on treatment with Metadichol®.

Metadichol® is a nanoemulsion of long-chain alcohols that is nontoxic. It is a mixture of long-chain alcohols derived from food. The work presented here is about the effect of Metadichol® on Telomerase expression profile in Umbilical cord cells. Our results using q-RT-PCR show increases of mRNA telomerase expression by Sixteen-fold at one picogram but down-regulates expression at higher concentrations of 100 pg, 1 ng, 100 ng and one microgram per ml concentration. Western blot studies showed expression of Telomerase protein which is slightly higher than control at one picogram, i.e., Telomerase protein expression continues at replacement level. Since it is devoid of toxic effects, it can be directly tested on humans and is in use today as an immune boosting supplement. Metadichol® increases expression of Klotho an anti-aging gene expression in cancer cell lines by Four to Ten-fold, and Klotho gene has been documented to inhibits the growth of cancer cells. Metadichol® also inhibits TNF, ICAM1, CCL2, and BCAT1 which that is associated with proliferation in yeast and increased metastatic potential in human cancers. It paves the way for safe clinical testing and research and study of Telomerase biology and its use in humans.

Keywords: Telomerase; mRNA expression; h-TERT; VDR; Inverse agonist; Metadichol; UBC; Stem cells; Nano-emulsion; Aging cancer; Chronic diseases; Cell division

Introduction

A lot of research work has been ongoing to understand how chromosomes are protected by telomeres and the enzyme telomerase [1,2]. The human chromosome has a unique component at their ends that provide stability called Telomere. Telomerase is a ribonucleic reverse transcriptase needed for synthesizing telomeric DNA repeats at the 3' ends of linear chromosomes. Telomerase enzyme complex consists of two components known as TERC and TERT. TERC (h-TR) makes the repeat sequence of DNA, i.e., TTAGGG and TERT that adds to the ends of chromosomes. H-TR is ubiquitously expressed in embryonic and somatic tissues, expression of hTERT is tightly regulated and is not seen somatic cells and is the rate-limiting step in telomerase activity [3].

In the nucleus of human cells are 46 chromosomes, which carry our genetic information derived from our ancestors. During cell division, human telomeres lose about 80-100 base pairs in their telomeric DNA after each mitosis. Telomeres shorten as a cell divides, and once telomeres reach a critically short length, there is cell apoptosis, or it stops dividing and senesces [4-6].

At birth, humans do not have every cell our body needs. There is a need for new cells replacement regularly like skin cells and those that line our intestines. Without Telomerase, cell division would not

be possible for cells to reproduce. Telomeres have a defensive role in guarding key genetic material against being lost when cells divide. When the cell divides, the ends are not copied, and the telomeres are a little shorter, leading to a situation of short telomeres, and no cell division occurs known as the Hayflick limit [7-9]. Broken chromosomes result in DNA damage. Unrepaired DNA ends will prevent any cell division and can result in apoptosis [10].

Somatic cells are the majority of the cells in the human body and are devoid of any telomerase activity unlike that of stem cells [11]. Older cells have very short telomeres, and this can lead to cancer and other age-related diseases [12-15]. Drugs that increase telomerase activity within stem cells for disease treatment, as well as anti-aging therapies, are what it is needed today.

Some of the factors that cause Telomere shortening are shown in

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Table 1. In addition to cancer, telomeres are involved in many diseases, and these are shown in Table 2. Failure to repair restore telomere damage of hyper telomerase activity are the causes of many diseases and overcoming these hurdles could result in novel therapies. Long telomeres have a large number of protective proteins. Critically short telomeres have few protective proteins available. There has research that suggests that Telomerase causes cancer as it is active in 85% of cancer cells. However, other research suggests that the median increase in telomere length from diagnosis to remission is an overall powerful predictor of survival. Several SNPs have been identified with longer Telomeres, and the same SNPs has been shown to correlate with cancer. Telomeres play diverse roles in different cancers, and short telomeres may be risk factors for the tumors [16-19].

Many dietary compounds have been shown to regulate telomerase activity [20]. Telomerase inhibitors derived from food like retinoic acid, 1, 25(OH)₂ Vitamin D3 polyphenols, fatty acids, tocotrienol, and sulforaphane have been shown to inhibit telomerase. Kasiappan et al. [21] provided evidence that 100 nm of 1, 25(OH)₂ Vitamin D3 treatment led to telomerase inhibition. A green tea extract, EGCG suppresses tumor size and shortens telomere length. On the other hand, Genistein and Amadori-PE induce telomerase activity in Cancer cells [22]. Geron, a biopharmaceutical Company is developing Imetelstat [23] a telomerase inhibitor against hematologic myeloid malignancies like Myelodysplastic Syndrome (MDS). A commercially available telomerase activator in use today is TA-65 [24]. It is a dietary extract derived from traditional Chinese medicine. It has shown improvements in biomarkers of aging, like cardiovascular, metabolic, bone, and inflammatory markers, without significant signs of toxicity [25-27]. Most of these dietary components work in doses varying from hundreds of mg to grams.

Human Mesenchymal Stem Cells (hMSCs) display multipoint properties in differentiation and are useful in cell and gene therapy. Zimmermann et al. [28] showed that telomerase activity is not detectable in human mesenchymal stem cells. This has been confirmed by Karimi et al. [29] who detected no telomerase activity in UCB-MSCs from several passages. One can introduce h-TERT into telomerase inactive cells to restore telomerase activity and potentially increase cellular lifespan. Liang and their co-workers have suggested UC-MSCs could be immortalized by transduction with a lentiviral vector carrying hTERT into hepatocyte-like cells [30]. The transfected hUCMSCs cells overexpressed the h-TERT gene and up-regulated their telomerase activity. Ramunas

et al. [31] were able to show that transient delivery of TERT mRNA comprising modified nucleotides increased telomerase activity, telomere length, and proliferative capacity without immortalizing cells.

Modulating telomerase enzymatic activity and telomere maintenance *in vivo* is essential both for our understanding of telomere biology and telomerase dysfunction in disease pathogenesis. The work presented will show that Metadichol could be useful in overcoming the problems facing Telomerase researchers.

Materials and Methods

Gene regulation of telomerase in Umbilical Cord Blood-Mesenchymal Stem cells (UCB-MSCs) treated with Metadichol

All work was outsourced and carried out by Skanda Labs Pvt. Ltd., Bangalore, India.

Cell line: Umbilical Cord Blood-Mesenchymal Stem cells (UCB-MSCs) sourced from Lonza, USA was used for the study. RNase free (Thermo Fisher, cat #AM2694), TRIzol (Sigma, cat #T9424 200 ml), DEPC (Thermo Fisher, cat #RO581), chloroform (Sigma, cat #C7559), isopropanol (SRL 67-63-0), DEPC treated water (Thermo Fisher, cat #RO581) was used. All the consumables were treated with DEPC water and autoclaved. Human Wharton's Jelly Mesenchymal Stem cells (UCB-MSCs) isolated from Wharton's Jelly of human umbilical cords (HiMEDIA cat #21736) cultured in Dulbecco's Modified Eagle Medium (DMEM from HiMEDIA cat #1782) supplemented with 10% Fetal Bovine Serum (HiMEDIA cat #15500). Cells were maintained at 37°C with 5% CO₂ supplement. The cells were conventionally subcultured and counted using Hemocytometer. 1 × 10⁶ cells were grown in P35 dish for 24 hours Cells were treated with varying concentrations of test sample Metadichol (1 pg/ml, 100 pg/ml, 1 ng/ml and 100 ng/ml) and incubated for 24 hrs for RNA isolation and 48 hrs for protein isolation. Fresh media was used as a control.

Sample preparation and RNA isolation

Total RNA from UCB-MSCs cells was extracted using TRIzol Reagent (Sigma) as per manufacturer's instruction. Cells were washed twice with PBS and centrifuged at 425x g for 5 min. To the cell pellet, 1 ml of TRIzol (per p35 dish) added in 1.5 ml microcentrifuge tube and vortexes. Samples were allowed to stand for 5 minutes at room temperature. To the reaction mixture, 0.2 ml of chloroform is added and vigorously mixed for 15 seconds. The tube was allowed to stand at room temperature for 5 minutes, centrifuged the resulting mixture at 10621x g for 15 min at 4°C. The upper aqueous phase is transferred to a new sterile microcentrifuge tube and treated with 0.5 ml of isopropanol. The resultant mixture is mixed gently by inverting the contents five times and incubated at room temperature for 5 minutes. Samples were centrifuged at 10621x g for 10 min at 4°C. The supernatant was discarded, and the RNA pellet washed by adding 1 ml of 70% ethanol. The sample was mixed gently by inverting a few times, centrifuged for 5 min at 20817 at 4°C. The supernatant was discarded by inverting the tube on a clean tissue paper. Later, the pellet was dried by incubating in a dry bath for 5 min at 55°C. The pellet was then resuspended in 25 µl of DEPC treated water.

RT-PCR

A semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using a Techno Prime system to determine the levels of telomerase and β-Actin mRNA expressions. The cDNA

| | Obesity | Oxidized LDL |
|---------------------------------|------------------|-------------------------------|
| Coronary heart disease | Smoking | Decreased Nitric oxide levels |
| Diabetes, Myocardial infraction | Oxidative stress | Mitochondrial DNA damage |
| Insulin resistance | Homocysteine | Lack of estrogen |

Table 1: Factors that can shorten telomeres.

| Cardiovascular | Cell and tissue Transplants |
|-----------------------|-------------------------------|
| Cancer | AIDS |
| Alzheimers | Progeria |
| Osteoarthritis | Dyskeratosis Congenita |
| Rheumatoid Arthritis | Idiopathic Pulmonary Fibrosis |
| Osteoporosis | Down Syndrome |
| General Immunity | Liver Cirrhosis |
| Skin aging | Muscular Dystrophy |
| Muscular Degeneration | COPD |

Table 2: Diseases caused by telomeres shortening.

was synthesized from 2 µg of RNA using the Verso cDNA synthesis kit (Thermo Fischer Scientific) with oligo dT primer according to the manufacturer's instructions. The reaction volume was set to 20 µL, and cDNA synthesis was performed at 42°C for 60 min, followed by RT inactivation at 85°C for 5 min (Table 3).

Polymerase Chain Reaction (PCR)

The PCR mixture (final volume of 20 µL) contained 1 µL of cDNA, 10 µL of Red Taq Master Mix 2x (Amplicon) and 1 µM of each complementary primer specific for Telomerase and β-Actin (internal control) sequence. The samples were denatured at 94°C for 5 min and amplified using 35 cycles of 94°C for 30 sec, and for Telomerase annealing temperature was set to 49°C and for β-Actin the annealing temperature was set to 55°C for 30 sec and elongation at 72°C for 1 min followed by a final elongation at 72°C for 10 min. The optimal numbers of cycles have been selected for amplification of these genes experimentally so that amplifications were in the exponential range and have not reached a plateau. Instrument CFX96 real-time PCR, Bio-Rad used for qPCR. 10 µL of the final amplification product was run on a 2% ethidium-stained agarose gel and photographed. Quantification of the results was by measuring the optical density of the bands, using the computerized imaging program Image J. The values were normalized to β-Actin intensity levels.

Isolation of protein

The cells, post-harvesting, were washed twice using 1XPBS. The cell pellets suspended in 500 µl of RIPA buffer with 1X Protease Inhibitor (Sigma; P-8340). The cells were incubated for 30 mins by gentle mixing every 5mins. Post incubation, the cells were centrifuged at 10621x g for 12-15 minutes. The protein lysates in the supernatant were transferred to fresh sterile tubes and stored in -20°C until further use.

Western blot and SDS-PAGE procedure

A 140 µg protein sample from each cell lysate was mixed with 5X loading dye and heated for 6 min at 98°C (Figure 1). Protein samples

were loaded and separated on 12% SDS-PAGE gel using Mini protean Tetra cell (Bio-Rad). Methanol activated 0.2 µM PVDF membrane was pre-wet in transfer buffer for 10 min at RT. Protein transfer was done for 10 min in Turbo Transblot (Bio-Rad) apparatus. Blot was blocked in 5% BSA+TBST for 1 hr at RT. Blot was incubated with 10 Ab (SAB4502945, Sigma Aldrich) at dilution: 1:1000 for overnight at 40°C on a shaker. Washed three times with TBST for 5 min at RT. Blot was incubated with 20 Ab (Goat-anti-Rabbit HRP- IgG; Ab6721) at dilution 1:1000 for 1 hr at RT. Washed three times with TBST for 5 min at RT. Blot was rinsed with ECL reagent (two-component systems) for 1 min in the dark and image was captured with 40-sec exposure in Chemidoc MP imaging system (Bio-Rad) (Figure 2).

Results

The internal control β-Actin was used to normalize the gene expression. Results showed that the cells at the lowest concentration of 1 pg/ml showed 2.11 fold up-regulation compared to the highest treated concentration of 1 µg/ml with 1.18 folds (Table 4).

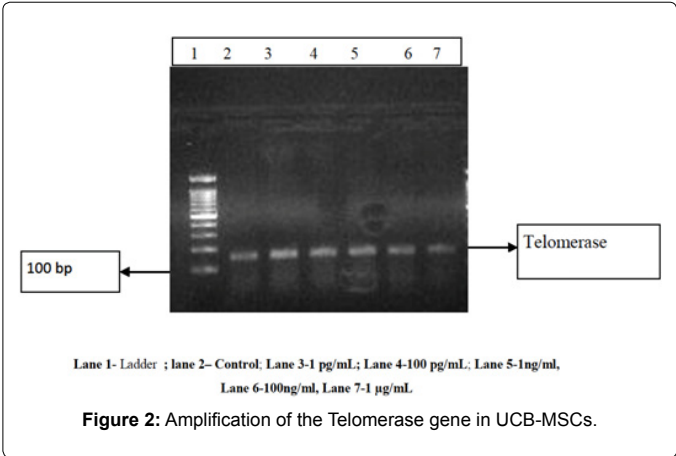
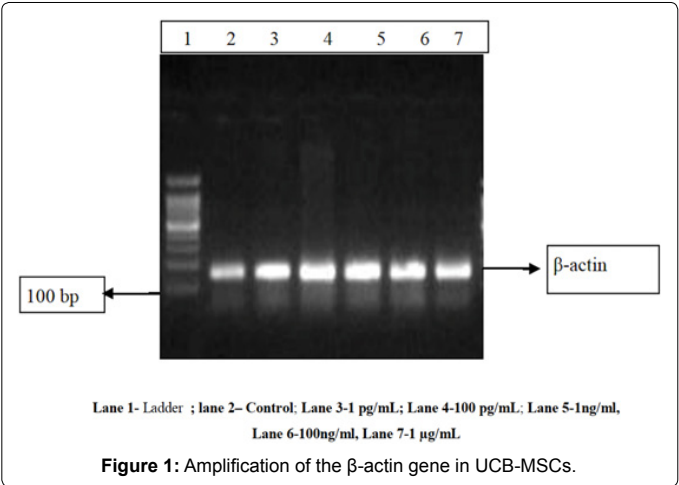
The effect of sample Metadichol on the expression of Telomerase (TERT)

Figure 3 shows the semi-quantitative relative gene expression which one picogram is 2.11 and decreasing with increasing concentrations. Figure 4 shows q-RT-PCR where the TERT expression in the cells treated with 1 pg/ml is increased 16.68 fold increase compared to control (Tables 5 and 6). Whereas, in the cells treated with higher concentrations, the expression was found to be gradually down-regulated. This is seen clearly in the Log scale plot in Figures 5-7 and Table 7.

The cells treated with various concentrations of test sample Metadichol and the results suggest that the relative expression of telomerase was found to be 1.05 fold at 1 pg/ml treatment compared to control whereas, the cells treated with other concentrations have shown no expression (Figure 6).

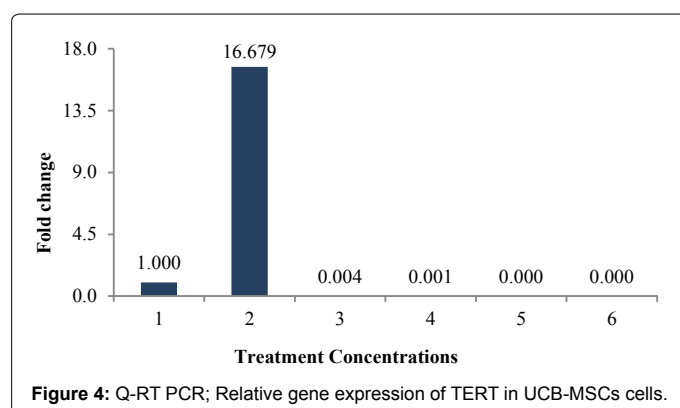
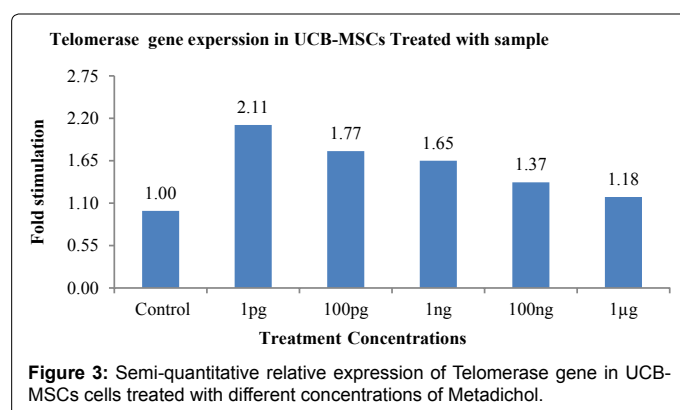
| Gene | Primer pair | Sequence | Tm | Product size (bp) |
|-------------------|-------------|-------------------------|-------|-------------------|
| B-actin | FP | TCCTCCTGAGCGCAAGTACTCT | 62.1 | 153 |
| | RP | GCTCAGTAACAGTCCGCCTAGAA | 62.4 | |
| Telomerase (TERT) | FP | GGGAGGTCAGGTGTCCATTG | 55.88 | 142 |
| | RP | TGCTCTCGGATAGTCACCA | 53.83 | |

Table 3: Primer details for β-actin and Telomerase.



| Samples | Band intensity of PCR amplicon of genes | | Normalized | Relative gene expression |
|-----------|-----------------------------------------|------------|------------|--------------------------|
| | β -actin | Telomerase | | |
| Control | 18938.05 | 8150.83 | 0.43 | 1.00 |
| 1 pg | 20880.71 | 18999.86 | 0.91 | 2.11 |
| 100 pg | 22188.10 | 16936.35 | 0.76 | 1.77 |
| 1 ng | 21099.88 | 14985.93 | 0.71 | 1.65 |
| 100 ng | 19137.88 | 11295.45 | 0.59 | 1.37 |
| 1 μ g | 18060.52 | 9162.23 | 0.51 | 1.18 |

Table 4: Relative expression of Telomerase gene in UCB-MSCs treated with different concentrations of Metadichol.



| Sample | Conc. | Relative Telomerase gene expression | |
|------------|-----------|-------------------------------------|----------------------|
| | | Fold change | C _q Value |
| Metadichol | Control | 1.000 | 36.52 |
| | 1 pg | 16.68 | 26.07 |
| | 100 pg | 0.00 | 38.85 |
| | 1 ng | 0.00 | 42.89 |
| | 100 ng | 0.00 | 50.24 |
| | 1 μ g | 0.00 | 72.54 |

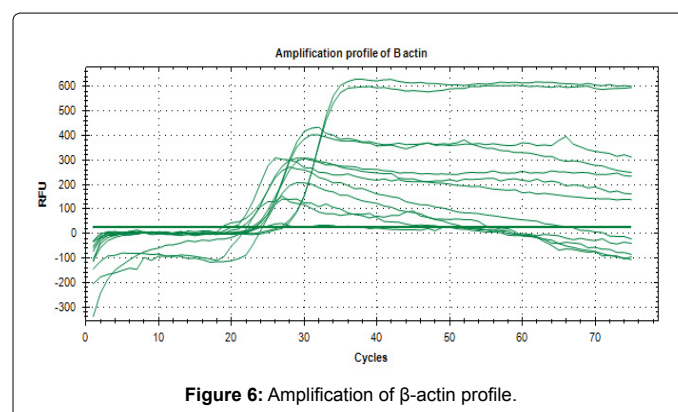
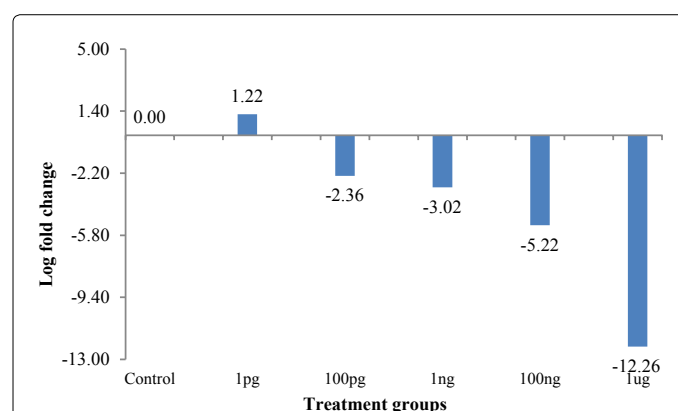
Table 5: Q-RT-PCR analysis of Telomerase in UCB cells at showing the fold change and C_q value in USB-MSCs cells treated with different concentrations of Metadichol.

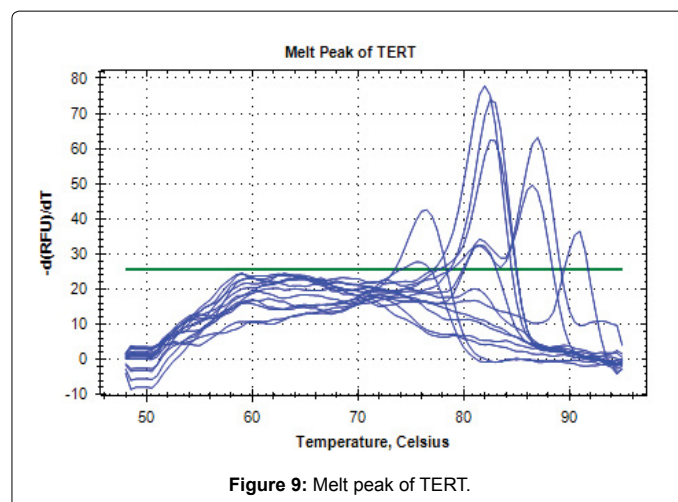
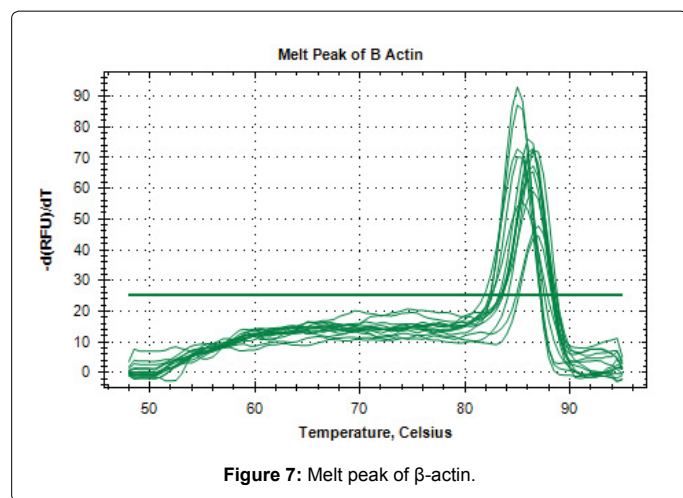
Discussion

From the data, Figure 4, Metadichol® increases Telomerase expression sixteen-fold at one picogram/ml. At higher concentrations, there is hardly any expression. Figure 5 in log format shows down-regulation at higher concentrations.

| Fluor | Target | Treatment | C _q | C _q Mean | C _q Std. Dev. |
|-------|------------|-----------|----------------|---------------------|--------------------------|
| SYBR | Telomerase | Control | 37.55 | 36.52 | 1.457 |
| | | | 35.49 | | |
| | | 1 pg | 27.31 | 26.07 | 1.754 |
| | | | 24.83 | | |
| | | 100 pg | 40.29 | 38.85 | 2.036 |
| | | | 37.41 | | |
| | | 1 ng | 42.32 | 42.89 | 0.806 |
| | | | 43.46 | | |
| | | 100 ng | 50.5 | 50.24 | 0.368 |
| | | | 49.98 | | |
| | | 1 ug | 74.01 | 72.54 | 2.079 |
| | | | 71.07 | | |
| SYBR | Actin | Control | 26.99 | 26.77 | 0.311 |
| | | | 26.55 | | |
| | | 1 pg | 18.57 | 20.38 | 2.560 |
| | | | 22.19 | | |
| | | 100 pg | 21.14 | 21.26 | 0.170 |
| | | | 21.38 | | |
| | | 1 ng | 23.67 | 23.1 | 0.806 |
| | | | 22.53 | | |
| | | 100 ng | 23.05 | 23.15 | 0.141 |
| | | | 23.25 | | |
| | | 1ug | 19.84 | 22.06 | 3.140 |

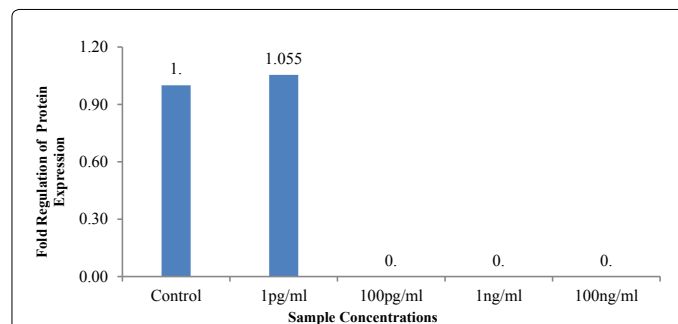
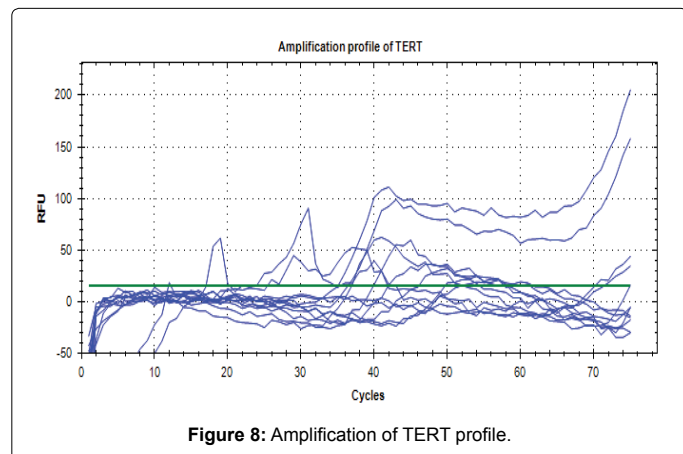
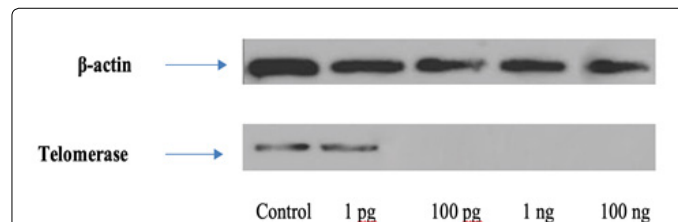
Table 6: Data of C_q values and fold change of Telomerase old change in real-time PCR in USB-MSCs treated with different concentrations of Metadichol.





| Metadichol (Conc.) | Band intensity proteins | | Normalised 0.88 | Relative gene expression |
|--------------------|-------------------------|------------|-----------------|--------------------------|
| | β-actin | Telomerase | | |
| Control | 19386.59 | 17149.7 | 0.93 | 1.00 |
| 1 pg/ml | 24.00 | 18399.48 | 0.00 | 1.05 |
| 100 pg/ml | 15812.68 | 0.00 | 0.00 | 0.00 |
| 1 ng/ml | 18091.9 | 0.00 | 0.00 | 0.00 |
| 100 ng/ml | 18601.63 | 0.00 | 0.00 | 0.00 |

Table 7: Relative gene expression of Telomerase protein in Metadichol treatment in USB-MSCs.

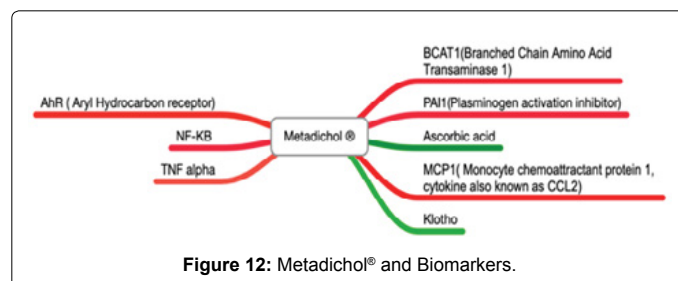


The western blot studies show post-translation that there is Telomerase activity and expression of the protein is similar to that seen in control, suggesting post-translational regulation of telomerase activity is being maintained at replacement levels of cell division (Figures 9-11).

Metadichol® shows dual properties like increasing insulin and also decreasing Insulin [32,33] and besides acts on key biomarkers as shown in Figure 12 (red is inhibition or decrease, and green is an increase in biomarker levels). All these biomarkers affect the expression of Telomerase activity and expression.

Metadichol® and VDR

Metadichol is an inverse agonist of Vitamin D receptor (VDR). Vitamin D3 (1,25 OH)D3 and its analogs inhibit h-TERT expression



and telomerase activity in leukemic cells [21]. Metadichol® binds to VDR as an inverse agonist. It is not surprising that it has different effects compared to that of the agonist Vit D3. Inverse agonists bind to the same site as the natural agonist but have different effects [34].

Metadichol® is the only known VDR inverse agonist today.

Consensus Path DB [35] a software program that integrates gene interaction networks and generates the shortest interaction paths between 2 genes. Metadichol binding to VDR leads to the expression of MYC genes which in turn activates the Telomerase gene as shown in Figure 13. This pathway has its roots in the work of Wang et al. [36] who showed that MYC activates Telomerase gene. Zviran et al. [37] showed that Myc activity is indispensable for conducive IPS (induced pluripotent stem cells) formation from somatic cells.

VDR is widely expressed in many tissues [38], including hematopoietic progenitor cells and the culture of human CD34+ hematopoietic progenitor cells. Addition of 1,25-dihydroxy Vitamin D3 (vitamin D3) induces massive monocyte recruitment *in vitro* [39,40]. Vitamin D3 is needed for definitive hematopoiesis and suggests potential therapeutic utility in HSPC expansion [41]. Metadichol' has been shown earlier in *ex vivo* study to enriches CD34+ and also CD33+ cells using umbilical cord cells [42,43]. VD3 and analogs inhibit malignant cells growing in the blood [44], brain [45] and other cancers as well [46].

Metadichol® and AhR and other cytokines

AHR inhibition leads to an expansion of human umbilical cord blood-derived HSPCs when stimulated by cytokines. AHR inhibition leads to *ex vivo* HSC expansion and could be useful for the clinical use of HSC therapy [47,48]. Metadichol is an inverse agonist of AhR [49].

TNF alpha activates NF-KB, and this targets Telomerase by modulating its nuclear translocation [50-52]. Telomere shortening results from increased levels of cytokines PAI-1, ICAM-1, MCP-1 [53,54]. Moreover, Metadichol inhibits all these biomarkers. Elevated PAI-1 levels are involved in many diseases including cancer and lead to accelerated aging and cellular senescence. PAI-1 is a downstream target of p53 in the induction of senescence [55]. Stem cell dysfunctions are the result of a deficiency of Klotho that lead to telomere shortening [56]. Metadichol increases klotho expression in cancer cell lines [57]. Free radical production can lead to oxidative stress

and telomere shortening [58]. Also, antioxidants like ascorbic acid can mitigate this. Vitamin C increasing intracellularly is the key to the suppression of oxidative stress leading to telomere length maintenance. Metadichol increases Ascorbic to levels far above what can be achieved by oral Vitamin C supplementation. Metadichol increases Glut-4 expression tenfold, and this can recycle oxidized ascorbic acid [59]. In some cases increased telomerase activity is correlated with upregulation of Telomerase (h-TERT) mRNA [60]. Metzger, et al. [61] have shown that hTERT mRNA expression but not telomerase activity is associated with improved 5-year survival cancer rates.

Also, BCAT1 (Branched-chain amino acid transaminase 1) is associated with proliferation in yeast and increased metastatic potential in human cancers, and it is also inhibited by Metadichol [62].

Using a gene enrichment analyzer program Topp Cluster [63] one can generate the cluster of diseases that can be targeted by Metadichol [64] and this is shown in Figure 14. This approach aimed at multiple targets offers superior efficacy because to tackle diseases, multiple receptors and pathway need to be impacted. The idea of one disease, one gene, one target, and one drug is no longer a viable concept to be pursued and the concept emerging is what is referred to as poly-pharmacology [65-67], and Metadichol' is the first example of a new class of molecules that prove the viability of this emerging concept.

Conclusion

Metadichol® at one picogram per ml leads to a sixteen-fold increase in mRNA expression followed by an expression of the Telomerase protein, no expression is seen with increased concentration. mRNA-based therapies require a systemic application, safety, and sufficient concentrations of the therapeutic protein, meaning high quantities of mRNA expression and these are achieved by use of Metadichol.

The advantage that Metadichol has over other telomerase activators is that it is safe and can be tested directly in humans. The present goal being pursued in tissue engineering research is to overcome organ failure by enriching cells with telomerase. This could lead to its use in conditions where telomere attrition has well known medical

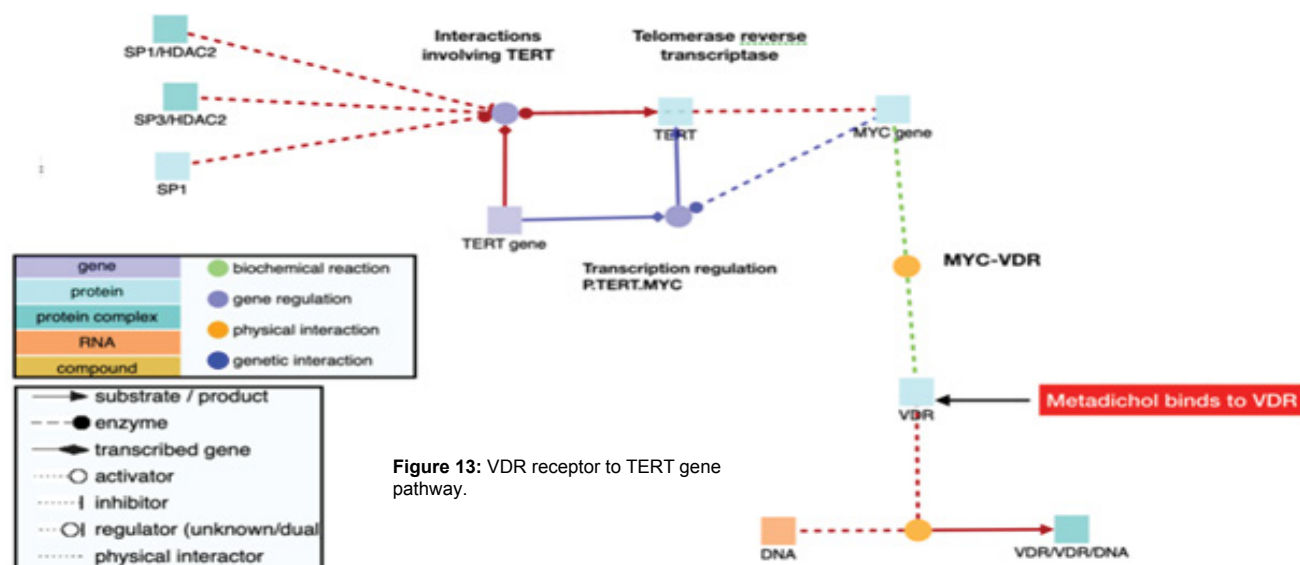




Figure 14: Gene cluster and disease network.

consequences. An approach in use today is a patient donates cells that are enriched with Telomerase in culture. These cells are then injected back in the patient to correct the deficiency. The limitation is the lifespan of most cells, and this is more pronounced in cells from older patients. This inability to proliferate can be overcome using Metadichol. Results of ongoing work on a small subset of Patients who have been using Metadichol for over five years and the effect on Telomere lengths will be reported in due course. Telomerase activation using Metadichol could potentially lead to immortalizing human cells *in vivo* and mass producing *in vitro* any human cell that can lead to an unlimited supply of normal human cells.

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