

Transcriptional Up-Regulation of TERT and TERC necessarily not mean higher Telomerase activity: A Report of Telomerase Inhibition with higher Transcription of TERT, TERC and TERF2 in A549 cells treated with Staurosporine

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Abstract:

Targeting telomerase is one of the approaches to kill cancer cells since almost 90% cancer are telomerase-positive. TERT and TERC are essential subunits of telomerase and a number of reports available regarding telomerase inhibitions due to inhibition of either of the essential subunits. Here, we present inhibition of telomerase activity with up-regulation of TERT and TERC in A549 cells treated with staurosporin, a potent protein kinase inhibitor. Staurosporin was known to inhibit telomerase and we also observed time-dependent telomerase inhibition by staurosporin. Notably, staurosporin up-regulates the transcription of TERT and TERC with time. This data implicates higher expression of TERT and TERC does not enhance telomerase activity always and there must be other post-transcriptional factors that regulate telomerase activity. Furthermore, staurosporine enhances TERF2 expression in a time-dependent manner implicating that it may alter native telomere structure. Staurosporine induced apoptosis is well-established fact. Here, we compared staurosporine induced telomerase inhibition and apoptosis induction in a time frame to elucidate whether these two events are independent or not in our case. We observed that significant apoptosis induction (12h) was earlier event than significant telomerase inhibition (24h) after staurosporine treatment. Our data suggests, staurosporine transcriptionally elevates TERT and TERC but reduces telomerase activity and may alter telomere native structure via up-regulation of TERF2. Higher transcription of essential subunits of telomerase does not assure higher telomerase activity. Since induction of apoptosis is earlier event than telomerase inhibition, staurosporine induces apoptosis independent of telomerase inhibition

Keywords: Staurosporine, Telomerase, TERT, TERC, TERF2, TRAP Assay, Apoptosis, Caspase-3, Nuclear Fragmentation

Introduction:

Telomerase, a ribonucleoprotein complex that maintains telomere length, is highly activated in more than 90% tumor cells and generally absent in somatic cells. Activation of telomerase enzyme and telomere stabilization is an important step in tumorigenesis [1]. Telomerase consists of an RNA component (TR or TERC) that acts as template for synthesis of telomere DNA, and a reverse transcriptase unit (TERT) having catalytic activity of formation of telomeric DNA copying RNA template. These two components are necessary and sufficient for in vitro telomerase activity but several other accessory factors such as Dyskerin, TEP1 (or

TP1), P23, HSP90, etc. play a role in regulating in vivo activity [2, 3]. Telomere shortening after each cycle of cell division can be prevented either by telomerase or by recombination-based mechanism known as alternative lengthening of telomere (ALT) [4, 5]. Telomere dysfunction has been implicated to genomic instability, cancer and aging [6–8]. In particular, inhibition of telomerase and telomere shortening below a critical length triggers apoptotic death in various cell types whereas induction of telomerase activity is associated with resistance to apoptosis [9, 10]. The ectopic expression of TERT subunit increases the life span of cells by maintaining the telomere length [11, 12]. Since TERT and TERC are essential subunits of telomerase a number of

reports are there where people targeted TERT or TERC to inhibit telomerase activity to kill cancer. A number of small molecules not only inhibit catalytic activity but also reduce transcription of TERT and thereby kill cancer cells [13, 14]. BIBR 1532 is a catalytic inhibitor of TERT also reduced TERT expression [15]. Similarly, anti-sense RNA against TERC, which is in clinical trial, is also effective to kill various kinds of cancer via cleavage of TERC transcript [16, 17]. However, since telomerase is a nucleoprotein complex there should have post-transcriptional events which may regulate telomerase activity. Full length expression of TERT and post-transcriptional alternating splicing of TERT modulates telomerase activity and are the main rate-limiting step in telomerase regulation [18, 19]. Furthermore, a number of reports showed that TERT and TERC have other non-telomeric functions in cells [20]. So, TERT and TERC expression could be major factor for telomerase regulation.

In searching for mechanism of telomerase inhibition by staurosporine, we got an inverse-correlation between telomerase activity and transcript of TERT / TERC. Staurosporine is well-known inhibitor of various protein kinases such as PKA, PKC, PKG etc. [21, 22] and it can inhibit telomerase activity and induce apoptosis [23]. Since staurosporine is reported to have telomerase inhibitory effect and apoptotic effect, question remains whether these two events are linked or independent to each other. Here we present the data to resolve whether telomerase inhibition and apoptosis induction by staurosporine is independent to each other. As such there is no report of its effect in telomere-associated proteins and telomere native structure. Here, we report role of staurosporine in regulation of telomerase and telomere proteins in A549 cells.

Materials & methods:

2.1. Chemicals and antibodies:

RNase A and Hoechst dye were obtained from Sigma Chemicals (USA) and Proteinase K was from Life Technologies, (USA). Reverse transcriptase, first strand buffer and oligo dT were from Life Technologies (USA), gene specific oligonucleotides for PCR amplification were from Eurofins (India). Caspase-3 assay kit was from PharMingen (USA). Other molecular biology grade fine chemicals were procured locally from SRL, India.

2.2. Cell culture

A549 cells, the epithelial cell line of Human lung carcinoma was obtained from NCCS, Pune, India. A549 cells were routinely grown as monolayer in plastic Petri dishes using DMEM supplemented with 10% FBS (complete medium) at 37°C in humidified atmosphere containing 5% CO₂ [24].

2.3. MTT assay:

The cell viability was measured by MTT assay after treatment with staurosporine. In a 96 well plate, 3000 cells per well in 100 µl fresh medium were seeded for overnight and then treated with different concentrations of staurosporine (0.005-20nM) for 48h and the MTT assay was done using standard protocol [25]. After

treatment, the medium was replaced with fresh medium. Then MTT (0.5mg/ml) was added and incubated in dark for 2 hours. After incubation medium discarded again and 100 µl of DMSO added into the wells. Absorbance was recorded at 595 nm in a Thermo MULTISKAN ES micro plate reader.

2.4. Determination of telomerase activity by PCR based telomerase repeat amplification protocol (TRAP):

Methods for preparation of cell extract and detection of telomerase activity were similar to described earlier [26]. In brief, telomeric substrate (TS) [5'-AATCCGTCGAGCAGAGTT-3'] was allowed to extend by cell extract (0.5 µg of total protein) for 30min in TRAP reaction mixture [20 mM Tris.HCl, 1.5 mM MgCl₂, 78 mM KCl, 0.0005 % Tween-20, 1 mM EGTA, 50 µM dNTP, 0.1 µg TS, 2.5 µg BSA] in a total volume of 50 µl. The telomeric product was amplified by PCR (94°C for 30 sec, 52°C for 30 sec, 72°C for 45 sec; 26- 30 cycles) using the reverse primer (ACX) [5'-CCCTTACCCTTACCCTTACCCTTA-3']. The internal standards TSNT and NT primers were also used. The telomeric products were resolved on 10% non-denaturing PAGE and silver stained following standard procedure.

The image of gel was then documented with BioRad gel documentation system. Image was analyzed with ImageJ and Telomere Products Generation (TPG) were calculated using the formula as described by Kim et. al. [27]. The intensities of bands in control lane is taken as 100% and accordingly the telomerase activity was calculated in treated lanes. The mean telomerase activity ± standard deviations were calculated and plotted. Each experiment was repeated at least 4 times. The p-values were calculated using one way ANOVA with a post-hoc test such as the Dunnett's test.

2.5. Real time PCR of TERC, TERT and TERF2:

The quantitative expression of hTERT, hTERC and TERF2 were monitored after treatment with staurosporine in at different time point by Real time PCR using Taqman assay (TERT, Hs00972656_m1; TERC, Hs03454202_s1; TERF2, Hs00194619_m1). The reactions were performed under standard assay programme (95 °C for 10 min and then 40 cycles of 95 °C for 15 s, followed by annealing and extension at 60 °C for 1 min). The threshold fluorescence signal was set up manually and the corresponding Ct values were determined. The expression levels were normalized using 18S rRNA (Hs99999901_s1) as endogenous control by $\Delta\Delta C_t$ method.

2.6. Detection of nuclear fragmentation:

Methodology used for the detection of nuclear fragmentation was same as described earlier [28]. In brief, cells were grown on coverslip and treated with staurosporine. Then the cells were washed with PBS and fixed with 70% ethanol for 1 hour at 4 °C. After the fixation cells were stained with 1 mM Hoechst in phosphate buffered saline (PBS) in the dark at room temperature for 5 min. and observed under a fluorescence microscope (Zeiss, Axioscope A1). 200 cells counted each time and analyzed to

determine the fraction of apoptotic cells with fragmented nuclei.

2.7. Caspase-3 activation:

Caspase-3 activity assay was performed according to the protocols recommended by the manufacturers. In short, cells treated with different concentrations of staurosporine for different time point like (0 - 72h), washed twice with PBS and lysed in lysis buffer. The total protein was estimated in the cell lysate. In caspase-3 activity assay mixture, 300µg of total proteins and 10 µl of reconstituted Ac-DEVD-AFC, the substrate for caspase-3, was added and incubated for 1 h at 37°C. The fluorescence of released AFC was measured in fluorescence spectrophotometer (Agilent Cary Eclipse Fluorescence Spectrophotometer) with an excitation at 400 nm and an emission at 505 nm. The inhibitors of the caspase were used together with their respective substrates during the assay to check the enzyme-substrate specificity.

Results:

3.1. Staurosporine induces dose-dependent cell death in A549 cells:

Survival of A549 cells decreased significantly in a dose dependent manner after treatment with various doses of Staurosporine (0.005-20 nM) for 48h. Data is shown in Figure 1. The % of survival at 0.25nM and 1nM were 73% ($p= 4.3E-3$) and 66% ($p= 1.9E-4$)

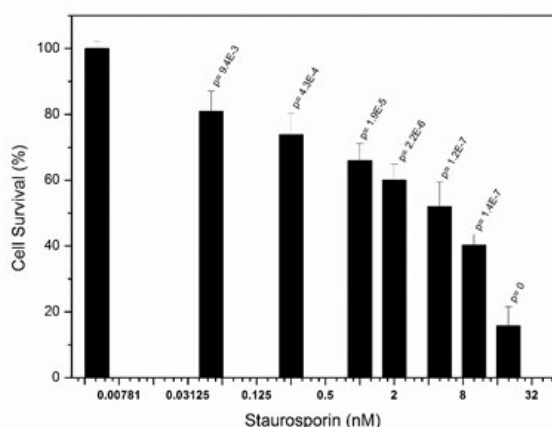


Figure 1: Survival fraction of A549 cells in MTT assay after 48 hours treatment with different concentration of Staurosporine.

respectively. At 0.5nM staurosporine the %survival of A549 cells is about 70% which is significantly lower than untreated control.

3.2. Telomerase inhibition by staurosporine in A549 cells as detected by TRAP assay:

We have treated A549 cells with 0.5 nM of staurosporine for different time interval (0-72h). We observed time dependent decrease of telomerase and decrease is significant at each time point as shown in Figure 2A. However, there was very less significant of telomerase inhibition before 24h treatment of staurosporine (data not shown). So, telomerase inhibition occurred by 0.5nM staurosporine at the earliest after 24h treatment of staurosporine. Amount of telomerase activity at each time point with respect to

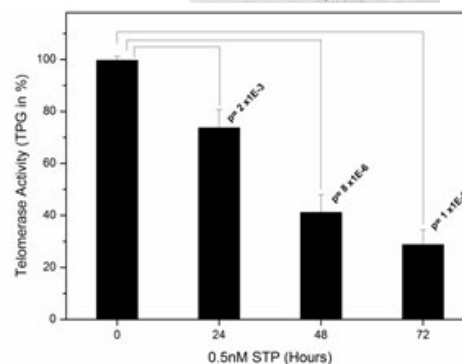
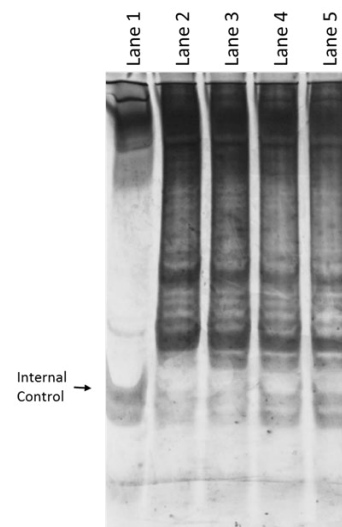


Figure 2: Telomerase activity after treatment with staurosporine. (A) Reduction of telomerase activity in A549 cells at different time interval, lane 1 for negative control, lane 2 for 0 hours, lane 3 for 24 hours, lane 4 for 48 hours, lane 5 for 72 hours; (B) Amount of telomerase activity calculated by TPG; P-values were calculated at each time with respective to or zero time point. the control (0 hour, 100%) is shown in bar diagram in Figure 2B. This data represents that staurosporine inhibits telomerase activity.

3.3. Expression of TERT, TERC and TERF2:

Since telomerase activity was decreased by staurosporine, we have checked mRNA expression of two essential subunits of telomerase (TERT and TERC) and key shelterin protein TERF2 using q-PCR. Notably, TERT expression increased significantly at each time point and time-dependent expression profile is shown in Figure 3A. TERC expression was also observed significantly higher at each time point, shown in Figure 3B. Expression of TERC first increases at 24h, then a slight decrease was observed at later time points. This data indicates that staurosporine treatment increases expression of essential subunits of telomerase but decreases telomerase activity. Furthermore, we observed higher expression of key shelterin protein- TERF2 after staurosporine treatment (Figure 3C). This data implicates that staurosporine may change

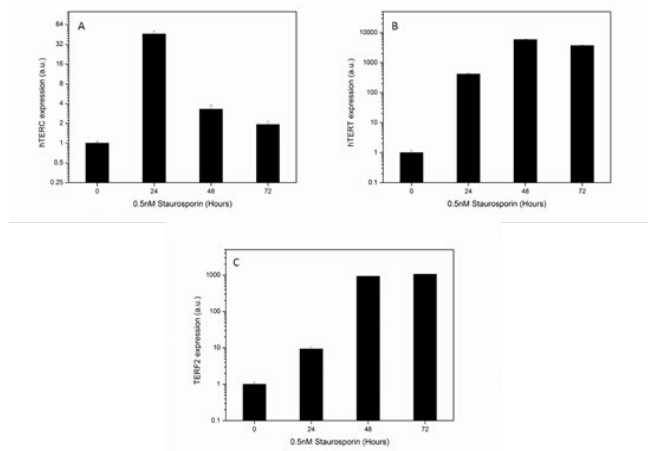


Figure 3: mRNA expressions from qRT-PCR; (A) Expression of TERC, (B) TERT and (C) TERF2 in A549 cells after time dependent treatment of staurosporine. native telomere structure and/or ALT pathway.

3.4. Apoptosis induction by staurosporine as detected by nuclear fragmentation and caspase-3 activation:

Apoptotic death of A549 cells were measured after treatment with staurosporine by nuclear fragmentation and caspase-3 activation. The typical fragmented nucleus was shown in left panel of Figure 4A and morphology of cultured cells was also shown side by side in the right panel of the same picture. The cells were severely

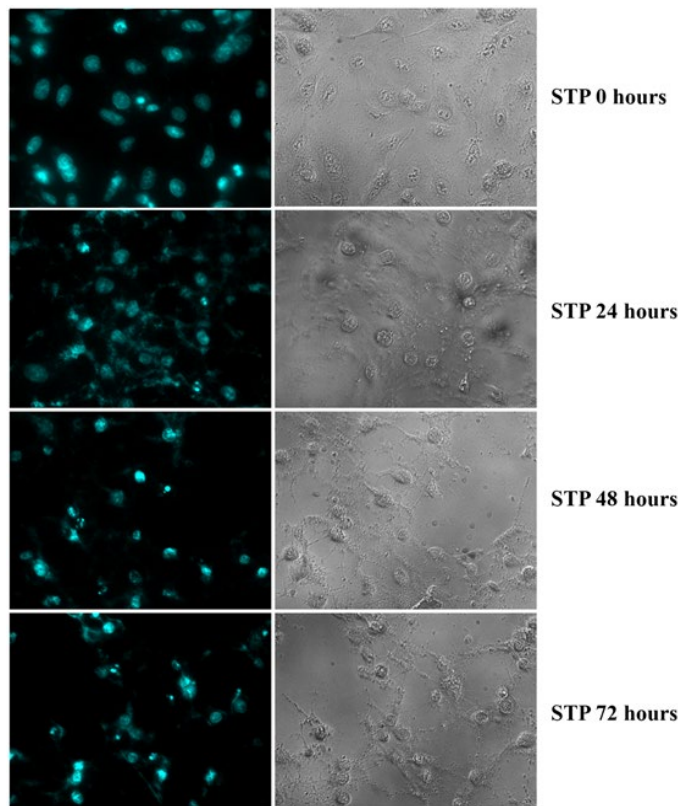


Figure 3: mRNA expressions from qRT-PCR; (A) Expression of TERT, (B) TERC and (C) TERF2 in A549 cells after time dependent treatment of staurosporine.

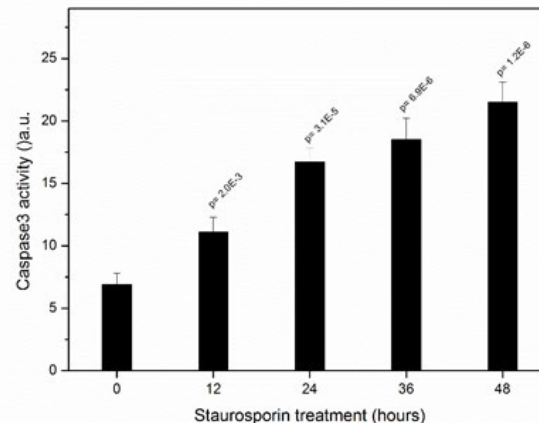


Figure 4: Induction of apoptosis in A549 cells after treatment with staurosporine; (A) Nuclear morphology of cells after treatment at different time, (B) Caspase-3 activation as detected using fluorescence assay damaged as seen from the morphology in the right panel of Figure 4A.

Apoptotic cells were counted under fluorescence microscope and data was shown in Table 1. % of apoptotic cells increased significantly at each time point. Caspase-3 activation was also increased significantly at each time point as shown Figure 4b. This data implicates that staurosporin induces apoptosis in A549 cells

Staurosporine treatment	% of apoptotic cell death
0 Hours	1.98 ± 0.11
24 Hours	32.06 ± 4.23
48 Hours	55.71 ± 6.18
72 Hours	69.82 ± 9.03

Table 1. Fraction of Apoptotic cells after treatment with staurosporine and significant induction of apoptosis started as early as 12h of treatment with staurosporine.

Discussion:

Here we observed that staurosporine treatment inhibited telomerase activity along with increase of transcripts of TERT and TERC. Literature reviews show that telomerase inhibition can be achieved by reducing transcription of TERT/TERC or post-transcription cleavage of mRNA of TERC as discussed in introduction section. But reverse is not true – i.e., higher expression of TERT/TERC transcript is not always associated with higher telomerase activity. So, post-transcriptional processing, modification and telomerase assembly may be the rate-limiting step of telomerase regulation. This data is also corroborated with other reports of non-telomerase

function of TERT and TERC [29]. Thus TERT and TERC are not only involved in telomerase activity. Notably, our data raises interesting question regarding mechanism of telomerase inhibition by staurosporine. It is well-known that phosphorylation can modulate gene expression and staurosporine may induce transcription of telomerase subunits via inhibition of protein kinases. However, we did not check phosphorylation status of the regulatory genes. Furthermore, PKC can regulate TERT transcript [19]. Notably, phosphorylation of TERT subunit by PKC favors its nuclear localization and assembly of telomerase holoenzyme [30, 31]. Thus, in spite of increase of TERT/TERC transcript, telomerase assembly may decrease due to inhibition of PKC by staurosporine in our case. Furthermore, TERT phosphorylation at ser/thr residue is required for its activity [32]. So, here inhibition of telomerase activity by staurosporine via inhibiting phosphorylation of hTERT subunit cannot be ruled out. TERF2 is a key shelterin protein to retain native capped structure at telomere and depletion of TERF2 makes the telomere open, leading to induce DNA damage signal [33]. On the other hand, telomere damage induces up-regulation of TERF2 [34]. We also observed up-regulation of TERF2 and TERF1 after telomere damage by bleomycin or g-quadruplex ligand (communicated). Here, we observed TERF2 up-regulation after treatment with staurosporine. So, staurosporine may increase TERF2 via telomere damage. However, we did not check telomere damage by staurosporine in our experimental condition. As we mentioned earlier that phosphorylation can be a switch for modulation of gene expression. So, increase of TERF2 expression due to inhibition of phosphorylation by staurosporine cannot be ruled out.

Phosphorylation of target proteins may activate or deactivate number of proteins that are involved in apoptosis. One common example is phosphorylation of bcl-2 family members, which consists the anti-apoptotic proteins bcl-2, bcl-xLII and pro-apoptotic proteins bax and bad. Phosphorylation modifies the function of bcl-2 family members with changing pattern of dimerization [35, 36]. The various caspases that gets activated in cells after receiving death signal are also regulated by phosphorylation apart from apoptotic stimulus [37, 38]. We did not check the phosphorylation status of those proteins involved in apoptotic pathways. So, apoptosis induction by staurosporine may be via inhibition of several protein kinases. Again, telomerase inhibition may lead to telomere shortening that may induce DNA damage induced apoptosis [39]. So, induction of apoptosis by staurosporine may be resulted due to inhibition of telomerase [9] or may be independent of telomerase regulation such as inhibition of phosphorylation of bcl-2 protein [35, 36]. To distinguish whether apoptosis induction was related or unrelated to telomerase regulation we have carried out time kinetics of apoptosis induction and telomerase activity after treatment with staurosporine. Here, we observed appreciable apoptosis induction after 12h of treatment where as significant reduction of telomerase activity was observed after 24h treatment of staurosporine. The result demonstrate that the apoptosis induction was earlier event than telomerase inhibition. Hence, staurosporine independently induce apoptosis reduce telomerase activity.

Conclusion:

Staurosporine reduces telomerase activity with increase of TERT and TERC transcription. This data implicates that up-regulation of TERT and TERC transcription does not imply higher telomerase activity. It also increases expression of key shelterin TERF2 indicating that it can alter telomere native structure or ALT pathway. Staurosporine also induces apoptosis independent of telomerase inhibition in A549 cells.

Conflict of interest:

There is no conflict of interest in publishing this work.

Acknowledgement:

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