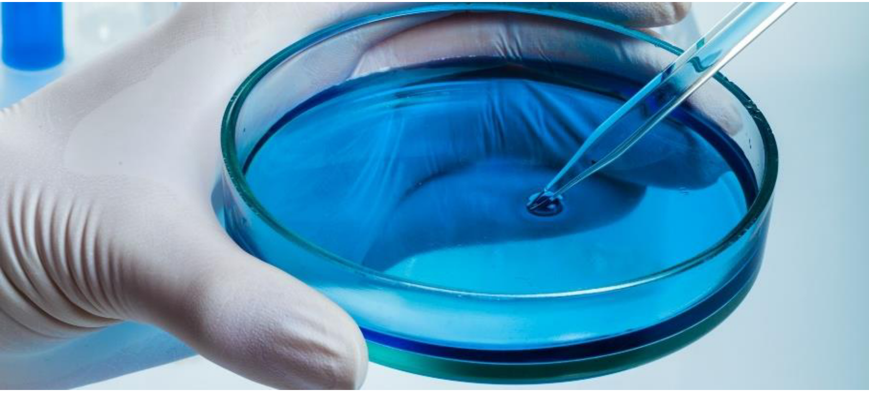
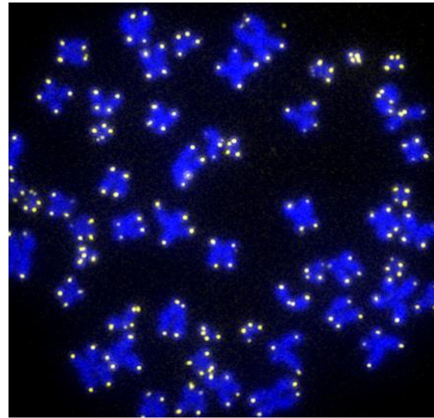
LIFE LENGTH





STUDY REPORT

TOM MCCORKLE

LIFE LENGTH

11/07/2022

STUDY REPORT FOR TOM MCCORKLE

Analysis of the effect of 3 compounds on telomerase activity in vitro in a PC3 cell line

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STUDY OBJECTIVE

Determination of the toxicity by MTT assay and evaluation of the effect of Enterolactone (O), M-enantiomer A (A), M-enantiomer B (B) on telomerase activity by Q-TRAP in cultures of PC3

1. MTT ASSAY

1.1 TECHNIQUE OVERVIEW

The MTT toxicity test is a colorimetric assay to measure cells' metabolic activity by serving as a substrate of cellular enzymes that reduce the tetrazolium orange dye, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to its insoluble formazan, giving a purple color. The rate of conversion is directly proportional to cells´ mitochondrial metabolic activity via NAD (P) H-dependent cellular oxidoreductase enzymes and serves as a surrogate marker of cell viability.

Cells and culture conditions

Cultures of PC3 (Human Prostate Cancer Cell Line) cells working stock (5 passages) were established under standard culture conditions in in Kaighn´s modification of Ham´s F-48 medium (F-48K) (ATCC).

F-48K is supplemented with 10% FBS, 2mM Glutamine and and Penicillin 0.01mg/ml.

Toxicity assay (MTT)

Cells previously expanded were seeded in 96 well plates (Nunc) at 1.9 $\times 10^5$ and 9.6 $\times 10^4$ cells/plate for 24- and 72-hours treatment respectively. This concentration is known to show the best window for the MTT assay, with better sensitivity and low variability.

The compounds were provided as dry powder by the client and were kept in optimal conditions until its use. A quantity of 5 mg of the compounds (powder) were dissolved in 500 µl of DMSO at RT. Eight-point curves, 1/3 dilutions, (highest concentration 125 µM) were prepared. Final concentration of DMSO was 0.5% (this concentration does not affect the cells)

Twenty-four hours following the seeding process, the cells were washed once with PBS and treated with the respective compounds in cell culture media. Each condition was tested in triplicates. For positive and negative controls, 8mM methyl methane sulfonate (MMS) and DMSO 100% were used respectively.

Following compound addition, the plates were incubated for 24 and 72 hours. After the treatment period, cells were washed twice with PBS and media was replaced with MTT reagent at 0.5 mg/ml in DMEM without phenol red. The plates were gently shaken and incubated for 4 hours. After the incubation the medium was removed and replaced by DMSO. The plates were gently shaken to solubilize the formazan crystals.

Absorbance was measured using an Envision multiplate reader at a wavelength of 570 nm.

1.2 RESULTS

Toxicity Assay (MTT) in PC3 cells at 24 and 72 hours

Eight serial dilutions were prepared (125.00, 41.67, 13.89, 4.63, 1.54, 0.51, 0.17 and 0.06 µM to evaluate the compound.

Triplicates per concentration were prepared to evaluate the compound. MTT results showing cell death values above 20% are considered as having a significant toxic effect.

Tables 1-6. *MTT assay results expressed as the percentage of cell death after treatments.*

24 hours - Enterolactone					
Concentration (µM)	**Cell death #1 (%)**	**Cell death #2 (%)**	**Cell death #3 (%)**	**MEAN**	**SD**
125.00	19.65	12.01	15.10	15.59	3.84
41.67	-32.30	-17.98	-35.59	-28.62	9.36
13.89	-33.85	-41.29	-40.62	-38.59	4.12
4.63	-41.49	-38.59	-32.20	-37.42	4.75
1.54	-30.56	-47.58	-25.82	-34.65	11.45
0.51	-29.78	-35.88	-8.98	-24.88	14.10
0.17	-12.56	-5.41	-10.24	-9.40	3.65
0.06	-3.37	-20.79	-22.43	-15.53	10.56

24 hours – Enantiomer A					
Concentration (µM)	**Cell death #1 (%)**	**Cell death #2 (%)**	**Cell death #3 (%)**	**MEAN**	**SD**
125.00	32.42	28.74	33.00	31.39	2.31
41.67	-6.18	-0.76	-12.85	-6.60	6.06
13.89	-26.49	-20.40	-18.56	-21.82	4.15
4.63	-17.98	-21.37	-15.27	-18.21	3.05
1.54	-29.20	-24.37	-23.78	-25.78	2.97
0.51	-2.70	-15.18	-10.44	-9.44	6.30
0.17	-27.17	-17.50	-8.79	-17.82	9.19
0.06	-10.92	-13.82	-11.02	-11.92	1.65

24 hours – Enantiomer B					
Concentration (µM)	**Cell death #1 (%)**	**Cell death #2 (%)**	**Cell death #3 (%)**	**MEAN**	**SD**
125.00	6.23	3.54	4.64	4.80	1.36
41.67	-68.77	-35.80	-33.35	-45.97	19.78
13.89	-71.22	-63.50	-63.74	-66.15	4.39
4.63	-72.93	-63.74	-39.60	-58.76	17.22
1.54	-58.35	-51.85	-43.77	-51.32	7.31
0.51	-15.09	-34.33	-34.94	-28.12	11.29
0.17	-22.81	-20.24	-14.35	-19.13	4.33
0.06	-14.60	-12.15	-1.24	-9.33	7.11

72 hours - Enterolactone					
Concentration (µM)	**Cell death #1 (%)**	**Cell death #2 (%)**	**Cell death #3 (%)**	**MEAN**	**SD**
125.00	35.94	32.46	22.11	30.17	7.19
41.67	-35.45	-32.21	-34.95	-34.20	1.74
13.89	-60.86	-51.02	-63.98	-58.62	6.76
4.63	-74.57	-70.83	-81.17	-75.52	5.24
1.54	-63.98	-69.71	-46.41	-60.03	12.14
0.51	-71.45	-77.56	-73.70	-74.24	3.09
0.17	-49.03	-43.54	-39.93	-44.17	4.58
0.06	-39.68	-40.18	-37.32	-39.06	1.53

72 hours – Enantiomer A					
Concentration (µM)	**Cell death #1 (%)**	**Cell death #2 (%)**	**Cell death #3 (%)**	**MEAN**	**SD**
125.00	75.56	74.94	74.82	75.11	0.40
41.67	9.78	23.24	14.02	15.68	6.88
13.89	-45.54	-47.91	-30.21	-41.22	9.60
4.63	-76.31	-66.59	-54.63	-65.85	10.86
1.54	-46.91	-52.39	-53.51	-50.94	3.53
0.51	-63.73	-69.58	-63.23	-65.51	3.53
0.17	-43.42	-40.55	-38.31	-40.76	2.56
0.06	-39.43	-40.43	-33.95	-37.94	3.49

72 hours – Enantiomer B					
Concentration (µM)	**Cell death #1 (%)**	**Cell death #2 (%)**	**Cell death #3 (%)**	**MEAN**	**SD**
125.00	64.88	61.78	57.24	61.30	3.85
41.67	11.03	11.96	14.65	12.55	1.88
13.89	-33.31	-22.56	-34.75	-30.21	6.66
4.63	-45.81	-40.03	-52.74	-46.19	6.37
1.54	-52.64	-40.34	-51.19	-48.05	6.72
0.51	-47.57	-52.84	-52.74	-51.05	3.01
0.17	-33.20	-39.41	-29.79	-34.13	4.87
0.06	-22.56	-30.41	-23.49	-25.49	4.29

1.3 OBSERVATIONS AND CONCLUSIONS

- Morphological analysis through optical microscopy visualization (20x) did not show any deleterious effect at any concentration.
- No compound precipitation was observed at concentrations tested.
- Based on the results the concentrations chosen for the Q-TRAP and HERT assays are 40, 20, 10, 5, 2.5 µM.

2. TELOMERASE ACTIVITY DETERMINATION BY Q-TRAP

In this assay telomerase activity is determined by Q-TRAP in PC3 after 24, 48 and 72 hours treatment and at the concentrations mentioned before. Cells were seeded at 300.000 (24 hours), 200.000 (48 hours), 150.000 (72 hours) cells/well in Eagle´s minimum essential medium (EMEM) (ATCC).

2.1 TECHNIQUE OVERVIEW

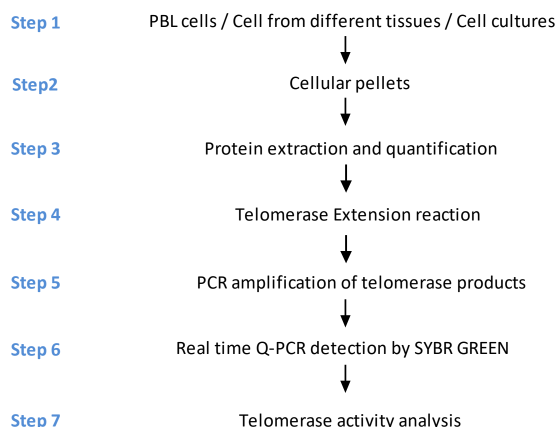
Q-TRAP can measure relative telomerase activity by the Telomeric Repeat Amplification Protocol (TRAP), modified for real-time, quantitative PCR analysis (Q-TRAP). This method has the advantages of exquisite sensitivity, rapidity, and a high-throughput format in respect to the regular TRAP assay. We assess telomerase enzyme activity in whole cell lysates from cell cultures.

The general mechanism of the Q-TRAP technique involves cellular pellets being lysed for protein extraction which is then subsequently quantified and stored under specific conditions to avoid protein degradation. Protein obtained in the process was used inside the following 72 hours, samples were stored at 4°C. Telomerase protein extracts are then incubated with a specific oligonucleotide substrate to allow the enzymatic addition of telomeric DNA repeats by endogenous telomerase.

Following the enzymatic reaction. telomerase extension products are then amplified and quantified by real-time qPCR. In real time PCR, a positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for fluorescence to cross the threshold (i.e.: exceeds background levels). The telomerase-positive standard dilution series is plotted against the telomere protein concentration (r^2 > 0.9) as a standard curve of Ct values.

To ensure that the data are both reproducible and quantitative. we routinely perform the assay in triplicate. The mean and standard deviation (SD) from each triplicate is calculated which include both positive (Lymphoid cell line standard Curve) and negative controls (inactivated by heat) are included.

Data are reported as **RTA (Relative Telomerase Activity)** and the general workflow scheme can be seen below.



2.2 RESULTS

***Table 7**. Abbreviations used throughout the report*

Treatment	*Abbreviation*
Control untreated group	*Ctrl*
Treatment with Enterolactone at 40 µM	O_40
Treatment with Enterolactone at 20 µM	O_20
Treatment with Enterolactone at 10 µM	O_10
Treatment with Enterolactone at 5 µM	O_5
Treatment with Enterolactone at 2.5 µM	O_2.5
Control untreated group	*Ctrl*
Treatment with A enantiomer at 40 µM	A_40
Treatment with A enantiomer at 20 µM	A_20
Treatment with A enantiomer at 10 µM	A_10
Treatment with A enantiomer at 5 µM	A_5
Treatment with A enantiomer at 2.5 µM	A_2.5
Control untreated group	*Ctrl*
Treatment with B enantiomer at 40 µM	B_40
Treatment with B enantiomer at 20 µM	B_20
Treatment with B enantiomer at 10 µM	B_10
Treatment with B enantiomer at 5 µM	B_5
Treatment with B enantiomer at 2.5 µM	B_2.5

Quality control parameters

Before plating and conducting Q-TRAP protocol samples were assessed for:

- Protein concentration: Protein quantification is performed in each sample using the Biorad protein assay. A minimum of 0.3 µg/µl protein concentration is required to proceed with the analysis of the samples to guarantee consistent results.
- Regression Curve: Internal controls are included, and a regression analysis is performed for each run/plate. Those plates in which regression curves have an R^2 below 0.9 are repeated.
- Replicates: Samples with less than 2 valid replicates are discarded.
- Cycle amplification: Signals obtained after cycle #35 are considered unspecific amplification.

Protein concentrations

ENTEROLACTONE

Table 8. *Protein concentration results for samples treated with* ***Enterolactone*** *analyzed by Q-TRAP. Each column shows the concentration of each triplicate.*

Treatment	Time Point (h)	Protein (µg/µl)
Control – Time 0 – Replicate 1	0	0.31
Control – Time 0 – Replicate 2	0	0.30
Control – Time 0 – Replicate 3	0	0.30
Control – 24 hours – Replicate 1	24	0.32
Control – 24 hours – Replicate 2	24	0.34
Control – 24 hours – Replicate 3	24	0.33
O_40 – 24 hours – Replicate 1	24	0.34
O_40 – 24 hours – Replicate 2	24	0.30
O_40 – 24 hours – Replicate 3	24	0.30
O_20 – 24 hours – Replicate 1	24	0.31
O_20 – 24 hours – Replicate 2	24	0.33
O_20 – 24 hours – Replicate 3	24	0.32
O_10 – 24 hours – Replicate 1	24	0.30
O_10 – 24 hours – Replicate 2	24	0.37
O_10 – 24 hours – Replicate 3	24	0.35
O_5 – 24 hours – Replicate 1	24	0.30
O_5 – 24 hours – Replicate 2	24	0.31
O_5 – 24 hours – Replicate 3	24	0.35
O_2.5 – 24 hours – Replicate 1	24	0.34
O_2.5 – 24 hours – Replicate 2	24	0.34
O_2.5 – 24 hours – Replicate 3	24	0.30
Control – 48 hours – Replicate 1	48	0.40
Control – 48 hours – Replicate 2	48	0.33
Control – 48 hours – Replicate 3	48	0.38
O_40 – 48 hours – Replicate 1	48	0.43
O_40 – 48 hours – Replicate 2	48	0.42
O_40 – 48 hours – Replicate 3	48	0.54
O_20 – 48 hours – Replicate 1	48	0.43
O_20 – 48 hours – Replicate 2	48	0.47
O_20 – 48 hours – Replicate 3	48	0.41
O_10 – 48 hours – Replicate 1	48	0.41
O_10 – 48 hours – Replicate 2	48	0.39
O_10 – 48 hours – Replicate 3	48	0.48

O_5 – 48 hours – Replicate 1	48	0.45
O_5 – 48 hours – Replicate 2	48	0.48
O_5 – 48 hours – Replicate 3	48	0.48
O_2.5 – 48 hours – Replicate 1	48	0.40
O_2.5 – 48 hours – Replicate 2	48	0.33
O_2.5 – 48 hours – Replicate 3	48	0.38
Control – 72 hours – Replicate 1	72	0.34
Control – 72 hours – Replicate 2	72	0.30
Control – 72 hours – Replicate 3	72	0.30
O_40 – 72 hours – Replicate 1	72	0.31
O_40 – 72 hours – Replicate 2	72	0.33
O_40 – 72 hours – Replicate 3	72	0.32
O_20 – 72 hours – Replicate 1	72	0.30
O_20 – 72 hours – Replicate 2	72	0.37
O_20 – 72 hours – Replicate 3	72	0.35
O_10 – 72 hours – Replicate 1	72	0.30
O_10 – 72 hours – Replicate 2	72	0.31
O_10 – 72 hours – Replicate 3	72	0.35
O_5 – 72 hours – Replicate 1	72	0.34
O_5 – 72 hours – Replicate 2	72	0.34
O_5 – 72 hours – Replicate 3	72	0.30
O_2.5 – 72 hours – Replicate 1	72	0.34
O_2.5 – 72 hours – Replicate 2	72	0.30
O_2.5 – 72 hours – Replicate 3	72	0.30

ENANTIOMER A

Table 9. *Protein concentration results for samples treated with* ***<u>Enantiomer A</u>*** *analyzed by Q-TRAP. Each column shows the concentration of each triplicate.*

Treatment	Time Point (h)	Protein (µg/µl)
Control – Time 0– Replicate 1	0	0.31
Control – Time 0 – Replicate 2	0	0.30
Control – Time 0 – Replicate 3	0	0.30
Control – 24 hours – Replicate 1	24	0.32
Control – 24 hours – Replicate 2	24	0.33
Control – 24 hours – Replicate 3	24	0.34
A_40 – 24 hours – Replicate 1	24	0.30
A_40 – 24 hours – Replicate 2	24	0.31
A_40 – 24 hours – Replicate 3	24	0.31
A_20 – 24 hours – Replicate 1	24	0.30
A_20 – 24 hours – Replicate 2	24	0.31
A_20 – 24 hours – Replicate 3	24	0.35

A_10 – 24 hours – Replicate 1	24	0.33
A_10 – 24 hours – Replicate 2	24	0.30
A_10 – 24 hours – Replicate 3	24	0.30
A_5 – 24 hours – Replicate 1	24	0.38
A_5 – 24 hours – Replicate 2	24	0.32
A_5 – 24 hours – Replicate 3	24	0.34
A_2.5 – 24 hours – Replicate 1	24	0.33
A_2.5 – 24 hours – Replicate 2	24	0.37
A_2.5 – 24 hours – Replicate 3	24	0.34
Control – 48 hours – Replicate 1	48	0.38
Control – 48 hours – Replicate 2	48	0.38
Control – 48 hours – Replicate 3	48	0.36
A_40 – 48 hours – Replicate 1	48	0.33
A_40 – 48 hours – Replicate 2	48	0.36
A_40 – 48 hours – Replicate 3	48	0.35
A_20 – 48 hours – Replicate 1	48	0.28
A_20 – 48 hours – Replicate 2	48	0.28
A_20 – 48 hours – Replicate 3	48	0.35
A_10 – 48 hours – Replicate 1	48	0.29
A_10 – 48 hours – Replicate 2	48	0.31
A_10 – 48 hours – Replicate 3	48	0.35
A_5 – 48 hours – Replicate 1	48	0.36
A_5 – 48 hours – Replicate 2	48	0.33
A_5 – 48 hours – Replicate 3	48	0.29
A_2.5 – 48 hours – Replicate 1	48	0.46
A_2.5 – 48 hours – Replicate 2	48	0.40
A_2.5 – 48 hours – Replicate 3	48	0.39
Control – 72 hours – Replicate 1	72	0.32
Control – 72 hours – Replicate 2	72	0.33
Control – 72 hours – Replicate 3	72	0.34
A_40 – 72 hours – Replicate 1	72	0.30
A_40 – 72 hours – Replicate 2	72	0.31
A_40 – 72 hours – Replicate 3	72	0.31
A_20 – 72 hours – Replicate 1	72	0.30
A_20 – 72 hours – Replicate 2	72	0.31
A_20 – 72 hours – Replicate 3	72	0.35
A_10 – 72 hours – Replicate 1	72	0.33
A_10 – 72 hours – Replicate 2	72	0.30
A_10 – 72 hours – Replicate 3	72	0.30
A_5 – 72 hours – Replicate 1	72	0.38
A_5 – 72 hours – Replicate 2	72	0.32
A_5 – 72 hours – Replicate 3	72	0.34

A_2.5 – 72 hours – Replicate 1	72	0.33
A_2.5 – 72 hours – Replicate 2	72	0.37
A_2.5 – 72 hours – Replicate 3	72	0.34

ENANTIOMER B

Table 10. *Protein concentration results for samples treated with* ***Enantiomer B*** *analyzed by Q-TRAP. Each column shows the concentration of each triplicate.*

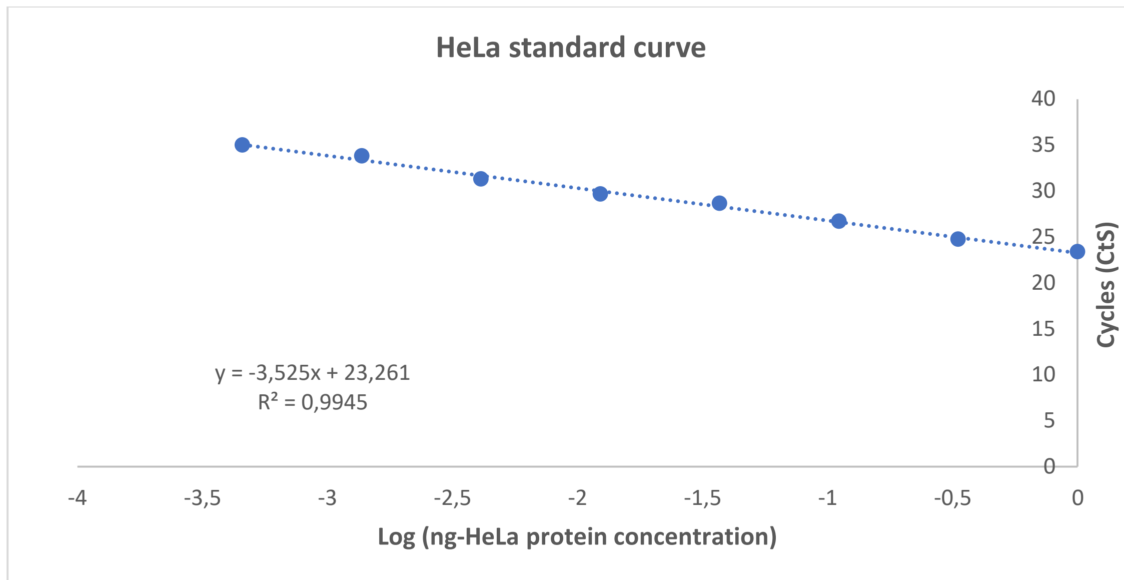
Treatment	Time Point (h)	Protein (µg/µl)
Control – Time 0 – Replicate 1	0	0.31
Control – Time 0 – Replicate 2	0	0.30
Control – Time 0 – Replicate 3	0	0.30
Control – 24 hours – Replicate 1	0	0.37
Control – 24 hours – Replicate 2	0	0.37
Control – 24 hours – Replicate 3	0	0.34
B_40 – 24 hours – Replicate 1	0	0.36
B_40 – 24 hours – Replicate 2	0	0.32
B_40 – 24 hours – Replicate 3	0	0.30
B_20 – 24 hours – Replicate 1	24	0.34
B_20 – 24 hours – Replicate 2	24	0.33
B_20 – 24 hours – Replicate 3	24	0.32
B_10 – 24 hours – Replicate 1	24	0.35
B_10 – 24 hours – Replicate 2	24	0.32
B_10 – 24 hours – Replicate 3	24	0.37
B_5 – 24 hours – Replicate 1	24	0.38
B_5 – 24 hours – Replicate 2	24	0.40
B_5 – 24 hours – Replicate 3	24	0.40
B_2.5 – 24 hours – Replicate 1	24	0.30
B_2.5 – 24 hours – Replicate 2	24	0.32
B_2.5 – 24 hours – Replicate 3	24	0.31
Control – 48 hours – Replicate 1	24	0.49
Control – 48 hours – Replicate 2	24	0.52
Control – 48 hours – Replicate 3	24	0.62
B_40 – 48 hours – Replicate 1	24	0.57
B_40 – 48 hours – Replicate 2	24	0.52
B_40 – 48 hours – Replicate 3	24	0.44
B_20 – 48 hours – Replicate 1	48	0.44
B_20 – 48 hours – Replicate 2	48	0.45
B_20 – 48 hours – Replicate 3	48	0.39
B_10 – 48 hours – Replicate 1	48	0.51
B_10 – 48 hours – Replicate 2	48	0.52
B_10 – 48 hours – Replicate 3	48	0.48

B_5 – 48 hours – Replicate 1	48	0.54
B_5 – 48 hours – Replicate 2	48	0.51
B_5 – 48 hours – Replicate 3	48	0.58
B_2.5 – 48 hours – Replicate 1	48	0.52
B_2.5 – 48 hours – Replicate 2	48	0.57
B_2.5 – 48 hours – Replicate 3	48	0.58
Control – 72 hours – Replicate 1	48	0.37
Control – 72 hours – Replicate 2	48	0.37
Control – 72 hours – Replicate 3	48	0.34
B_40 – 72 hours – Replicate 1	48	0.36
B_40 – 72 hours – Replicate 2	48	0.32
B_40 – 72 hours – Replicate 3	48	0.30
B_20 – 72 hours – Replicate 1	72	0.34
B_20 – 72 hours – Replicate 2	72	0.33
B_20 – 72 hours – Replicate 3	72	0.32
B_10 – 72 hours – Replicate 1	72	0.35
B_10 – 72 hours – Replicate 2	72	0.32
B_10 – 72 hours – Replicate 3	72	0.37
B_5 – 72 hours – Replicate 1	72	0.38
B_5 – 72 hours – Replicate 2	72	0.40
B_5 – 72 hours – Replicate 3	72	0.40
B_2.5 – 72 hours – Replicate 1	72	0.30
B_2.5 – 72 hours – Replicate 2	72	0.32
B_2.5 – 72 hours – Replicate 3	72	0.31

Telomerase Activity results

Only those samples with sufficient amount of protein were analyzed (> 0.3 mg/ml). Standard curve results: generated by graphing Threshold cycles (Ct values) of HeLa cell line standards against log of 1000, 333, 111, 37.03, 48.34, 4.11, 1.37 and 0.45 ng of protein (whole cell extract).

Figure 1. *Standard curve of Ct values of protein from HeLa cells against log of protein (ng). The cycle number at the threshold (Ct value) for each sample is interpolated in the curve. in order to calculate the relative telomerase activity (RTA).*



Measurements were performed in triplicates to calculate coefficients of variation and mean amplification signals.

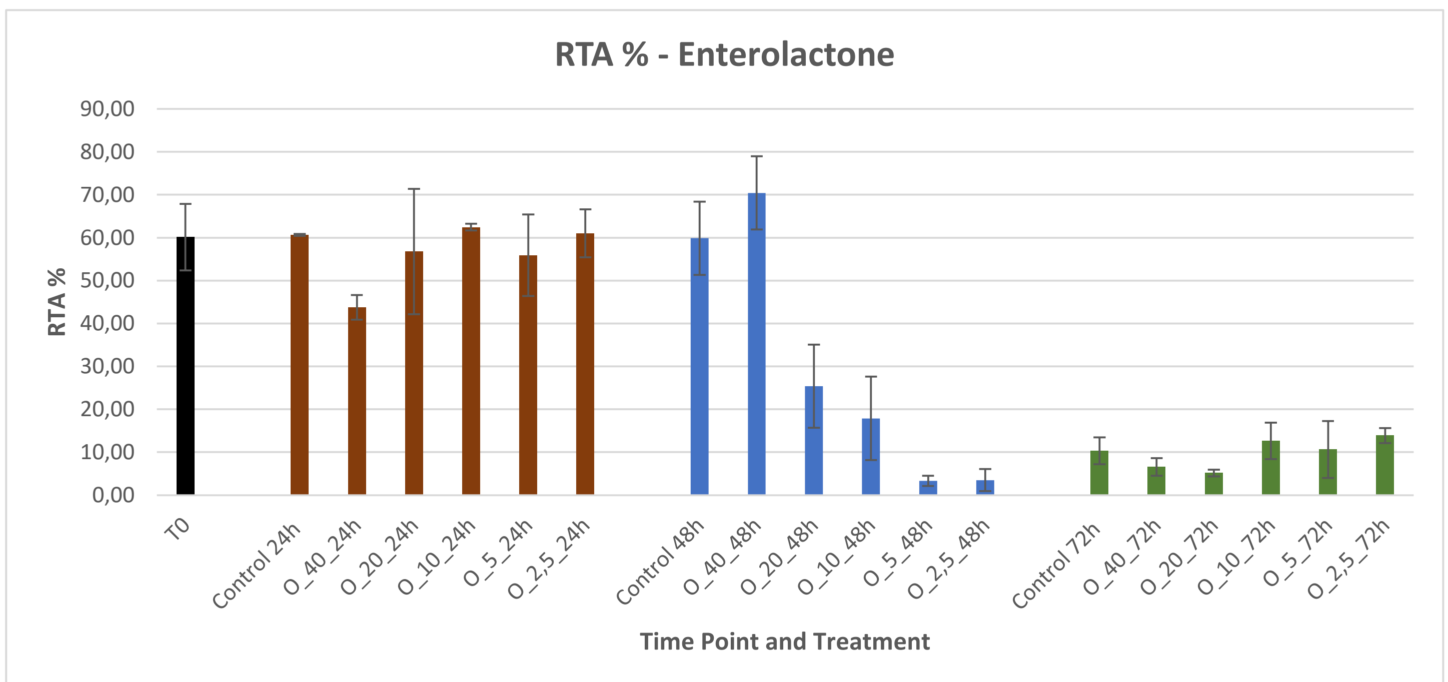
The table below summarize the average of triplicate data. mean and SD after normalization against Hela control reported as Relative Telomerase Activity (RTA) for each sample.

ENTEROLACTONE

Table 11. *RTAs values for all* ***<u>Enterolactone</u>*** *treated samples analyzed*

Telomerase activity (RTA%) Enterolactone								
	0 Hours		24 Hours		48 Hours		72 Hours	
	RTA (%)	SD	RTA (%)	SD	RTA (%)	SD	RTA (%)	SD
Control	60.14	7.76	60.64	0.21	59.88	8.53	10.32	3.11
O_40			43.75	2.88	70.42	8.54	6.57	2.04
O_20			56.77	14.63	25.39	9.70	5.15	0.81
O_10			62.45	0.76	17.89	9.69	12.63	4.23
O_5			55.90	9.52	3.31	1.17	10.65	6.64
O_2.5			61.02	5.60	3.50	2.56	13.88	1.73

Figure 2. *Graphs for Q-TRAP results (RTA) from in vitro treatment with* ***<u>Enterolactone</u>****.*



Q-TRAP analysis for PC3 at different times were compared. Data were grouped by time point of treatment. Statistical analysis for grouped data was applied using t test comparing with control condition.

Table 12. *T-Student analysis indicates if there are significant differences among the Q-TRAP results (RTA) compared to control condition. Significant differences are indicated in the "Significance" column. From lowest to highest significance: No: non-significant; Yes (*): p<0.05; Yes (**): p<0.01; Yes (***): p<0.001; Yes (****): p<0.0001.*

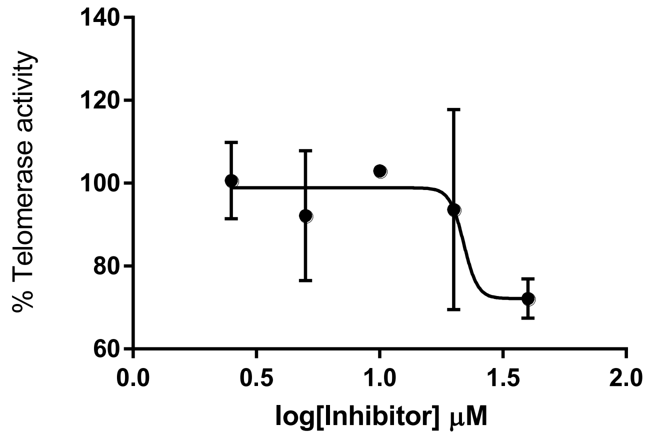
T-Student Analysis – Relative Telomerase Activity Enterolactone		
24 hours	**RTA (p Value)**	**Significance**
Control vs. O_40	0.0143	Yes (*)
Control vs. O_20	0.7441	No
Control vs. O_10	0.0839	No
Control vs. O_5	0.5544	No
Control vs. O_2.5	0.9329	No
48 hours	**RTA (p Value)**	**Significance**
Control vs. O_40	0.2688	No
Control vs. O_20	0.0098	Yes (**)
Control vs. O_10	0.0049	Yes (**)
Control vs. O_5	0.0003	Yes (***)
Control vs. O_2.5	0.0004	Yes (***)
72 hours	**RTA (p Value)**	**Significance**
Control vs. O_40	0.2381	No
Control vs. O_20	0.1156	No
Control vs. O_10	0.5239	No
Control vs. O_5	0.9419	No
Control vs. O_2.5	0.1580	No

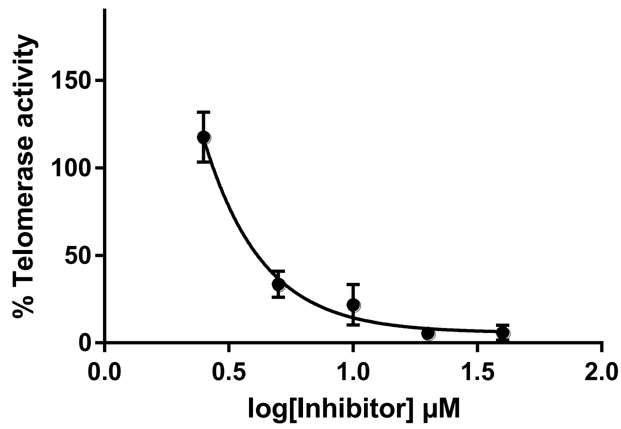
Log-Dose vs Response Enterolactone at 24, 48 and 72 h

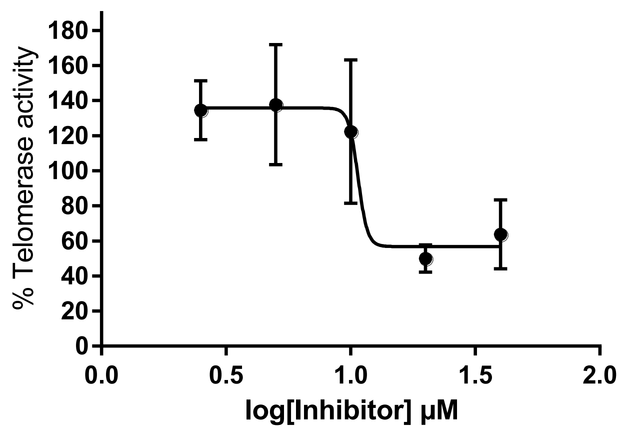
Figure 3. *Graphs of IC50 of* ***Enterolactone*** *at 24, 48 and 72 hours (Graphs were generated using GraphPad Prism 6) using a nonlinear regression (curve fit); log [inhibitor] vs. response. The curve has a variable slope.*

IC50 µM - Enterolactone			
	24h	**48h**	**72h**
Enterolactone	21.92	0.05	10.71

The average of telomerase activity from replicates were calculated for each concentration of enterolactone at 24, 48 and 72h and compared to the average value of the no-drug control to generate the percent (%) of telomerase activation and plotted against the treatment concentration to determine the drug concentrations for each formulation to achieve 50% inhibition (IC_{50} value).


Enterolactone 24h
140
120
100
80
60
% Telomerase activity
0.0
0.5
1.0
1.5
2.0
log[Inhibitor] μM


Enterolactone 48h
150
100
50
0
% Telomerase activity
0.0
0.5
1.0
1.5
2.0
log[Inhibitor] μM

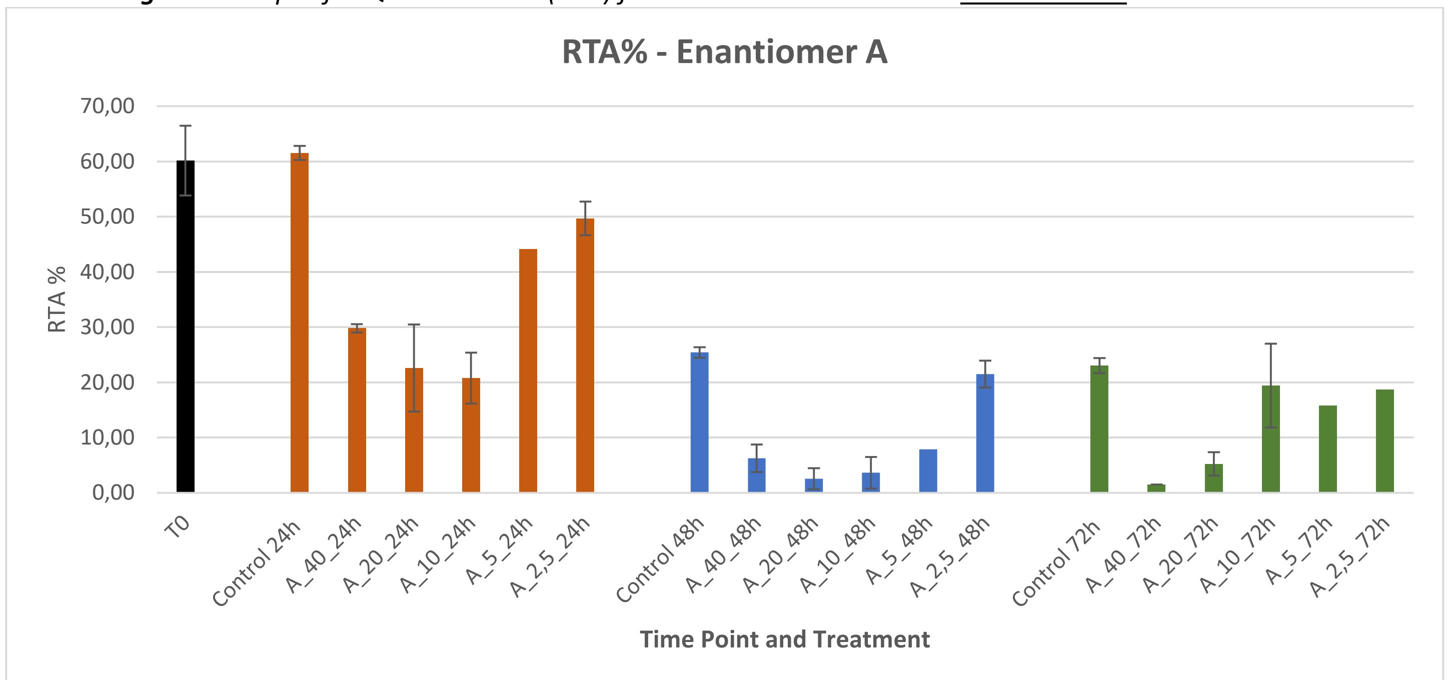

Enterolactone 72h
180
160
140
120
100
80
60
40
20
0
% Telomerase activity
0.0
0.5
1.0
1.5
2.0
log[Inhibitor] μM

ENANTIOMER A

Table 13. *RTAs value for all* ***Enantiomer A*** *treated samples analyzed.*

Telomerase Activity (RTA%) – Enantiomer A								
	0 Hours		24 Hours		48 Hours		72 Hours	
	RTA (%)	SD	RTA (%)	SD	RTA (%)	SD	RTA (%)	SD
Control	60.14	7.76	61.53	6.32	25.39	3.05	23.04	2.45
A_40			29.78	8.38	6.23	2.75	1.48	0.31
A_20			22.61	1.29	2.51	0.98	5.20	1.36
A_10			20.76	0.75	3.60	2.47	19.40	0.00
A_5			44.11	7.88	7.86	1.91	15.77	2.12
A_2.5			49.67	4.59	21.48	2.87	18.63	7.61

Figure 4. *Graphs for Q-TRAP results (RTA) from in vitro treatment with* ***Enantiomer A****.*



Q-TRAP analysis for PC3 at different times were compared. Data were grouped by time point of treatment. Statistical analysis for grouped data was applied using t test comparing with control condition.

Table 14. *T-Student analysis indicates if there are significant differences among the Q-TRAP results (RTA) compared to control condition. Significant differences are indicated in the "Significance" column. From lowest to highest significance: No: non-significant; Yes (*): p<0.05; Yes (**): p<0.01; Yes (***): p<0.001; Yes (****): p<0.0001.*

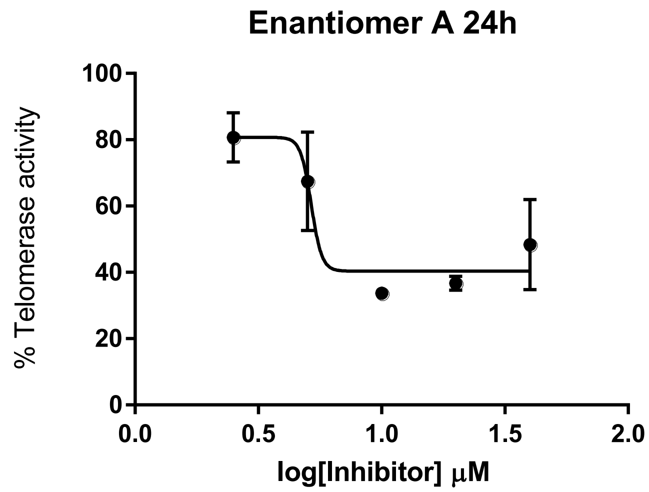
T-Student Analysis – Relative Telomerase Activity Enantiomer A		
24 hours	**RTA (p Value)**	**Significance**
Control vs. A_40	0.0063	Yes (**)
Control vs. A_20	0.0005	Yes (***)
Control vs. A_10	0.0033	Yes (**)
Control vs. A_5	0.0405	Yes (*)
Control vs. A_2.5	0.1112	No
48 hours	**RTA (p Value)**	**Significance**
Control vs. A_40	0.0222	Yes (*)
Control vs. A_20	0.0010	Yes (**)
Control vs. A_10	0.0030	Yes (**)
Control vs. A_5	0.0204	Yes (*)
Control vs. A_2.5	0.3178	No
72 hours	**RTA (p Value)**	**Significance**
Control vs. A_40	0.0065	Yes (**)
Control vs. A_20	0.0122	Yes (*)
Control vs. A_10	0.1708	No
Control vs. A_5	0.0379	Yes (*)
Control vs. A_2.5	0.5173	No

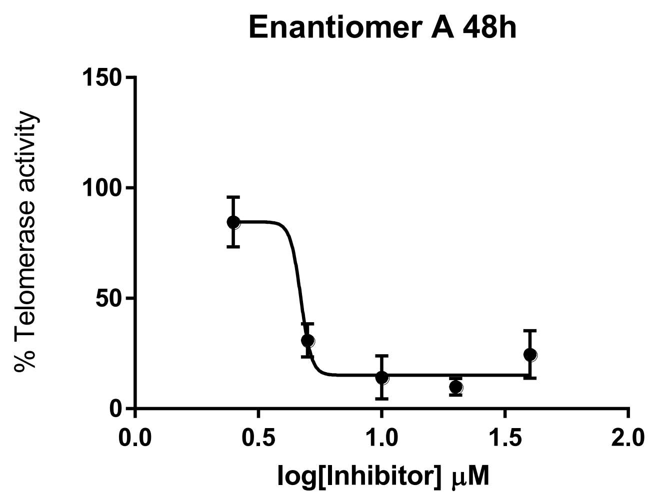
Log-Dose vs Response Enantiomer A at 24, 48 and 72 h

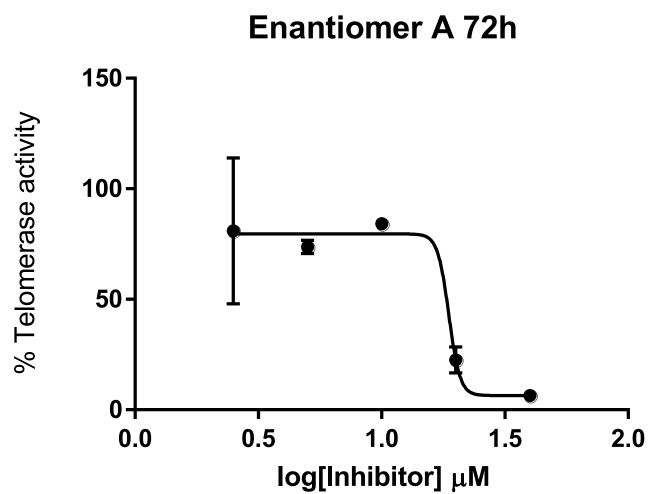
Figure 5. *Graphs of IC50 of **Enantiomer A** at 24, 48 and 72 hours (Graphs were generated using GraphPad Prism 6) using a nonlinear regression (curve fit); log [inhibitor] vs. response. The curve has a variable slope.*

IC50 µM - Enantiomer A			
	24h	**48h**	**72h**
Enantiomer A	5.18	4.71	18.73

The average of telomerase activity from replicates were calculated for each concentration of enantiomer A at 24, 48 and 72h and compared to the average value of the no-drug control to generate the percent (%) of telomerase activation and plotted against the treatment concentration to determine the drug concentrations for each formulation to achieve 50% inhibition (IC_{50} value).


Enantiomer A 24h
100
80
60
40
20
0
% Telomerase activity
0.0
0.5
1.0
1.5
2.0
log[Inhibitor] μM


Enantiomer A 48h
150
100
50
0
% Telomerase activity
0.0
0.5
1.0
1.5
2.0
log[Inhibitor] μM

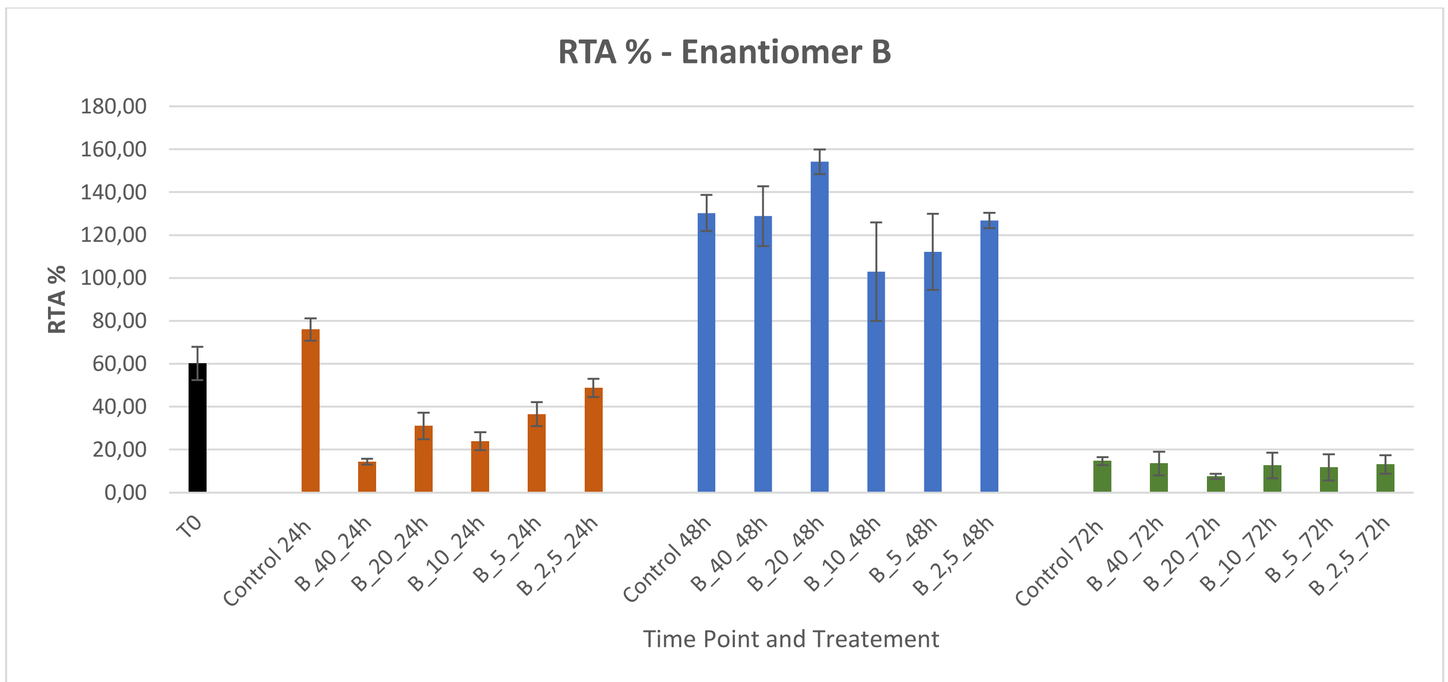

Enantiomer A 72h
150
100
50
0
% Telomerase activity
0.0
0.5
1.0
1.5
2.0
log[Inhibitor] μM

ENANTIOMER B

Table 15. *RTAs value for all* ***Enantiomer B*** *treated samples analyzed.*

Telomerase activity (RTA%) – Enantiomer B								
	0 Hours		24 Hours		48 Hours		72 Hours	
	RTA (%)	SD	RTA (%)	SD	RTA (%)	SD	RTA (%)	SD
Control	60.14	7.76	75.92	5.23	130.28	8.44	14.62	1.85
B_40			14.44	1.35	128.83	13.91	13.49	5.47
B_20			31.05	6.16	154.17	5.77	7.53	1.18
B_10			23.87	4.18	102.96	22.92	12.58	5.96
B_5			36.48	5.61	112.17	17.77	11.67	6.14
B_2.5			48.73	4.20	126.77	3.54	13.05	4.29

Figure 6. *Graphs for Q-TRAP results (RTA) from in vitro treatment with* ***Enantiomer B****.*



Q-TRAP analysis for PC3 at different times were compared. Data were grouped by time point of treatment. Statistical analysis for grouped data was applied using t test comparing with control condition.

Table 16. *T-Student analysis indicates if there are significant differences among the Q-TRAP results (RTA) compared to control condition. Significant differences are indicated in the "Significance" column. From lowest to highest significance: No: non-significant; Yes (*): p<0.05; Yes (**): p<0.01; Yes (***): p<0.001; Yes (****): p<0.0001.*

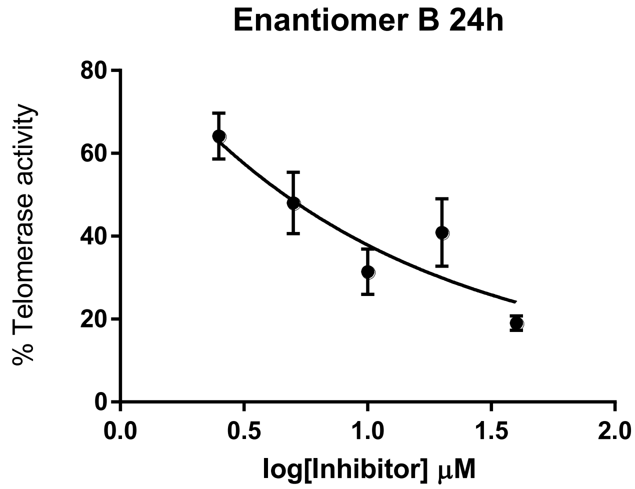
T-Student Analysis – Relative Telomerase Activity Enantiomer B		
24 hours	**RTA (p Value)**	**Significance**
Control vs. B_40	0.0002	Yes (***)
Control vs B_20	0.0036	Yes (**)
Control vs. B_10	0.0011	Yes (**)
Control vs. B_5	0.0184	Yes (**)
Control vs. B_2.5	0.0291	Yes (*)
48 hours	**RTA (p Value)**	**Significance**
Control vs. B_40	0.9058	No
Control vs B_20	0.0807	No
Control vs. B_10	0.2545	No
Control vs. B_5	0.2854	No
Control vs. B_2.5	0.6412	No
72 hours	**RTA (p Value)**	**Significance**
Control vs. B_40	0.8074	No
Control vs B_20	0.0446	Yes (*)
Control vs. B_10	0.6847	No
Control vs. B_5	0.5731	No
Control vs. B_2.5	0.6818	No

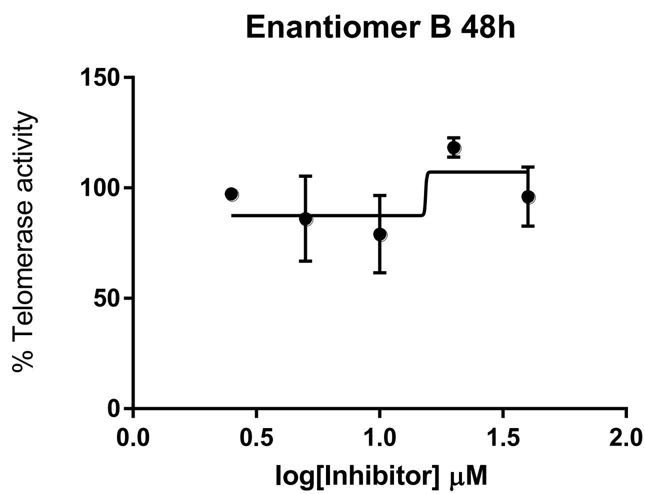
Log-Dose vs Response Enantiomer B at 24, 48 and 72 h

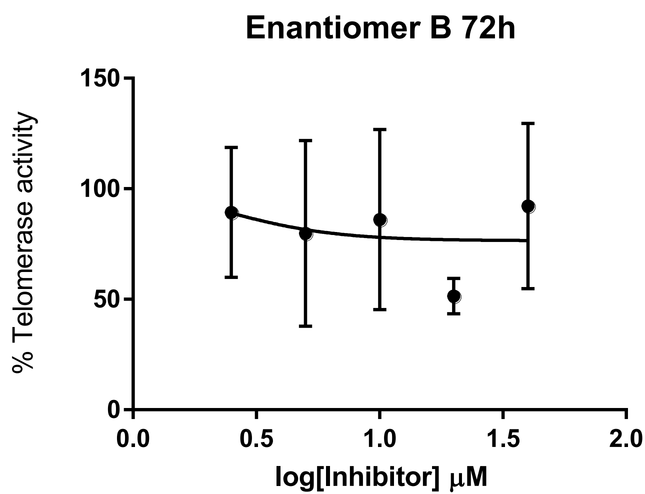
Figure 7. *Graphs of IC50 of **Enantiomer B** at 24, 48 and 72 hours (Graphs were generated using GraphPad Prism 6) using a nonlinear regression (curve fit); log [inhibitor] vs. response. The curve has a variable slope.*

IC50 µM - Enantiomer B			
	24h	48h	72h
Enantiomer B	2.987	15.38	2.384

The average of telomerase activity from replicates were calculated for each concentration of Enantiomer B at 24, 48 and 72h and compared to the average value of the no-drug control to generate the percent (%) of telomerase activation and plotted against the treatment concentration to determine the drug concentrations for each formulation to achieve 50% inhibition (IC_{50} value).


Enantiomer B 24h
% Telomerase activity
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
log[Inhibitor] μM


Enantiomer B 48h
% Telomerase activity
150
100
50
0
0.0
0.5
1.0
1.5
2.0
log[Inhibitor] μM


Enantiomer B 72h
% Telomerase activity
150
100
50
0
0.0
0.5
1.0
1.5
2.0
log[Inhibitor] μM

2.3 OBSERVATIONS AND CONCLUSIONS

Quality Control Results

- Protein extraction yields from all samples rendered suitable amount of protein (> 0.3 µg/µl) to perform the Q-TRAP assay.
- The regression curve's coefficient of determination (HeLa Cells) was higher than 0.9 (R^2 = 0.99).

Enterolactone

- At 24 hours three treated groups showed lower telomerase activity levels compared to control however only the highest concentration presented a significant difference.
- At 48 hours four treated groups presented a reduction in RTA compared to the control untreated group. All of these changes were found to be significant.
- At 72 hours no significant differences were detected between control and treated groups.

Enantiomer A

- At 24 hours, all treated groups showed lower telomerase activity levels compared to control. The differences were significant for all groups except for the lowest concentration.
- At 48 hours, all treated groups showed lower telomerase activity levels compared to control. The differences were significant for all groups except for the lowest concentration.
- At 72 hours, all treated groups showed lower telomerase activity levels compared to control. The differences were significant for the two highest concentrations.

Enantiomer B

- At 24 hours, all treated groups showed lower telomerase activity levels compared to control with all of the differences being significant.
- At 48 hours, the groups treated with concentrations 40, 10, 5 and 2,5 showed lower telomerase activity levels compared to control. However, the differences were not found to be significant.
- At 72 hours, the telomerase activity dropped to basal levels without any particular differences between the treated and control groups.

Taking into account the data obtained it can be concluded that Enantiomer A presents a telomerase inhibition effect in a PC3 cell line that is more prolonged throughout the different time points. Enantiomer B presented a telomerase inhibition effect observed at 24 hours.

3. REFERENCES

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