LIFE LENGTH

STUDY REPORT

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11/07/2022
STUDY REPORT FOR TOM MCCORKLE

Analysis of the effect of 3 compounds on telomerase activity in vitro in a MCF7 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 MCF7 cells.

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 MCF7 (Human Breast Cancer Cell line) cells working stock (5 passages) were established under standard culture conditions in Eagle´s minimum essential medium (EMEM) (ATCC).

EMEM is supplemented with 10% FBS, 2mM Glutamine and Pen/Strep, and Penicillin 0.01mg/ml.

Toxicity assay (MTT)

Cells previously expanded were seeded in 96 well plates (Nunc) at 1.9×10^{5} and 9.6×10^{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.

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1.2 RESULTS

Toxicity Assay (MTT) in MCF7 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	-13.51	-8.13	7.64	-4.66	10.99
41.67	-30.05	-30.43	-40.43	-33.64	5.89
13.89	-10.05	-8.13	-3.51	-7.23	3.36
4.63	-5.05	-19.28	-25.82	-16.71	10.62
1.54	-16.20	-16.97	-21.20	-18.13	2.69
0.51	2.26	-23.51	-19.28	-13.51	13.82
0.17	-14.66	-12.36	-5.43	-10.82	4.80
0.06	-11.97	-41.20	-33.13	-28.77	15.10

24 hours – Enantiomer A					
Concentration (µM)	Cell death #1 (%)	Cell death #2 (%)	Cell death #3 (%)	MEAN	SD
125.00	98.41	95.72	100.72	98.29	2.50
41.67	-83.51	-78.89	-45.05	-69.15	21.00
13.89	-61.20	-35.05	-25.05	-40.43	18.67
4.63	-58.89	-57.74	-34.66	-50.43	13.67
1.54	-48.89	-51.59	-43.51	-48.00	4.11
0.51	-57.74	-18.51	-45.82	-40.69	20.11
0.17	-27.36	-44.28	-55.05	-42.23	13.96
0.06	-25.82	-37.36	-35.43	-32.87	6.18

24 hours – Enantiomer B					
Concentration (µM)	Cell death #1 (%)	Cell death #2 (%)	Cell death #3 (%)	MEAN	SD
125.00	63.85	47.46	63.13	58.15	9.26
41.67	-211.84	-192.61	-204.01	-202.82	9.67
13.89	-132.06	-174.09	-134.91	-147.02	23.49
4.63	-134.19	-163.40	-138.47	-145.35	15.77
1.54	-115.67	-156.99	-114.96	-129.21	24.06
0.51	-124.22	-120.66	-95.01	-113.30	15.93
0.17	-146.30	-115.67	-91.45	-117.81	27.49
0.06	-90.03	-85.75	-79.34	-85.04	5.38

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72 hours - Enterolactone					
Concentration (µM)	Cell death #1 (%)	Cell death #2 (%)	Cell death #3 (%)	MEAN	SD
125.00	88.19	90.54	89.20	89.31	1.18
41.67	-22.36	-24.04	-22.70	-23.03	0.89
13.89	-48.83	-38.44	-39.11	-42.13	5.81
4.63	-53.85	-66.92	-69.26	-63.34	8.30
1.54	-70.27	-54.19	-60.89	-61.78	8.08
0.51	-66.25	-61.22	-61.89	-63.12	2.73
0.17	-67.59	-62.23	-65.91	-65.24	2.74
0.06	-30.40	-23.70	-42.46	-32.19	9.51

72 hours – Enantiomer A					
Concentration (µM)	Cell death #1 (%)	Cell death #2 (%)	Cell death #3 (%)	MEAN	SD
125.00	99.92	99.92	99.58	99.80	0.19
41.67	15.49	4.10	2.76	7.45	7.00
13.89	-43.13	-52.85	-46.15	-47.38	4.97
4.63	-90.37	-113.15	-79.31	-94.28	17.25
1.54	-57.87	-73.62	-58.88	-63.46	8.81
0.51	-79.98	-51.84	-72.61	-68.15	14.59
0.17	-56.87	-58.21	-50.17	-55.08	4.31
0.06	-51.17	-29.40	-11.64	-30.74	19.80

72 hours – Enantiomer B					
Concentration (µM)	Cell death #1 (%)	Cell death #2 (%)	Cell death #3 (%)	MEAN	SD
125.00	99.00	99.52	99.00	99.17	0.30
41.67	-18.96	-6.08	17.10	-2.65	18.27
13.89	-23.34	-30.55	-9.69	-21.20	10.60
4.63	-32.87	-34.42	-30.04	-32.44	2.22
1.54	-22.57	-37.77	-19.48	-26.60	9.79
0.51	-28.49	-52.45	-56.31	-45.75	15.07
0.17	-74.60	-57.08	-52.45	-61.38	11.68
0.06	-31.84	-27.21	-56.05	-38.37	15.49

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.

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2. TELOMERASE ACTIVITY DETERMINATION BY Q-TRAP

In this assay telomerase activity is determined by Q-TRAP in MCF7 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).
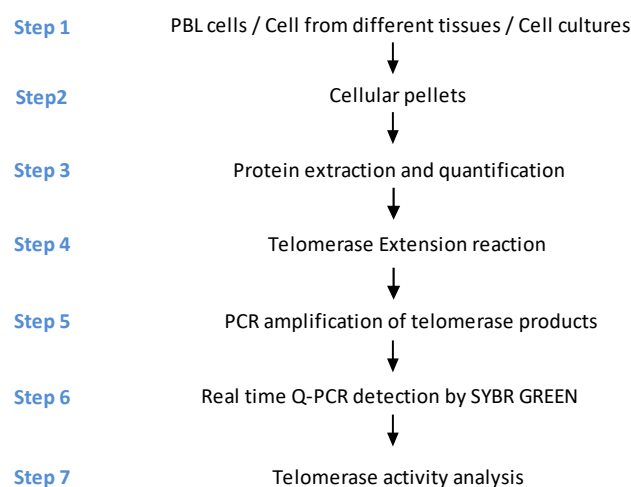
2.1 TECHNIQUE OVERVIEW

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.

Step 1	PBL cells / Cell from different tissues / Cell cultures
	↓
Step2	Cellular pellets
	↓
Step 3	Protein extraction and quantification
	↓
Step 4	Telomerase Extension reaction
	↓
Step 5	PCR amplification of telomerase products
	↓
Step 6	Real time Q-PCR detection by SYBR GREEN
	↓
Step 7	Telomerase activity analysis

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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.

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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	0.34
Control – Time 0 – Replicate 2	0	0.35
Control – Time 0 – Replicate 3	0	0.30
Control – 24 hours – Replicate 1	24	0.33
Control – 24 hours – Replicate 2	24	0.39
Control – 24 hours – Replicate 3	24	0.39
O_40 – 24 hours – Replicate 1	24	0.39
O_40 – 24 hours – Replicate 2	24	0.33
O_40 – 24 hours – Replicate 3	24	0.42
O_20 – 24 hours – Replicate 1	24	0.38
O_20 – 24 hours – Replicate 2	24	0.39
O_20 – 24 hours – Replicate 3	24	0.35
O_10 – 24 hours – Replicate 1	24	0.32
O_10 – 24 hours – Replicate 2	24	0.40
O_10 – 24 hours – Replicate 3	24	0.46
O_5 – 24 hours – Replicate 1	24	0.36
O_5 – 24 hours – Replicate 2	24	0.37
O_5 – 24 hours – Replicate 3	24	0.47
O_2.5 – 24 hours – Replicate 1	24	0.37
O_2.5 – 24 hours – Replicate 2	24	0.44
O_2.5 – 24 hours – Replicate 3	24	0.41
Control – 48 hours – Replicate 1	48	0.34
Control – 48 hours – Replicate 2	48	0.33
Control – 48 hours – Replicate 3	48	0.35
O_40 – 48 hours – Replicate 1	48	0.33
O_40 – 48 hours – Replicate 2	48	0.31
O_40 – 48 hours – Replicate 3	48	0.31
O_20 – 48 hours – Replicate 1	48	0.33
O_20 – 48 hours – Replicate 2	48	0.31
O_20 – 48 hours – Replicate 3	48	0.30
O_10 – 48 hours – Replicate 1	48	0.34
O_10 – 48 hours – Replicate 2	48	0.33
O_10 – 48 hours – Replicate 3	48	0.36
O_5 – 48 hours – Replicate 1	48	0.34

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O_5 – 48 hours – Replicate 2	48	0.35
O_5 – 48 hours – Replicate 3	48	0.33
O_2.5 – 48 hours – Replicate 1	48	0.32
O_2.5 – 48 hours – Replicate 2	48	0.33
O_2.5 – 48 hours – Replicate 3	48	0.37
Control – 72 hours – Replicate 1	72	0.40
Control – 72 hours – Replicate 2	72	0.34
Control – 72 hours – Replicate 3	72	0.39
O_40 – 72 hours – Replicate 1	72	0.36
O_40 – 72 hours – Replicate 2	72	0.34
O_40 – 72 hours – Replicate 3	72	0.35
O_20 – 72 hours – Replicate 1	72	0.36
O_20 – 72 hours – Replicate 2	72	0.54
O_20 – 72 hours – Replicate 3	72	0.45
O_10 – 72 hours – Replicate 1	72	0.41
O_10 – 72 hours – Replicate 2	72	0.47
O_10 – 72 hours – Replicate 3	72	0.39
O_5 – 72 hours – Replicate 1	72	0.37
O_5 – 72 hours – Replicate 2	72	0.40
O_5 – 72 hours – Replicate 3	72	0.37
O_2.5 – 72 hours – Replicate 1	72	0.41
O_2.5 – 72 hours – Replicate 2	72	0.41
O_2.5 – 72 hours – Replicate 3	72	0.40

ENANTIOMER A

Table 9. *Protein concentration results for samples treated with **Enantiomer A** 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	0.43
Control – Time 0 – Replicate 2	0	0.45
Control – Time 0 – Replicate 3	0	0.46
Control – 24 hours – Replicate 1	24	0.38
Control – 24 hours – Replicate 2	24	0.47
Control – 24 hours – Replicate 3	24	0.40
A_40 – 24 hours – Replicate 1	24	0.62
A_40 – 24 hours – Replicate 2	24	0.57
A_40 – 24 hours – Replicate 3	24	0.39
A_20 – 24 hours – Replicate 1	24	0.75
A_20 – 24 hours – Replicate 2	24	0.46
A_20 – 24 hours – Replicate 3	24	0.43
A_10 – 24 hours – Replicate 1	24	0.43

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A_10 – 24 hours – Replicate 2	24	0.46
A_10 – 24 hours – Replicate 3	24	0.31
A_5 – 24 hours – Replicate 1	24	0.73
A_5 – 24 hours – Replicate 2	24	0.50
A_5 – 24 hours – Replicate 3	24	0.53
A_2.5 – 24 hours – Replicate 1	24	0.45
A_2.5 – 24 hours – Replicate 2	24	0.48
A_2.5 – 24 hours – Replicate 3	24	0.50
Control – 48 hours – Replicate 1	48	0.35
Control – 48 hours – Replicate 2	48	0.36
Control – 48 hours – Replicate 3	48	0.32
A_40 – 48 hours – Replicate 1	48	0.33
A_40 – 48 hours – Replicate 2	48	0.34
A_40 – 48 hours – Replicate 3	48	0.30
A_20 – 48 hours – Replicate 1	48	0.34
A_20 – 48 hours – Replicate 2	48	0.35
A_20 – 48 hours – Replicate 3	48	0.34
A_10 – 48 hours – Replicate 1	48	0.35
A_10 – 48 hours – Replicate 2	48	0.38
A_10 – 48 hours – Replicate 3	48	0.30
A_5 – 48 hours – Replicate 1	48	0.35
A_5 – 48 hours – Replicate 2	48	0.35
A_5 – 48 hours – Replicate 3	48	0.34
A_2.5 – 48 hours – Replicate 1	48	0.34
A_2.5 – 48 hours – Replicate 2	48	0.34
A_2.5 – 48 hours – Replicate 3	48	0.36
Control – 72 hours – Replicate 1	72	0.42
Control – 72 hours – Replicate 2	72	0.38
Control – 72 hours – Replicate 3	72	0.38
A_40 – 72 hours – Replicate 1	72	0.46
A_40 – 72 hours – Replicate 2	72	0.34
A_40 – 72 hours – Replicate 3	72	0.35
A_20 – 72 hours – Replicate 1	72	0.42
A_20 – 72 hours – Replicate 2	72	0.39
A_20 – 72 hours – Replicate 3	72	0.45
A_10 – 72 hours – Replicate 1	72	0.41
A_10 – 72 hours – Replicate 2	72	0.43
A_10 – 72 hours – Replicate 3	72	0.45
A_5 – 72 hours – Replicate 1	72	0.41
A_5 – 72 hours – Replicate 2	72	0.40
A_5 – 72 hours – Replicate 3	72	0.43
A_2.5 – 72 hours – Replicate 1	72	0.40

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Treatment	Time Point (h)	Protein (µg/µl)
A_2.5 – 72 hours – Replicate 2	72	0.47
A_2.5 – 72 hours – Replicate 3	72	0.44

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	0.34
Control – Time 0 – Replicate 2	0	0.35
Control – Time 0 – Replicate 3	0	0.30
Control – 24 hours – Replicate 1	24	0.47
Control – 24 hours – Replicate 2	24	0.72
Control – 24 hours – Replicate 3	24	0.47
B_40 – 24 hours – Replicate 1	24	0.49
B_40 – 24 hours – Replicate 2	24	0.54
B_40 – 24 hours – Replicate 3	24	0.45
B_20 – 24 hours – Replicate 1	24	0.48
B_20 – 24 hours – Replicate 2	24	0.46
B_20 – 24 hours – Replicate 3	24	0.38
B_10 – 24 hours – Replicate 1	24	0.44
B_10 – 24 hours – Replicate 2	24	0.49
B_10 – 24 hours – Replicate 3	24	0.51
B_5 – 24 hours – Replicate 1	24	0.44
B_5 – 24 hours – Replicate 2	24	0.49
B_5 – 24 hours – Replicate 3	24	0.54
B_2.5 – 24 hours – Replicate 1	24	0.52
B_2.5 – 24 hours – Replicate 2	24	0.48
B_2.5 – 24 hours – Replicate 3	24	0.46
Control – 48 hours – Replicate 1	48	0.36
Control – 48 hours – Replicate 2	48	0.43
Control – 48 hours – Replicate 3	48	0.36
B_40 – 48 hours – Replicate 1	48	0.31
B_40 – 48 hours – Replicate 2	48	0.31
B_40 – 48 hours – Replicate 3	48	0.30
B_20 – 48 hours – Replicate 1	48	0.34
B_20 – 48 hours – Replicate 2	48	0.35
B_20 – 48 hours – Replicate 3	48	0.35
B_10 – 48 hours – Replicate 1	48	0.39
B_10 – 48 hours – Replicate 2	48	0.38
B_10 – 48 hours – Replicate 3	48	0.37
B_5 – 48 hours – Replicate 1	48	0.40

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B_5 – 48 hours – Replicate 2	48	0.38
B_5 – 48 hours – Replicate 3	48	0.37
B_2.5 – 48 hours – Replicate 1	48	0.32
B_2.5 – 48 hours – Replicate 2	48	0.34
B_2.5 – 48 hours – Replicate 3	48	0.33
Control – 72 hours – Replicate 1	72	0.40
Control – 72 hours – Replicate 2	72	0.58
Control – 72 hours – Replicate 3	72	0.43
B_40 – 72 hours – Replicate 1	72	0.40
B_40 – 72 hours – Replicate 2	72	0.39
B_40 – 72 hours – Replicate 3	72	0.40
B_20 – 72 hours – Replicate 1	72	0.43
B_20 – 72 hours – Replicate 2	72	0.44
B_20 – 72 hours – Replicate 3	72	0.40
B_10 – 72 hours – Replicate 1	72	0.45
B_10 – 72 hours – Replicate 2	72	0.43
B_10 – 72 hours – Replicate 3	72	0.47
B_5 – 72 hours – Replicate 1	72	0.47
B_5 – 72 hours – Replicate 2	72	0.46
B_5 – 72 hours – Replicate 3	72	0.50
B_2.5 – 72 hours – Replicate 1	72	0.54
B_2.5 – 72 hours – Replicate 2	72	0.44
B_2.5 – 72 hours – Replicate 3	72	0.46

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Telomerase Activity results and Data analysis

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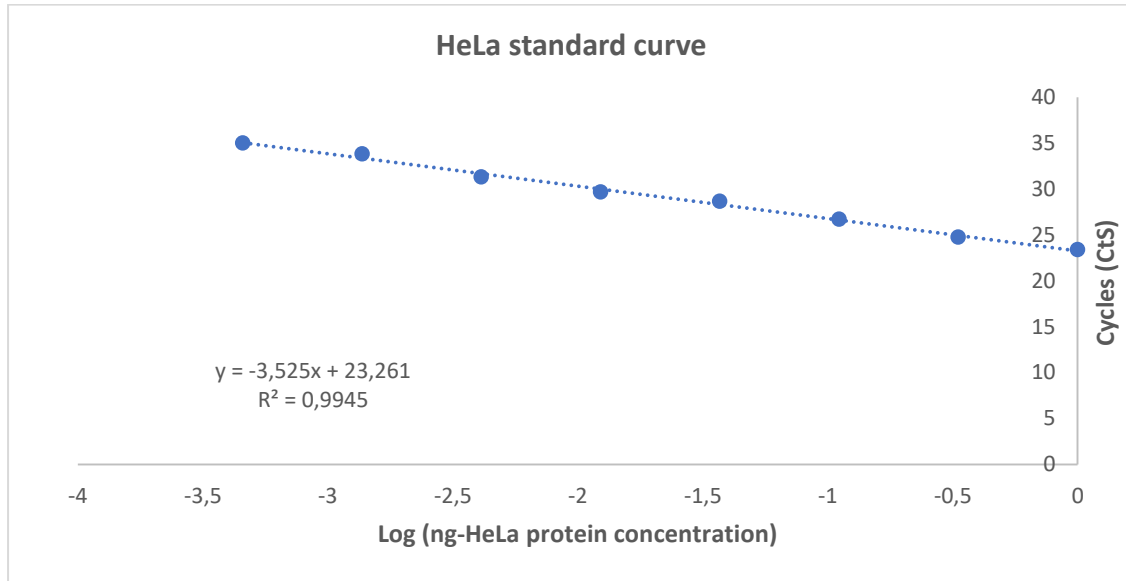
Measurements were performed in triplicates to calculate coefficients of variation and mean amplification signals.

Table 11. *RTAs values for all __Enterolactone__ treated samples analyzed*

Telomerase activity (RTA%) Enterolactone								
	0 Hours		24 Hours		48 Hours		72 Hours	
	RTA (%)	SD	RTA (%)	SD	RTA (%)	SD	RTA (%)	SD
Control			135.17	41.98	66.24	8.21	12.67	2.42
O_40			190.19	0.53	50.82	1.00	11.24	0.85
O_20	49.71	4.22	154.35	7.76	47.81	5.53	12.30	1.33
O_10			171.54	3.65	63.65	0.90	13.17	2.33
O_5			143.76	19.19	62.82	0.65	11.68	3.50
O_2.5			161.70	40.18	60.29	4.93	10.17	1.67

Figure 2. *Graphs for Q-TRAP results (RTA) from in vitro treatment with __Enterolactone__.*
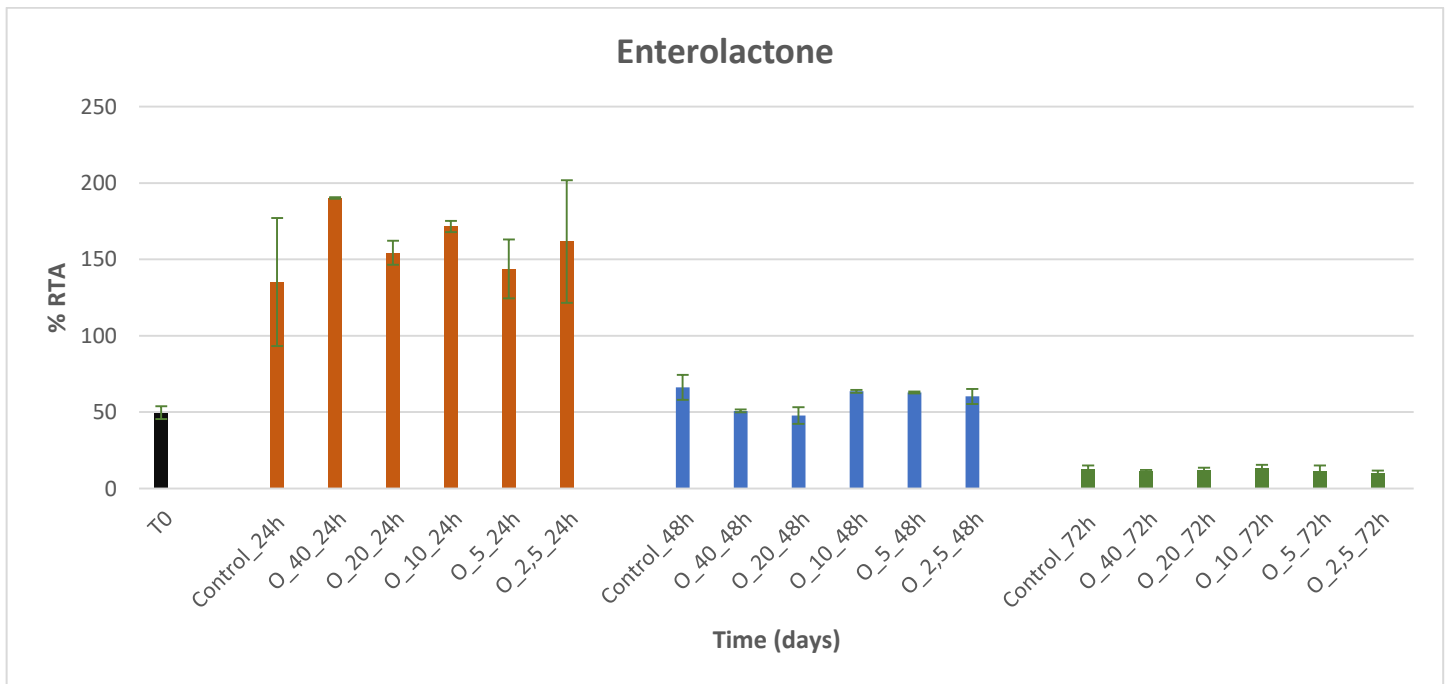
Q-TRAP analysis for MCF7 at different times were compared. Data were grouped by time point of treatment. Statistical analysis for grouped data was applied using t test comparing with control condition.

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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.*
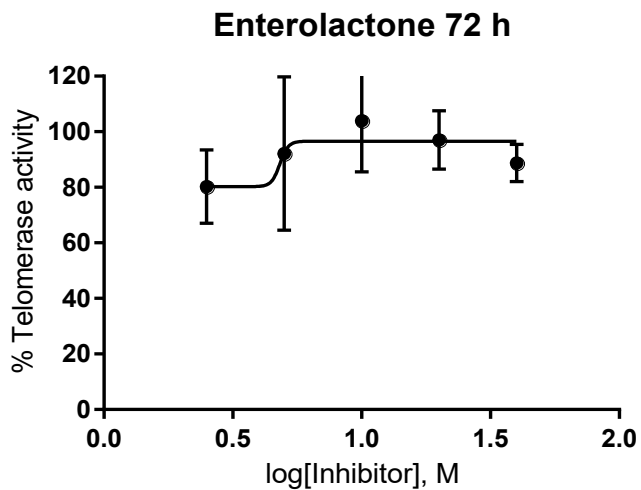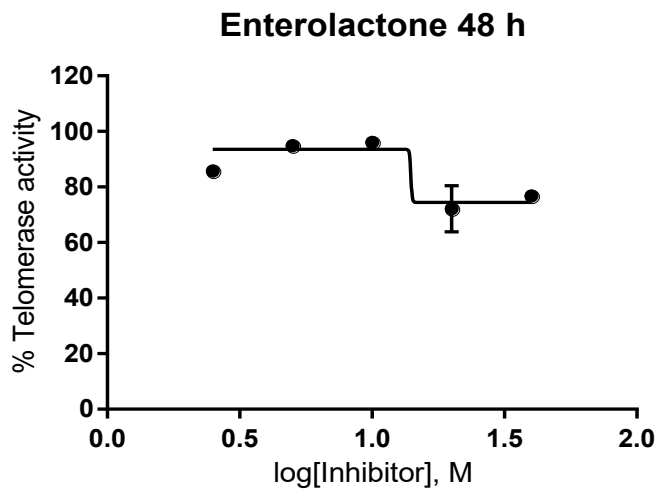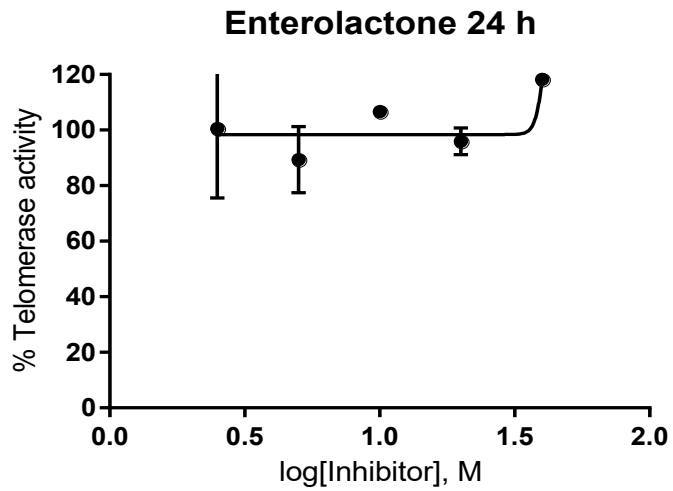
T-Student Analysis – Relative Telomerase Activity Enterolactone		
24 hours	**RTA (p Value)**	**Significance**
Control vs. O_40	0.205	No
Control vs. O_20	0.590	No
Control vs. O_10	0.347	No
Control vs. O_5	0.817	No
Control vs. O_2.5	0.528	No
48 hours	**RTA (p Value)**	**Significance**
Control vs. O_40	0.119	No
Control vs. O_20	0.119	No
Control vs. O_10	0.701	No
Control vs. O_5	0.617	No
Control vs. O_2.5	0.472	No
72 hours	**RTA (p Value)**	**Significance**
Control vs. O_40	0.390	No
Control vs. O_20	0.828	No
Control vs. O_10	0.809	No
Control vs. O_5	0.707	No
Control vs. O_2.5	0.215	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	>40	>40	>40

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).

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Enterolactone 24 h

Enterolactone 48 h

Enterolactone 72 h

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*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			124.84	15.84	61.23	10.44	14.77	3.80
A_40			62.53	3.35	36.82	0.68	0.86	0.76
A_20	49.71	4.22	25.50	15.27	41.20	2.49	5.82	3.45
A_10			40.24	6.05	28.47	3.16	15.87	0.72
A_5			35.54	15.64	29.14	0.43	14.77	4.27
A_2.5			115.47	17.18	49.73	10.70	15.70	5.76

*Figure 4. Graphs for Q-TRAP results (RTA) from in vitro treatment with **Enantiomer A**.*
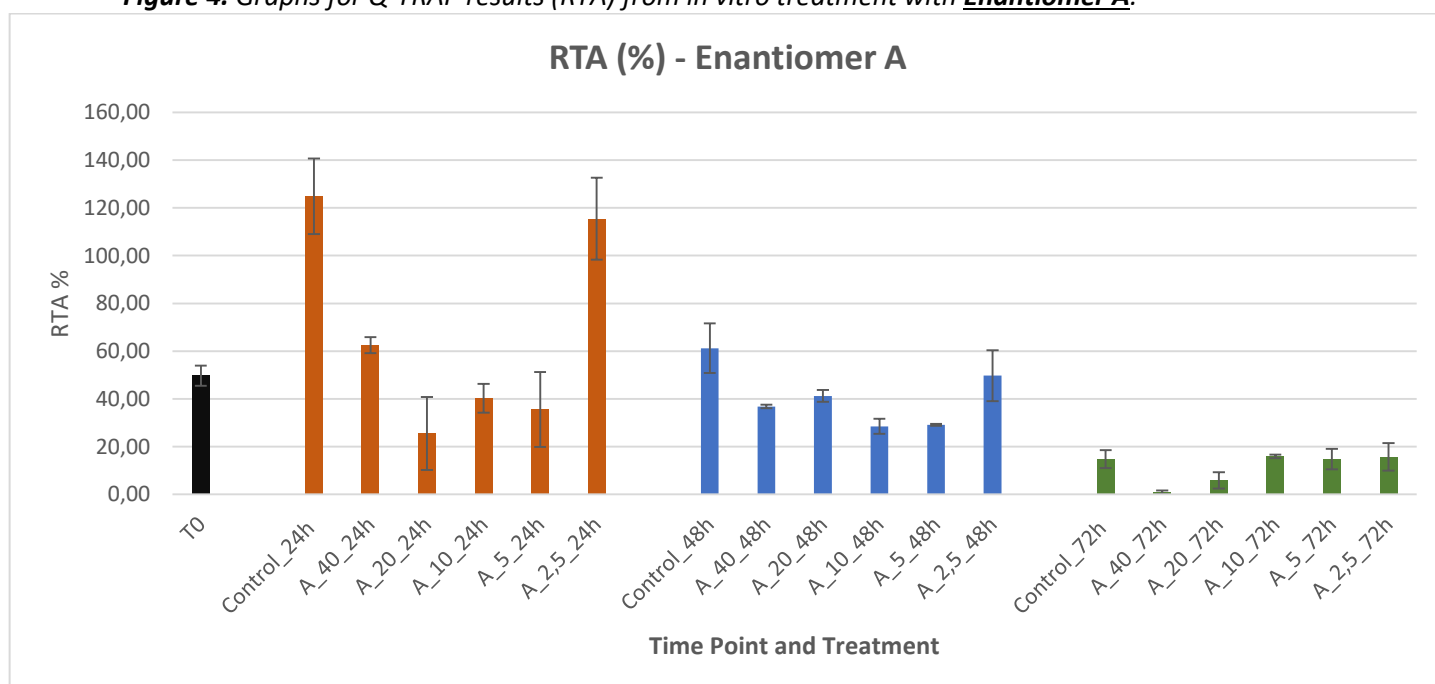
Q-TRAP analysis for MCF7 at different times were compared. Data were grouped by time point of treatment. Statistical analysis for grouped data was applied using t test comparing with control condition.

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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	0.032	YES (*)
Control vs. A_20	0.024	YES (*)
Control vs. A_10	0.019	YES (*)
Control vs. A_5	0.030	YES (*)
Control vs. A_2.5	0.628	NO
48 hours	**RTA (p Value)**	**Significance**
Control vs. A_40	0.052	YES (*)
Control vs. A_20	0.085	NO
Control vs. A_10	0.007	YES (**)
Control vs. A_5	0.026	YES (*)
Control vs. A_2.5	0.317	NO
72 hours	**RTA (p Value)**	**Significance**
Control vs. A_40	0.003	YES (**)
Control vs. A_20	0.076	NO
Control vs. A_10	0.726	NO
Control vs. A_5	0.999	NO
Control vs. A_2.5	0.827	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	~ 2.49	2.23	19.79

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).
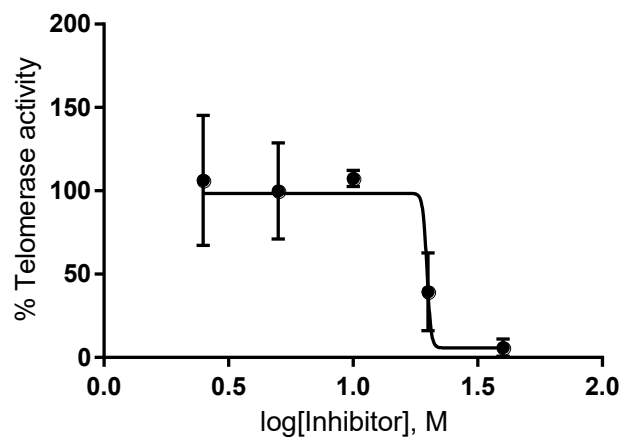
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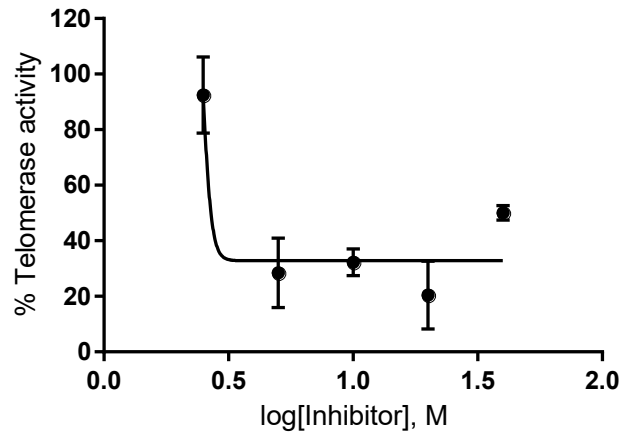
Enantiomer A 24 h

Enantiomer A 48h

Enantiomer A 72h

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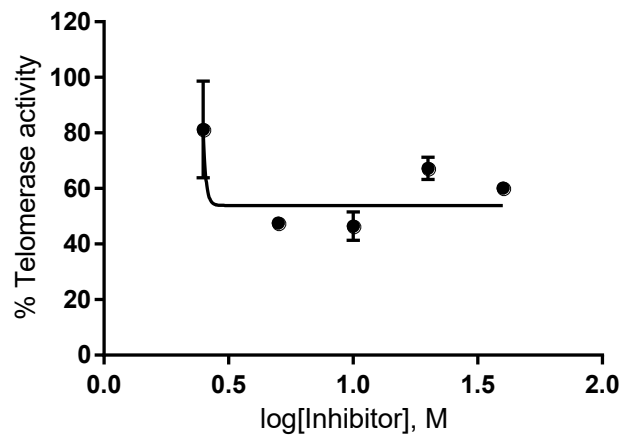
ENANTIOMER B

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			61.47	2.53	61.23	10.44	17.16	0.49
B_40			53.63	7.32	26.82	7.60	10.17	2.75
B_20	49.71	4.22	44.48	7.66	44.82	6.57	16.69	1.45
B_10			59.90	1.49	32.76	2.25	16.26	3.01
B_5			48.30	6.02	31.70	11.45	14.68	3.13
B_2.5			47.79	7.30	23.71	3.41	15.49	0.02

Figure 6. *Graphs for Q-TRAP results (RTA) from in vitro treatment with __Enantiomer B__.*
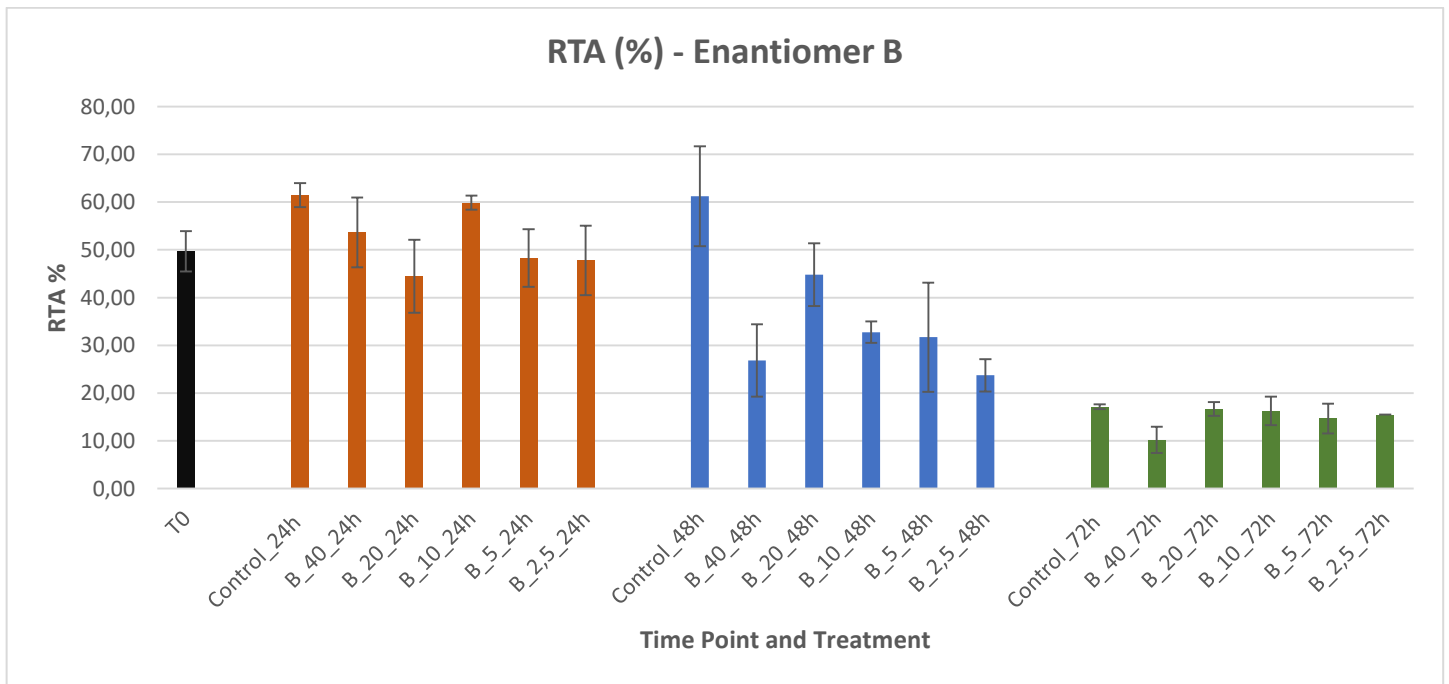
Q-TRAP analysis for MCF7 at different times were compared. Data were grouped by time point of treatment. Statistical analysis for grouped data was applied using t test comparing with control condition.

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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	0.289	YES (*)
Control vs B_20	0.097	YES (*)
Control vs. B_10	0.528	YES (*)
Control vs. B_5	0.104	YES (*)
Control vs. B_2.5	0.129	NO
48 hours	**RTA (p Value)**	**Significance**
Control vs. B_40	0.029	YES (*)
Control vs B_20	0.150	NO
Control vs. B_10	0.036	YES (*)
Control vs. B_5	0.058	NO
Control vs. B_2.5	0.018	YES (*)
72 hours	**RTA (p Value)**	**Significance**
Control vs. B_40	0.072	NO
Control vs B_20	0.710	NO
Control vs. B_10	0.718	NO
Control vs. B_5	0.384	NO
Control vs. B_2.5	0.041	YES (*)

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	>40	5.165	>40

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).
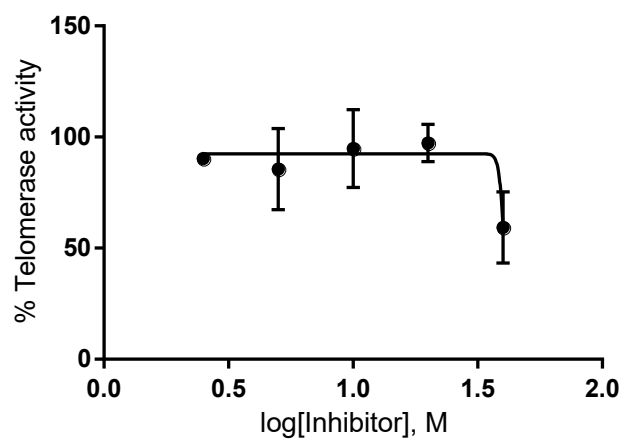
Enantiomer B 24h
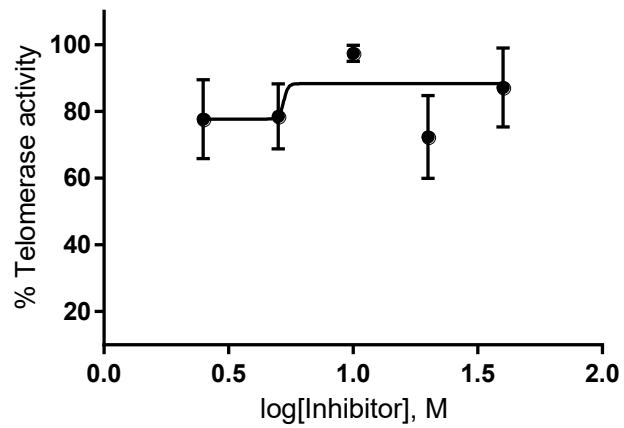
Enantiomer B 48h

Enantiomer B 72h

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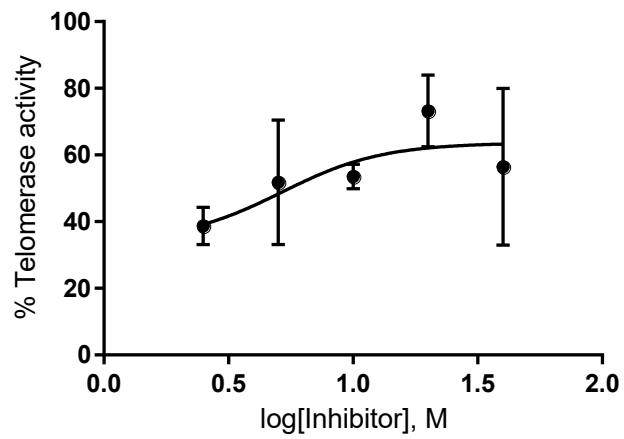
2.3 OBSERVATIONS AND CONCLUSIONS

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 all treated groups showed higher telomerase activity levels compared to control.
- At 48 hours the groups treated with the two highest concentrations showed a small reduction in RTA compared to the control untreated group.
- At 72 hours all RTA returned to basal levels and no particular differences are observed.
- 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 three of the treated groups.
- At 72 hours, although all RTA levels dropped to basal levels it can still be observed that the groups treated with the two highest concentrations presented further lower RTA levels with the difference between the control group and the highest concentration being statistically significant.

Enantiomer B

- At 24 hours, all treated groups showed lower telomerase activity levels compared to control with various differences being significant.
- At 48 hours, all treated groups showed lower telomerase activity levels compared to control, even more pronounced than 24 hours, with various differences being significant.
- At 72 hours, all RTA levels dropped to basal levels. Nevertheless, the group treated with the highest concentration presented lower RTA levels.

Taking into account the data obtained it can be concluded that both Enantiomer A and Enantiomer B presented a telomerase inhibition effect ina a MCF7 cell line.

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3. REFERENCES

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