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CELLULAR AND MOLECULAR BIOLOGY

Cytotoxic effect of combining two antisense oligonucleotides against telomerase rna component (hTR and mRNA of centromere protein B (CENP-B) in hepatocellular carcinoma cells

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Abstract: Telomerase is a ribonucleoprotein enzyme that plays a crucial role in maintaining the malignancy and is responsible for cellular immortality and tumorigenesis. On another hand, Centromere protein B (CENP-B) plays an important role in cell cycle regulation and helping in the high rate proliferation of cancer cells. Our study is designed to evaluate the effect of using combined antisense oligonucleotides (ASOs) targeting (hTR) and mRNA of CENP-B on liver cancer cells. Compared with a single treatment, combination treatment with Locked Nucleic Acid (LNA) ASO (hTR) and (CENP-B) (6.25 nM from each) exhibit the maximum synergistic cytotoxic effect. hTR and CENP-B mRNA was abrogated while hTERT expression was disappeared. Caspase-3, Bax, and Bcl-2 were not detected, indicating caspase-independent cell death. A significant reduction in [Tumor necrosis factor (TNF-α) and Transforming growth factor (TGF-β)] coincides with elevation in Nitric oxide (NO) secretions was observed. Taken together; our data suggest that combination treatment with LNA ASO (hTR) and (CENP-B) could provide a promising strategy for cancer treatment by controlling many pathways concurrently. This might open a new prospective application of antisense in cancer therapy.

Key words: Telomerase, hTR, CENP-B, antisense oligonucleotide.

INTRODUCTION

The telomere is a TTAGGG repeat that protects chromosomes against degradation and end-to-end fusion. Telomerase, a ribonucleoprotein enzyme (Chen et al. 2006), is responsible for the immortality of tumor cells by shielding the telomere (Asghari-Kia et al. 2017). It has been reported that telomerase expression and activation is a crucial step for cancer cells to prevent aging (Ye et al. 2005). Telomerase is made up of three main subunits; the RNA component (hTR) serves as a template for elongation of the telomere, the enzymatic active part [the telomerase reverse transcriptase (hTERT)], and telomerase-associated protein (TP1) (Yatabe et al. 2002). Several studies have shown that although telomerase is found in most cancer cell types, it is not detected in normal cells (Chen et al. 2006). While telomeres shortened with each cell division in normal human cells, they continuously elongated in tumor cells (Yu et al. 2015). Thus, researches focused on telomerase due to its pivotal role to be used as a selective target for new anticancer drugs (Yatabe et al. 2002, Yu et al. 2015).

In normal cell proliferation, microtubules attach to Centromere/Kinetochore in the segregation of paired sister chromatids during cell division (Qianjin et al. 2002, Tan et al. 2014).

Centromere/kinetochore complex is the area in that DNA combines with centromere proteins, which are required to stabilize the intact microtubes in bi-orientation chromosomes (Song et al. 2005, Okada et al. 2007). Centromere protein B (CENP-B) is 80 kD constitutive protein that passes in the inner domain of Centromere/Kinetochore complex by binding with 17 bp CENP-B box in α-satellite DNA (Song et al. 2005). However, CENP-B is highly expressed during cancer cell proliferation, and its down-regulation leads to a reduction in the cancer DNA replication activity and, consequently, cancer cell death (Song et al. 2005), the absence of CENP-B in normal cells does not markedly affect the cell division. CENP-B null mice seemed normal, and its functional centromere/kinetochore complex is maintained (Okada et al. 2007, Tan et al. 2014).

Antisense oligodeoxynucleotides (ASOs) are designed molecules in a sequence-specific earmarked manner to be complemented with targeted nucleic acids (RNA), this hybridization prompts call up and initiates RNase H which degrades RNA/ASO duplex (Yatabe et al. 2002, Chen et al. 2006). Locked Nucleic Acid (LNA) GapmeR is a single-strand effective antisense agent that exhibits a high selective binding affinity to the targeted RNA (Grünweller et al. 2003). It is considered a promising therapeutics with a selective, potent knockdown activity (Kauppinen et al. 2005).

One of the crucial steps in cancer therapy is to develop therapeutic agents capable of silencing cancer-dependent gene(s). Antisense-based strategies against hTR have endeavored in various cancer cells, and it is more effective in blocking telomerase activity than targeting hTERT mRNA (Asghari-Kia et al. 2017). To further improve its response, we explored the combining effect of two Antisense LNA™ GapmeR (ASOs) targeting human telomerase RNA (hTR), and mRNA of the Centromere B protein simultaneously on the liver cancer cell line (HepG2). Anti-tumor and anti-apoptotic activity of combined ASOs as well as changes in cytokine secretion levels and nitric oxide (NO) production was investigated.

MATERIALS AND METHODS

Ethical approval

All experiments were performed according to the principles of the ethics committee of the University of Sadat City. Ethics Approval No: SCU_0034722.

Oligonucleotide design and synthesis

The LNA antisense oligonucleotides (LNA ASO) against hTR with the sequence: 5'-GCTCTAGAATGAACGG- 3' complement to the region between residues 151 and 166, LNA ASO against mRNA of CENP-B with the sequence: 5'-CACGCGGTCATCAATG-3' complement to the region between residues 1893 and 1908 were designed and synthesized by Exiqon A/S, Denmark. Negative control LNA ASO with sequence 5'-AACACGTCTATACGC-3' was run in parallel (Exiqon). They are nucleotides long GapmeR with phosphorothioate backbone modification.

Cell cultures

Hepatic HepG2 cancer cells (American Type Culture Collection,(ATCC) Manassas, VA) were maintained 90% confluent in a complete medium consisting of RPMI-1640 supplemented with L-glutamine (200 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), sodium pyruvate 100 mM (Lonza, Basel, Switzerland), HEPES buffer (1M) and 10% heat-inactivated, mycoplasma and virus-free fetal bovine serum (FBS) at 37°C in a humidified incubator. All tissue culture chemicals purchased from Biowest (Nuaillé, France) except mentioned.

Cell viability assay

The effect of antisense oligonucleotides on tumor cells was evaluated by colorimetric 3,4,5-Dimethylthiazol-2,5-Diphenyltetrazolium bromide (MTT) assay (Sigma Aldrich, St. Louis, MO, USA). Cells were seeded in a 96-well plate to be 6000 cells/well before LNA ASOs treatment. HepG2 cells were treated with hTR LNA ASO and CENP-B LNA ASO alone and in combination in different doses (6.25nM, 12.5nM, and 25nM) using EzWay™ Transfection Reagent (Koma Biotech, Seoul, Korea) for 24 and 48 h according to manufacturer's instructions. Cells were cultured in quadrants for each treatment. Negative control LNA ASO was run in parallel. Cell density was measured by its ability to convert tetrazolium salt to insoluble formazan crystals by mitochondrial dehydrogenase, which is solubilized by acidified Isopropanoland the absorbance was measured by ELISA reader (SunriseTM, Tecan Group. Männedorf/Switzerland) at 570 nm (Talaat et al. 2015). The relative cell viability was calculated as follows: (mean absorbance of treated cells/ mean absorbance of untreated control cells) X100.

The synergistic effects and combination index (CI)

To evaluate the interaction between hTR and CENP-B ASOs, the combination index (CI) was calculated using CompuSyn Software (http://www.combosyn.com/) depending on the results of cytotoxicity after 48 hours according to the classic isobologram equation: CI=(D)1/(Dx)1+(D)2/(Dx)2, where (Dx)1 and (Dx)2 indicates the individual dose of hTR ASO and CENP-B ASO; respectively, required to inhibit a given level of viability index. (D)1 and (D)2 are the doses of hTR ASO and CENP-B ASO; respectively, necessary to produce the same effect in combination (Chou 2010).

HepG2 transfection in 6-well plates

Cells were seeded in 6 well culture plates and allowed to grow on the day before transfection with ASOs. Cell confluence reached Ѷ80% after overnight culture. Transfection of HepG2 cells with different doses of LNA ASO (hTR) and LNA ASO (CENP-B) (6.25 nM. 12.5 nM, 25 nM) either alone or in combination was performed according to the instructions of EzwayTM transfection reagent. After 48 h incubation at 37ºC, supernatants were collected and stored at -80°C for the later measurement of cytokine levels, cells were trypsinized, and total RNA was extracted using TRIzolTMreagent (Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

Estimation of transforming growth factor (TGF-β) and tumor necrosis factor (TNF-α)

TGF-β and TNF-α were measured in collected supernatants by Duoset enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc., Minneapolis, MN) according to the manufacturer protocol. Absorbencies were measured at 450 nm using the UV-max ELISA plate reader (SunriseTM). The ELISA reader-controlling software (Magellan, V 7.1) readily processes the digital data of raw absorbance value into a standard curve from which cytokine concentrations of unknown samples can be derived directly. Results are expressed as a pictogram of cytokine per milliliter of the supernatant.

Estimation of nitric oxide (NO)

Evaluation of NO concentration in the collected supernatant was performed using a nitric oxide assay kit (Biodiagnostic Co., Egypt) as described in the kit user guide. The assay depends on the reduction of nitrate by vanadium (III) chloride (VCl3) combined with detection by the acidic Griess reaction, which leads to bright reddish azo dye. Absorbencies were measured at 540 nm

by the microplate ELISA reader (SunriseTM), and the results for Nitrite in samples were referred to nitrate standard and calculated in µmol/L (mean absorbance of sample/mean absorbance of the standard) X 50.

Apoptosis evaluation

Apoptotic gene expression was performed as previously described by (Talaat et al. 2019). In brief, extracted RNA was dissolved in RNase/DNase free water and quantified spectrophotometrically using NanoDrop 2000c spectrophotometer (Thermo Fischer Scientific, USA) and stored at - 80°C degrees. Verso one-step RT-PCR Ready Mix kit (Thermo Fisher Scientific) was used to detect the expression of desired genes; hTR, CENP-B (primers designed by primer-blast), caspase-3 (Fidan-Yaylalı et al. 2016), Bax, and Bcl-2 (Huang et al. 2011). The effect of hTR LNA (ASO) on telomerase activity is also estimated by hTERT gene expression (Kim et al. 2016). Primer sets and PCR cycling conditions were summarized in (Table I). The PCR products were separated on agarose gel containing ethidium bromide solution (Sigma-Aldrich), and visualized by transilluminator Gel Documentation 3UV Benchtop System, and analyzed by DOC-IT (MSA) program (Ultra-Violet Products Ltd., UK). β-actin RNAs are used as the internal control for normalization (Li et al. 2016).

Statistical analysis

All the statistical analyses were performed with SPSS19.0 (SPSS, Inc., Chicago, IL). Cytotoxicity data were conducted in a quadrate and repeated three times and presented as mean (x) with the corresponding relative standard deviation (RSD) [RSD = (SX100) / $\bar{x}$ where S=standard deviation and $\bar{x}$ = mean]. Cytokine data are expressed as mean ± standard error (x ± SE). Differences between groups were analyzed using a one-way analysis of variance of nonparametric data (Kruskal-Wallis test). Mann-Whitney U test was used as a post-hoc test. $P < 0.05$ was considered statistically significant.

Table I. PCR Primers sequence and conditions.

Primer	Sequence (5' → 3')	Product size (bp)	Annealing temperature(°C)	Cycles
β-actin	F:5'-GAGACCTTCAACAACCCAGCC-3' R: 5'-GGATCTTCATGAGGTAGTCAG-3'	205	56	40
hTR*	F:5'-AACCCTAACTGAGAAGGGCG-3' R: 5'-GACTCGCTCCGTTCCTCTTC-3'	342	58	35
hTERT	F:5'-AGAGTGTCTGGAGCAAGTTGC-3' R: 5'-CGTAGTCCATGTTCACAATCG-3'	183	57	40
CENP-B*	F:5'-CCAGTGCCCTACTCTGCATT-3' R: 5'-TTCCAAGTGGAGGATGTGGC-3'	238	56	35
Caspase-3	F:5'-GCAGCAAACCTCAGGGAAAC-3' R: 5'-TGTCGGCATACTGTTTCAGCA-3'	354	60	40
Bax	F:5'-TCTGACGGCAACTTCAACTG-3' R: 5'-TTGAGGAGTCTCACCCAACC-3'	188	58	35
Bcl-2	F:5'-TCCATGTCTTTGGACAACCA-3' R: 5'-CTCCACCAGTGTTCCCATCT-3'	203	58	35

***Primers designed used primer blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).**

RESULTS

Evaluation of cytotoxic properties for LNA ASO against hTR and LNA ASO against CENP-B mRNAs

To assess the potential cytotoxicity of the LNA ASOs, cell viability was determined by MTT. HepG2 cells were treated individually with 6.25 nM, 12.5 nM, and 25 nM LNA ASO (hTR) or LNA ASO (CENP-B) for 24 h and 48 h (Figure 1). Compared to the control groups, reduction in cell proliferation was time and concentration-dependent (Supplementary Material - Table SI). The concentration of LNA ASO (hTR) leading to a 50% reduction in cell viability (IC50) was 38.25 nM and 26.1 nM for LNA ASO (CENP-B) after 24 h. Looking at the cytotoxic effect of LNA ASO (hTR) and LNA ASO (CENP-B) after 48 h, a severe cytotoxic effect was observed leading to less than 50% viability in all concentrations (27.47, 24.58, 18.55, and 41.2, 35.66, 26.51 for hTR and

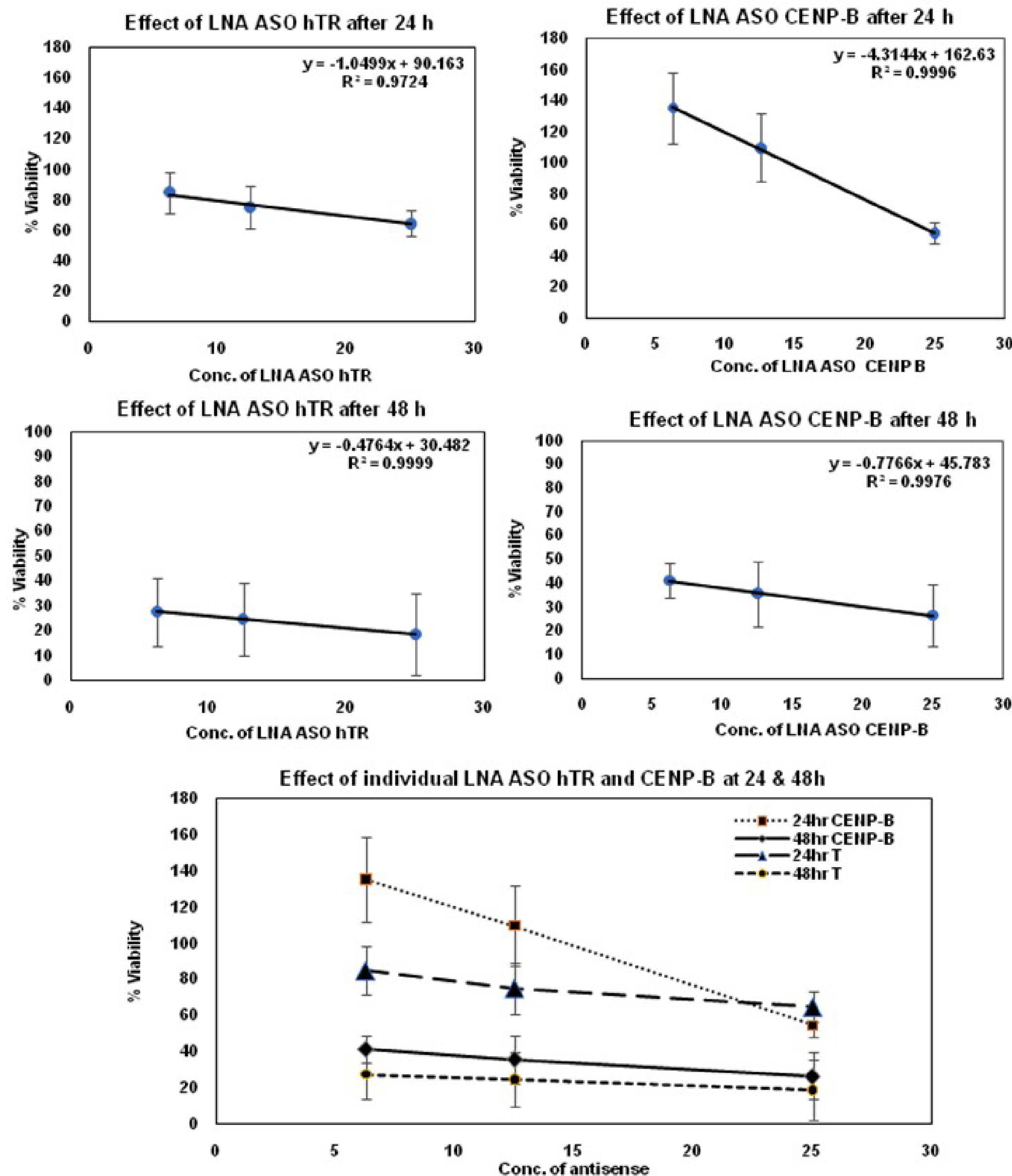

Figure 1. Cytotoxic effect of individual LNA ASO hTR and LNA ASO CENP-B after 24 h and 48 h on cell viability. The experiment was repeated three times.

CENP-B at 6.25, 12.5, 25 nM; respectively). On the other hand, it appears that LNA ASO (hTR) has a stronger antiproliferative effect than LNA ASO (CENP-B) in all-time intervals (24 and 48 h).

The combination of LNA ASO (hTR) and LNA ASO (CENP-B) examined for their cytotoxic activity on HepG2 cells (Table SII). We found that LNA ASO CENP-B could potentiate the anti-cancer property of LNA ASO (hTR) in some concentrations. As shown in (Figure 2), some combination of LNA ASO (hTR) and LNA ASO (CENP-B) was more effective in inhibiting cell viability as compared to their individual effect. The maximum inhibition in cell viability was estimated at 48 h post-treatment with LNA ASO (hTR) and LNA ASO (CENP-B) at 6.25nM combination from each.

To investigate to what extent LNA ASO (hTR) and LNA ASO (CENP-B) act synergistically, we used Chou T.C. Combination Index by CompuSyn software (Theorem), which calculated the combination index (CI) values. CI < 1, >1, and =1 indicate synergism, antagonism, and additive effect; respectively. The fraction-affect (FA)

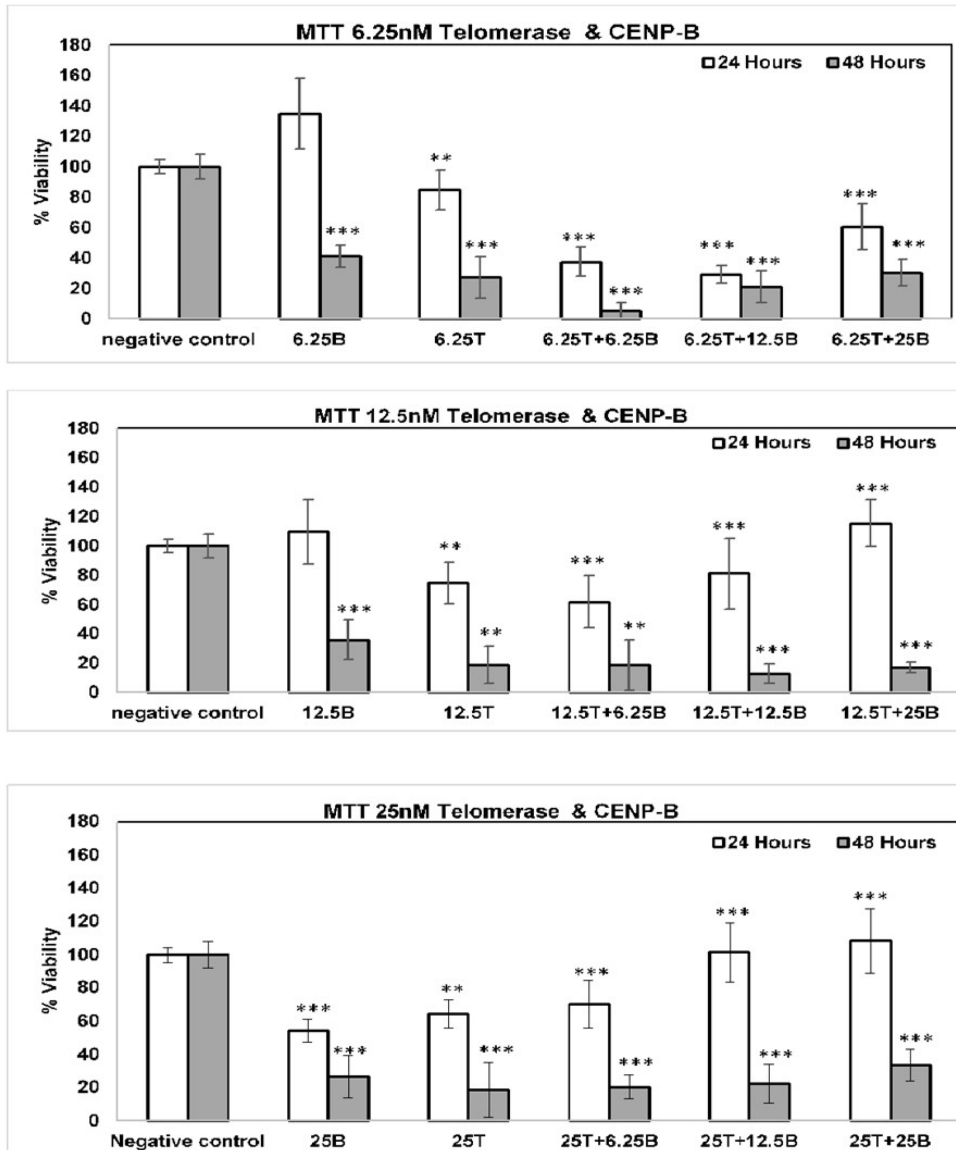

Figure 2. Cytotoxic effects comparison of individual and in-combination LNA antisense oligonucleotides hTR ASO (T) and CENP-B ASO (B). The experiment was repeated three times. Values are given as mean ± RSD. P ≤ 0.05, (**) P ≤ 0.01, and (***) P ≤ 0.001 represent significant changes from negative control.

versus log (CI) analysis revealed that all the concentration points, which are below the line of additive effects exhibited a synergistic anti-proliferative effect (Figure 3a). In agreement with the CI analysis, the isobologram analysis also demonstrated the synergistic cytotoxic effect of LNA ASO (hTR) when combined with LNA ASO (CENP-B) (Figure 3b). The synergistic and antagonist concentrations are shown in (Table SIII).

The effect of LNA ASO (hTR) and ASO LNA (CENP-B) on TGF-β and TNF-α

We assessed TGF-β and TNF-α secretion behavior 48 hours after treatment of HepG2 with LNA ASO (hTR) and LNA ASO (CENP-B) individually or combined with different concentrations. Figure 4 showed a reduction in TGF-β after treating cells with 6.25 nM, 12.5 nM, or 25 nM LNA ASO (hTR) and ASO LNA (CENP-B) either alone or in combination. The reduction was statistically significant for ASO LNA (CENP-B) alone or in combinations with LNA ASO (hTR), except for 6.25 nM LNA ASO (hTR). As compared with untreated cells, a slight, insignificant reduction was observed with all solitary concentrations of LNA ASO (hTR).

No change in TNF-α secretion was observed in 6.25 nM and 12.5 nM LNA ASO (hTR). In contrast, treating cells 25 nM of LNA ASO hTR displayed a significant ($p<0.01$) reduction in TNF-α. In ASO LNA (CENP-B), the elevation of TNF-αdetected followed by a significant reduction in 12.5nM and 25 nM as compared with untreated cancer cells. Considering the dual treatment of HepG2 cells with both antisenses, a reduction in TNF-α notified.

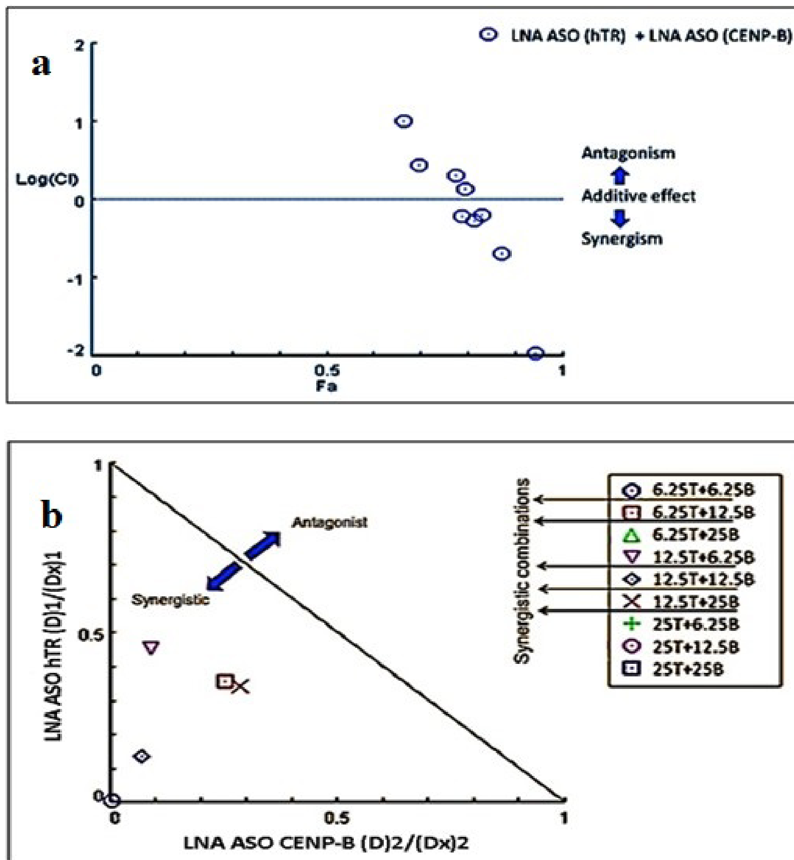

Figure 3. (a) Logarithmic Combination Index Plot and **(b)** Isobologram are for visual determination of synergism or antagonism calculated according to the classic isobologram equation: CI=(D)1/(Dx)1+(D)2/(Dx)2, where (Dx)1 and (Dx)2 indicates the individual dose of hTR ASO (T) and CENP-B ASO (B); respectively, required to inhibit the given level of viability index. (D)1 and (D)2 are the doses of hTR ASO and CENP-B ASO; respectively, necessary to produce the same effect in combination.

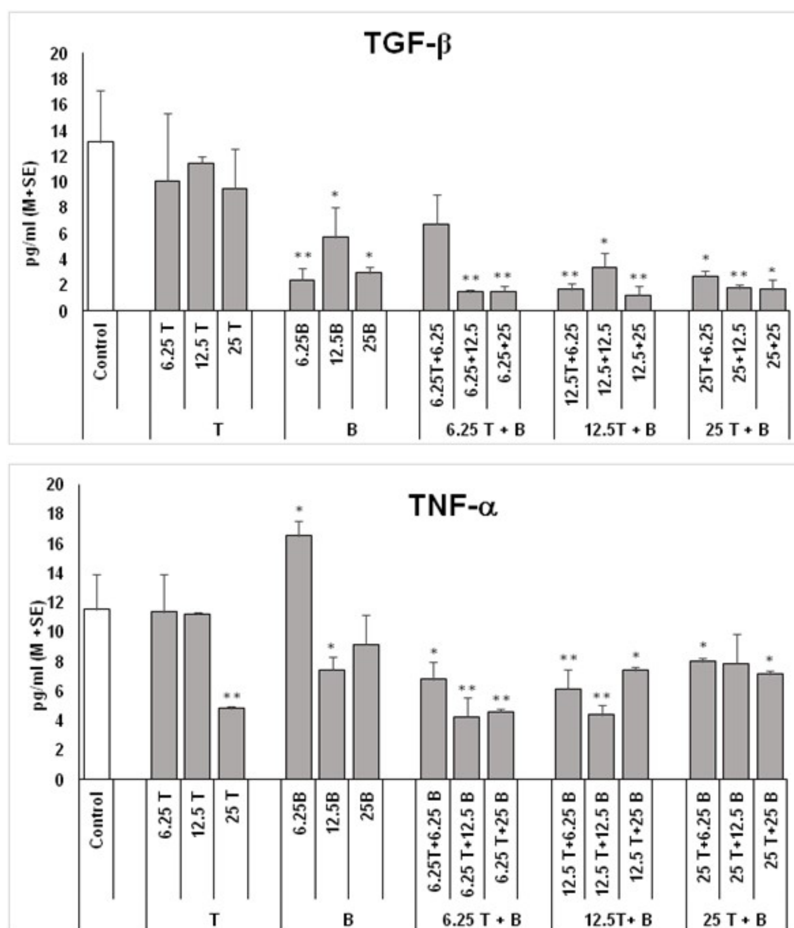

Figure 4. TGF-β and TNF-α expression levels in the supernatant after 48 h from exposure to LNA ASOs. Values are given as mean ± S.E. (*) P≤0.05 and (**) P≤0.01 represents significant changes from the negative control.

Estimation of (NO) secretion

The difference in NO secretion was evaluated in the collected supernatant 48 h after treating cancer cells with LNA-GapmeR antisense oligonucleotide either alone or combined. Our results demonstrated a significant elevation in NO level in all concentrations alone or in combinations excluding 25 nM LNA ASO (hTR) + 25 nM ASO LNA (CENP-B) (Figure 5).

Effect of LNA-GapmeR antisense oligonucleotides on RNAs

To assist the efficacy of LNA-GapmeR antisense oligonucleotides on target RNAs (hTR and mRNA (CENP-B), total RNAs extracted 48 h post-treatment were tested for the presence of hTR and mRNA CENP-B by RT-PCR. Compared with untreated cells, all concentrations of LNA-GapmeR antisense oligonucleotides (6.25 nM, 12 nM, and 25 nM) showed potent degradation for hTR, and mRNA CENP-B. Telomerase activity was measured by detection of the expression level of hTERT gene as a reflection of anticancer activity of LNA ASO (hTR), and ASO LNA (CENP-B) combined or individually. Inhibition of hTERT level after transfection with antisense (solo or combined) comparing to control HepG2 cells was found. Apoptosis evaluated by measuring the expression of Caspase-3, Bax, and Bcl-2. No detected expression for three apoptotic genes, indicating cell death via caspase-3 independent pathway by our LNA-GapmeRs. Antisense data was compared with HepG2 cells treated with the Tamoxifen drug (20μM) as a caspase-dependent apoptotic pathway (Figures S1(a,b,c,d) and S2(a, b, c)).

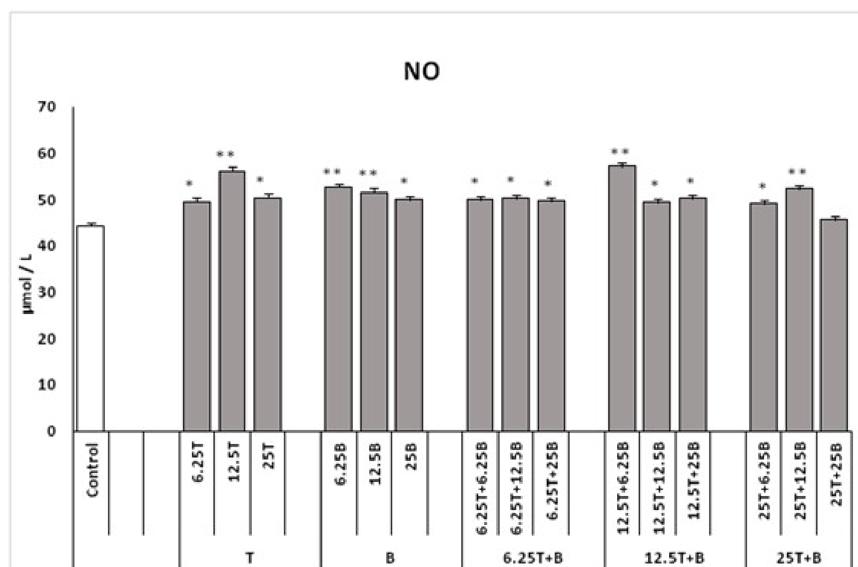

Figure 5. Nitric oxide secretion level in the supernatant after 48 h from exposure to LNA ASOs. Values are given as mean ± S.E. (*) P≤0.05 and (**) P≤0.01 represents significant changes from the negative control.

DISCUSSION

Antisense oligonucleotide treatment shows promising results in clinical trials. The latest accomplishments in the application of antisense oligonucleotides in clinical trials have revolutionized therapeutic methodologies for humans especially for treating cancers which require specific focuses at cell levels (Rinaldi & Wood 2017, Shen & Corey 2018). In this study, we aimed to evaluate the cytotoxic effect of using two LNA™ GapmeR antisense oligonucleotides (ASO) simultaneously targeting (hTR) and mRNA of CENP-B on HepG2 cancer cells.

Antisense LNA GapmeRs with phosphorothioate backbone modification were used as these molecules had the desirable characteristics of binding affinity, specificity, intracellular penetration, and stability compared to different antisense techniques (Grünweller et al. 2003, Kauppinen et al. 2005). In our study, LNA ASO against hTR is used to get the maximum selectivity and efficacy to inhibit telomerase activity. The results obtained from previous studies showed that targeting the hTR is more direct and efficient than targeting hTERT mRNA or hTERT protein (Natarajan et al. 2004, Li et al. 2011). Our data revealed that treating HepG2 cells with LNA ASO (hTR) reduces its survival rate and induces time- and concentration-dependent cytotoxic effects. These results are consistent with several documented studies (Koga et al. 2001, Yatabe et al. 2002, Asghari-Kia et al. 2017). Of note, we have demonstrated that LNA ASO (hTR) completely inhibits telomerase enzyme and diminish hTERT mRNA and hTR effectively.

CENP-B has a significant control effect on kinetochore composition and the centromeres function (Okada et al. 2007, Fachinetti et al. 2015). Interestingly, knockout of CENP-B was reported to reduce the cancer cell proliferation by lowering the progress of the cell cycle, while the cancer cell proliferation indexes back to normal after the effect of antisense CENP-B ended (Qianjin et al. 2002, Song et al. 2005, Tan et al. 2014). In the agreement, we demonstrated that LNA ASO (CENP-B) induced a cytotoxic effect, in time- and dose-dependent, on the viability of HepG2 cells. Notably, LNA ASO (CENP-B) was active in abrogating CENP-B mRNA.

Previous efforts have tried to increase the potency of chemotherapy or radiation by combining it with anti-telomerase agents. They indicated that inhibition of telomerase could potentiate cytotoxicity and decrease the side

effects of chemotherapeutic agents (Berardinelli et al. 2017, Barczak et al. 2018). It is interesting to discover the impact of treating cells with more than one ASOs targeting cancer genes at the same time. Surprisingly, targeting HepG2 cells with both LNA ASO (hTR) and (CENP-B) in combinations exhibited a different cytotoxic effect manner compared to each one individually. A synergistic cytotoxic effect in low concentration combinations has been demonstrated while the impact is reversely antagonist by increasing the concentrations. Also, the cells have undergone caspase-independent apoptosis after treatment with LNA ASO (hTR) or (CENP-B) individually and in combination.

Trying to get an idea about changes in some mediators released by HepG2 in response to ASOs, we have measured TGF-β, TNF-α and NO which play an important role in cancer apoptosis, proliferation, and metastasis (Fukumura et al. 2006, Liu et al. 2017). As increasing the level of TGF-β and TNF-α mediate metastasis, several approaches were trying to block their pathways have been conducted (Sasi et al. 2012, Neuzillet et al. 2015, de Gramont et al. 2017, Wu et al. 2017). We demonstrated that using LNA ASO (hTR) alone has not affected the secretion levels of TGF-β and TNF-α. On the contrary, at high concentration (25 nM), decreased TNF-α level has been observed. Interestingly, the addition of LNA ASO (CENP-B) to LNA ASO (hTR) led to a dramatic reduction in both TGF-β and TNF-α secreted by HepG2 making this combination a promising approach to target cancer cells.

Of particular interest, we found that combining both ASOs [LNA ASO (CENP-B) and LNA ASO (hTR)] elevated the level of NO secreted from HepG2 cells. NO is an important messenger that can regulate the tumor microenvironment through a variety of physiological and cellular pathways such as vasodilatation, cell proliferation, apoptosis, and cell cycle. Increasing NO production may recruit more anticancer drugs via increasing the blood supply to cancer cells (Somasundaram et al. 2018). NO can play two roles either tumor promoter or cancer suppressor (Fukumura et al. 2006). The role of NO as a potent anticancer agent is also documented in some reports (Huerta 2015).

CONCLUSION

Taken together, our results elucidate that a combination of ASOs could induce different patterns of cytotoxic effects than individual ones. LNA ASO (CENP-B) could potentiate the anti-cancer effect of LNA ASO (hTR) in a caspase-independent mechanism. Co-treatment of cancer cells by LNA ASO (CENP-B) and LNA ASO (hTR) has displayed a significant reduction in TGF-β and TNF-α coincides with an elevation of NO. Overall, the outcome data brace our hypothesis that combined LNA ASO (CENP-B) with LNA ASO (hTR) could augment the anti-tumor effect and provide a novel promising strategy for cancer treatment. Further investigations, including in vivo studies, are recommended to improve our results.

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SUPPLEMENTARY MATERIAL

Table SI. Percent viability results after 24 h and 48 h from exposure to LNA ASOs. Values are given as mean ± RSD.

Table SII. Percent viability results after 24 h and 48 h from exposure to combination of LNA ASOs. Values are given as mean ± RSD.

Table SIII. The combination index (CI) was calculated using CompuSyn Software was calculated according to the classic isobologram equation depending on the results of cytotoxicity after 48 hours. CI <1: synergism and, CI>1: antagonism.

Figure S1. RT-PCR for (a) β-actin (housekeeping gene), (b) hTR, (c) CENP-B, (d) hTERT gene.
Figure S2. Expression of Caspase 3(a), Bax (b) and Bcl-2 (c). Antisense data was compared with Tamoxifen drug (20μM).

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This work was carried out in collaboration between all authors: EL-Desoky AM and Talaat RM: designed the research, contributed new reagents/analytic tools; : EL-Desoky AM and Ali YBM: performed the experiment; Talaat RM and EL-Desoky AM analyzed and interpreted the data. All authors wrote, read, and approved the final manuscript and agreed to publish it.