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Research Article
Novel Levothyroxine HIV-TAT Nanoconjugates Suppressing HeLa Cell Lines Growth in Management of Cervical Cancer

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Abstract

Background and Objective: Thyroid hormones play an important role in normal development, growth and metabolism. Thyroid hormones have been linked to the pathophysiology of different cancer types for over a century, the aim of this work is to potentiation the anticancer activity of L-thyroxine (LT) by conjugating with Trans-Activator of Transcription (TAT) peptide against cervical cancer. **Materials and Methods:** To enhance the anticancer activity of L-thyroxine (LT) for ovarian cancer cells, LT was conjugated with TAT then characterized for size, Zeta potential and PDI. Biological evaluation as cytotoxicity, cell cycle analysis, annexin-V and caspase-3 were evaluated. **Results:** The prepared formula was conjugated with HIV-TAT peptide, the prepared formula observed to be in nanometer scale and low PDI size (0.232) with 140.3 nm size. There was a 2.3-fold increase in apoptosis and a 4.3-fold increase in necrosis of the HeLa cells when incubated with the LT-TAT formula when compared to control cells. This increased apoptosis was also associated with increased caspase-3 in treated cells compared to the raw-LT group. Evaluation of cell cycle data showed a substantial arrest of the G2-M phase of the HeLa cells when incubated with LT-TAT NPs. At the same time, a dramatically increased number of pre-G1 cells showed the full apoptotic potential of the LT when administered via the proposed formulation. statistical analysis used in the study was the one-way analysis of variance (ANOVA), followed by the Tukey test. **Conclusion:** The upregulation of caspase-3 established the superiority of the LT-TAT approach to ovarian cancer. In summary, the outcomes achieved LT-TAT efficient delivery tool for controlling the growth of ovarian cancer cells for improved efficacy.

Key words: Hormones, cell-cycle analysis, cationic peptides, apoptosis, HeLa cell lines, ovarian cancer, zeta potential

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nowadays, one of the leading causes of death from cancer in women is cervical cancer. Cervical cancer is considered the fourth most widely diagnosed malignancy worldwide in women. Moreover, it occurs in an approximated 530,000 new incidents with about 270,000 deaths per year[1,2]. Cervical cancer appears in underdeveloped or emerging nations with around 85%. This disease's mortality rate is 18 times higher in countries that have low income and middle income than in affluent countries[3,4]. Different countries have the highest rates of occurrence, including the Caribbean, Central and South America, Southern Asia and Sub Saharan Africa. There were estimated cases (12,990) and deaths (4120) from cervical cancer in the United States in 2016[5-7].

Moreover, the average age of patients at the moment of diagnosis is about 47 years[8]. Patients who have cervical cancer at the early stage is usually treated with a different procedure, including radical hysterectomy and lymph node dissection and/or radiation with or without chemotherapy[9,10]. Furthermore, the suffering patients from local progressed cervical cancer are generally treated with external beam radiotherapy and concomitant cisplatin-based chemotherapy with brachytherapy. Brachytherapy is essential for the treatment of cervical cancer and its effects are inferior when it is replaced by external beam radiotherapy[11,12]. The local management average for women in three years with early and advanced-stage cervical cancer is 87-95 and 74-85%, respectively, with state-of-the-art staging and management. The cervical cancer survival rate (3-f years) in several underdeveloped countries is less than 50% for all stages combined. Local disease development also involves mortality from cervical cancer, resulting in severe suffering, involving fistulas, pain, ureteral obstruction and discomfort. The main aim of this study was to extensively analyze cervical cancer biology, preventive methods, management and advocacy, with a focus on the global effects of these complicated issues[13-20].

Thyroid Hormones (THs) are primarily involved in natural development, growth and metabolism. Over a century of studies, an association between THs and the pathophysiology of different forms of cancer has been confirmed[21-23]. The effect of THs (T3 and T4) on angiogenesis, cancer growth, invasiveness and apoptosis has been shown in *in vitro* experiments and examination in animal models. THs facilitate their influences on cancer cells via many non-genomic ways, such as activation of the plasma membrane receptor integrin αvβ3. Moreover, cancer's growth and progression are impaired by local TH bioavailability dysregulation[24,25].

HIV-1 transactivator of transcription peptide (TAT) is known as a cationic Cell-Penetrating Peptide (CPP)[26,27]. This cell-penetrating peptide has earned some attention over the last years. This kind of protein can combine different compounds with therapeutic potentials, such as small peptides, nucleic acids, liposomes and antibodies nanoparticles. TAT's ability to enter cells successfully helps it to distribute different exogenous compounds efficiently. Also, TAT can reduce cancer cells' growth and it acts as a precious carrier of chemotherapeutic materials. Consequently, the aim of the analysis in this work is to decide if the optimized LT to TAT electrostatic conjugation would increase the antiproliferative action against HeLa cells.

MATERIALS AND METHODS

Study area: This research project was carried out at Nanotechnology and Cell Culture labs at the Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia, from September, 2020-January, 2021.

Materials: The LT was gifted from the Egyptian International Pharmaceutical Industries Company EIPICO (10th of Ramadan City, Cairo, Egypt). TAT protein was acquired from (Chengdu Youngshe Chemical Co., Chengdu, China). HeLa Cell Line human was bought from (Sigma-Aldrich Co., St. Louis, MO, USA). Chemicals were of analytical grade.

Preparation of LT-TAT formulations: The LT-TAT formulation was formulated as mentioned before[28,29]. Total 0.1 M of both LT and TAT was added in a 0.01 M phosphate buffer (20 mL) with pH 7. Subsequently, the preparation was vortexed for dissolution for 2 min. Then, an amount (1 mL) of the prepared complexes was withdrawn and then diluted in a similar buffer that contains 10 mL to determine the particle size and zeta potential.

Particle size and zeta potential determination: In the beginning, the formulated LT-TAT nanoparticles were dispersed in water. After that, the prepared nanoparticles were measured utilizing a particle size analyzer machine (Zetatrac; Microtrac Inc., Montgomeryville, PA, USA) to determine the zeta potential and particle size of the prepared nanoparticles. Three repeated readings, the average particle size and zeta potential were measured[30].

Cell culture: To increase the stock culture, a whole amount of culture medium (10 mL) (RPMI-1640) (Gibco, Waltham, MA, USA) was used. Moreover, glutamine and Fetal Bovine

Serum (FBS) with 10% were placed in the medium[31]. After that, the medium was changed every two days. The cells in the medium were detached by a solution of trypsin-EDTA (ethylenediaminetetraacetic acid) (0.25%) (Gibco, Waltham, MA, USA). Moreover, a sterile 96-well microtiter plate was used to seed the cells. The density of the cells in every well was between 30,000-50,000. Cell incubation was performed at 37°C with 5% CO_2.

Antiproliferative activity: A colorimetric MTT assay was utilized to measure the cell metabolic activity. This compound is non-soluble and violet. In this method, an MTT assay kit (Sigma-Aldrich, St. Louis, MO, USA) was utilized to investigate the antiproliferative activity of the prepared formulations (*in vitro*), which are TAT, LT and LT-TAT, in HeLa cells. A Combosyn (ComboSyn, Inc., Paramus, NJ, USA) was used to determine a Combination Index (CI). When the CI is <1, it suggests that the mixture has a synergistic effect. However, when the CI is equal to 1 or >I, it indicates that the combination has an additive or antagonistic effect, respectively. Furthermore, the IC_{50} values were performed in non-target cells of non-tumorigenic sources for LT, TAT and LT-TAT preparations.

Analysis of cell cycle progression: In this method, a flow cytometer instrument (FACSCalibur, BD Bioscience, Franklin Lakes, NJ, USA) was used to determine the distribution of the cell cycle DNA. This method was described previously. In Brief, six-well cell culture plates were utilized to seed around 3×10^5 cells/well. Then, 0.1 μM LT-TAT concentration was added to the seeded cells for one day with equal amounts of TAT and LT. After that, to analyze the cell cycle, Reagent Kits named CycleTEST PLUS DNA (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) were performed. Then, the DI (DNA index) was determined of each examined formulation in comparison to cells with a fixed DNA content (PBMCs). Propidium Iodide (PI) was performed for staining. Finally, the cell cycle distribution was studied by software, which is CELLQUEST software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Annexin V assay: Translocation or externalization of phosphatidylserine (PS) occurs before the loss of the integrity of the membrane due to cell death stages produced by apoptosis or necrosis. Consequently, annexin V staining is performed using PI dye. It performed to identify cells in the early and late apoptosis stage. This dye is not capable of penetrating the viable cells that have undamaged membranes. However, this agent can penetrate the damaged membranes and dead cells. Consequently, the cells that give negative PI and annexin V stains are considered as viable. Conversely, the positive cells for annexin V and negative cells for PI stains are in early apoptosis, while the cells that are positive for PI and annexin V stains are dead or in late apoptosis. In this method, the assay was conducted utilizing a kit (BioVisionResearch Products, Mountain View, CA, USA) for detection of annexin V-FITC apoptosis.

Assay of caspase 3 enzyme: In the beginning, full growth medium Dulbecco's Modified Eagle's Medium (DMEM) was utilized with FBS (10%) in culturing of HeLa. This method was performed on a 96-well plate for 24 hrs. After that, each plate contains 1.8×10,000 cells/well. After that, the cultured cells were subjected to 0.1 μM LT-TAT, with equal amounts of TAT or LT. Then, the treated cells were lysed using a buffer for cell extraction. After that, a sample (100 μL) was used for assaying caspase 3 from each incubation utilizing a commercial kit (USCN Life Science Inc., Wuhan, Hubei, China). Then, another kit named CASP3 ELISA Kit (USCN Life Science Inc., Wuhan, China) was utilized to verify the content of the cleaved caspase 3. According to the instructions from the manufacture, no interference or cross-reactivity between analogues and caspase 3. At a wavelength of 450 nm, the caspase-substrate reaction, which has occurred in intense colour, was measured by spectrophotometry.

Statistical analysis: In this study, data were produced at three separate replicates. After that, the data are shown as mean±SD. Then, a statistical analysis program was used, which named IBM SPSS statistics version 25 (SPSS Inc., Chicago, IL, USA). Student's t-tests were used to compare the Means (for cellular uptake investigation). Moreover, the one-way analysis of variance (ANOVA), followed by the Tukey test, unless otherwise stated, is a *post hoc* measure. The $p<0.05$ result was found to be significant.

RESULTS

The prepared nanoparticles particle size was found 140.4 nm as represented in Fig. 1. The zeta potential values were found 4.8 mV for all tested formula. Different concentrations of LT, TAT and LT-TAT formulations were used to evaluate their antiproliferative action on HeLa cells.

According to the results illustrated in Fig. 2, the IC_{50} values of the treated groups were for the TAT as 12.65±2.4 μM and LT as 41.66±5.54 μM. But, the conjugated formula of prepared LT-TAT displayed significantly enhanced, proliferation-inhibiting activity (1:1, LT/TAT, respectively) and the IC_{50} value was 8.76±1.25 μM.

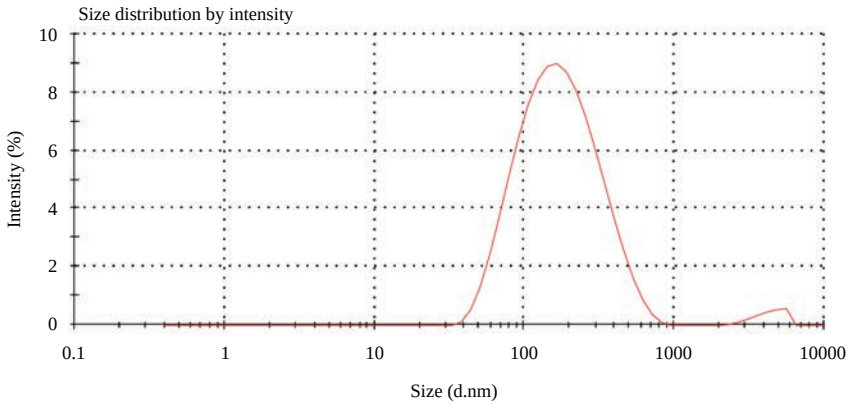

Fig. 1: LT-TAT NPs size distribution intensity

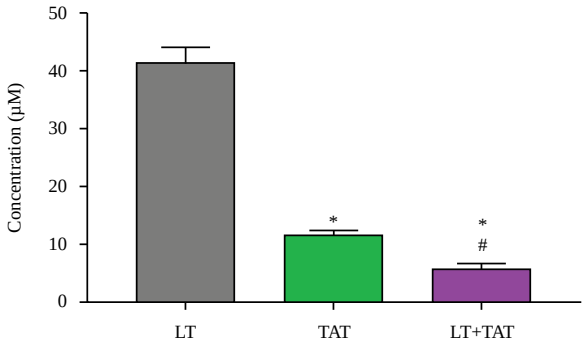

Fig. 2: Cell viability following *in vitro* MTT assay of the cells treated with LT, raw TAT and LT-TAT

Values are expressed as mean±SD (n = 3). *Significantly different from LT at p<0.05. #Significantly different from TAT at p<0.05

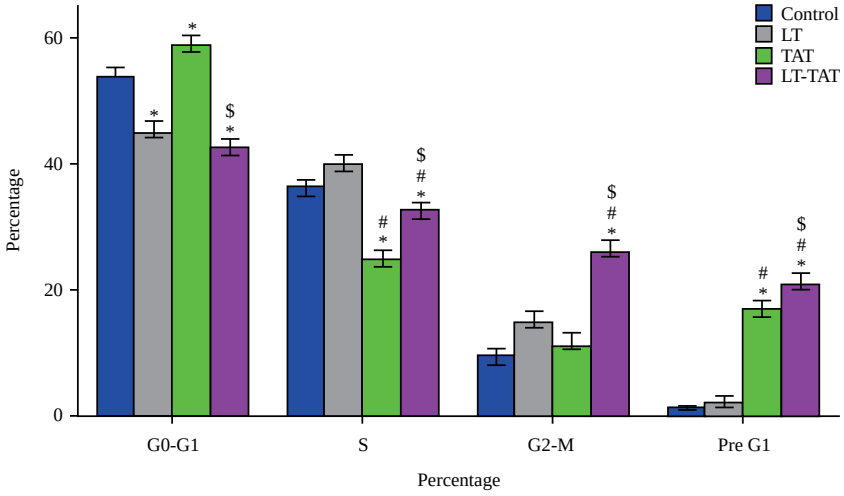

Fig. 3: Cell cycle analysis of LT cells, TAT and LT-TAT in HeLa cell line

Values are expressed as Mean±SD (n = 3). *Significantly different from untreated cells at p<0.05. #Significantly different from LT at p<0.05, $Significantly different from TAT at p<0.05

The results in Fig. 3 showed that the control cells, which is HeLa cells, displayed proliferative properties, with 26.87±2.8% at the S phase, 51.8% at the G0/G1 phase, 11.6±1.2% at the G2-M phase and just about 1.36±0.02% at pre-G1 phase. The incubation in TAT (1 µM), LT (0.1 µM) and LT-TAT (0.1-1 µM, respectively) reduced the proliferation of the

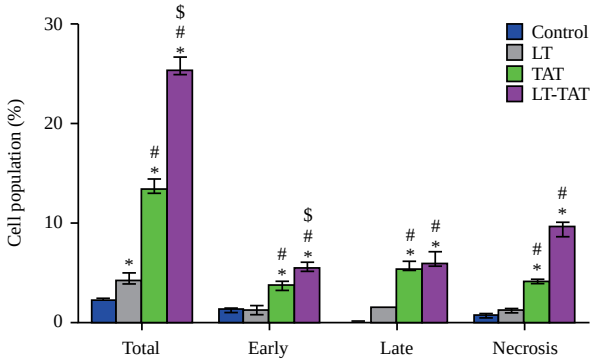

Fig. 4: Apoptotic and necrotic assessment of LT cells, TAT and LT-TAT in HeLa cell line

Cells were exposed to the samples for 24 hrs and stained with Annexin-V/FITC and propidium iodide. Values are expressed as Mean±SD (n = 3). *Significantly different from untreated cells at p<0.05. #Significantly different from LT at p<0.05. $Significantly different from TAT at p<0.05

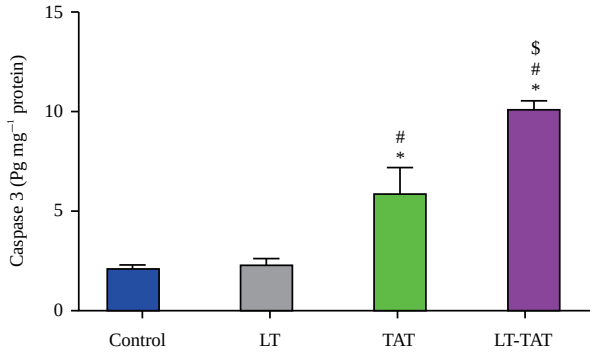

Fig. 5: Caspase 3 expression in HeLa cells after treatment with LT, TAT and LT-TAT

Values are expressed as Mean±SD (n = 3). *Significantly different from untreated cells at p<0.05. #Significantly different from LT at p<0.05, $Significantly different from TAT at p<0.05

utilized cells (HeLa). Also, at the pre-G1 apoptotic, it induced their accumulation phase. For the prepared formulations LT, TAT and LT-TAT, the accumulation amounted to 18.36±0.5,16.12±1.7 and 25.76±2.9%, respectively in the G2-M process, LT-TAT-treated cells showed substantial accumulation. Moreover, In Fig. 3, various cell cycle phases are compared.

In this part, an annexin V test was performed to confirm the observed apoptosis. For the control, the percentage of the stained positive HeLa cells was determined for the formulated groups of the LT, TAT and LT-TAT (Fig. 4). It is clear that compared to the other observed groups, LT-TAT had a noticeable rise in early, late and complete death of a cell.

The results in Fig. 5 represent the evaluation of caspase-3 concentration in HeLa cells. It suggests a significantly elevated amount of the LT-TAT enzyme in comparison with the LT, TAT and control groups' raw materials. The Caspase-3 concentration value was 2.6±0.3, 6.2±2.1, 10.2±1.7 pg mL^{-1} protein for LT, TAT and LT-TAT, respectively.

DISCUSSION

The present study revealed that LT-TAT conjugated nano complexes improved the cytotoxicity of LT significantly in comparing with raw LT, this cytotoxicity in HeLa cells confirmed by cell cycle analysis and annexin-V staining test which revealed accumulation of the tested cells in the G2-M phase. The caspase-3 assay showed an increase in the caspase-3 by 140% in HeLa cells treated with LT-TAT in comparison with raw LT. Proliferation, differentiation, apoptosis and metabolism are all regulated by Thyroid Hormones (TH). TRH induces the pituitary gland to produce and secrete Thyroid-Stimulating Hormone (TSH), which stimulates TH synthesis and secretion in the thyroid gland. Specific iodothyronine deiodinases catalyze the conversion of tetraiodothyronine (T4) to triiodothyronine (T3), the primary hormone synthesized in the thyroid gland. T3 is the primary thyroid hormone that controls metabolic function by forming complexes with nuclear thyroid hormone receptors alpha (TR) and beta (TR)[25,32,33]. Thyroid hormone reaction elements on particular genes are bonded by this nuclear T3-receptor complex, which controls their transcription. Diseases caused by an accumulation of thyroid hormone (hyperthyroidism) or a deficiency of thyroid hormone (hypothyroidism) are normal and have distinct clinical symptoms[23,34].

CPPs have been applied to the intracellular carrier as applicable tools for drug delivery, plasmid DNA and RNA and proteins. Specifically, it has been proven that HIV-1 TAT-derived CPPs are beneficial for delivering different drug molecules types. The main aim of this study is to explore the potential enhancement of HeLa cell death by loading the LT compound in the TAT.

The capacity to translocate numerous therapeutic molecules into living cells was shown by TAT as a CPP. Moreover, more attention has been obtained from the focus on CPPs as an alternative approach to deliver the therapeutic load against several diseases to address drug delivery limitations. As a positively charged peptide, TAT could enhance LT cell translocation through the attraction of cell membranes, which stimulates a receptor-independent pathway through endocytosis mediated by receptors[35-38].

In this work, Important proliferation-inhibiting activity was observed in the LT-TAT formula. A previous study examined the cell-penetrating peptides cytotoxicity for TAT versus after conjugation with Fluvastatin sodium which supported this concept[37]. Our results on the arrest of the G2-M phase by the action of LT is similar to the reported literature[39]. Yet, an increased level of cell cycle arrest in the G2-M phase was associated with the novel formulation developed in this study. Besides, there was a significant increase in the number of cells that were in the pre-G1 phase ($p<0.05$) when compared to cells in the control group or the blank micelle group. The increased numbers of cancerous cells in the pre-G1 phase could be also seen in the existing literature[39]. The effect on the G0-G1 and S phase of the cell cycle by the free drug and drug-loaded micelle is not significant. Interestingly, a characteristic sign of an apoptotic potential is the increased percentage of cells in the pre-G1 phase of the cell cycle[40]. Thus, the induction of apoptosis and the alterations observed in the cell cycle stages could indicate increased cytotoxicity of the LT-TAT formula. This study highlighted the importance of LT in the management of cervical cancer especially after conjugation with TAT. Mechanistic studies and *in vivo* studies are recommended to evaluate the efficacy of LT-TAT nanoconjugates.

CONCLUSION

The conjugated LT-TAT formula was in nanoscale. The conjugated LT-TAT formula significantly enhances its cytotoxic activities against HeLa cells. This is mediated, at least partly, by enhanced apoptosis as evidenced by cell cycle analysis, annexin V staining and determination of caspase 3 which improved cytotoxicity of LT.

SIGNIFICANCE STATEMENT

The importance of nano conjugates as a new system for better delivery and increase the effects of LT on cancer therapy was demonstrated in this study. The results showed improved cytotoxic effects compared to raw LT. This can be helpful for the treatment of cervical cancer as LT is approved as a safe substance and has minimal side effects. The study will illustrate the importance of nanocarriers in cancer therapy by scientists and oncologists.

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