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Research Article
Augmentation of Alendronate Cytotoxicity Against Breast Cancer Cells by Complexation with Trans-activating Regulatory Protein

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Abstract

Background and Objective: Alendronate sodium (ALS) is a member of the class bisphosphonates used to treat osteoporosis. Recent reports suggested that ALS may induce cell death and inhibit the proliferation of estrogen-sensitive MCF-7 breast cancer cells. The aim of this work was the combination of ALS and trans-activating regulatory protein (TAT) loaded nanostructured lipid carriers (NLCs) to potentiate ALS cytotoxicity against MCF-7 breast cancer cells. **Materials and Methods:** The ALS and TAT were prepared and loaded in NLCs. The formed NLCs were characterized for entrapment efficiency (%) (EE %), particle size and zeta potential. Cell viability was also carried out for the prepared NLCs. **Results:** The results revealed that the prepared NLCs were spherical, with particle sizes of 285.2±14.1 nm and polydispersity index of 0.51. Zeta potential of ALS-TAT-NLCs was +21.1±3.4 mV. The ALS EE percentage of the prepared NLCs was 12.2±1.1%. At concentration of 10 μg mL^{-1}, the investigated formulations showed cell survival percentage of 6.86, 20.34, 97.53 and 91.55% for ALS-TAT NLCs, raw ALS, raw TAT and plain NLCs, respectively. The IC_{50} values for the treatments were estimated as follows: ALS-TAT NLCs, 1.98±0.17 μg mL^{-1}, raw ALS, 2.77±0.36 μg mL^{-1}, raw TAT, 1066.67±21.22 μg mL^{-1} and plain NLCs, 10816.2±112.32 μg mL^{-1}. **Conclusion:** The combination of ALS with TAT protein loaded NLCs offered a dual mechanism of ALS activity potentiation and a promising candidate for breast cancer therapy.

Key words: Alendronate sodium, breast cancer, ALS cytotoxicity, nanostructured lipid carriers, nanoparticles, bisphosphonates, cancer therapy, trans-activating regulatory protein

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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

Alendronate sodium (ALS) is a potent inhibitor of farnesyl pyrophosphate synthase. The ALS is a bisphosphonate drug that is used for the treatment of osteoporosis. The ALS is a specific inhibitor of osteoclast mediated bone resorption. The ALS is more effective compared with the first generation biphosphates in reducing bone disposition. The anti-osteoclastic activity mechanism has enabled alendronate to be used as palliative agents for metastatic bone disease, particularly for breast and prostate cancer[1]. It was reported that ALS could be used as a cytotoxic agent via inhibition of tumor growth and migration with synergistic effects dependent on its side chain on ALS chemical structure[2,3]. In addition, some clinical trials showed patients observed the positive effects of coadministration of ALS with chemotherapeutics on the occurrence of bone metastases and cancer recurrence[4,5]. For a long time, researchers try to rationalize the molecular mechanisms of the anti-tumor activity of ALS. It is postulated that the change in the bone microenvironment stemming from ALS that leads to this cytotoxic property[6,7].

Trans-activating regulatory protein (TAT) is a protein encoded in HIV virus which aids viral transduction into cell membrane. Cell penetrating peptide as TAT have the ability to solve drug delivery barriers via the interaction between negative cell membrane and the positively charged catatonic peptide[8,9].

Nanostructured lipid carriers (NLCs) are nanosized carrier systems in which solid particles consisting of both solid and liquid lipids core matrix stabilized in the aqueous phase by surfactants[10,11]. The NLCs were developed to overcome previously developed nano-lipid carrier (e.g., nanoemulsion and liposomes) obstacles and to be better tolerated than polymeric nanoparticles. The NLCs offers improved solubility, enhanced storage stability, improved permeability and bioavailability, reduced adverse effects, prolonged half-life and tissue-targeted delivery compared to the previously developed nano-lipid carriers[12-15].

Therefore; the aim of this study was the potentiation of ALS-NLCs cytotoxicity and improving its cellular uptake by the utilization of cell-penetrating peptides TAT that overcome the lipophilic barrier of cell membrane and ALS delivery barriers, hence improving its activity.

MATERIALS AND METHODS

The study of the manuscript was carried out during the period October, 2017-September, 2018 in the Nanotechnology Laboratory, Department of Pharmaceutics, Faculty of Pharmacy, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia.

The ALS was obtained as kind gift from EIPICO. Pharmaceuticals (10th of Ramadan city, Egypt). HIV-1 TAT protein was purchased from Chengdu Youngshe Chemical Co., Ltd., (Mainland, China). Compritol ATO and Gelucire® 44/14 were a kind gift from Gattefossé (Saint Priest, Lyon, France). The L-alpha-phosphatidylcholine, almond oil, ethyl acetate and chloroform were purchased from Sigma-Aldrich (St. Louis, MI, USA).

Preparation of ALS-TAT NLCs: The hot emulsification-ultrasonication method was used to prepare ALS-TAT NLCs according to the method used by El-Helw and Fahmy[16]. Briefly, ALS (10 mg), almond oil (1%), compritol ATO (3%) and L-alpha-phosphatidylcholine (0.5%) were added to 20 mL of ethyl acetate and chloroform (1:1) mixture which were then heated together at 50°C. The organic solvent was evaporated at 40°C using a rotatory evaporator (Büchi rotavapor, Büchi Laboratoriums-Technik, Flawil, Switzerland). Gelucire® 44/14 (1%) and TAT (100 mg) were dissolved in warm water (20 mL), which was then poured to re-constitute the lipid layer. The mixture was ultrasonicated by probe sonicator (Sonics VC750, Newtown, CT, USA) for 5 min. Finally, the formulated ALS-TAT NLCs were ultra-centrifugated using the Hitachi model E ultracentrifuge (Hitachi, Ltd., Tokyo) at 100,000 rpm for 30 min.

Particle size and zeta potential analysis: The mean particle size and zeta potential of the prepared ALS-TAT-NLCs were investigated using a Malvern ZPS (Malvern Instruments, Malvern, UK).

Study of ALS-TAT NLCs using transmission electron microscopy: The prepared ALS-TAT-NLCs were investigated using transmission electron microscope (TEM) after loading, drying and staining with uranyl acetate solution.

Entrapment efficiency percentage (EE %) determination: The ALS entrapped in NLCs was quantified indirectly. The ALS-TAT NLCs were ultra-centrifugated at 90,000 rpm for 45 min using a Thermo Scientific™ Sorvall™ (Pittsburgh, PA, USA). The supernatant was diluted and analyzed by a high-performance liquid chromatography (HPLC, Agilent 1200 series, Germany) system using Al Deeb *et al.*[17] method after modification. The EE% was calculated from the following equation:

$$EE\ (\%) = \frac{\text{Initial amount of drug added} - \text{Free drug}}{\text{Initial amount of drug added}} \times 100$$

Stability of ALS-TAT NLCs: Particle size, zeta potential and EE% of ALS-TAT NLCs were measured after three cycles of free and thaw (-20°C and +25°C).

ALS-TAT NLCs diffusion study: The study was carried out using a Franz diffusion cell apparatus (Microette Plus™, Hanson Research, Chatsworth, USA). Samples of the raw ALS and ALS-TAT NLCs were placed in the donor chamber to pass through a dialysis membrane with cutoff molecular weight of 12 kDa. Phosphate-buffered saline (pH 7.0) was used as a diffusion medium and samples were withdrawn automatically at 1, 2, 4, 6, 8, 12 and 24 h. The ALS diffused was measured by HPLC using the same method described in the above-mentioned EE (%) determination section.

***In vitro* cytotoxicity screening of ALS-TAT NLCs:** The cytotoxicity of ALS-TATNLCs, ALS, TAT and plain formula using the same drug formula concentrations were tested against MCF-7 cells by sulforhodamine B (SRB) assay as previously described by Skehan *et al.*[18] and Vichai and Kirtikara[19]. Briefly, exponentially growing cells were trypsinized by 0.25% Trypsin-EDTA and seeded in 96-well plates at 2000-5000 cells/well. Cells were treated with ALS-TAT NLCs, ALS, TAT and plain formula with concentrations of 0.1-1000 μg mL^{-1} for 72 h and then fixed with TCA (10%) for 2 h at 3°C. Cells were washed with water several times and then exposed to 0.4% SRB solution in the dark at room temperature. Cells were then washed with 1% glacial acetic acid. Tris-HCl was used to dissolve the SRB stained cells after the plates dried overnight and color intensity was measured at 540 nm with an ELISA microplate reader.

Statistical analysis: One-way ANOVA with Dunnett's multiple comparison test used to compare IC_{50} data using GraphPad Prizm software (La Jolla, CA, USA). A $p<0.05$ indicated significant difference of the investigated data.

RESULTS

Preparation and characterization of ALS-TAT NLCs: The prepared ALS-TAT-NLCs particle size was 285.2±14.1 nm, with a polydispersity index of 0.51 and zeta potential of +21.1±3.4 mV as measured by Malvern ZPS nanosizer (Malvern Instruments, Malvern, UK). The ALS EE% of the prepared NLCs was 12.2±1.1%. The TEM image of the prepared ALS-TAT-NLCs revealed discrete spherical vesicles as shown in Fig. 1.

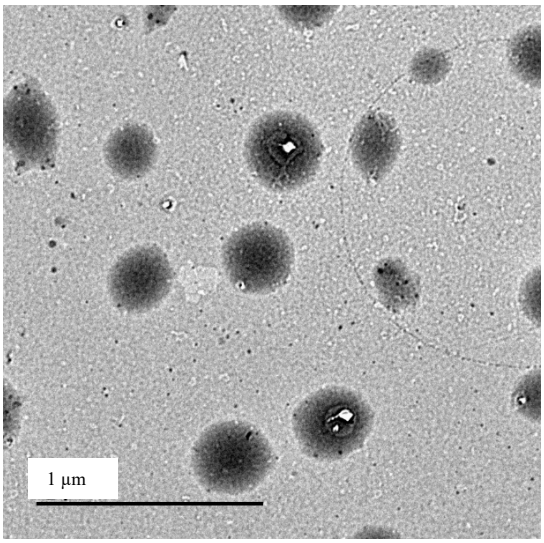

Fig. 1: TEM image of ALS-TAT NLCs

TEM: Transmission electron microscope, ALS: Alendronate sodium, TAT: Trans-activating regulatory protein, NLCs: Nanostructured lipid carriers

ALS-TAT NLCs diffusion study: The results in Fig. 2 shows diffusion profile of raw ALS and ALS-TAT NLCs. Raw ALS shows a fast dissolution rate with in the first hour, which is attributed to the water solubility of ALS. On the other hand, ALS-TAT-NLCs experienced an initial burst effect followed by slow release.

***In vitro* cytotoxicity screening of ALS-TAT NLCs:** Four treatments (ALS, ALS-TAT-NLCs, TAT and plain formula) with concentrations range from 0.1-1000 μg mL^{-1}, for each preparation were applied to breast carcinoma cell line MCF-7. As presented in Fig. 3, all treatments reduced cell viability in a dose-dependent manner. At concentration of 10 μg mL^{-1}, the investigated formulations showed cell survival percentage of 6.86, 20.34, 97.53 and 91.55% for ALS-TAT NLCs, raw ALS, raw TAT and plain NLCs, respectively as shown in Fig. 3. The IC_{50} values for the treatments were estimated as follows: ALS-TAT NLCs, 1.98±0.17 μg mL^{-1}, raw ALS, 2.77±0.36 μg mL^{-1}, raw TAT, 1066.67±21.22 μg mL^{-1} and plain NLCs, 10816.2±112.32 μg mL^{-1} as shown in the Table 1.

DISCUSSION

The results revealed slightly higher particle size than the average size reported in literature that could be attributed to the inclusion of TAT peptide that augment cellular delivery of ALS-NLCs[20,21]. The image in Fig. 1 revealed spherical particles that have regular edges. The biphasic release behavior for ALS-TAT-NLCs could be attributed to the components of NLCs (solid and liquid lipids). The higher melting point (Solid) lipid crystallizes first with the drug that forms lipid core[21]. Most of the liquid lipids are localized at the outer NLCs shell, which is rich in drug, releases the drug in a fast (burst) pattern at the initial stage[21]. Deep ALS entrapped in the NLCs is affected by factors such as the nature of the lipid carrier and formula composition, the pH of the medium and the particles size.

The enhanced cellular cytotoxicity and penetration of ALS delivered with TAT were utilized to enhance the efficacy of the standard ALS dose against MCF-7 cancer cells and attenuate ALS side effects[22]. The TAT peptides up taken by human cells via several pathways that can arise concurrently[23]. The TAT as positively charged molecule, increased inclusion of ALS -NLCs and improved cellular ALS uptake through attracting with the negatively charged cell membrane and triggering a receptor-independent pathway, which result in entrance to the cellular cytoplasm[24,25]. In more details, the interface between TAT and the cellular membrane included the attraction between simple amino acids and negatively charged proteoglycans[26]. In addition, TAT stimulated intracellular signaling cascades that enhanced the organic pathway of the uptake process[27]. Endocytosis is a natural, energy-structured, procedure that could begin with

Table 1: IC_{50} values for all the treatments

Treatments	IC_{50} values (μg mL^{-1})
ALS-TAT NLCs	1.98±0.17
Raw ALS	2.77±0.36
Raw TAT	1066±21.22
Plain NLCs	10816±112.32

ALS: Alendronate sodium, TAT: Trans-activating regulatory protein, NLCs: Nanostructured lipid carriers

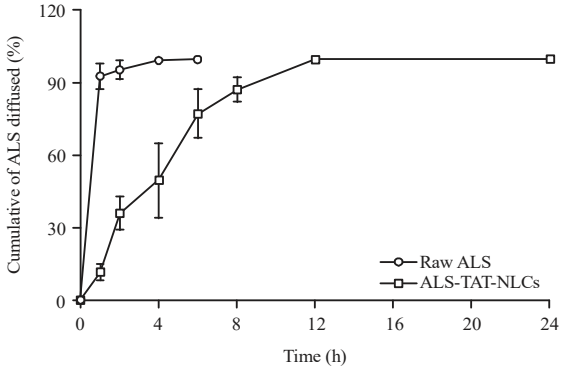

Fig. 2: Release profile of ALS-TAT NLCs and raw ALS

ALS: Alendronate sodium, TAT: Trans-activating regulatory protein, NLCs: Nanostructured lipid carriers

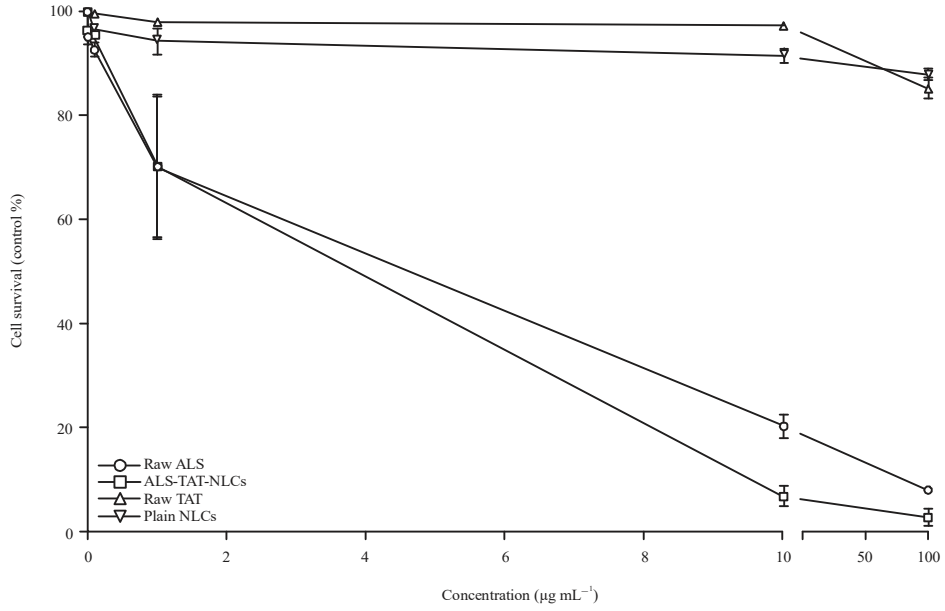

Fig. 3: Cell viability of MCF-7 breast cancer cells for raw ALS, raw TAT, plain NLCs and ALS-TAT NLCs

ALS: Alendronate sodium, TAT: Trans-activating regulatory protein, NLCs: Nanostructured lipid carriers

interactions between positive and negative charges which allocated in proteoglycans on the surface of the cell and the TAT peptide which affected the lipid bilayer stability[28,29]. The TAT penetrated cells using distinctive endocytic pathways and especially pinocytosis along with macropinocytosis, clathrin-mediated endocytosis, caveolae or Q7 lipid-raft-mediated endocytosis and clathrin or caveolae-impartial endocytosis[27]. The choice among endocytic uptake mechanisms depends on peptide properties, NLCs load- features and cell-unique goal characters[30-32]. Transport of ALS is confined with the aid of the extracellular carrier system metabolism, which involved peptide uptake and intracellular degradation in endocytic vesicles[33]. Endosomal escape is important stage to keep away from degradation in lysosomes and permit the TAT-NLCs load (ALS) to reach its biological target[34]. Other reports revealed the role of TAT and NLCs in carrying small agents especially water-soluble drugs[35-37]. Cellular membrane could be considered a physical barrier for its lipid nature, for hydrophilic ALS. So, NLCs play a critical role in modification of ALS nature[38,39]. The medical impacts of the drug loaded NLCs might be related to the internalization and extended contact time of the NLCs by the cancer cells[34].

Combination of NLCs and TAT represented a new way for drug delivery, due to the specific characters of both carriers. The NLCs carried and released the drug in controlled manner, which showed good stability and distribution. This study confirmed the improvement of ALS cytotoxicity which facilitated ALS cellular entrance, higher contact ability to the cellular membrane and delayed ALS release.

CONCLUSION

The study revealed the synergistic effect of ALS and TAT on ALS cytotoxicity against MCF-7 breast cancer cells. Moreover, particle size showed an impact on cellular cytotoxicity. The prepared NLCs achieved a relatively high ALS EE% with a relatively extended release profile. Cell viability confirmed cytotoxicity augmentation. Finally, loading ALS and TAT on NLCs could provide a novel strategy for targeting MCF-7 cells in breast cancer therapy.

SIGNIFICANCE STATEMENT

This study represents a novel combination of ALS and TAT in form of NLCs for the treatment of breast cancer. This combination in nanosized lipid carrier achieved an efficient ALS cellular uptake with efficient cytotoxic effect on MCF-7 breast cancer cells. Our study confirmed the vital role of cationic peptides in targeting cancer cells which open the way for other researchers to evaluate role of other peptides in different cancer cells. Finally loading ALS and TAT on NLCs could provide a novel strategy for targeting MCF-7 cells in breast cancer therapy.

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