RESEARCH ARTICLE

Folate-Targeted Nanostructured Lipid Carriers (NLCs) Enhance (Letrozol) Efficacy in MCF-7 Breast Cancer Cells

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Abstract

Objective: Targeted-drug-delivery based lipid nanoparticles has emerged as a new and effective approach in cancer chemotherapy. Here, we investigated the ability of folate-modified nanostructured lipid carriers (NLCs) to enhance letrozol (LTZ) efficacy in MCF-7 breast cancer cells. **Methods:** New formulations were evaluated regarding to particle size and scanning electron microscope (SEM) features. Anti-proliferative effects of LTZ loaded nanoparticles were examined by MTT assay. To understand molecular mechanisms of apoptosis and cell cycle progression, flow cytometric assays were applied. **Results:** Optimum size of nanoparticles was obtained in mean average of 98 ± 7 nm with a poly dispersity index (PDI) of 0.165. The IC50 value was achieved for LTZ was 2.2 ± 0.2 μM. Folate-NLC-LTZ increased the percentage of apoptotic cells from 24.6% to 42.2% compared LTZ alone ($p<0.05$). Furthermore, LTZ loaded folate targeted NLCs caused marked accumulation of cells in the subG1 phase. **Conclusion:** Taken together, our results concluded that folate targeted LTZ can be considered as potential delivery system which may overcome limitations of clinical application of LTZ and improve drug efficacy in tumor tissue.

Keywords: Apoptosis- Folate- Nano structured lipid carriers- LTZ- LTZ

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Introduction

Side effects of anti-cancer agents to normal cells and also resistance of some patients to response to treatment have remained as the major obstacle in breast cancer chemotherapy (Kuchuk et al., 2013; Bartucci et al., 2015; Wicaksono et al., 2015). Despite substantial progress in new and advanced protocols, it is appears chemotherapy with conventional agents still remained as a one of major line of treatment procedures. So, modification of anti-cancer agents along with novel targeted delivery systems could be considered alternative strategy to diminish side effect of chemotherapy for recent decade(Lu et al., 2013; Amirijavid et al., 2015; Liu et al., 2016). Letrozol (LTZ) is considered constitute of third-generation of Non-steroidal aromatase blockage which uses in treatment of hormone receptor positive breast cancer in postmenopausal women. In contrary to serious side effects of LTZ including, vaginal dryness, hot flashes, irritability, breast tenderness and also un-stability in circulation, LTZ is still applied in clinical. Furthermore, pervious study has reported acquired resistance to LTZ after during treatment cycle in some patients led to significant failure in treatment protocols(Osborne and Schiff, 2011). To enhance therapeutic efficiency and diminish harmful effects of LTZ, some investigations has been reported that perhaps a biodegradable and stable delivery system, can provides a potent and localized dose of LTZ to the tumor position(Nair et al., 2011; Siddiqa et al., 2014). Nanostructured lipid carriers have recently emerged as a multifunctional platform for drug delivery in cancer therapy (Beloqui et al., 2016; Han et al., 2016; Jiang et al., 2016). Many advantages of this delivery systems such as high entrapment efficiency, adequate stability and sustained release of drugs at the interval certain rhythmic reported in pervious study (Awotwe-Otoo et al., 2012; Kazemi et al., 2016).In this study we formulated LTZ in mentioned nanoparticles that modified with folate and evaluated the impact of this formulation in induction of apoptosis and also cell cycle progression in MCF-7 breast cancer cells.

Materials and Methods

Chemicals

Roswell Park Memorial Institute medium (RPMI) 1,640 medium, Poloxamer407, (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), tetra methylene ether) glycol (PTMEG, Mn=2,000 g mol−1) was supplied from Sigma-Aldrich (Germany). Dimethylolpropionic acid (DMPA), triethanolamine (TEA) and phenol were obtained from Merck (Germany). Compritol® ATO 888 was provided from Gattefossè (Saint Periest Cedex, France). Miglyol 812 (caprylic/capric triglycerides) was

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delivered from Sasol (Witten, Germany). The human breast cancer (MCF-7) cell line was purchased from Pasteur Institute Cell Culture Collection (Tehran, Iran). Fetal bovine serum (FBS), dimethyl sulfoxide (DMSO) glutamine, streptomycin, and penicillin G, were obtained from Invitrogen Life Technologies (Auckland, New Zealand). Annexin V-fluorescein isothiocyanate (FITC), propidium iodine (PI) was supported from (E-bioscience, USA). LTZ was donated as gift by IRAN Hormone Pharmaceutical Company.

Preparation of folate targeted LTZ-NLCs as carriers

Modified hot homogenization along with ultra-sonication methods were applied to prepare new formulation of NLCs (Patel et al., 2015; Müller et al., 2016). To reach for this purpose, LTZ was dissolved in oil (Miglyol) and the mixture was added into melted solid lipid (compritol). Then, the hot aqueous surfactant solution (poloxamer 4%) at the same temperature with melted lipids were added drop wise into the lipid phase under homogenization at 20000 rpm for 20 minutes. Then the sample immersed into a sonicated bath and sonicated for 5 min. The formed hot o/w nano-emulsion was cold down in the ambient temperature resulting in the lipid phase re-crystallization, and finally the nano complex was produced.

Characterization of LTZ-NP

The average size distribution of LTZ-loaded NLCs were measured by the dynamic light scattering technique using a particle size analyzer (SALD 2101, Shimadzu, Japan) at room temperature after appropriate dilution with distilled water. To investigate of the morphology of the particles, we were following dilution sample with purified water in ratio of 1:5. Then samples were poured in microscopic lamel. Finally, after evaporation of water, samples were monitored under scanning electron microscope (MV2300, Vega Tescan, Czech Republic).

Cytotoxicity study

The MCF-7 breast cancer cells were rendered in RPMI medium with supplemented 15% FBS, at 37 °C with 5% CO2 in 96-well plates in incubator. The inhibitory effect of void NPs, free LTZ, and LTZ-NLCs on the cell proliferation and its least cytotoxicity were evaluated after 24 h of treatment at concentrations of 0.5 up to 5μM. The cells were substituted with 200 μL fresh media containing 20 μL of MTT solution (2 mg/ml) and incubated for additional 4 h at 37˚C. Finally the absorbance was measured at 570 nm after shaking plates for 15 min by means of ELIZA reader (Biotek, ELX 800, USA)(Tupal et al., 2016).

Determination of apoptotic cells by flow cytometry

The cells (3×10^5) per well were incubated in the medium containing free LTZ, LTZ loaded NLCs and folate-NLC-LTZ in the concentration of 2μM of LTZ at 37°C, for 24 hours. MCF-7 cells were detached, washed twice in PBS, and re-suspended in PBS at room temperature. Finally FITC and PI were added and followed by using flow-cytometry (FACS Calibur flow cytometer; BD Biosciences, San Jose, CA) by the annexin V FITC apoptosis detection kit (E-bioscience, USA)(Sabzichi et al., 2016).

Cell cycle arrest analysis

The cells were seeded at a density of 3×10^5 per well and after treatment with desired concentrations for 24h, the cells were gathered and washed twice with Phosphate Buffer Saline, and then under cold ethanol(70%) at 4°C overnight was fixed. Cells were then exposed with PI and ribonuclease A for 30 min. Cell population in each phase of cell cycle was investigated by flow-cytometer(Fujii et al., 2013).

Statistical analysis

Our results were analyzed as mean ± standard deviation of three independent experiments. $P< 0.05$ was considered as statistically significant analyzed with (T-Test) Graph Pad Prism software V6.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Characterization of LTZ-Loaded NLCs

Particle size analysis displayed almost straitened size distribution of NLC formulations in the range of 60 to 135 nm that confirmed by scanning electron microscope (SEM) (Figure 1a, b). With a poly dispersity index (PDI) of 0.165, indicating a narrow particle size distribution.

Evaluation of LTZ-loaded NLCs on Cell Proliferation

The MTT assay was carried out to justify the influence of LTZ-loaded NLCs in MCF-7 breast cancer cells after 24 h incubation with desired concentrations of LTZ. As shown in Fig. 2a, the IC50 values for LTZ were 2.2 ±0.2 μM. LTZ-loaded NLCs suppressed proliferation of MCF-7cells more effectively than LTZ alone ($p<0.05$). There were no marked differences between the cancer cells incubated with NLCs alone and control cells which demonstrated the biocompatibility and low cytotoxicity of these carriers (Figure 2b).

LTZ loaded NLCs Induced Apoptosis in MCF-7 Cancer Cells

Our finding exposed that no substantial change between the cells treated with carriers alone and control

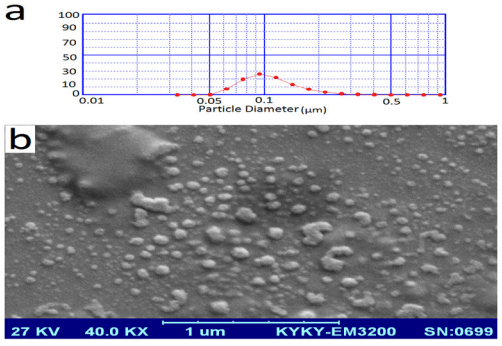

Figure 1. Formulation and Characterization of the LTZ-loaded Nano Lipid Structured Carriers (NLCs). a) Size Distribution, b) Scanning Electron Microscopic Images of LTZ-Loaded NLCs

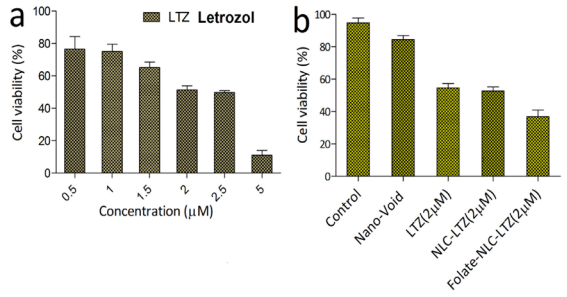

Figure 2. Cytotoxicity Effect of LTZ and LTZ- Loaded Nano Lipid Structured Carriers (NLCs) on Growth of MCF-7 (Breast Cancer Cell Line). IC50 Value Measured for LTZ, with Different Concentrations from 0.5 up to 5 µM (a) and formulation, nano-viod, LTZ and folate-NLC-LTZ associated with 2 µM LTZ (b). The results was calculated as the mean ± standard deviate on (n=3)

cells ($p>0.05$) (Figure 3a, b). LTZ showed cell population in apoptotic phase 24.64% (Figure 3c).Our results demonstrated that folat-LTZ-loaded NLCs increased cell population in apoptotic phase 42 ±3.45 %($p<0.05$) (Figure 3 e).

Apoptosis was associated with Cell Cycle Arrest in Response to LTZ-NLCs

Incubation MCF-7 breast cancer cells with void nanoparticles showed no apoptotic cells which was comparable to control performance (Figure 4a, b). Exposed of the cancer cells with LTZ led to accumulation of cells in subG1 population up to 13 ± 3.2%, (Figure 4 c). Treatment of the cells with LTZ-NLCs for 24 h caused 30 ± 2.8% apoptosis that was accompanied with 34 ± 0.7% G2/M arrest (Figure 4d). Applying folate-LTZ-NLCs enhanced the percentage of cancer cells in sub-G1population up to 26±3.11 (Figure 4 e).

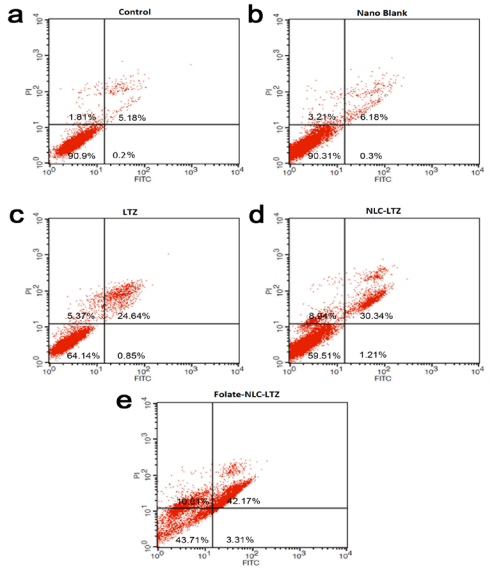

Fig 3. Effects of LTZ-Loaded Folate Targeted Nano Lipid Structured Carriers (NLCs) on the Rates of Apoptosis of MCF-7 (Breast Cancer Cells). a) Untreated group as a negative control, b) Nano lipid structured Carriers (NLCs), c) LTZ (as a positive control), d) LTZ-loaded NLCs, and e) folate-NLC-LTZ

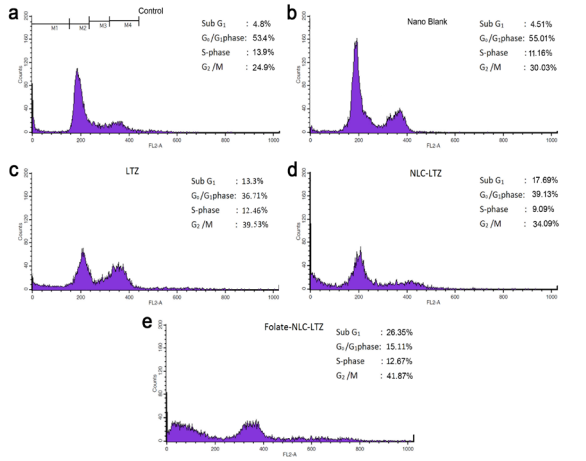

Figure 4. Effects of LTZ-Loaded Folate Targeted Nano Structured lipid Carriers (NLCs) on Cell Cycle Distribution of MCF-7Cells. a) Untreated group as a negative control, b) Nano Structured lipid Carriers (NLCs), c) LTZ (as a positive control), d) LTZ-loaded NLCs, and e) folate-NLC-LTZ

Discussion

One of the significant objectives of cancer exploration is to formulate of the anti-cancer drugs into an effective and safe delivery system that can be selective penetrated in tumor cells with less systemic side effects (Ghanbarzadeh et al., 2014; Rasaie et al., 2014; Amiri et al., 2016). In this attempt, LTZ targeted with folate was formulated in nano structured lipid carriers (NLC) to improve efficacy of LTZ in MCF-7 breast cancer cells. NLC as new generation of lipid based nanoparticles that currently is attracted in cancer therapy protocols(Selvamuthukumar and Velmurugan, 2012; Shao et al., 2015). Hot homogenization technic was applied to reach the final formulation of LTZ. The optimum and ideal nanoparticles was successfully decorated with folate to achieve targeting purpose. The size of nanoparticles demonstrated narrow and uniformity of particles that is essential to entrance of this formulation to cancer cells due to enhanced permeability and retention effect (Prabhakar et al., 2013). To better understand of size and also shape of particles, we monitored the morphology of nanoparticles with scanning electron microscopy. SEM results confirmed size and homogeneity of particles that is provided guaranty of reproducibility of this formulation. Cytotoxicity study was performed to indicate LTZ incorporated in NLC could enhance rate of cell death in cancer cells due to it's equipped with folate ligand. Folate-NLC-LTZ showed highest toxicity compared to NLC and NLC-LTZ in 2µM concentration. We also found NLC-void did not cytotoxicity behavior as alone. Our achievement demonstrated that alteration in dosage form of LTZ can be considered as an alternative compared to conventional systemic methods which normal cell is safe and respect. Furthermore, we analyzed apoptosis and cell cycle arrest of cancer cells with new formulation to understand of molecular mechanism of cell death. LTZ alone revealed intensification in the percentage of apoptotic cells. The population of early apoptotic cells augmented from 5 ± 0.2% to 24 ± 1.5% after 24 h incubation with 2 µM LTZ. Incubation of the MCF-7 breast cancer cells with

LTZ-loaded NLCs for 24 h exhibited a minor growth in the population of late apoptotic cells. Our results confirmed that the effects of folate-LTZ-loaded NLCs on activation of apoptosis are accompanied with changes in the cell cycle progression by generating Sub-G1 arrest. Our outcomes are consistent with the notion that the progression of cell cycle and apoptosis are related with high endocytosis and ligand-receptor profile in response to folate-LTZ formulation.

Targeted cancer therapy currently is designed as an advanced and effective strategy to combat cancer tissue with respect to normal cell. Nano structured lipid carriers improved efficacy of LTZ in MCF-7 breast cancer cells. These nano systems enhanced induction of apoptosis in cancer cell and altered cell cycle progression. In conclusion, this system can be considered as alternative protocol compared to conventional methods. In vivo study need to investigate all clinical dimension of this system to reach clinical trials.

Statement conflict of Interest

The authors announce that there are no conflicts of interest in this project.

Acknowledgments

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