Original Article

The efficiency of Zoledronic acid loaded liposomal nanoparticles on breast cancer: an in vitro study.

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Abstract
Breast cancer as the most common cancer among women annually causes one million new cases in the world. Chemotherapy is considered to be one of the main treatments for the disease. Zoledronic acid (Zol) is a bisphosphonate agent used in the treatment of osteoporosis. However, many studies have recently shown the anticancer potency of the drug. We here encapsulated Zol in liposome nanoparticles (NPs) and evaluated its efficacy on mouse breast cancer cell line 4T1. The size, size distribution and zeta potential of the drug loaded NPs were 157 nm, 0.285 and -43 mV, respectively. Zol entrapment efficiency was also calculated and was found to be 9%. Cytotoxicity evaluation indicated the superior anticancer activity of nanodrug compare to standard drug by 2.5 folds enhancement. Finally, the results of study suggested in vivo evaluation of formulation.

Keywords: Breast cancer, Zoledronic acid, Liposome, Drug delivery, MTT.

1. Introduction
Breast cancer-the most common cancer among women-causes one million new cases diagnosed each year worldwide (Bonilla et al., 2015). Chemotherapy is one of the main treatments for breast cancer; however it comes with some serious side effects, such as nonspecific cytotoxicity, poor aqueous solubility and bioavailability (Parhi & Sahoo, 2015). It has been recently demonstrated that nanotechnology has a great potential for cancer therapy. Cancer nanotechnology is a promising field with extensive applications, and provides a new approach against cancer through target-specific drug therapy (Misra et al., 2010). Liposome is a small nanoscale vesicle which is produced by suspending natural and synthetic lipids in an aqueous environment.

Conjugation of polyethylene glycol (PEG) to the surface of liposomes is a major advancement in the technology of targeted drug delivery approaches (Chandrasekaran & King, 2014; Wang et al., 2012; Xiao et al., 2010). Zoledronic acid (Zol)—a Bisphosphonate agent—is the first line therapy of osteoporosis. It inhibits the osteoclast-mediated bone resorption and increases Bone Mineral Density (BMD) (Black et al., 2015). This compound also inhibits the activity of farnesyl diphosphate synthase (FPP synthase). FPP synthase is needed for the production of isoprenoid lipids requisite for prenylation of small GTPases. In addition, the inhibition of FPP synthase activity indirectly blocks protein prenylation resulting in a loss of osteoclast function and apoptosis (Koto et al., 2010). Many studies have shown the potential of Zol in preventing the growth of various cancerous cells including multiple myeloma (Iguchi et al., 2003), leukemia (Kuroda et al., 2003), prostate...
cancer (Lee et al., 2001) and chondrosarcoma (Gouin et al., 2006). In this study, Zol was loaded on liposome NPs (NPs) by reverse phase evaporation technique, and NPs efficiency was investigated on breast cancer cell line 4T1 in vitro.

2 Materials and Methods

2.1 Materials
Lecithin and cholesterol (Chol) were purchased from Sigma Company (USA). Ethanol 96% and PEG 3350 were obtained from Fanavaran Arvand CO. (Iran) and Kimia Garan Emrooz Chemical Ind. (Iran), respectively. Zol was purchased from Novartis (Novartis, Basilea, Svizzera). 4T1 cell line was prepared from Pasteur Institute of Iran.

2.2 Preparation of NPs
NPs were prepared by reverse phase evaporation technique. To synthesize Zol loaded pegylated liposomal NPs, PEG3350, cholesterol and phosphatidylcholine (1:3:7 molar ratio) were dissolved in 20 ml of ethanol 96%. Then, Phosphate Buffer Saline (PBS) (pH 7.2, 0.1 M) containing Zol (735 µM) was added to viscous gel and stirred (300 rpm, 30 min, room temperature). A control solution was also prepared without the drug. Later, the two formulations were sonicated (Bandelin Sonorex Digits, 60 HZ) for 10 minutes to reduce the size of liposomes.

2.3 NPs characterization
The size, size distribution and zeta potential of NPs were determined by Zetasizer device (Malvern Instruments LTD). The stability of NPs was also evaluated, regarding the size and size distribution of Zol loaded NPs.

2.4 Entrapment efficiency
To estimate drug encapsulation efficiency, 2 ml of nanodrug was centrifuged (21000 rpm, 45 min, 4°C) and the absorbance was measured at 328 nm by Spectrophotometric method (UV-160PC, Shimadzu, Japan), and entrapment efficiency was calculated through following equation.

\[ \text{Encapsulation efficiency} = \frac{\text{Actual amount of drug loaded in NPs}}{- \text{Amount of drug loaded in NPs}} \times 100 \]

2.5 Cytotoxicity assay
To evaluate the cytotoxicity of Zol loaded NPs, 100 µl of 4T1 cells suspension contained 10000 cells was added to the wells of 96-well plates and incubated (37°C, 5% CO₂). After 24 h, the culture medium was removed and substituted the various concentrations of different drug formulations (Standard Zol and Zol loaded NPs), and then incubated at the mentioned conditions for a further 24 h. Later, culture supernatants were removed, and MTT solution (0.5 mg/ml) was added. After 3 h incubation, the amethyst crystal (formazan) was dissolved in 100 µl isopropanol. Then, the absorbance was measured at 570 nm, and Inhibitory Concentration of 50 (IC₅₀) was determined using Pharm software.

2.6 Statistical analysis
The data showed the mean values from triplicate experiments. They were also statistically analyzed by one-way analysis of variance using IBM Statistics SPSS 19, and significant difference was set at p < 0.05.

3 Results
3.1 NPs characterization
The size, size distribution and Zeta potential of drug loaded NPs were found to be 157 nm, 0.285 and -50 mV, and the values for blank NPs were estimated 147 nm, 0.18 and -50 mV, respectively.

3.2 Entrapment efficiency
Entrapment efficiency was calculated to be 9% using spectrophotometric method.

3.3 Cytotoxicity assay
The cytotoxicity of Zol loaded NPs relative to standard drug was determined (Figure 1).

![Figure 1: Viability values based on optical density from Zol loaded liposomal NPs, Zol and blank liposome NPs.](image-url)
As the figure shows cell viability with nanodrug significantly decreases compare to the free drug. The results have presented as mean ± 5% error from three independent experiments. IC50 values were determined to be 18 µM and 45 µM for Zol loaded liposomal NPs and Zol, respectively.

4. Discussion
4.1 NPs characterization
One of the main techniques for preparation of liposome NPs is reverse phase evaporation (Akbarzadeh et al., 2013). Mara et al., used this method to encapsulate Zol into the liposome NPs. They obtained NPs with a size equal to 270 nm (2012). In addition, Porrru et al., encapsulated Zol in liposome NPs via hydration film method, and the size of NPs was about 147 nm (2014). Moreover, the stability test confirmed the appropriate stability of NPs, in which size and size distribution after 9 months were not significantly changed relative to the first day of construction.

4.2 Entrapment efficiency
Based on the materials and methods used for preparation of Zol loaded liposome NPs, various encapsulation efficiencies of Zol were reported in the literatures (Mara et al., 2012; Choi et al., 2013). Mara et al., used EPC/Chol/DSPE-PEG (1:0.32:0.30 weight ratio) in the structure of liposome and obtained encapsulation efficiency of 6% (2012). Choi et al., prepared transferrin conjugated pegylated liposome encapsulating Zol with encapsulation efficiency of 10% (2013). We here obtained encapsulation efficiency of 9% that confirmed the suitability of method for preparation of NPs.

4.3 Cytotoxicity assay
Bisphosphonates (BPs) are potent growth-suppressive of various cancer cell lines in vitro (Caraglia et al., 2006) by the inhibition of farnesyl diphosphate (FPP) synthase which is a key enzyme of mevalonate pathway (De Rosa et al., 2013). However, anticancer activity of BPs in vivo is approximately ineffective, because of their preferential accumulation in the skeleton after intravenously administration (Chen et al., 2002). About Zol, the maximum plasma concentration of drug is approximately 10-100 times less than that needed to exert anticancer activity in vitro (Chen et al., 2002). However, nanomedicine has a potential to increase the extra skeletal bioavailability of drugs and consequently increase their efficacy (De Rosa et al., 2013). Various nanoparticulate platforms were used as Zol carrier to treat breast cancer (Mara et al., 2012; Schillrreff et al., 2014). We here successfully prepared Zol loaded pegylated liposome NPs. Using PEG in the structure of NPs make them stealth and unrecognized by Reticulendothelial system (RES) and increase the bioavailability and possibility of drug delivery to tumor tissues (Wang et al., 2008). The cytotoxicity of Zol loaded NPs relative to standard drug was determined. It was firstly appeared that blank NPs have no cytotoxicity effect higher than the concentration of applied nanodrug. The cytotoxicity results showed the association of Zol with liposome NPs potentiates the anticancer activity of drug. In the presence of nanodrug, IC50 was decreased by 2.5 fold compare to the standard drug (18 µM versus 45 µM).

5 Conclusion
The reverse phase evaporation technique was confirmed as an appropriate method. In the present study, the size, size distribution, Zeta potential, encapsulation efficiency and the cytotoxicity of Zol loaded NPs were evaluated. The results showed that the new formulation has a significant cytotoxicity against cancerous cells. This study recommends evaluating the formulation in vivo environment.

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