

# Repositioning of zoledronic acid for breast cancer using transferrin-conjugated liposome

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**Abstract** Zoledronic acid (ZOL) is one of the most potent nitrogen containing bisphosphonates (N-BP) which are used for cancer-induced skeletal disease by inhibiting osteoclast-mediated bone resorption. It acts by inhibiting the farnesyl diphosphate synthase which is one of several key enzymes in the mevalonate pathway consequently leading to osteoclast apoptosis. From recent studies, direct anti-cancer effects of bisphosphonates, particularly ZOL, was demonstrated in breast cancers. The synergic anti-cancer effects of ZOL treated with other anti-cancer agents in breast cancer cells were also reported. However, pharmacokinetic properties of ZOL have limited its cytotoxic activities because almost 60 % of injected ZOL are disposed into bone and 40 % of the dose cleared rapidly from blood, so that not enough dose of ZOL delivered to the cancer cells. For this reason, we introduced transferrin-conjugated long-circulating liposomes as targeted delivery system for enhancing the anti-cancer effect of ZOL. Transferrin receptors have been explored as a target to deliver therapeutics into cancer cells due to their increased expression on malignant cells. Liposomes were prepared by a reverse-phase evaporation vesicle method with aqueous ZOL solution. Transferrins were attached to the neutral liposomes via free thiols (–SH) to the PDP-end of the pyridylthiopropionylamino-PEG-dis-tearoyl-phosphatidylethanolamine (PDP-PEG-DSPE). Liposomes were characterized by measuring size distribution and zeta potential. The mean diameter of liposomes was 190 nm, and the encapsulation efficiency of ZOL was about

10 %, which was determined by HPLC analysis. Based on the 50 % inhibitory concentration (IC<sub>50</sub>) of ZOL against breast cancer cell MDA-MB-231, increased anti-cancer effect of transferrin-conjugated liposomal ZOL was evaluated compared to free ZOL and non-conjugated liposomal ZOL. In conclusion, transferrin-conjugated liposome can enhance the anti-cancer effect of ZOL and it is suggested that ZOL can be used as an anti-cancer agent with this formulation in breast cancer patients.

**Keywords** Zoledronic acid · Breast cancer · Transferrin · Liposome · Anticancer drugs

## Introduction

Nitrogen containing bisphosphonates (N-BPs) are used for osteoclast-mediated bone loss caused by osteoporosis, metastatic breast cancer, and Paget's disease. N-BPs induce apoptosis of osteoclast, thereby showing anti-resorptive effects in bone (Drake et al. 2008). Recently, direct anti-cancer effects of N-BPs, especially zoledronic acid (ZOL), have been demonstrated in many researches. In these researches, ZOL exerts not only its direct anti-cancer effects such as inhibition of cell growth and induction of apoptosis, but its indirect effects such as inhibition of invasive and angiogenic properties in various cancer cells including breast cancer (Yamada et al. 2009; Aft 2011; Winter et al. 2008; Holen and Coleman 2010; Caraglia et al. 2010), prostate cancer (Clyburn et al. 2010; Lee et al. 2001), melanoma (Forsea et al. 2004), pancreatic cancer (Tassone et al. 2003), and other solid tumors. In various breast cancer cell lines, ZOL induced the apoptosis and inhibited cell growth in a time- and dose-dependent manner, with a 50 % inhibitory concentration (IC<sub>50</sub>) in the

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range of 10–100  $\mu\text{M}$  (Senaratne and Colston 2002; Senaratne et al. 2000). The synergic anti-cancer effects of ZOL treated with other anti-cancer agents such as paclitaxel (Jagdev et al. 2001), doxorubicin (Ottewell et al. 2012), letrozole (Neville-Webbe et al. 2010) in breast cancer cells (Zhao et al. 2012) were also reported. Moreover, there are some clinical evidences of direct anti-cancer effects of ZOL added to neoadjuvant chemotherapy in breast cancer (Coleman et al. 2010). It is already known that adjuvant use of bisphosphonates reduces the rates of recurrence and death in patients with early-stage breast cancer (Coleman et al. 2011).

The ZOL exerts the anti-cancer effect on cancer cells through inhibition of farnesyl pyrophosphate synthase which is the one of key enzymes in mevalonate pathway. Accordingly, ZOL suppresses prenylation of small GTPases including Ras, Rho and Rac that regulate the proliferation, invasive properties and proangiogenic activity of human cancer cells (Goffinet et al. 2006; Luckman et al. 1998).

Even with these potentials of ZOL as an anti-cancer agent, the pharmacokinetic properties of ZOL have made difficult to evaluate its anti-cancer effects in vivo. After i.v. administration, approximately 60 % of the dose were rapidly accumulated within bone matrix, hydroxyapatite, and about 40 % of the dose were eliminated from plasma by kidney within 24 h (Chen et al. 2002; De Luca et al. 2011; Asikoglu and Durak 2009; Weiss et al. 2008). Because of these properties, very low concentrations of ZOL reaches into non-skeleton tissues including tumor tissues, so that ZOL can't exert enough anti-cancer effects. And administration of high ZOL concentrations has the possible risks of side effects such as osteonecrosis of the jaws (ONJ) (Haidar et al. 2009; Mehrotra and Ruggiero 2006). From this consideration, we introduced one of the most studied delivery system, liposome, for enhancing the anti-cancer effects of ZOL and overcoming its non-favorable pharmacokinetic properties.

There are some researches about other bisphosphonates encapsulated in liposomes, including alendronate (N-BPs) and clodronate (BPs) targeting monocytes and macrophages (Epstein-Barash et al. 2010; Zeisberger et al. 2006). However, in these studies, liposome vehicles enhanced the half-life of drugs in plasma but were captured in reticulo-endothelial system (RES) randomly. It is suggested that conventional liposomal formulation is not enough to deliver the ZOL specifically into the tumor tissues.

“Stealth liposomes” (PEGylated liposomes) are the liposomes modified with hydrophilic polymer, polyethylene glycol (PEG), on their surface. They have long-circulating property and increased the half-life in the plasma compared with conventional liposomes (non-PEGylated liposomes). The mechanism of long-circulating effect is the evasion of RES by reducing opsonization affected by

hydrophilicity and steric hindrance effect of PEG (Epstein-Barash et al. 2010; Maruyama 2011). Thereby, stealth liposomes can be accumulated passively in solid tumor tissues much more than normal tissues by enhanced permeability and retention (EPR) effect (Matsumura and Maeda 1986; Maeda 2001). This phenomenon explains the leakage of nano-particles to tumor tissues through leaky vasculature (100–780 nm pore size) and retain in that tissues due to the lack of lymphatic drainage system when the particle size is in range of 90–200 nm (Liu et al. 1992).

On the other hand, enough concentration of ZOL for anti-cancer effects is higher than other conventional anti-cancer agents, for example,  $\text{IC}_{50}$  range of ZOL was  $\mu\text{M}$  range compared with the nM range of other anti-cancer agents like paclitaxel, letrozole (Jagdev et al. 2001; Neville-Webbe et al. 2010). Thus, active targeting strategy is needed for delivery of ZOL into the tumor tissues more selectively and efficiently, in addition to passive targeting by PEGylation.

Transferrin receptor ( $\text{T}_\text{f}\text{R}$ , CD71) is a type II transmembrane glycoprotein (180 kDa) significantly overexpressed in various malignant cells (Faulk et al. 1980; Shindelman et al. 1981) that require large amount of iron for proliferation. Besides, cancer cells with high metastatic properties show higher expression of  $\text{T}_\text{f}\text{R}$  (Inoue et al. 1993). In malignant breast cells,  $\text{T}_\text{f}\text{R}$  expression was detected up to 4–5 times higher than normal breast cells (Walker and Day 1986). And transferrin ( $\text{T}_\text{f}$ ) is a monomeric glycoprotein (76–81 kDa) transporting iron into cells via  $\text{T}_\text{f}\text{R}$  by receptor-mediated endocytosis. In this regard, there have been many studies on targeted delivery of therapeutics into various cancer cells using  $\text{T}_\text{f}$ -modified delivery systems (Hong et al. 2010; Ishida et al. 2001; Li et al. 2009). So, we conjugated  $\text{T}_\text{f}$  to the distal end of the PEG chains of the PEGylated liposomes for targeting the tumors.

In this study, we described the procedures of preparing  $\text{T}_\text{f}$ -conjugated PEGylated liposomes encapsulating ZOL and evaluated their characteristics such as particle size, zeta potential, and drug encapsulation efficiency. After optimizing properties of delivery system, anti-cancer effect of ZOL with this formulation compared to free ZOL was determined in malignant breast cancer cell MDA-MB-231.

## Materials and methods

### Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol (Chol), human transferrin, 4-(*p*-maleimidophenyl) butyric acid *N*-hydroxysuccinimide ester (SMPB), dithiothreitol (DTT), tetrabutylammonium bromide (TBA),

3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), chloroform, Sephadex G-75 were purchased from Sigma Chemical Co. (St. Louis Mo, USA). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy-(polyethylene glycol)-2000] (DSPE-mPEG<sub>2000</sub>), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[PDP-(polyethylene glycol)-2000] ammonium salt (DSPE-PEG<sub>2000</sub>-PDP) were purchased from the Avanti Polar Lipids (Alabaster, AL, USA). Diethyl ether, methanol (HPLC grade), acetonitrile (HPLC grade), dimethylsulfoxide (DMSO) were purchased from Duksan Co., LTD (Ansan, Korea). Zoledronic acid (Zometa<sup>TM</sup>, Novartis, Switzerland) was purchased from Taeyoung Pharm. Co., LTD (Seoul, Korea). MDA-MB-231, human breast cancer cell, was obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea). RPMI 1640 medium (RPMI 1640), fetal bovine Serum (FBS), trypsin-EDTA, penicillin/streptomycin and Dulbecco's phosphate buffered saline (DPBS) were purchased from WelGENE Inc. (Daegu, Korea). BSA protein assay agent was purchased from Bio-Rad (BIO-RAD Lab., CA, USA). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from BD Pharmingen (San Diego, CA). All other reagents were of analytical grade and used without further purification.

#### Preparation of PEGylated liposome

PEGylated liposomes were prepared by the reverse-phase evaporation (REV) method (Szoka and Papahadjopoulos 1978). The lipid mixture composed of DPPC:Chol:DSPE-mPEG2000:DSPE-PEG2000-PDP (6.7:3.3:0.1:0.06 molar ratio) were dissolved in 1 ml of chloroform. The organic solvent was removed by a rotary evaporator (Laborota 4000, Heidolph, Italy) under nitrogen gas flow. The dry lipid film was resuspended in 1.5–2 ml of fresh diethyl ether then 1 ml of ZOL solution was added. The emulsion was sonicated in a bath type sonicator (Branson 2200, USA) for 3 min in cold water. And the resulting one-phase semi-solid gel suspension was evaporated for removing organic solvent. Unencapsulated ZOL was removed by passing the liposome suspension through Sephadex G-75 column. The liposome suspension was downsized by extrusion through 0.2  $\mu$ m cellulose membrane filter (Whatman Int. Ltd., England) 10 times using Lipex<sup>TM</sup> extrusion device (Northern Lipids Inc., Vancouver, Canada).

#### Conjugation of transferrin to PEGylated liposome

The liposome containing 0.6 mol% DSPE-mPEG-PDP was incubated with DTT (25 mM in final solution) for 30 min at room temperature (20–25 °C), then separated from unreacted DTT through Sephadex G-75 column. The transferrin (T<sub>f</sub>) dissolved in DPBS (0.2 mM) was reacted with SMPB

solution (25 mM) dissolved in dimethylformamide to give a 1:20 molar ratio of T<sub>f</sub>:SMPB at room temperature for 1 h, and MPB-T<sub>f</sub> was separated from unreacted SMPB through Sephadex G-75 column. The thiolated liposome, reduced by DTT, and MPB-T<sub>f</sub> were mixed and incubated overnight at room temperature. After incubation, unbound MPB-T<sub>f</sub> was removed using Sephadex G-75 column. In this gel chromatography, 500  $\mu$ l of final liposome was collected in clean plastic tube and each sample was measured absorbance at 280 nm wavelength which is  $\lambda_{\text{max}}$  of proteins.

#### Size distribution and zeta potential analysis

The mean diameter and zeta potential of liposomes were measured using dynamic laser-light scattering system (NICOMP 380ZLS, Inc., Santa Barbara, CA, USA). Each sample was diluted in DPBS and analyzed scattering angle was 90°. All measurements were performed at room temperature.

#### Encapsulation efficiency of zoledronic acid into liposomes

The concentration of ZOL was calculated by high performance liquid chromatography (HPLC). The chromatographic separation was achieved on a CAPCELLPAK C18 UG120 column (150 mm  $\times$  4.6 mm, I.D. 5  $\mu$ m) using a mobile phase composed of 5 mM phosphate buffered saline, methanol, and acetonitrile in ratio of 97:2:1 vol% (pH 7.4). And 7 mM tetrabutylammonium (TBA) bromide was added as a ion-pairing reagent for retention of ZOL in a reverse-phase column (Rao et al. 2005; Jiang et al. 2004). The flow rate of mobile phase was 1 ml/min and the injection volume was 15  $\mu$ l. Separated ZOL was detected at 215 nm wavelength by Waters 486 tunable absorbance detector (Waters, MA, USA). The chromatograms were analyzed by Millennium 32 program (Waters). ZOL encapsulated in the liposome samples was extracted using non-ionic surfactant Triton X-100 which solubilizes the lipid membrane into aqueous phase (Goni et al. 1986; Chern et al. 2006). Briefly, 5  $\mu$ l of Triton X-100 added into the 100  $\mu$ l of prepared liposomes (5 % of liposomal sample in volume ratio). The mixture was mixed for 1 h by vortexing, and centrifuged for 20 min at 14,000 rpm. Finally, lipids in the sample fell down and hydrophilic materials including ZOL were contained in supernatant, so that aqueous supernatant was analyzed by HPLC. The amount of ZOL encapsulated in liposome sample was determined from standard curve of ZOL. And the encapsulation efficiency of ZOL (%) was calculated using the following formula;

Encapsulation efficiency of ZOL (%) = Amount of ZOL in the supernatant from liposome/Initial loading amount of ZOL in preparing process  $\times$  100

## Protein assay

The amount of  $T_f$  attached to the liposomes was calculated by Bradford Protein assay (Bradford 1976). The standard solution was prepared by dissolving  $T_f$  with DPBS, and diluted in the concentration between 0 and 1 mg/ml, which were used in preparing for the standard curve. The 1:80 dilution of  $T_f$ -conjugated liposome was used as a sample. Non-conjugated liposome was used as a control group. To each 800  $\mu$ l sample, 200  $\mu$ l of 1:5 dilution Bradford reagent (Bio-RAD Protein assay reagent, BIO-RAD Lab., CA, USA) was added. The mixture was incubated at room temperature for 15 min. Absorbance was measured at 595 nm using a UV/VIS spectrophotometer.

## Lipid assay

The concentration of phospholipids was measured by the Bartlett's phosphorus assay (Bartlett 1959). The standard solution for phosphorus assay was  $KH_2PO_4$  solution. Standard solution between 0.1 and 0.4  $\mu$ M of phosphorus were prepared by adding 50–200  $\mu$ l of the stock solution which contains 1 mg/ml phosphorus (2.195 g  $KH_2PO_4$  in 500 ml DPBS) in clean borosilicate glass tubes. Sample phospholipids were extracted from liposome by the Bligh and Dyer (1959) method. To each tube of standard and sample, 400  $\mu$ l of 10 N sulfuric acid was added and the tube were heated in a heating block at 165  $^{\circ}C$  for 30 min. After 30 min, the tubes were cooled down, and 30  $\mu$ l of 30 % hydrogen peroxide was added to each tube, which were then heated again at 165  $^{\circ}C$  for 30 min. After further cooling, 4.6 ml of 22 % (w/w) ammonium molybdate solution was added to each tube, followed by 0.2 ml of Fiske–Subba Row reagent with immediate mixing. These tubes were heated in boiling water for 10 min, and after cooling, the absorbance was measured at 830 nm wavelength using a spectrophotometer.

## Number of transferrin attached to a liposome

In order to calculate the number of  $T_f$  attached to each liposome, some assumptions were made;

1. the thickness of lipid bilayer of each liposome was 40  $\text{\AA}$
2. the area occupied by each phospholipid was 70  $\text{\AA}$
3. the size of liposome was 200 nm in diameter

Using these assumptions, the number of liposomes of 200 nm diameter per 1  $\mu$ M lipid was  $1.8 \times 10^{12}$ . And the molecular weight of  $T_f$  is 76–81 kDa, which means each  $T_f$  weight is  $1.26 \times 10^{-19}$ – $1.35 \times 10^{-19}$  g. With these informations, we could calculate the actual number of  $T_f$  on a

liposome from the result of Bradford protein assay and Bertlett's phosphorus assay.

## Cell culture

MDA-MB-231 human breast cancer cells were purchased from the Korea Cell Line Bank (KCLB, Seoul, Korea) and maintained in RPMI 1640 medium with 10 % fetal bovine serum (FBS), 25 mM HEPES, L-glutamine and 100 unit/ml penicillin/streptomycin at 37  $^{\circ}C$  in a 5 %  $CO_2$  incubator (Sanyo, Electric Co. Ltd., Japan). Cells were grown in 25 or 75  $cm^2$  polystyrene tissue culture flask (Nunc, Denmark). The cells were washed with serum free medium, trypsinized and collected from culture flask. The trypsinized cells were centrifuged and cell pellet was resuspended with fresh medium containing FBS, then seeded in culture flask or dishes. The culture medium was replaced every other day with fresh one.

## Inhibition of proliferation in MDA-MB-231 cells

For cell density optimization, MDA-MB-231 cells were plated at various cell densities in 96-well plates. The proper cell density which allowed the cells to grow at the same rate for 3 days was determined by MTT assay. Growth inhibition of the cells by zoledronic acid was also evaluated using the MTT assay. MDA-MB-231 cells (3000 cells/well) were plated in 96-well plate and incubated at 37  $^{\circ}C$ , 5 %  $CO_2$  condition. After 24 h, medium was suctioned out and the cells were treated with DPBS (control) and various formulations of ZOL, such as in free drug, conventional liposomes (Conv-Lipo), and PEGylated liposomes (PEG-Lipo). And the toxicity of liposome itself was evaluated by empty liposomes which is the formulation not encapsulating ZOL. After 48 h incubation, the treatments were suctioned out and MTT solution (1 mg/ml in serum free medium) was added to each well and incubated for additional 4 h in a  $CO_2$  incubator. The MTT containing medium was aspirated off and 100  $\mu$ l of DMSO was added to dissolve the formed crystal formazan. The absorbance was measured at 570 nm wavelength using an ELISA reader (EL800, BIO-TEK, USA). The relative cell viability of drug treated cells was presented in percentage of control (DPBS).

## Apoptosis assay by FACS

The apoptosis of MDA-MB-231 breast cancer cells was analyzed by FACS which detects the externalized phosphatidylserin (PS) by fluorescein isothiocyanate (FITC) conjugated annexin-V and integrity of cellular membrane by propidium iodide (PI). MDA-MB-231 cells ( $1 \times 10^5$  cells/well) were plated in 6-well plates and incubated at 37  $^{\circ}C$ , 5 %  $CO_2$  condition. After 24 h, medium was



removed and the cells were treated with DPBS, empty liposomes, free form of ZOL, conventional liposomal ZOL, PEGylated liposomal ZOL, and T<sub>f</sub>-conjugated PEGylated liposomal ZOL with fresh serum-free medium for 24 h. Then, the cells were washed with DPBS, and trypsinized and collected. After centrifugation, the cell pellets were resuspended to the concentration of  $1 \times 10^6$ /ml in  $1 \times$  binding buffer and stained with 5  $\mu$ l of FITC annexin-V and 5  $\mu$ l of PI using FITC annexin-V apoptosis detection kit (BD Pharmingen™, San Diego, CA). After incubation of cells in the dark at room temperature for 15 min, 100  $\mu$ l of the cells were diluted with 400  $\mu$ l of  $1 \times$  binding buffer according to the manufacturer’s instructions, and measured the fluorescence intensity by dual color FACS analysis using FACSCanto II (BD Biosciences, San Jose, CA). Viable cells with intact cellular membranes excluded PI, while membranes of dead or damaged cells were permeable to PI. For this reason, we can consider both the FITC annexin-V and PI negative cells as viable. On the other hand, FITC annexin-V positive and PI negative cells were considered as early apoptotic; and FITC annexin-V positive and PI positive cells were considered as late apoptotic or necrotic ones.

Statistical analysis

The results were expressed as a mean  $\pm$  standard deviation (SD).

Results

Conjugation of transferrin to the PEGylated liposome

The final transferrin-conjugated liposomal zoledronic acid (T<sub>f</sub>-Lipo-ZOL) collected from gel chromatography was measured using the absorbance at 280 nm (Fig. 1). The

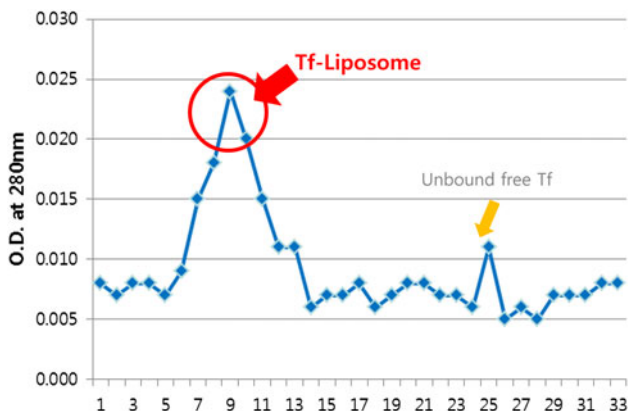


Fig. 1 Separation of the T<sub>f</sub>-Lipo from unbound free T<sub>f</sub> using the Sephadex G-75 column

collection fraction 9 and 10 showed the highest absorbance and were used for the following experiments. The collection fraction 25 was estimated as an unbound T<sub>f</sub>.

Size distribution and zeta potential analysis

The mean diameter of T<sub>f</sub>-Lipo-ZOL was  $189.5 \pm 69.9$  nm (Fig. 2a). And the average zeta potential of the liposomes was  $-0.04$  mV (Fig. 2b). There was no significant changes of size (between 150 and 250 nm) and aggregation or precipitation of liposomal ZOL during the storage at 4 °C environment up to a month (data not shown).

Encapsulation efficiency of zoledronic acid into liposomes

The encapsulation efficiency of ZOL was calculated by HPLC analysis. In this HPLC condition, retention time of ZOL was 2.46 min (Fig. 3). The encapsulation efficiency of ZOL extracted from liposomes was determined from the calibration curve of standard free ZOL solution with the initial loading amount of ZOL. The encapsulation efficiency of ZOL encapsulated in the T<sub>f</sub>-Lipo was about 9.16 %. On the other hands, the encapsulation efficiency of

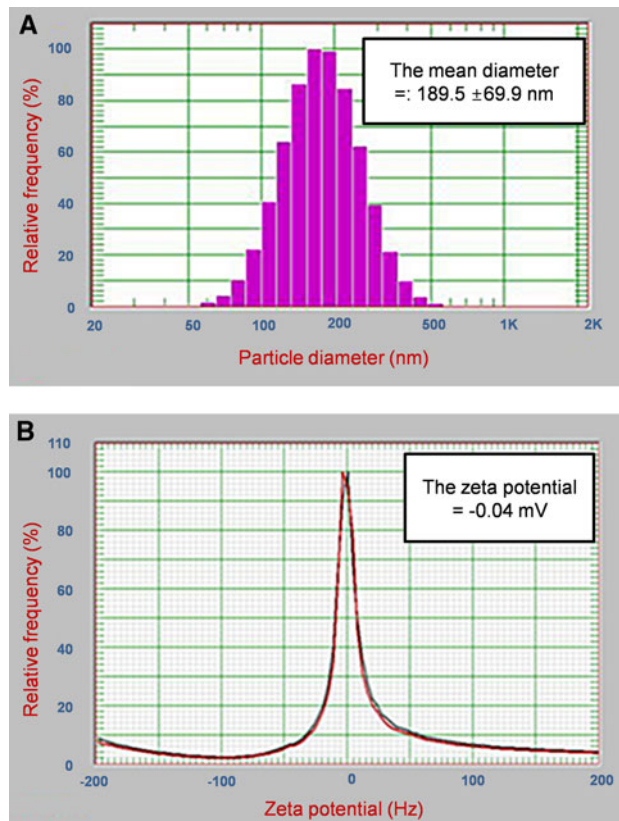
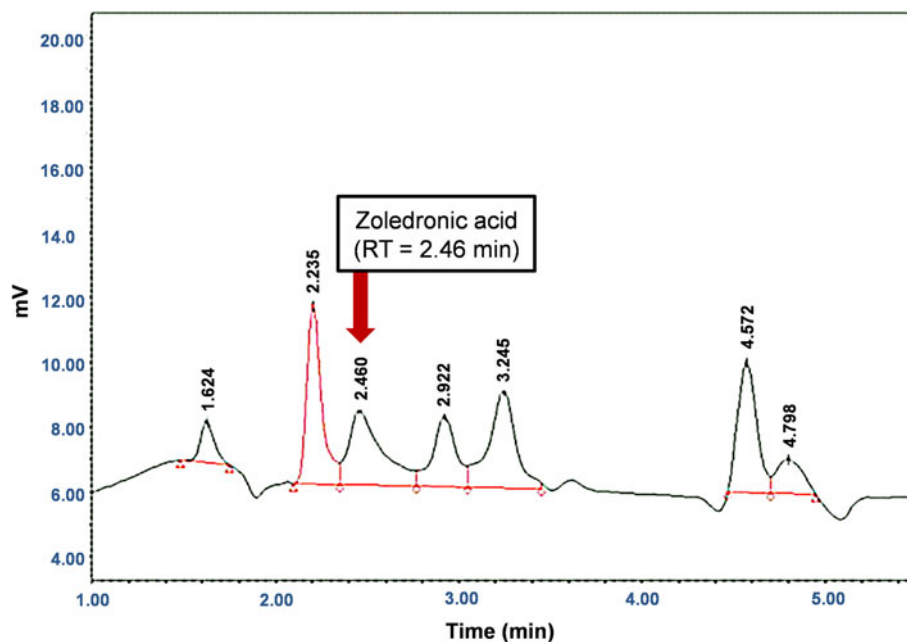


Fig. 2 Size distribution (a) and zeta potential (b) of liposomes, showing the mean diameter and zeta potential value of  $189.5 \pm 69.9$  nm and  $-0.04$  mV, respectively

**Fig. 3** Representative chromatograms of ZOL from T<sub>f</sub>-Lipo. Retention time of ZOL was 2.46 min and the encapsulation efficiency calculated from standard curve of free ZOL was 9.16 %



**Table 1** The mean diameter, zeta potential and encapsulation efficiency of various liposomal formulations

	Mean diameter $\pm$ SD (nm)	Zeta potential $\pm$ SD (mV)	Encapsulation efficiency $\pm$ SD (%)
Conv-Lipo-ZOL	160.5 $\pm$ 37.3	-1.14 $\pm$ 0.18	14.94 $\pm$ 0.59
PEG-Lipo-ZOL	182.9 $\pm$ 38.5	-0.63 $\pm$ 1.39	15.24 $\pm$ 1.08
T <sub>f</sub> -PEG-Lipo-ZOL	190.9 $\pm$ 24.5	-0.27 $\pm$ 0.32	9.16 $\pm$ 2.10

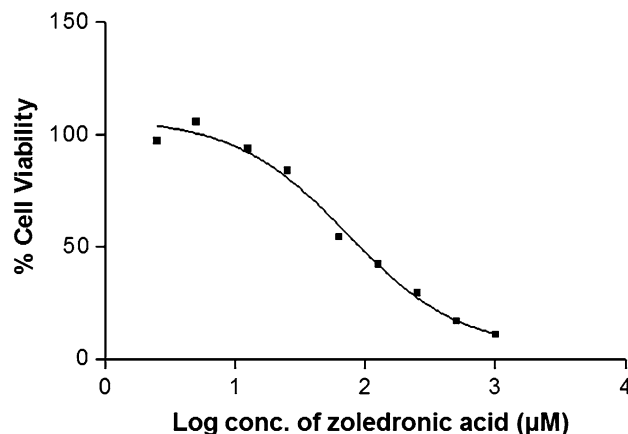
conventional liposomal ZOL (Conv-Lipo-ZOL) and PEGylated liposomal ZOL (PEG-Lipo-ZOL) was about 15 % (Table 1). It is considered that reduced encapsulation efficiency of ZOL in T<sub>f</sub>-Lipo is due to the dilution during several separation processes of unreacted materials in the T<sub>f</sub>-conjugation procedure.

#### Number of transferrin attached to a liposome

The number of T<sub>f</sub> attached to each liposome was optimized by changing the concentration of T<sub>f</sub> solution (data not shown). Finally, 0.2 mM T<sub>f</sub> solution and 0.06 molar ratio of DSPE-PEG<sub>2000</sub>-PDP: total lipids were used for proper number of T<sub>f</sub> attachment. According to the Bradford protein assay,  $2.063 \times 10^{-4}$  g T<sub>f</sub> was conjugated to 1 ml of T<sub>f</sub>-Lipo. And the 1 ml of T<sub>f</sub>-Lipo contained  $4.878 \times 10^{-5}$  mol phosphorus, which was determined by the Bartlett's phosphorus assay. Therefore, the actual number of T<sub>f</sub> molecules attached to each liposome is about 17 ~ 19.

#### Inhibition of proliferation in MDA-MB-231 cells

For the in vitro experiments, the seeding density of MDA-MB-231 cells should be determined. The optimum cell

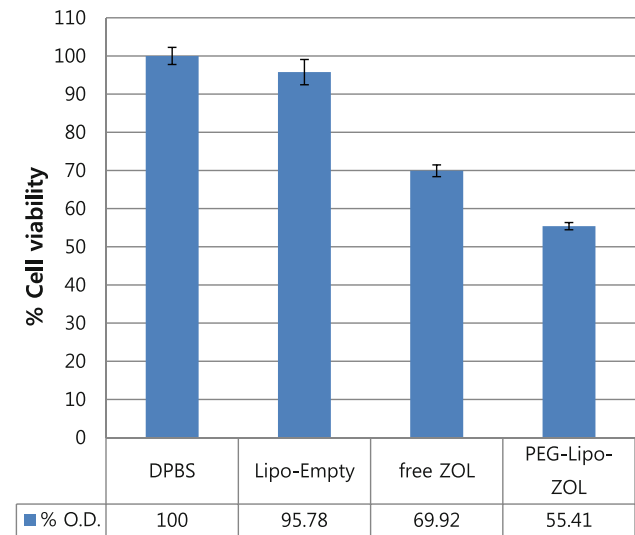


**Fig. 4** Result of inhibitory concentration assay of free zoledronic acid in MDA-MB-231 cells

density was between 2,500 and 5,000 cells in a 96-well flat-bottomed microassay plate, so that we chose 3,000 cells for the future study (data not shown). The concentration of treated ZOL was determined by inhibitory concentration assay using free form of ZOL in MDA-MB-231 cells (Fig. 4). The IC<sub>50</sub> of free ZOL was about 75  $\mu$ M. The anti-proliferative effect of ZOL was enhanced with the liposomal formulation compared with free form of ZOL (Fig. 5).

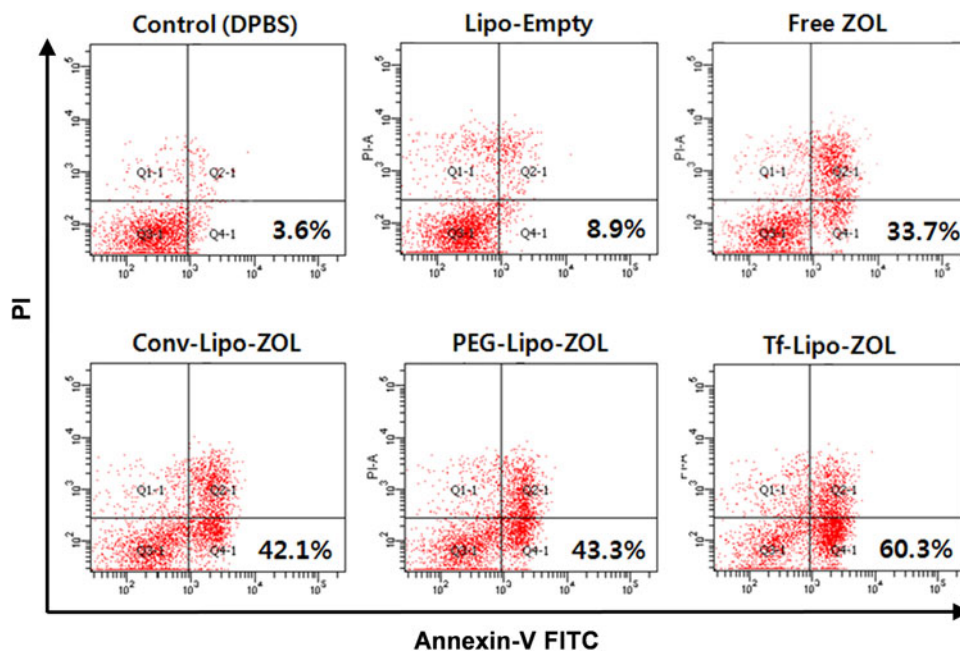
Effect of transferrin-conjugated liposomal zoledronic acid on apoptosis

As shown in Fig. 6, most of the cells treated with empty liposome were not positively labeled with FITC annexin-V, indicating a low fraction of apoptotic cells. When the cells were treated with transferrin-conjugated liposomal zoledronic acid ( $T_f$ -Lipo-ZOL), the apoptotic population was enhanced (60.3 %) 2-times more than free ZOL (33.7 %) and 1.5-times more than non-conjugated liposomal ZOL (about 43 %).



**Fig. 5** Anti-proliferative effect of liposomal zoledronic acid treated for 48 h on MDA-MB-231 cells. Cell proliferation evaluated by MTT assay. The data represent a mean  $\pm$  SD and reported as the percentage of control (DPBS)

**Fig. 6** FACS analysis of apoptosis induced by various formulation of ZOL; control (DPBS), empty liposome (*Lipo-Empty*), free zoledronic acid (*Free ZOL*), conventional liposomal zoledronic acid (*Conv-Lipo-ZOL*), PEGylated liposomal zoledronic acid (*PEG-Lipo-ZOL*), transferrin-conjugated liposomal zoledronic acid ( $T_f$ -Lipo-ZOL). The spots appeared in lower left quadrant (FITC<sup>-</sup>, PI<sup>-</sup>) represent viable cells, in lower right quadrant (FITC<sup>+</sup>, PI<sup>-</sup>) represent early-apoptotic cells, and upper right quadrant (FITC<sup>+</sup>, PI<sup>+</sup>) represent necrotic cells



Discussion

The most potent nitrogen containing bisphosphonate, zoledronic acid, has been used for osteoclast-mediated bone disease and used for combination therapies with anti-cancer agents in adjuvant and neoadjuvant cancer therapy (Fox 2010; Coleman et al. 2010). Beside the adjuvant anti-cancer effect, many studies have demonstrated the direct anti-cancer effects of ZOL in vitro and the molecular pathway for anti-cancer effect is similar to that observed in osteoclasts (Winter et al. 2008). Through this mechanism, ZOL inhibited proliferation and induced apoptosis in the various cancer cells (Gnant 2011; Clyburn et al. 2010; Tassone et al. 2003). But the poor pharmacokinetic property of rapid elimination from the plasma and accumulation into the bone limited the study and evaluation of anti-cancer effect of ZOL in vivo (Chen et al. 2002). In this regard, we introduced liposomal delivery system for reducing bone affinity and delivering of ZOL selectively to the tumor tissues.

In this study, we prepared liposomal ZOL and evaluated its characteristics and the in vitro anti cancer effect on the breast cancer cells. The mean diameter of  $T_f$ -Lipo-ZOL was about 190 nm, which is optimum for being accumulated in tumor tissues by EPR effect (Maeda 2001). And with the size, neutral zeta potential ( $-0.03$  mV) is favorable to evade from the RES. In the study of clodronate encapsulated in liposomes, neutral liposomes were less detected to macrophages or monocytes (Epstein-Barash et al. 2010). The transferrins attached to liposomes are also key strategy for targeting and internalizing in cancer cells. The targeting efficiency is dependent on targeting moiety

content of the liposome. Less number of  $T_f$  per liposome can lead to lower targeting effect and too high concentration of proteins can result in aggregation of liposome in blood stream. The actual number of transferrin attached to a liposome was calculated as 17–19, and this number seems to be optimal as a drug targeting moiety.

From the results of in vitro anti-cancer effect, liposomal formulation can be evaluated as a safe and less toxic delivery system. The empty liposome showed less than 10 % cytotoxic effects (4.22 % of anti-proliferation effect and 8.9 % of apoptosis inducing). On the other hand, anti-cancer effects of the liposomal ZOL were higher than free ZOL but not significant, especially in non-conjugated liposomal ZOL. Therefore it is considered that active targeting is essential for evaluating the anti-cancer effect in vivo study.

From these results, we suggest that the potential use of ZOL as an anti-cancer agents can be materialized with the targeted delivery systems such as liposomes. Moreover, with the additional properties of ZOL, such as tolerable side effects and well known anti-resorptive activity, ZOL encapsulated in targeted liposomes can be a promising therapeutic for malignant breast cancer and other metastatic cancer patients by dual effects of enhanced anti-cancer effects in tumor sites and anti-resorptive effect in bone.

**Conflict of interest** All authors (M Choi, DH Shin and JS Kim) declare that they have no conflict of interest.

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