Coencapsulation of alendronate and doxorubicin in pegylated liposomes: a novel formulation for chemoimmunotherapy of cancer

Hilary Shmeeda, Yasmine Amitay, Jenny Gorin, Dina Tzemach, Lidia Mak, Stephan T. Stern, Yechezkel Barenholz and Alberto Gabizon

Abstract

We developed a pegylated liposome formulation of a dissociable salt of a nitrogen-containing bisphosphonate, alendronate (Ald), coencapsulated with the anthracycline, doxorubicin (Dox), a commonly used chemotherapeutic agent. Liposome-encapsulated ammonium Ald generates a gradient driving Dox into liposomes, forming a salt that holds both drugs in the liposome water phase. The resulting formulation (PLAD) allows for a high-loading efficiency of Dox, comparable to that of clinically approved pegylated liposomal doxorubicin sulfate (PLD) and is very stable in plasma stability assays. Cytotoxicity tests indicate greater potency for PLAD compared to PLD. This appears to be related to a synergistic effect of the coencapsulated Ald and Dox. PLAD and PLD differed in in vitro monocyte-induced IL-1β release (greater for PLAD) and complement activation (greater for PLD). A molar ratio Ald/Dox of ~1:1 seems to provide an optimal compromise between loading efficiency of Dox, circulation time and in vivo toxicity of PLAD. In mice, the circulation half-life and tumor uptake of PLAD were comparable to PLD. In the M109R and 4T1 tumor models in immunocompetent mice, PLAD was superior to PLD in the growth inhibition of subcutaneous tumor implants. This new formulation appears to be a promising tool to exploit the antitumor effects of aminobisphosphonates in synergy with chemotherapy.

Introduction

Combination therapies for the treatment of cancer have increased in recent years in an attempt to address the multiple pathways of oncogenesis that have been discovered. A powerful tool for multiple drug delivery are nanoparticles containing coencapsulated drugs with different mechanisms of action, targeting different pathways of the oncogenic scenario and with nonoverlapping toxicities [1]. In this study, we aim at the codelivery of aminobisphosphonates and anthracyclines, by co-encapsulation in the classical Stealth liposome formulation.

Bisphosphonates are used primarily to increase bone mass and reduce the risk of fracture in patients with osteoporosis, to slow bone turnover in patients with Paget’s disease of the bone, and to treat bone metastases and hypercalcemia in patients with cancer. Aminobisphosphonates (alendronate, pamidronate, ibandronate, risedronate, zoledronic acid) are a subclass of bisphosphonates with potent inhibitory activity of farnesyl pyrophosphate synthase, a key enzyme of the mevalonate pathway [2]. During the past decade, it has been demonstrated that zoledronic acid, alendronate and other aminobisphosphonates can interfere with critical processes in cell signaling and growth at nanomolar concentrations. Antiangiogenic effects have also been demonstrated [3]. Preclinical studies have indicated synergistic antitumor effects when doxorubicin is followed by zoledronic acid treatment [4]. Furthermore, aminobisphosphonates have been shown to cause expansion and activation of a subpopulation of T lymphocytes occurring in pri-

mates, known as γδ T (GD-T) cells, particularly the Vγ9Vδ2 T-cell subset, which have natural tumoricidal effects [5–7]. Clinical studies have demonstrated systemic antitumor effects of aminobisphosphonates [8], and notably effective prevention of disease recurrence in patients with breast cancer when zoledronic acid is given together with adjuvant chemohormonal therapy after resection of the primary tumor [9]. This has been confirmed in a recent and comprehensive analysis demonstrating an antitumor effect of bisphosphonates on skeletal as well as nonskeletal bone metastases [10].

In previous studies, we have shown that intracellular delivery of zoledronic acid (Zol) encapsulated into folate-targeted liposomes results in highly potent in vitro tumoricidal activity [11]. We also found that liposomal encapsulation of Zol reduces its rapid renal clearance and increases its accumulation in the tumor by the EPR (enhanced permeability and retention) effect [12]. However, liposomal Zol is very toxic in vivo, causing deaths of injected mice at doses ~50-fold lower than the LD50 of free Zol, particularly when Zol is delivered in the most stable and long-circulating pegylated formulations [12]. Although the dose-limiting toxicity of free bisphosphonates is in most settings hypocalcemia and nephrotoxicity, the reason for the lethal toxicity of liposomal Zol appears to be cytokine activation [12]. Subsequent studies with liposomal alendronate (Ald) have demonstrated much less toxicity in vivo than with liposomal Zol (authors’ unpublished results). Furthermore, liposomal Ald was found to have powerful in vivo activity as a GD-T-cell booster [13], suggesting that Ald is a valuable aminobisphosphonate candidate for delivery in liposomes.

In this study, we describe a new formulation of Ald and doxorubicin (Dox) coencapsulated in pegylated liposomes and have
tested its potential application to cancer therapy. Some of the studies presented here are the result of a collaboration between the Shaare Zedek Oncology Institute and the Nanotechnology Characterization Laboratory (NCL) to characterize the formulations developed at Shaare Zedek Oncology Institute. NCL characterization included measuring various physicochemical properties, in vitro cytotoxicity, and other tests related to the inflammasome pathway, complement activation and coagulation cascade. This new formulation appears to have new and valuable properties that differentiate it from clinically approved pegylated liposomal doxorubicin sulfate (PLD) and make it a potentially attractive tool for chemoinmunotherapy of cancer.

Materials and methods

**Liposome components and sources**

Hydrogenated soybean phosphatidyl-choline (HSPC) (Lipoid, Germany), methoxy-polyethylene glycol-distearoyl-phosphatidyl-ethanolamine (mPEG2000-DSPE) (Bio-lab, Jerusalem, Israel), cholesterol (Chol) (Sigma, St. Louis, MO), alendronic acid (Teva, Israel, and Tokyo Chemical Industry Co Ltd, Japan), radioactive $^3$H-alendronate as sodium salt (Moravek, CA), doxorubicin (Teva, Israel), pegylated liposomal doxorubicin (PLD) (Janssen Pharmaceuticals, NJ, commercial name Doxil™ or Caelyx™).

**Animals**

Female Balb/C and Sabra, 8–11 weeks old, were obtained from Harlan Biotech (Jerusalem, Israel). Experiments in mice were performed at the Shaare Zedek Medical Center or at the Animal Facility of the Hebrew University-Hadassah Medical School. All animal experiments were approved by the Animal Ethics Committee of the Hebrew University – Hadassah Medical School.

**Liposomal formulation and encapsulation of alendronate**

Liposomes were prepared by the standard method of ethanol injection into an aqueous buffer, followed by extrusion through polycarbonate membrane filters down to 0.05 µm pore size. The lipid components: HSPC, mPEG-DSPE and Chol at mole ratios of 55%, 40% and 5%, respectively, were weighed and dissolved in ethanol. The buffer was a solution of 250 mM Ald acid dissolved in double-distilled water (DDW) and adjusted to pH 6.0 with 25% ammonium hydroxide at 60 °C to form a salt of (NH$_4$)$_2$-Ald. The resulting liposomes were then processed by serial size extrusion in a high-pressure extruder device (Lipex Biomembranes, Vancouver, BC), with temperature control at 60 °C, through filters with pore sizes from 1 µm to 0.05 µm. Nonencapsulated Ald was removed by dialysis against a buffer of 5% dextrose with 15 mM histidine, pH 7.0, followed by passage over a Dowex anion-exchange resin. The liposomes were sterilized by filtration through 0.22-µM filters and stored in Vacutainer™ tubes at 4 °C. These pegylated liposomes containing Ald are referred to as PLA.

Phospholipid (PL) and Ald content were determined after Folch extraction (8:4:3 chloroform/methanol/WWV/sample) where the lower lipophilic phase contains the organic phosphate (PL), and the upper phase contains the inorganic phosphate (Ald). Samples of each phase were assayed by the Bartlett method to determine phosphorus. Ald quantification was based on the Bartlett method to determine phosphorus. Ald quantification was based on the Bartlett method to determine phosphorus. Ald quantification was based on the Bartlett method to determine phosphorus. Ald quantification was based on the Bartlett method to determine phosphorus.

**Gradient-induced loading of doxorubicin**

PLA, prepared as described earlier, was incubated with a solution of 10 mg/ml Dox in 5% dextrose, at Ald/Dox molar ratios ranging between 2 and 0.5. The incubation was with gentle shaking for 60 min at a temperature of 60 °C, followed by ice-water cooling immediately upon end of incubation. The resulting liposome suspension was passed through a Dowex cation-exchange resin to remove any nonencapsulated Dox and was sterilized using 0.22 µm filters. Dox was measured spectrophotometrically by absorbance at 480 nm after dilution and disruption of the liposomes in acidified (0.075N HCl) 10:90 water/t-butanol solution. The final concentration of Dox in the formulation was adjusted to 2.0 mg/ml by further dilution with the dialysis buffer (5% dextrose/15 mM histidine). PLA loaded with Dox is referred to as PLAD. Dox quantitation was confirmed at NCL by a method of reverse phase HPLC described in Supplement 2.

**Characterization of the coencapsulated liposomes**

The following tests were done to characterize the PLAD formulation.

1. The liposome particle size distribution was measured by dynamic light scattering (DLS) using a Malvern High Performance ParticleSizer. Two samples were tested in triplicate runs. The targeted Gaussian mean vesicle diameter by DLS was 60–130 nm with a polydispersity index (PDI) < 0.2. The osmolarity was determined using an Osmomat 030-D (Genotec) osmometer. Zeta potential was determined in four diluted replicate sample of PLA and PLAD using a Zetamaster (Malvern, UK) instrument at 25 °C, with voltage maintained at 150 V. For DLS, liposomes were diluted in physiologic saline. For zeta potential, liposomes were diluted in the dextrose–histidine buffer.

2. The liposome morphology was examined by cryotransmission electron microscopy analysis at the Moskowitz Center for Nano-Imaging of the Weizmann Institute of Science. For details on sample processing, see Supplement 3.

3. Ammonium concentration was determined in one of our PLAD batch preparations as described by Silverman and Barenholz [14].

4. For encapsulation efficiency and detection of residual free drug (Dox or Ald), gel chromatography fractionation (Sepharose 6B) was used to separate liposomal material from nonencapsulated, low-molecular-weight material, as reported previously [15].

5. The long-term chemical integrity of Dox was evaluated by an accelerated stability test with exposure of PLAD. Dox was analyzed by HPLC [16].

6. For plasma stability testing, PLAD (100 µl) was incubated with 80% human plasma (400 µl) at 37 °C for 1 h to 24 h and assessed for the release of Ald and/or Dox by gel chromatography with Sepharose 6B. The protein fractions elute after the liposome fractions and before the free drug fractions.

**Cytotoxicity**

We performed two types of cytotoxicity assays: one assay with subconfluent cells growing rapidly at a near-exponential rate (Shaare Zedek Oncology Institute), and the other assay with near-confluent cells under static growth conditions (NCL). At Shaare Zedek Oncology Institute, growth rate was assessed colorimetrically based on either methylene blue staining or the MTT assay (Promega kit) after 72 h of continuous cell exposure to drugs, as previously described [11]. At NCL, cytotoxicity was determined as described in
PLA and PLD were tested at concentrations ranging were diluted to the desired assay concentrations in cell culture media. PLAD and PLD were tested at concentrations ranging 2–500 μM Dox. PLA was tested at equivalent concentrations of alendronate to PLAD. Placebo liposomes were tested at equivalent concentrations of phospholipid to PLAD. Min-u-sil 5 (US Silica, Frederick, MD) was used as a positive control at a concentration in the range of 1.5 ng/mL to 100 μg/mL. THP-1 cells were plated at 4 × 10⁵ cells/mL in 96-well, microtiter plate format. Cells were preincubated with 50 nM PMA for 24 h prior to test material addition, reaching an approximate confluence of 80%. Cells were then exposed to test material for 48 h in the dark. In order to determine NLRP3 inflammasome activation, IL-1β secretion was measured in 48 h culture media using the human IL-1β/IL-18 Quantikine ELISA kit according to manufacturer’s instructions.

Interleukin 1β release

In this assay, we used the THP-1 cell line of monocytic lineage, pre-treated with 50 nM phorbol myristate acetate (PMA). Test materials were diluted to the desired assay concentrations in cell culture media. PLAD and PLD were tested at concentrations ranging 2–500 μM Dox. PLAD was tested at equivalent concentrations of alendronate to PLAD. Placebo liposomes were tested at equivalent concentrations of phospholipid to PLAD. Min-u-sil 5 (US Silica, Frederick, MD) was used as a positive control at a concentration in the range of 1.5 ng/mL to 100 μg/mL. THP-1 cells were plated at 4 × 10⁵ cells/mL in 96-well, microtiter plate format. Cells were preincubated with 50 nM PMA for 24 h prior to test material addition, reaching an approximate confluence of 80%. Cells were then exposed to test material for 48 h in the dark. In order to determine NLRP3 inflammasome activation, IL-1β secretion was measured in 48 h culture media using the human IL-1β/IL-18 Quantikine ELISA kit according to manufacturer’s instructions.

Complement activation

The procedure described in http://ncl.cancer.gov/working_assay-cascade.asp (ITA-5.2) was followed. PLAD was analyzed at several dilutions, 0.4, 2, 10 and 100 μg/mL of Dox. Vehicle liposomes (PLC) and PLA were analyzed at dilutions resulting in equivalent phospholipid or alendronate concentrations, respectively. PLD was included as an additional control. It was tested at equivalent Dox concentrations as those used for PLAD, and directly from the commercial stock, resulting in a final concentration of 0.7 mg/mL.

Leukocyte procoagulant assay

The objective of this assay was to evaluate the effect of nanoparticle treatment on human plasma coagulation in vitro following NCL protocol described in http://ncl.cancer.gov/working_assay-cascade.asp (ITA-12 Coagulation Assay). PLA was analyzed at several dilutions, 0.4, 2, 10 and 100 μg/mL of Dox. Placebo liposomes and PLA were analyzed at dilutions resulting in equivalent phospholipid or alendronate concentrations, respectively.

In vivo toxicity

The toxicity of PLAD was tested at dose levels of 5–15 mg/kg based on Dox content in 8–10-week-old female Sabra mice. Mice were injected i.v. up to three times with a dose interval of at least one week. Mice were inspected and weighed three times weekly. PLA was tested at doses up to 14 mg/kg. The toxicity of formulations with various Ald:Dox ratios (2:1, 1:1, 1:2) was tested at a Dox dose of 10 mg/kg. In addition, blood samples of Sabra mice injected i.v. with PLA or PLAD were obtained by puncture of the retro-orbital venous sinuses at day 7 after treatment and complete blood counts (CBC) were performed in an automatic blood counting machine (Sysmex XE-2100, Japan).

Pharmacokinetics

We followed the pharmacokinetics (PK) of PLAD based on plasma levels of doxorubicin in either BALB/c or Sabra mice. Mice were injected i.v. with 10 mg/kg of PLAD based on Dox content, and compared to PLD. Different ratios of Ald:Dox were tested in Sabra mice at a dose of 10 mg/kg. One to 48 h after injection, mice were anesthetized by isoflurane inhalation, bled by eye enucleation (≥1 ml blood per mouse) and immediately sacrificed by cervical dislocation. Blood was collected in heparinized tubes and centrifuged immediately to separate plasma from blood cells. Plasma levels of doxorubicin were measured fluorimetrically after extraction of Dox from plasma by mixing 100 μL of plasma with 900 μL of acifidil isopropanol [18]. PLAD formulations containing trace amounts of radioactive tritiated Alendarone (3H-Ald) were also tested in Sabra mice. For the quantitation of 3H cpm, 50 μL of plasma was added to 5 ml of Quicksafe and radioactivity was measured by scintillation counting in a Wallac 1409 scintillation counter.

Therapeutic efficacy

The therapeutic activity of PLA and PLAD was compared to PLD in two mouse tumor models (M109R Dox-resistant lung carcinoma, and 4T1 breast carcinoma implanted in 10-week-old female BALB/c mice), and two human tumor models (KB cervix carcinoma, and IGROV-1 ovarian carcinoma implanted in 10-week-old athymic nude female mice). Tumor cells were injected in the right hind footpad (10⁵ M109R, 10⁶ 4T1, 10⁵ KB) or inoculated intraperitoneally (10⁵ IGROV-1). PLAD, PLD and other comparator treatments were injected i.v. in the tail vein once weekly at the indicated doses based on Dox content. The schedule of treatment given in figure legends. PLA was injected at the equivalent concentration to that of the PLAD formulation (Ald dose, 12.5–14.3 mg/kg). Tumor growth was followed by measurement with precision calipers. Cages were inspected daily. Mice were monitored for survival, body weight and tumor growth twice or thrice per week. Kaplan–Meier curves and log-rank test (Graphpad Prism, ver. 6) were used to plot and analyze time to treatment failure and survival.

Results

Liposomal formulation

A schematic representation of the process of liposome formulation and Dox loading is shown in Figure 1. Dox was remote loaded into the PLA formulation to form PLAD. Encapsulation efficiency of Ald was low (~3%) as expected from passive encapsulation of a watersoluble drug in small liposomes. The characteristics of a typical preparation of the coencapsulated liposomes loaded at an initial Ald/Dox ratio of 1:1 are presented in Table 1. Dox was loaded at high efficiency (>80%) resulting in a preparation approaching the
drug/phospholipid ratio of PLD. The final formulation was adjusted to 2 mg/ml of doxorubicin. Mean diameter ranged between 75–100 nm with PDI values of 0.10. Both PLA and PLAD had a weakly electronegative surface potential (zeta potential between 15.6 mV and 14.9 mV). In the initial buffer, the ammonium concentration was 500 mEq/L. After dialysis of PLA and before Dox loading, the ammonium concentration dropped to 17 mEq/L, and after Dox loading the final formulation of PLAD contains 6.8 mEq/L of ammonium.

To optimize the Ald/Dox ratio, we compared formulations obtained with three initial ALD/Dox molar ratios of 1:0.5, 1:1 and 1:2. Final Ald/Dox ratios after loading were greater than initial ratio values depending on the efficiency of Dox encapsulation and drug losses. At an initial Ald/Dox molar ratio of 1:2, the efficiency of Dox loading was substantially lower than at 1:1 and 1:0.5 (not shown). Coencapsulation of Ald and Dox in PLAD was validated using gel chromatography fractionation in which we separate liposome material from low molecular weight material (nonencapsulated drugs) as described in “Methods” section. Liposome-associated drugs elute in fractions #5–7, while free, nonencapsulated drugs elute in fractions #10–12 (Figure 2). As can be seen, radiolabeled Ald eluted with the liposome fractions (#5–7) and later fractions contained negligible amounts of Ald (Figure 2(A)). In addition, as shown in Figure 2(B), doxorubicin eluted with the liposome fractions (#5–7) and later fractions contained negligible amounts of drug. The patterns of elution of both drugs, Ald and Dox, overlap with each other confirming that they are co-encapsulated in the liposomes.

Further, as shown in Figure 3(A–B), PLAD, after 2-h exposure to human plasma, demonstrates excellent retention of Ald and Dox within liposomes, as neither protein fractions (#9–10), nor free drug fractions (#10 and further) show any significant amounts of Ald or Dox.

The long-term stability of PLAD was assessed by Sepharose 6B chromatography (as described earlier). PLAD appears to be stable at 4°C with no particle size growth, and no drug leakage for >1 year. Currently a two-year-old formulation of PLAD stored at 4°C has demonstrated excellent size stability and drug retention based on Dox content and phosphorus assay-based Ald content. There is no decline in cell cytotoxic potency in in vitro assays. The potency retention of PLAD was evaluated by HPLC analysis of the chemical stability of Dox and found intact for >6 months of storage. No adverse effects of alendronate on Dox chemical stability were detectable (data not shown).

CryoTEM revealed PLAD liposomes containing a crystalline rod-like precipitate (Figure 4(B)) similar to the precipitate detectable in cryoTEM photographs of PLD. However, PLAD liposomes appear round rather than the oval (coffee-bean) shape characteristic of PLD (Figure 4(C)). The rods are not detectable in PLA liposomes without Dox (Figure 4(A)). Close-up view of the PLAD liposome and

---

**Table 1.** Formulation characteristics of a representative batch before (PLA) and after (PLAD) DOX loading. a

| Batch   | Ald concentration | PL concentration | DOX concentration | Size (nm) | Zeta potential |
|---------|-------------------|------------------|-------------------|-----------|---------------|
|         | µmol/ml           | mg/ml            | Recovery           | µmol/ml | Recovery | µmol/ml | mg/ml | Recovery | Mean | PDI | mV     |
| PLA     | 13.1              | 4.25             | 2.1%              | 23.3     | 93%     | N/A      | N/A      | N/A | 84.7 | 0.068 | −15.6 |
| PLAD    | 8.5               | 2.8              | N/A               | 16.3     | N/A     | 3.45     | 2.0     | 85% | 83.3 | 0.069 | −14.9 |

a pH was controlled between 6.0 and 7.0. Osmolarity was between 275 and 325 mOsm/L.
b Final Dox concentration adjusted to 2.0 mg/ml.
c Percent recovery is the end of process (output) amount of phospholipid or drug expressed as the fraction of the initial amount (input). N/A = not applicable.
its interior gel precipitate confirm that the rod-like precipitate is not in contact with the lipid bilayer and appears less well organized than the PLD rods (Figure 4(D)).

**Cytotoxicity of PLA and PLAD**

The cytotoxicity of PLA and PLAD were compared to that of free Dox, free Ald and PLD in various human tumor cell lines (Figure 5, Table 2). PLAD was more cytotoxic than PLD in most cell lines tested and remarkably approximates the activity of free DOX in some cell lines such as KB (Figure 5(C)). PLA and free Ald have essentially no cytotoxicity in any of these cell lines. The cytotoxicity advantage of PLAD over PLD required a long time of exposure (>48 h) to become apparent, in contrast to free Dox which reached near-maximal cytotoxicity within 3 h of incubation (Figure 6).

At NCL, cytotoxicity was tested in three cell lines (HEPG2, HL60, MDA-MB231) at near-confluency for 48, 72 or 96 h using the MTT and LDH release assays (NCL). As seen in Table 3, PLAD was significantly more cytotoxic than PLD in all three cell lines. As expected, IC50 values were much higher under conditions of near-confluency as compared to subconfluent cultures.

In drug uptake experiments with KB cells exposed to free Dox, PLD and PLAD, we found equally low-cell uptake of Dox when PLAD and PLD are compared (Figure 7(A)), indicating that the greater cytotoxicity of the former cannot be explained by a greater exposure to Dox and is probably related to the combined presence of Ald and Dox in the same liposome. Interestingly, when we compared uptake at 4°C to that at 37°C, there was no temperature dependency of uptake for PLAD and PLD; however, free Dox uptake was sharply diminished at low temperature (Figure 7(B)).
Figure 5. Cytotoxicity assays in subconfluent cultures of human cell lines: Cells were plated at 2–4 × 10^4 cells/mL in the 96-well plate format and pre-incubated for 24 h. Free Dox (red, filled symbols), PLAD (green, triangles) and PLD (Doxil) (red, empty symbols) were added at concentrations ranging from 0.05–100 μM doxorubicin. Free alendronate and PLA were added at an equivalent concentration of Ald to the PLAD formulation. Cells were treated for 72 h and processed as described in Methods. (A) MCF-7, (B) N-87, (C) KB, (D) IGROV-1.
Further control experiments for the cytotoxicity studies included a test of stability of PLAD in cell culture medium, and confocal fluorescence examination of cells treated with PLAD (see Supplement 5).

Inflammasome pathway

The ability of the PLAD formulation to activate the NLRP3 inflammasome pathway was assessed in PMA-treated human monocytic cells, THP-1, by measuring IL-1β secretion (Table 4) and monitoring cytotoxicity (not shown). PMA treatment was used to transform the THP-1 cells to a macrophage-like line, as well as to prime the NLRP3 pathway by inducing pro-interleukin 1 (IL-1) beta through NF-kappa-B signaling [19]. PLAD was compared to the controls free Ald, PLA, placebo PEYlated liposomes (PLC) and PLD (commercial Doxil). The cytotoxic potency of the various formulations on THP-1 cells followed the rank order of: PLD, PLC and Ald which were practically ineffective, followed by an increased cytotoxicity of PLAD and even more of PLA. This was almost identical to the rank order for IL-1β production (Table 4), except for the huge free Ald response. PLD ranks below PLC which is inferior to PLAD < PLA < Ald. PLD did not increase the inflammatory response over free Ald, while Dox, as in PLD and PLAD, appeared to antagonize the inflammatory response. Thus, PLAD and Ald + PLD had greatly reduced IL-1β release in comparison to PLA and Ald. This could not be explained by increased cytotoxicity.

Interaction with blood components

Interaction with blood components was assessed in vitro with PLA, PLD and PLAD. The tests included induction of hemolysis, complement activation, leukocyte procoagulant activity, platelet aggregation and blood clotting times. In vitro hemolysis was detectable only at the highest tested concentration, of PLAD and PLD. PLD, at Dox concentrations of 0.7 and 0.1 mg/mL, showed significant activation of complement as indicated by a sharp increase in iC3b

Table 2. Cell growth inhibition (IC₅₀) in subconfluent cell cultures of human and mouse tumor cell lines.

| Cell Line/Drug | Mouse J6456 | MouseM109R | Mouse4T1 | Human N-87 | Human Panc-1 | Human KB | Human IGROV-1 | HumanSKBR-3 | Human MCF-7 |
|---------------|-------------|-------------|-----------|-------------|--------------|---------|---------------|-------------|-------------|
| Free Ald      | >50         | >100        | Not done  | >100        | >100         | >20     | >20           | <50         | 48          |
| PLA           | >50         | >100        | Not done  | >100        | >100         | >20     | >20           | >50         | >50         |
| Free Dox      | 0.21        | 7.5         | 0.01      | 3.1         | 0.26         | 0.07    | 0.17          | 0.03        | 0.03        |
| PLD (Doxil)   | 6.8         | >100        | 0.56      | 32          | 94           | 1.4     | 5.8           | 0.3         | 0.5         |
| PLAD          | 1.1         | 91          | 0.26      | 9.9         | 25           | 0.65    | 1.6           | 0.09        | 0.08        |

Table 3. Cell growth inhibition (MTT) and LDH release assays in near-confluent cell cultures.

| Cell line (Incubation time) | MTT IC50 μM | MTT IC50 μM | Increase LDH release | Increase LDH release |
|-----------------------------|-------------|-------------|----------------------|----------------------|
| HL60 (48 h)                 | PLAD        | PLD         | PLAD                 | PLD                  |
| MDA-MB231 (72 h)            | 250         | >500        | Not done             | Not done             |
| Hep-G2 (96 h)               | 31          | >500        | 37%                  | 21%                  |

Cytotoxicity was tested after exposure to agents for 72 h as described in Methods. IC-50 values are the mean of 2 or more experiments, each in duplicate plates, expressed in μM of Dox, when Dox included as a test agent, and μM Ald when Dox is not included as a test agent.
tion with high ALD/Dox ratio (1:0.5) was toxic at this dose level, concentration, appears to be the MTD of the formulation. The formula-

Table 4. IL1β release from THP-1 monocytes.

| Sample testeda | Maximal IL-1β secretion in 48 h, % Increase |
|----------------|--------------------------------------------|
| Plain medium (baseline) | 100% (normalized) |
| Min-u-sil 5 (positive control) | 700% |
| Free alendronate | 11,137% |
| Free alendronate – PLD | 604% |
| Placebo liposomes | 439% |
| PLA | 10,773% |
| PLAD | 4,480% |
| PLD | 104% |

aPretreated for 24 h with phorbol myristate acetate (PMA) at 50 nM. Near-confluent cell cultures.

bMin-u-sil 5 (US Silica, Frederick, MD) tested at concentrations of 1.5–100 μg/ml; Other samples tested at 0.01–500 μM concentration of Dox, or Ald-equivalent, or phospholipid-equivalent concentrations.

Figure 8. In vitro analysis of complement activation in pooled plasma: PLD, PLAD, PLA and placebo (drug free) liposomes were tested at concentrations of 0.4, 1, 10 and 100 μg/ml of Dox for PLD and PLAD or at equivalent phospholipid concentrations for PLA and placebo. For clarity, only results for 100 μg/ml samples are presented since no responses were observed at lower concentrations. A sample of a PLD (Doxil stock) at 700 μg/ml and cobra venom factor (CVF) served as positive controls. PBS was used as negative control. Three independent samples were prepared and analyzed in duplicate (CV <20%). PLAD and PLA did not cause complement activation in contrast to PLD. Lower limit of detection is 0.14 μg/mL.

levels. However, in contrast to PLD, PLAD did not activate the complement system at any of the concentrations tested (Figure 8).

Leucocyte procoagulant activity was induced by free Dox but not by any of the tested formulations. None of the formulations induced detectable levels of platelet aggregation, significantly interfered with collagen-induced platelet aggregation, or affected plasma coagulation times (not shown).

In vivo toxicity

PLA was safe at doses of Ald<10 mg/kg but clearly toxic at ≥14 mg/kg in Sabra female mice causing deaths, without preceding weight loss, within 7–10 days, as previously described for much lower (~100-fold lower) doses of liposomal Zol [12]. There was no cumulative toxicity if a 2-week time window was maintained between the treatments (data not shown).

PLAD was tested in vivo in various mouse strains by i.v. injection in the dose range of 5–10 mg/kg based on Dox content. A 10 mg/kg dose level of the 1:1 Ald/Dox formulation, based on Dox concentration, appears to be the MTD of the formulation. The formulation with high ALD/Dox ratio (1:0.5) was toxic at this dose level, probably due to the higher levels of coinjected Ald (10–12 mg/kg). PLD given at 10 mg/kg did not cause significant toxicity (data not shown). Blood counts were tested 7 days after injection of PLA or PLAD. We found leukocytosis (Table 5), notably in PLA-treated mice, in agreement with previous results found for Zol liposomes [12].

Pharmacokinetics

PK studies after i.v. injection in Balb/C mice indicate PLAD and PLD have similarly slow plasma clearance profiles, with prolonged high Dox levels and estimated plasma half-lives of 15–20 h. Different ratios of Ald/Dox within the range tested here did not substantially affect the PK profile (Figure 9(A)). The clearance of Ald based on 3H-Ald label followed a similar profile in the plasma of Sabra mice to that of Dox (Figure 9(B)), indicating that the co-encapsulated drugs are cleared together from plasma in liposomal form.

In biodistribution experiments, PLAD resulted in significantly higher uptake in spleen and lower in liver than PLD. The reason for these differences is unclear. However, it did not have any impact on tumor uptake which was comparable for both formulations and nearly 9-fold greater than what was observed with free drug (Figure 9(C)).

Therapeutic efficacy

The therapeutic activity of PLAD was tested in the M109R model in immunocompetent BALB/c mice, an MDR tumor in which free Dox is inactive and PLD-Doxil has little efficacy [20]. Individual tumor growth curves and Kaplan–Meier curves demonstrate greater tumor inhibition and significantly longer time to tumor progression when PLAD is compared to PLD. PLAD was also superior to a combination of PLD and PLA (Figure 10(A)). Free Ald and PLA alone (Ald in PLA was given at the same dose as Ald in PLAD) were ineffective (data not shown). This experiment was repeated a second time with similar results.

In the 4T1 tumor model, also in BALB/c mice, PLAD was also superior to PLD (Figure 10(B)) in two successive experiments. However, here, the combination of PLA and PLD was as effective as PLD. Treatment was started early in the 4T1tumor model, between 24 to 48h after hours after tumor inoculation, because this tumor is highly aggressive, and rapidly growing.

Individual tumor growth curves of mice in experiments presented in Figures 10(A,B) can be found in Supplement 5 (Figure S3).

PLAD was also tested in the human KB and IGROV-1 tumor models in immunocompromised athymic nude mice. In the KB model, we followed the growth of subcutaneous (footpad) tumor implants, and in the IGROV-1 model, we followed ascites accumulation by weight increase and observation, and survival. In both models, the therapeutics effects of PLAD and PLD were comparable (Figure 10(C–D)). Both liposome-based treatments were greatly superior to free Dox (Figure 10(C)).
In our previous studies with liposome formulations of Zol, we demonstrated in vitro potent antitumor activity [11]; however, in vivo results were disappointing with lethal toxicity at surprisingly low doses [12]. We then replaced Zol with another aminobisphosphonate, Ald and found a much greater safe dose window in mice when Ald is formulated in long-circulating pegylated liposomes. Based on the elegant principle of remote loading with ammonium sulfate [26], we developed a pegylated liposome formulation of a dissociable salt of Ald with Dox using ammonium Ald to generate a gradient driving Dox into liposomes, and forming a stable salt with Ald in the liposome water phase (Figure 1). PLAD is thus a unique formulation containing two active principles (Ald and Dox) with different mechanisms of action and nonoverlapping toxicities, in which one agent is the driving force for encapsulation of the other by remote loading. PLAD allows for high-loading efficiency of Dox and high stability in biological fluids, comparable to that of PLD (Doxil™). Support for this codelivery approach comes also from studies with free drugs showing that aminobisphosphonates reinforce the effects of cytotoxic agents in a synergistic manner [27,28].

PLAD liposomes have an internal crystalline precipitate roughly similar to PLD; however, their shape remains round rather than adopting the characteristic oval shape of PLD. This may be related to different crystallization patterns for the Dox salts of sulfate and Ald, resulting in longer rods for the former that cause deformation of the spherical liposome. It should be noted that sulfate and the Ald pyrophosphate interact with up to two positively charged Dox molecules each, but while the former has two negative charges, the latter has four negative charges. New characterization tools, such as solution X-ray scattering recently applied to PLD [29], may help to further characterize and differentiate the structure and morphology of PLAD rods.

The PK profiles of Ald and Dox in PLAD are similar, suggesting nearly equal retention of both drugs (Figure 9). PLAD displays long circulating properties comparable to that of PLD, which allows for passive tumor targeting (Figure 9) based on the EPR effect [30]. However, noticeable differences were detected, when comparing PLAD and PLD, as follows:

- PLAD was a more potent cytotoxic agent than PLD in vitro at equal Dox concentration, at both subconfluent and near-confluent test conditions against a number of mouse and human tumor cell lines. Since in vitro Dox cell uptake was similar for PLAD and PLD, and PLA lacked any significant cytotoxicity, this argues for a synergistic effect of the codelivery of Ald and Dox.
- PLAD was a much more potent activator of the inflammasome pathway than PLD, leading to 40-fold greater secretion of IL-1β. Here, the contribution of Ald was obvious, as PLA was even more potent than PLAD on THP1 monocytes. The IL-1-mediated pro-inflammatory response of Ald with neutrophil recruitment is well known [31] and has been shown in vivo to be dependent on mast cells [32].
- In contrast to PLD, PLAD did not cause in vitro activation of the complement system in pooled human plasma. Pseudoallergic reactions during liposome infusions are related to complement activation and, albeit uncommon, constitute a major risk of liposome-based therapies [33,34]. There is no assurance that a negative result in this in vitro test precludes the occurrence of these reactions in patients. While the basis for this different test response to PLD and PLAD remains unclear, it is tempting to consider liposome encapsulated antitumor agents to skeletal tissue to facilitate treatment of metastatic bone tumors [24,25].

Discussion

NBPs are extensively used in combination therapies for various antitumor applications. It is likely that these applications do not reflect the full potential of NBPs as antitumor agents since as free agents they are rapidly eliminated from plasma by renal clearance, have minimal tissue distribution apart from bone, and have poor cellular permeability [2]. Recent work by a number of laboratories have explored the use of liposomal NBPs to overcome the above limitations and enhance their tissue targeting for a variety of applications including treatment of cancer, activation of the immune system, treatment of coronary re-stenosis, and other [11,13,21–23]. A few studies have utilized NBPs to target drug delivery platforms including liposome encapsulated antitumor agents to skeletal tissue to facilitate treatment of metastatic bone tumors [24,25].

**Figure 9.** Pharmacokinetic studies of PLAD. (A) PLAD formulations with different Ald/Dox ratios. Three PLAD formulations at Ald/Dox ratios of 2:1, 1:1 and 2:1, and PLD were injected into the tail veins of female Sabra mice at a dose of 10 mg/kg based on Dox content. Dox levels were measured in plasma at 1- and 24-h post-injection. Inset table presents the percent drop of [Dox] when the 24 and 1 hour levels are compared. (B) Pharmacokinetics of 3H-Ald-labeled PLAD: 3H-Ald-labeled PLAD was injected i.v. into Sabra mice at 1, 24 and 48 hours, Dox and 3H-Ald concentration were measured in plasma. Results are expressed as % injected dose to normalize the values. Parallel clearance of Ald and Dox is noticed. (C) Biodistribution of PLAD in BALB/c mice-bearing M109 tumors. Mice were sacrificed 2 hours after free Dox injection, and 48 h after PLD and PLAD injections. Results are expressed as % injected dose to normalize the values. The tumor levels of PLAD and PLD were comparable and much higher than those observed for free Dox. PLAD was found at higher levels in spleen than PLD.
Figure 10. Therapeutic efficacy of PLAD compared to PLD. The M109R lung Ca, and 4T1 breast Ca mouse tumor models and the KB cervical ca, and IGROV-1 ovarian ca human tumor models were assessed based on tumor size growth curves and/or Kaplan–Meier curves of time to treatment failure or time to death. A dose of 5–10 mg/kg based on Dox content was injected in the tail vein. PLA was injected at the equivalent concentration of Ald present in the PLAD formulation. Free Dox was tested only in the IGROV-1 model. In other models, it is much less active than PLD and was therefore not tested. N = 6–7 for untreated groups, N = 8–10 for treated groups. (A) M109R: 1 × 10^6 tumor cells were inoculated in the footpad of immunocompetent BALB/c f mice. A dose of 5 mg/kg Dox injected i.v. on days 7, 14 and 21. Statistical analysis (log-rank test): PLAD was significantly superior to PLD (p = 0.0001), and to PLA + PLD (p = 0.0101). (B) 4T1: 100,000 tumor cells were inoculated in the footpad of immunocompetent BALB/c f mice. A dose of 8 mg/kg Dox injected i.v. on days 7, 14 and 21. Statistical analysis (log-rank test): PLAD and PLD + PLA were statistically superior to PLD along various time points (e.g., on day 28: p = 0.0423 for PLAD, and p = 0.0137 for PLA + PLD). (C) IGROV-1: 1 × 10^6 tumor cells were inoculated in the peritoneal cavity of athymic nude mice. A dose of 8 mg/kg Dox injected i.v. on days 7 and 14. The survivals of PLD and PLAD-treated mice were significantly superior to that of free Dox-treated mice (p = 0.0008 and p = 0.0034, respectively, log-rank test). (D) KB: 1 × 10^6 tumor cells were inoculated in the footpad of athymic nude mice. A dose of 6 mg/kg Dox injected i.v. on days 9, 17 and 24. The effects of PLD, PLAD and PLD + PLA were not statistically different.
implicate the difference in particle shape as a possible contributory factor [34].

- The in vivo toxicity of a single-dose PLAD is somewhat greater than that of PLD, with the respective MTD's being approximately 10 and 15 mg/kg Dox equivalents. This difference in toxicity can be clearly accounted for by Ald. However, since Ald toxicity is reversible and noncumulative, it is reasonable to expect that there will be no significant difference in the maximal cumulative dose between PLAD and PLD.
- The therapeutic efficacy of PLAD was greater than that of PLD in two immunocompetent mouse tumor models and equal to that of PLD in the human lines tested in immunocompromised nude mice. Tumor-infiltrating macrophages may be involved in the antitumor effect elicited by combination of chemotherapy with zoledronic acid [35]. The superior effect of PLAD compared to PLD might be partly due to the macrophage polarization of tumor-associated macrophages from M2 to M1. In this context, PLA has been reported to modulate the polarization of macrophages toward M1 phenotype, and to abrogate the immunosuppressive and tumor-enhancing effects of drug-free liposomal carriers reported in some animal models [36,37].
- However, the most relevant aspect of the antitumor potential of NBPs in general and liposomal NBPs in particular is the enhancement of the antitumor immune response via accumulation of phosphoantigens in tumor cells and activation of $\gamma\delta$ T cells [38]. Athymic nude mice models probably underestimate the full therapeutic potential of PLAD. Even immunocompetent mice may be inadequate models as they lack the $V_\gamma9\delta2$ T-cell subset, which is characteristic of primates and has tumoricidal activity on cancer cells expressing phosphoantigens after exposure to aminobisphosphonates [39–41].

The application of bisphosphonates in cancer therapy will undoubtedly continue to be part of a combined modality approach including chemotherapy, hormonal therapy and other modalities. In breast cancer, which is one of the most explored clinical fields with bisphosphonates, doxorubicin is probably one of the best candidates for co-encapsulation and combined therapy since, together with taxanes, it is one of the most effective drugs. Coencapsulation facilitates synchronized codelivery of the two agents working on different intracellular targets with potentially synergistic antitumor effect. A further application of this platform would be the addition of targeting moieties such as the folate ligand [42], particularly to further improve the intracellular delivery of drugs with low cellular permeability such as aminobisphosphonates.

Acknowledgements
We thank Dr. Sharon Wolf (Weizmann Institute of Science) for the cryoTEM work. We thank the team of the NCL (Nanotechnology Characterization Lab, NIH/NCL) for their collaboration and feedback along several phases of this project.

Disclosure statement
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Funding information
This work was supported by grants from the Israel Cancer Research Fund, and Janssen Pharmaceuticals.

References
1. Liboiron BD, Mayer LD. Nanoscale particulate systems for multidrug delivery: towards improved combination chemotherapy. Ther Deliv 2014;5:149–71.
2. Green JR. Bisphosphonates: preclinical review. Oncologist 2004;9:3–13.
3. Streng V, Daubine F, Benzaid I, et al. Bisphosphonates in cancer therapy. Cancer Lett 2007;257:16–35.
4. Ottewell PD, Leffley DV, Cross SS, et al. Sustained inhibition of tumour growth and prolonged survival following sequential administration of doxorubicin and zoledronic acid in a breast cancer model. Int J Cancer 2010;126:522–32.
5. Girardi M, Oppenheim DE, Steele CR, et al. Regulation of cutaneous malignancy by gammadelta T cells. Science 2001;294:605–9.
6. Hayday AC. $\gamma\delta$-cells: a right time and a right place for a conserved third way of protection. Annu Rev Immunol 2000;18:975–1026.
7. Mattarollo SR, Kenna T, Nieda M, Nicol AJ. Chemotherapy and zoledronate sensitize solid tumour cells to $V_\gamma9\delta2$ T cell cytotoxicity. Cancer Immunol Immunother 2007;56:1285–97.
8. Coleman R, Grant M, Morgan G, Clezardin P. Effects of bone-targeted agents on cancer progression and mortality. J Natl Cancer Inst 2012;104:1039–67.
9. Grant M, Milneritsch B, Schipppinger W, et al. Endocrine therapy plus zoledronic acid in premenopausal breast cancer. N Engl J Med 2009;360:679–91.
10. Coleman R, Powles T, Paterson A, et al. Adjutant bisphosphonate treatment in early breast cancer: meta-analyses of individual patient data from randomised trials. Lancet 2015;386:1353–61.
11. Shmeeda H, Amitay Y, Gorin J, et al. Delivery of zoledronic acid encapsulated in folate-targeted liposomes results in potent in vitro cytotoxic activity on tumor cells. J Control Release 2010;146:76–83.
12. Shmeeda H, Amitay Y, Tzemach D, et al. Liposome encapsulation of zoledronic acid results in major changes in tissue distribution and increase in toxicity. J Control Release 2013;167:265–75.
13. Parente-Pereira AC, Shmeeda H, Whilding LM, et al. Adoptive immunotherapy of epithelial ovarian cancer with $V_\gamma9\delta2$ T cells, potentiated by liposomal alendronic acid. J Immunol 2014;193:5557–66.
14. Silverman L, Barenholz Y. In vitro experiments showing enhanced release of doxorubicin from Doxil® in the presence of ammonia may explain drug release at tumor site. Nanomedicine 2015;11:1841–50.
15. Gabizon A, Amitay Y, Tzemach D, et al. Therapeutic efficacy of a lipid-based prodrug of mitomycin C in pegylated liposomes: studies with human gastro-entero-pancreatic ectopic tumor models. J Control Release 2012;160:245–53.
16. Bandak S, Ramu A, Barenholz Y, Gabizon A. Reduced UV-induced degradation of doxorubicin encapsulated in polyethylene glycol-coated liposomes. Pharm Res 1999;16:841–6.
17. Chan FK, Moriwaki K, De Rosa MJ. Detection of necrosis by release of lactate dehydrogenase activity. Methods Mol Biol 2013;979:65–70.
18. Gabizon A, Horowitz AT, Goren D, et al. In vivo fate of folate-targeted polyethylene-glycol liposomes in tumor-bearing mice. Clin Cancer Res 2003;9:6651–9.
19. Kohro T, Tanaka T, Murakami T, et al. A comparison of differences in the gene expression profiles of phorbol 12-myristate
13-acetate differentiated THP-1 cells and human monocyte-derived macrophage. J Atheroscler Thromb 2004;11:88–97.

20. Gabizon AA, Tzemach D, Horowitz AT, et al. Reduced toxicity and superior therapeutic activity of a mitomycin C lipid-based prodrug incorporated in pegylated liposomes. Clin Cancer Res 2006;12:1913–20.

21. Marra M, Salzano G, Leonetti C, et al. Nanotechnologies to use bisphosphonates as potent anticancer agents: the effects of zoledronic acid encapsulated into liposomes. Nanomedicine 2011;7:955–64.

22. Gutman D, Epstein-Barash H, Tsuriel M, Golomb G. Alendronate liposomes for antitumor therapy: activation of γδ T cells and inhibition of tumor growth. Adv Exp Med Biol 2012;733:165–79.

23. Gutman D, Golomb G. Liposomal alendronate for the treatment of restenosis. J Control Release 2012;161:619–27.

24. Thamake SI, Raut SL, Gryczynski Z, et al. Alendronate coated poly-lactic-co-glycolic acid (PLGA) nanoparticles for active targeting of metastatic breast cancer. Biomaterials 2012;33:7164–73.

25. Bonzi G, Salmaso S, Scomparin A, et al. Novel pullulan bioconjugate for selective breast cancer bone metastases treatment. Bioconjug Chem 2015;26:489–501.

26. Haran G, Cohen R, Bar LK, Barenholz Y. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochim Biophys Acta 1993;1151:201–15.

27. Clezardin P, Ebetino FH, Fournier PG. Bisphosphonates and cancer-induced bone disease: beyond their antiresorptive activity. Cancer Res 2005;65:4971–4.

28. Ottewell PD, Brown HK, Jones M, et al. Combination therapy inhibits development and progression of mammary tumours in immunocompetent mice. Breast Cancer Res Treat 2012;133:523–36.

29. Schilt Y, Berman T, Wei X, et al. Using solution X-ray scattering to determine the high-resolution structure and morphology of PEGylated liposomal doxorubicin nanodrugs. Biochim Biophys Acta 2016;1860:108–19.

30. Maeda H, Nakamura H, Fang J. The EPR effect for macromolecular drug delivery to solid tumors: Improvement of tumor uptake, lowering of systemic toxicity, and distinct tumor imaging in vivo. Adv Drug Deliv Rev 2013;65:71–9.

31. Yamaguchi K, Motegi K, Iwakura Y, Endo Y. Involvement of interleukin-1 in the inflammatory actions of aminobisphosphonates in mice. Br J Pharmacol 2000;130:1646–54.

32. Norton JT, Hayashi T, Crain B, et al. Cutting edge: nitrogen bisphosphonate-induced inflammation is dependent upon mast cells and IL-1. J Immunol 2012;188:2977–80.

33. Szebeni J. Complement activation-related pseudoallergy: a stress reaction in blood triggered by nanomedicines and biologicals. Mol Immunol 2014;61:163–73.

34. Szebeni J, Muggia F, Gabizon A, Barenholz Y. Activation of complement by therapeutic liposomes and other lipid excipient-based therapeutic products: prediction and prevention. Adv Drug Deliv Rev 2011;63:1020–30.

35. Rogers TL, Wind N, Hughes R, et al. Macrophages as potential targets for zoledronic acid outside the skeleton-evidence from in vitro and in vivo models. Cell Oncol (Dordr) 2013;36:505–14.

36. Sabnani MK, Rajan R, Rowland B, et al. Liposome promotion of tumor growth is associated with angiogenesis and inhibition of antitumor immune responses. Nanomedicine 2015;11:259–62.

37. Rajan R, Sabnani MK, Wood LM et al. Pegylated liposomal alendronate: The impact of the drug cargo on carrier-induced immune modulation. In AACR Annual Meeting 2016; Abstract # 4008.

38. Benzaid I, Monkkonen H, Stresing V, et al. High phosphoantigen levels in bisphosphonate-treated human breast tumors promote Vγ9Vδ2 T-cell chemotaxis and cytotoxicity in vivo. Cancer Res 2011;71:4562–72.

39. Clezardin P. Bisphosphonates’ antitumor activity: an unravalled side of a multifaceted drug class. Bone 2011;48:71–9.

40. Clezardin P. Potential anticancer properties of bisphosphonates: insights from preclinical studies. Anticancer Agents Med Chem 2012;12:102–13.

41. Clezardin P. Mechanisms of action of bisphosphonates in oncology: a scientific concept evolving from antiresorptive to anticancer activities. Bonekey Rep 2013;2:267.

42. Gabizon AA, Shmeeda H, Zalipsky S. Pros and cons of the liposome platform in cancer drug targeting. J Liposome Res 2006;16:175–83.