International Journal of Pharmaceutics 403 (2011) 292–297

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Self-assembly nanoparticles for the delivery of bisphosphonates into tumors

G. Salzano[a], M. Marra[b], M. Porru[c], S. Zappavigna[b], A. Abbruzzese[b], M.I. La Rotonda[a], C. Leonetti[c], M. Caraglia[b], G. De Rosa[a,*]

[a] Department of Pharmaceutical and Toxicological Chemistry, Università degli Studi di Napoli Federico II, Via Montesano, 49, 80131 Naples, Italy
[b] Department of Biochemistry and Biophysics, Seconda Università degli Studi di Napoli, Via Costantinopoli, 16 80138 Naples, Italy
[c] Experimental Chemotherapy Laboratory, Regina Elena Cancer Institute, Via delle Messi d'oro, 156, 00158 Rome, Italy

ARTICLE INFO

Article history:
Received 9 August 2010
Received in revised form 20 October 2010
Accepted 26 October 2010
Available online 3 November 2010

Keywords:
Zoledronic acid
Self-assembly nanoparticles
Calcium phosphate
Cationic liposomes

ABSTRACT

Bisphosphonates (BPs) are molecules able to induce apoptosis in several cancer cell lines. However, their short half-life and the rapid uptake and accumulation within bone, limit its use as antitumor agent for extra-skeletal malignancies. Here we proposed a new delivery system to avoid BP accumulation into the bone, thus improving extra-skeletal bioavailability. In this work, we used the zoledronic acid (ZOL), a third generation bisphosphonate, able to induce apoptosis at micromolar concentration. We developed ZOL-containing self-assembly PEGylated nanoparticles (NPs) based on ZOL complexes with calcium phosphate NPs (CaPZ NPs) and cationic liposomes. PEGylation was achieved by two different strategies. CaPZ NPs were covered with PEGylated liposomes (pre-PLCaPZ NPs); alternatively, CaPZ NPs were previously mixed with cationic liposomes and then PEGylated by post-insertion method (post-PLCaPZ NPs). The NPs were fully characterized in terms of mean diameter and size distribution, morphology, ZOL loading, antiproliferative effect on different cell lines. Pre-PLCaPZ NPs showed the best technological characteristics, with a narrow size distribution and a high ZOL loading. Moreover, on different cancer cell lines, these NPs enhanced the antiproliferative effect of ZOL. Finally, in an animal model of prostate cancer, a significant reduction of tumor growth was achieved with pre-PLCaPZ NPs, while the tumor was unaffected by ZOL in solution.

1. Introduction

Bisphosphonates (BPs) are the most potent inhibitor of bone resorption and represent the treatment of choice for different diseases, such as osteoporosis, Paget's disease and bone metastases. In cancer treatment, their role in metastatic bone disease is well established, but there is increasing interest in their potential role in preventing cancer-induced bone loss and their possible direct anti-tumor effects (Caraglia et al., 2005). Increasing evidence is accumulating that BPs are able to directly affect tumor cells, in addition to their effects upon osteoclasts. The potency of their antitumor effects *in vitro* generally mirrors that one of the anti-resorptive ability with amino-BPs (NBPs); in particular, zoledronic acid (ZOL) is the most potent in both respects (Brown et al., 2004). NBPs induce apoptosis of tumor cells and inhibit tumor cell growth, *in vitro*, of a variety of tumor cell types, including breast (Senaratne et al., 2000), prostate (Lee et al., 2001), melanoma (Riebeling et al., 2002), osteosarcoma (Mackie et al., 2001; Sonnemann et al., 2001) and myeloma (Shipman et al., 1997) tumor cells. However, one of the most important limits of ZOL and NBPs in general, which makes their direct anti-cancer activity difficult to demonstrate *in vivo*, is their pharmacokinetic profile. In fact, ZOL is rapidly eliminated from plasma upon intravenous administration, due to renal excretion and rapid uptake and accumulation within bone (about 55% of the administered dose) (Caraglia et al., 2010). This disadvantageous pharmacokinetic profile strongly hampers the achievement of tumoricidal concentration of ZOL in non-skeletal tissues in which tumor can be usually localized. On the other hand, the use of high ZOL dose is certainly questionable, for the risks of side effects, such as osteonecrosis of the jaws, already observed at the concentrations used in the clinical practice (Caraglia et al., 2010; Scheper et al., 2009).

Previously, we demonstrated the direct antitumor activity of ZOL (data submitted for publication). In particular, in different animal models, a significant reduction of tumor growth and mice survival were found by using ZOL-encapsulating stealth liposomes; in the same experiments, any anti-cancer effect was found upon animal treatment with free ZOL.

In this paper, we described the development of a new delivery system for BPs, consisting in self-assembly PEGylated nanoparticles (NPs) based on calcium/phosphate NPs and cationic liposomes. The preparation conditions were optimized in order to achieve NPs easily prepared before use, with colloidal dimensions and high ZOL loading. Studies on different cancer cell lines allowed to com-

* Corresponding author. Tel.: +39 081 678 666; fax: +39 081 678 630.
E-mail address: gderosa@unina.it (G. De Rosa).

0378-5173/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2010.10.046

pare the anti-proliferative effect of ZOL-containing self-assembly NPs, compared with free ZOL. Finally, the *in vivo* efficacy of ZOL entrapped into the selected formulation, compared to free ZOL, was investigated on human prostate cancer xenografts.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-3-trimethylammonium-propane chloride (DOTAP) and 1,2-diacyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}) were kindly offered from Lipoid GmbH (Cam, Switzerland). Cholesterol (chol), tetrabutylammonium bromide (TBA), calcium chloride, sodium phosphate dibasic, potassium chloride, sodium chloride, were obtained from Sigma Chemical Co. (St. Louis Mo, USA). Zoledronic acid (ZOL) was kindly provided from Novartis (Novartis, Basel, Switzerland). Acetonitrile (HPLC grade) and analytical grade of methanol and chloroform were purchased from Carlo Erba (Milano, Italy). Culture media, bovine serum albumin (BSA) and foetal bovine serum (FBS) were purchased from Flow Laboratories (Milan, Italy).

2.2. Preparation of cationic liposomes

Liposomes consisting of DOTAP/chol (1:1 weight ratio) or DOTAP/chol/DSPE-PEG_{2000} (1:1:0.5 or 1:1:1 weight ratio) were prepared by hydration of a thin lipid film followed by extrusion. Briefly, the lipid mixture were dissolved in 1 ml of a mixture chloroform/methanol (2:1, v/v), the resulting solution was added to a 50 ml round-bottom flask, and the solvent was removed under reduced pressure by a rotary evaporator (Laborota 4010 digital, Heidolph, Schwabach, Germany) under nitrogen atmosphere. Then, the lipid film was hydrated with 1 ml of sterile water and the resulting suspension was gently mixed in the presence of glass beads until the lipid layer was removed from the glass wall, after that the flask was left at room temperature for still 2 h. The liposome suspension was then extruded using a thermobarrel extruder system (Northern Lipids Inc., Vancouver, BC, Canada) passing repeatedly the suspension under nitrogen through polycarbonate membranes with decreasing pore sizes from 400 to 100 nm (Nucleopore Track Membrane 25 mm, Whatman, Brentford, UK). After preparation, liposomes were stored at 4 °C. Each formulation was prepared in triplicate.

2.3. Preparation of calcium phosphate nanoparticles and their complexes with ZOL

CaP NPs were prepared as follows. An aqueous solution of calcium chloridre (18 mM) was added, dropwise and under magnetic stirring, to an aqueous solution on dibasic hydrogen phosphate (10.8 mM). The pH of both solutions was adjusted beforehand to 9.5 with NaOH. CaP NPs were obtained by filtration of the suspension through a 0.22 μm polycarbonate filter (MF-Millipore, Microglass Heim, Italy). CaP NPs were stored at 4 °C before use. CaP/ZOL-NPs complexes (CaPZ NPs) were prepared by mixing a CaP NPs dispersion with an aqueous solution of ZOL at different ZOL concentrations (10 mg/ml of ZOL in water or 50 mg/ml of ZOL in phosphate buffer at pH 9.5), at a volume ratio of 50:1.

2.4. Preparation of lipid-based self-assembly NPs containing ZOL

Lipid-based self-assembly NPs containing ZOL were prepared by two different techniques. The first one consisted on the post-insertion method (Ishida et al., 1999; Perouzel et al., 2003). Briefly, 500 μl of CaPZ NPs were mixed with 500 μl of DOTAP/chol liposomes, at a final ZOL concentration 0.25 or 0.05 mg/ml suspension (LCaPZ NPs 0.25 and LCaPZ NPs 0.05, respectively). One milliliter of a LCaPZ NPs suspension was then mixed with 50 μl micellar dispersion of DSPE-PEG_{2000} (47 mg/ml) and then incubated at 50–60 °C for 10 min. The resulting suspension (post-PLCaPZ NPs) was then allowed to cool to room temperature before use. Alternatively, equal volumes of suspensions containing DOTAP/chol/$DSPEG_{2000}$ liposomes and CaPZ NPs, respectively, were mixed in a glass tube and the resulting dispersion was allowed to stand at room temperature for 10 min (pre-PLCapZ NPs). By the same methods, self-assembly PEGylated NPs without ZOL, namely pre-PLCaP NPs and post-PLCaP NPs, were also prepared. Each formulation was prepared in triplicate.

2.5. Size and zeta potential of CaP NPs, cationic liposomes, post- and pre-PLCaP NPs

The mean diameter of cationic liposomes and PLCaPZ NPs, were determined at 20 °C by photon correlation spectroscopy (PCS) (N5, Beckman Coulter, Miami, USA). Each sample was diluted in deionizer/0.22 μm filtered water and analyzed with detector at 90° angle. As measure of the particle size distribution, polydispersity index (PI) was used. For each batch, mean diameter and size distribution were the mean of three measures. For each formulation, the mean diameter and PI were calculated as the mean of three different batches.

The zeta-potential (ζ) of the NPs surface was measured in water by means of a Zetasizer Nano Z (Malvern, UK). Data of ζ were collected as the average of 20 measurements.

2.6. Morphology of LCaPZ, pre- and post-PLCaP NPs

Morphological analysis of LCaPZ, post- and pre-PLCaPZ NPs were investigated by cold field emission gun-scanning electron microscopy (cFEG-SEM), as reported by De Rosa et al. (2008). For cFEG-SEM analysis, samples were prefixed in a mix of 4% formaldehyde and 1% glutaraldehyde in distilled water for 1 h. Then, samples were rinsed in distilled water by ultracentrifugation (80,000 rpm) and post-fixed by adding 1% OsO4 for 1 h. After a further washing with distilled water, pellets were filtered on a 0.1 μm polycarbonate filter (Nucleopore, Costar Corning, USA) in a Swinnex filtration apparatus (Millipore, USA). A second Nucleopore filter was placed over the first to form a sandwich in which liposomal pellets were trapped. Samples were then dehydrated in a graded alcohol series (10, 30 and 50% for 10 min, 70 and 80% for 30 min, 95% for 1 h and 100% overnight at 4 °C) and critically point dried. At the end of the treatment, the Swinnex was opened and both filters were placed on a stub cleaned with acetone to remove any grease. Double adhesive carbon disks (EMS, USA) were stuck onto the stub, and the filters containing the samples were placed over it. Finally, the stubs were sputter coated with a nanometric layer of gold. Observations were carried out by a cold cathode Field Emission Gun Scanning Electron Microscope (FEG Jeol 6700F, Jeol Ltd., Japan). The pictures of the NPs surface at higher magnifications were taken at 2–5 kV.

2.7. ZOL loading into CaPZ, pre- and post-PLCaP NPs

ZOL analysis was carried out by reverse phase high performance liquid chromatography (RP-HPLC). The HPLC system consisted of an isocratic pump (LC-10A VP, Shimadzu, Kyoto, Japan) equipped with a 7725i injection valve (Rheodyne, Cotati, USA), SPV-10A UV–Vis detector (Shimadzu) set at the wavelength of 220 nm. The system was controlled by a SCL-10A VP System Controller (Shimadzu) connected with a computer. Chromatograms were acquired and

analysed by a Class VP Client/Server 7.2.1 program (Shimadzu). The quantitative analysis of ZOL was performed on a Gemini 5 μm C_{18} column (250 mm × 4.60 mm, 110 Å Phenomenex, Klwid, USA) equipped with a security guard. The mobile phase was a mixture 20:80 (v/v) of acetonitrile and an aqueous solution (8 mM di-potassium hydrogen orthophosphate, 2 mM di-sodium hydrogen orthophosphate and 7 mM tetra-*n*-butyl ammonium hydrogen sulphate, adjusted at pH of 7.0 with sodium hydroxide). ZOL determination was performed in isocratic condition, at a flow rate of 1 ml/min at room temperature.

For CaPZ, post- and pre-PLCaPZ NPs the amount of uncomplexed ZOL was determined as follows: 1 ml of NPs dispersion was ultracentifugated (Optima Max E, Beckman Coulter, USA) at 80,000 rpm at 4 °C for 40 min. Supernatants were carefully removed and ZOL concentration was determined by RP-HPLC. The results have been expressed as complexation efficiency, calculated as

$$\frac{\text{theoretical ZOL}^* - \text{actual ZOL}^*}{\text{theoretical ZOL}^*} \times 100$$

where '*' referred to the ZOL concentration in the supernatant.

2.8. Cell culture and proliferation

The human multiple myeloma (MM) OPM2 cell line was kindly provided by Dr. Edward Thompson (University of Texas Medical Branch, Galveston, TX, USA). All the other cell lines were provided by ATCC and were grown in medium as suggested by ATCC in a humidified atmosphere of 95% air/5% CO_2 at 37 °C.

Proliferation of human cancer cell lines was performed in the presence of ZOL or ZOL loaded into pre- and post-PLCaP NPs by MTT assay as previously described (Caraglia et al., 1999).

2.9. In vivo experiments

CD-1 male nude (nu/nu) mice, 6–8 weeks old and weighing 22–24 g were purchased from Charles River Laboratories (Calco, Italy). All procedures involving animals and their care were conducted in conformity with the institutional guidelines, which are in compliance with national (D.L. No. 116, G.U., Suppl. 40, Feb. 18, 1992; Circolare No. 8, G.U., July 1994) and international laws (EEC Council Directive 86/609, OJ L 358. 1, Dec 12, 1987; Guide for the Care and Use of Laboratory Animals, United States National Research Council, 1996).

To compare the antitumor efficacy of free ZOL with ZOL-encapsulating NPs, immunosuppressed mice were injected intramuscularly (i.m.) into the hind leg muscles of mice at 5×10^6 cells/mouse. After 6 days (when a tumor mass of about 300 mg was evident) mice were randomized, divided in four groups and treatment started. The following groups were evaluated: (1) untreated; (2) blank NPs; (3) free ZOL; (4) ZOL-encapsulating NPs. Mice were treated intravenously (i.v.) with blank NPs or with 20 μg of free ZOL or ZOL-encapsulating NPs for three times a week for 3 consecutive weeks.

Tumor sizes were measured three times a week in two dimensions by a caliper and tumor weight was calculated using the following formula: $a \times b^2/2$, where a and b are the long and short diameter of the tumor, respectively. The following end-points were assessed: (a) percent tumor weight inhibition (TWI%), calculated as [1 − (mean tumor weight of treated mice/mean tumor weight of controls)] × 100; (b) tumor growth delay (TGD), evaluated as $T - C$, where T and C are the median times for treated and control tumors, respectively, to achieve equivalent size. Body weight was measured two times at week as control for treatment toxicity.

Table 1
Complexation efficiency of CaPZ, post-PLCaPZ and pre-PLCaPZ NPs, determined after ultracentrifugation of NPs and determination of ZOL concentration (ZOL actual concentration) into the supernatant. The complexation efficiency has been calculated as [(theoretical ZOL concentration − actual ZOL concentration)/theoretical ZOL concentration] × 100.

NP formulation	Final ZOL concentration (mg/ml)	Complexation efficiency (%±SD)
CaPZ NPs 0.2	0.2	100.0 ± 0.0
CaPZ NPs 1	1	69.0 ± 0.1
Post-PLCaPZ NPs	0.05	100.0 ± 0.0
Pre-PLCaPZ NPs	0.25	66.0 ± 1.0

2.10. Statistical analysis

The statistical difference of tumor weight among the different groups was determined by two-tailed Student's t-test assuming unequal variances. Differences were considered statistically significant when p was less than 0.05.

3. Results

In this study, we proposed a new self-assembly nanoparticulate system for the delivery of the NBP ZOL. In a preliminary phase of the work, we investigated ZOL behaviour in presence of cationic liposomes and we did not observe complex formation at different ZOL and cationic liposome concentrations (data not shown). Thus, ZOL was firstly complexed with CaP NPs at two different ZOL concentrations. In particular, ZOL was not found in the supernatant when complexed with CaP NPs at a final ZOL concentration of 0.2 mg/ml. At higher ZOL final concentrations, i.e. 1.0 mg ZOL/ml suspension, about 0.31 mg ZOL/ml was found in the supernatant, corresponding to a complexation efficiency of about 69% (Table 1). In the following step, we investigated the possibility to cover CaPZ NPs with PEG, by using two different approaches. In the first one, we mixed CaPZ NPs, prepared at concentration of 1.0 or 0.2 mg of ZOL/ml suspension, with cationic liposomes. The characteristics of these NPs (final ZOL concentration 0.25 or 0.05 mg/ml suspension) are reported in Table 2. The increase of ZOL concentration from 0.05 to 0.25 resulted in LCaPZ NPs with lower ζ (from about 38 to 24 mV), while the mean diameter and PI were not significantly affected. LCaPZ NPs were then PEGylated by mixing with micellar dispersion of DSPE-PEG_{2000} at a 20 mol%. In this case, when using LCaPZ NPs 0.25, large aggregates (mean diameter > micrometers) were observed. The use of LCaPZ NPs 0.05, resulted in post-PLCaPZ NPs with a mean diameter of about 309 nm with a broad size dis-

Table 2
Diameter, PI and zeta potential (ζ) of cationic liposomes, LCaPZ NPs, post- and pre-PLCaPZ NPs.

Formulation	Mean diameter (nm) ± SD	PI ± SD	ζ (mV) ± SD
DOTAP/Chol liposomes	166.6 ± 17.4	0.10 ± 0.03	47.4 ± 2.3
DOTAP/Chol/DSPE-PEG_{2000} liposomes	164.4 ± 21.0	0.11 ± 0.06	37.5 ± 1.5
LCaPZ NPs 0.05	181.7 ± 18.3	0.206 ± 0.06	38.2 ± 6.5
LCaPZ NPs 0.25	167.5 ± 16.9	0.186 ± 0.1	23.8 ± 2.4
Post-PLCaPZ NPs	309.1 ± 163.0	0.363 ± 0.2	10.7 ± 5.5
Pre-PLCaPZ NPs	147.5 ± 7.1	0.152 ± 0.06	17.5 ± 5.6

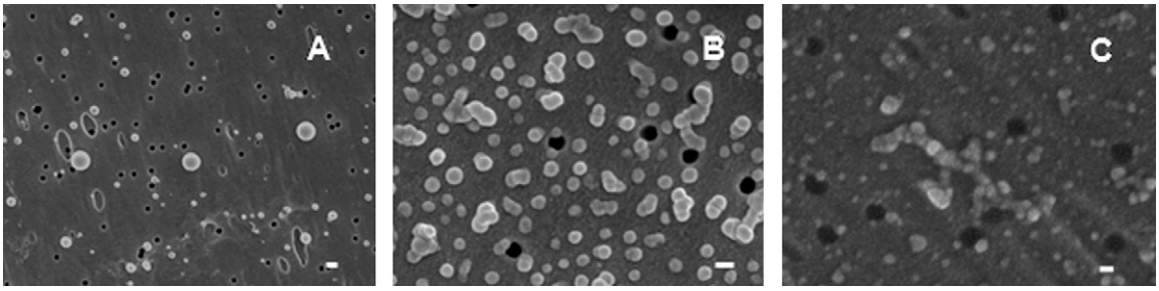

Fig. 1. Images from cFEG SEM analysis. (A) LCaPZ NPs, (B) Pre-PLCaPZ NPs, (C) Post-PLCaPZ NPs. Scale bar 100 nm.

tribution, with a PI of 0.36 (Table 2). Moreover, compared with the parent LCaPZ NPs, a reduction of the ζ from about 37 to about 10 mV was found, demonstrating a significant charge shielding. In alternative to the post-insertion method, PEGylated ZOL-containing NPs were prepared by mixing CaPZ NPs (ZOL concentration of 1 mg/ml) with DOTAP/chol/$DSPEG_{2000}$ cationic liposomes. In particular, when using cationic liposomes containing a $DSPE\text{-}PEG_{2000}$ at a molar ratio $\geq$10%, foaming of the suspension was observed and particles with a high size distribution (PI >1) were found. Thereafter, only liposomes containing 5% molar ratio of $DSPE\text{-}PEG_{2000}$ were used. These self-assembly NPs (pre-PLCaPZ NPs) were characterized by a mean diameter of about 147 nm with a very narrow size distribution (PI <0.2), while ζ was about 17.5 mV (Table 2). We also investigated morphological characteristics of LCaPZ NPs, pre- and post-PLCaPZ NPs by cFEG-SEM analysis (Fig. 1). In the case of LCaPZ NPs, regularly shaped NPs with a smooth surface were observed (Fig. 1A). On the other hand, the analysis of the formulation containing pre-PLCaPZ NPs showed irregularly shaped particles with homogeneous size distribution and a rather rough surface (Fig. 1B). The use of the post-insertion method, dramatically changed particles characteristics, as observed in Fig. 1C, in which the formulation appears formed by small particles mixed with large aggregates.

The amount of ZOL entrapped in the different self-assembly NPs was investigated and the results are reported in Table 1. In the case, of pre-PLCaPZ NPs, prepared at a final ZOL concentration of 0.25 mg/ml of suspension, about 66% of the total ZOL was entrapped into the particles. On the other hand, NPs prepared by post-insertion method, in which ZOL was used at 5-fold lower concentration, approximately 100% of the drug was encapsulated into the NPs.

Moreover, we studied the effect of increasing concentrations of ZOL encapsulated in pre- and post-PLCaP NPs on growth inhibition of different human cancer cell lines: prostate (PC3 and DU145), breast (MCF7, MDA-MB468, CG-5), lung (H1355), pancreas (BXPC3, PANC-1 and MIA PaCa), head/neck (KB) and multiple myeloma (RPMI, DOX, KMS, OPM2). For the analysis of the effects on cell growth we have used a MTT assay. In Table 3, the IC_{50} (50% inhibitory concentration) values of ZOL, free or encapsulated in pre- or post-PLCaP NPs, in the different cell lines analysed after 72 h, are reported. In all cases, when considering blank NPs, the cytotoxicity of post-PLCaP NPs was significantly higher than that observed for pre-PLCaP NPs. Moreover, when using ZOL-encapsulating NPs, we found a potentiation factor (PF) >1.0 in all tested cell lines, if compared with the free ZOL. The two tested formulations showed different efficiency to deliver ZOL to the cells, and this effect was strictly dependent on the cell line (Table 3), with the highest cell growth inhibition obtained on breast cancer cells. In particular, on MCF7, pre-PLCaPZ NPs caused an about 12-fold potentiation of ZOL-induced cytotoxicity, while a lower effect (about 8-fold) was found with post-PLCaP NPs.

Due to the good technological characteristic, together with a lower cytotoxicity and the highest potentiation of ZOL antiproliferative effect, pre-PLCaPZ NPs were used for further *in vivo* studies. Finally, we evaluated the *in vivo* efficacy of this formulation compared to free ZOL on human prostate cancer xenografts. To this purpose immunosuppressed mice were injected with PC-3

Table 3
IC_{50} values of ZOL, as free or encapsulated in PLCaP NPs in different cell lines. The concentrations are expressed in μM. The potentiation factor (PF) values (mean ± standard deviation from at least three separate experiments performed in quadruplicates) define the specific contribute of ZOL-containing PLCaP NPs evaluated as the ratio of the IC_{50} of free ZOL to the IC_{50} of encapsulated ZOL. In the case of blank pre- and post-PLCaP NPs, IC_{50} is referred as the theoretical ZOL concentration leading to 50% of cell dead.

Cell lines	Free ZOL	Post-PLCaPZ NPs	Blank post-PLCaP NPs	PF	Pre-PLCaPZ NPs	Blank pre-PLCaP NPs	PF
Prostate							
PC3	12.5	2.2	23	5.7	3.6	57.4	3.5
DU145	25	7.4	42	3.4	11.6	120	2.2
Breast							
MCF7	120	14.7	33.3	8.1	10.1	120	11.8
MDA-MB468	28.4	13	65	2.2	11.3	120	2.5
CG5	74	7.5	30	9.9	16	120	4.6
Head/neck							
KB	22.8	5.6	19.4	4.07	11.1	120	2
Lung							
H1355	65	11	35	5.9	9.5	86	6.8
Pancreas							
Panc	32	12.7	44.5	1.4	18.5	84.8	1.6
BX-PC3	12.3	11.8	44.6	1.04	9	22	1.4
MIA PaCa	55	16.8	46	3.3	42	120	1.3
Multiple myeloma							
RPMI	120	15.5	66	7.7	90	120	1.3
DOX	41.9	35	42	1.2	13.8	120	3
KMS	30	2.3	11.8	13	8	44.6	3.8
OPM2	83	35	69.7	2.4	33	95	2.5

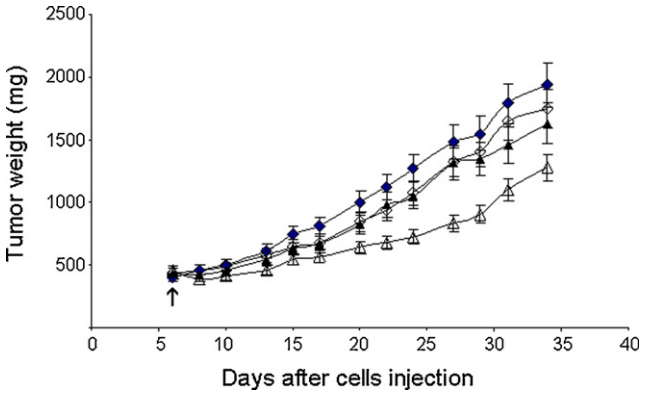

Fig. 2. Encapsulation in NPs increases antitumoral efficacy of ZOL against human prostate PC3 xenografts. Mice were injected i.m. with PC3 cells and starting from day 6 treated as follows: (♦) untreated; (◊) blank NPs; (▲) free ZOL; (△) ZOL encapsulating NPs. Points are means with SD (bars). Arrow indicates the start of treatment.

cells and starting from day 6 treated with free ZOL, pre-PLCaPZ NPs or with blank pre-PLCaP NPs three times a week for 3 consecutive weeks. As reported, in the Fig. 2, the administration of free ZOL was not effective in reducing the growth of tumors producing a maximal tumor weight inhibition of about 15% and the tumor mass was not significantly different ($p > 0.05$) compared to mice treated with blank pre-PLCaP NPs and untreated groups. On the contrary, the treatment of mice with pre-PLCaPZ NPs elicited a marked antitumor activity achieving an about 45% tumor weight inhibition. Moreover, the tumor mass of mice treated with this schedule, as evaluated at the nadir of the effect, was significantly reduced compared to untreated group ($p = 0.001$) and to mice treated with free ZOL or with blank NPs, respectively ($p = 0.041$ and 0.01, respectively). Moreover, the marked inhibition of tumor weight was accompanied by a tumor growth delay of 10 days significantly increased compared to untreated group ($p = 0.000$), to free ZOL (5 days, $p = 0.004$) and to blank NPs-treated group (5 days, $p = 0.009$).

Finally, it is interesting to note that all the treatments were well tolerated by animals since no body weight loss and toxic deaths have been observed.

4. Discussion

NBPs are synthetic analogs of the pyrophosphate, currently used in the clinical practice to inhibit bone resorption in different osteopenic diseases, such as bone metastases (Caraglia et al., 2005; Marra et al., 2009). ZOL is the most potent NBP, able to induce inhibition of the cell growth at very low concentrations (Caraglia et al., 2005; Santini et al., 2006). Many authors hypothesize that a direct anti-tumor activity should be responsible for the positive effect of ZOL on bone metastases. However, the direct anti-tumor effect of ZOL is still far to be demonstrated in the clinical setting even if preliminary data come from adjuvant treatment of early breast cancer (Gnant et al., 2009). The lack of clear evidence of ZOL-induced anti-cancer effects is likely due to its unfavourable pharmacokinetic profile. In fact, it accumulates almost exclusively in the bone and have a short serum half-life (only 15 min) not reaching active anti-tumor concentrations (Caraglia et al., 2010). In order to lower the anti-cancer active concentrations of ZOL, we have previously shown that ZOL anti-cancer activity can be potentiated both *in vitro* and *in vivo* on prostate and head and neck cancer cell lines through its combination with a farnesyltransferase inhibitor and that this effect is actually mediated by ras activity inhibition (Caraglia et al., 2007, 2004). An alternative way to potentiate anticancer properties of ZOL is its encapsulation in PEGylated liposomes in order to both improve its serum half-life escaping from bone tissue and to allow its diffusion through fenestrated vessels of neo-angiogenic vasculature of tumors. In a previous work, we have demonstrated that ZOL encapsulated in liposomes is efficient in inducing cancer cell growth inhibition both *in vitro* and *in vivo* and that liposomes encapsulating ZOL activity is much higher than that one of free ZOL (data submitted for publication). In the present work, we designed a new carrier to deliver ZOL to tumors. In particular, we developed self-assembly NPs, that can be easily prepared by mixing cationic liposomes, CaP NPs and ZOL, before use. CaP NPs have already been used by different authors to deliver nucleic acids (Sokolovaa et al., 2006; Pedraza et al., 2008), proteins (Gorbunoff, 1984a,b; Spenlehauer et al., 1989), anti-cancer drugs (Barroug et al., 2004) and antigens (He et al., 2000). The use of calcium phosphate ceramics offer advantages such, low cost, biocompatibility and non-toxic degradation product (Cheng and Kuhn, 2007). Here, CaP NPs were mixed with a ZOL aqueous solution to obtain a suspension containing CaPZ NPs. As expected, the yield of complexation depended on ZOL concentration. Then, in order to confer long circulating properties, CaP NPs were covered with PEG chains by mixing with PEGylated cationic liposomes or, alternatively, CaPZ NPs were mixed with cationic liposomes and then incubated with DSPE-PEG_{2000} micelles (post-insertion method). This last approach should allow to modify only the outer surface of the carrier (Uster et al., 1996; Li and Huang, 2009). Post-PLCaPZ NPs were only obtained when starting from LCaPZ NPs prepared at the lowest ZOL concentration (10 mg/ml). The very high surface charge of LCaPZ NPs (ζ of 38 mV) prepared at the lowest ZOL concentration, should prevent aggregation in presence of micelles when using the post-insertion method. When LCaP NPs were complexed with ZOL at a final drug concentration of 0.25 mg/ml, about 34% of the total drug remained uncomplexed in the aqueous medium, with consequent shielding of the highly positive surface charge of NPs. NPs prepared by post-insertion method were also characterized by a more heterogeneous size distribution, probably due to coexistence of two populations, namely DSPE-PEG_{2000} micelles and post-PLCaPZ NPs. This hypothesis was supported by FEG-SEM analysis in which two different populations were observed. The second approach used to self-assemble ZOL containing PEGylated NPs, consisted on the mixing of the negatively charged CaPZ NPs with PEGylated cationic liposomes. This method resulted in monodispersed NPs (PI <0.2) with a mean diameter of about 150 nm. Once more, FEG-SEM analysis confirmed these findings showing an homogeneous NP formulation.

Cell culture studies showed a potentiation of the cytotoxic effect of ZOL, when delivered by either pre- or post-PLCaPZ NPs. It is noteworthy that the maximal potentiation was observed in human breast cancer cell lines. In fact, ZOL is presently indicated in the treatment of bone metastases from breast cancer and for the prevention of the skeletal related events. Moreover, the first clinical evidence of a direct anti-tumor effect of ZOL comes specifically from the treatment of early breast cancer in the adjuvant setting (Gnant et al., 2009). The molecular targets of ZOL has been identified as critical enzymes of the mevalonate pathway, e.g. farnesyl diphosphate synthase, with an intracellular localization (Van Beek et al., 1999a,b). The increased cytotoxic effect of ZOL could be reasonably explained with a higher ZOL penetration into the cells, mediated by NPs. It is noteworthy that pre- and post-PLCaPZ NPs showed a different cytotoxicity. The cytotoxicity of cationic lipids is well documented (Lv et al., 2006), thus the development of a pharmaceutical formulation containing these additives should be used at very low concentrations. In this study, post-PLCaPZ NPs were characterized by a lower ZOL/cationic lipid ratio, compared with pre-PLCaPZ NPs. Thus, at the same ZOL concentration, a higher amount of cationic lipid was used, resulting in a higher cell toxicity. Moreover, the coexistence of DSPE-PEG_{2000} micelles together with post-PLCaPZ NPs, should contribute to the cytotoxicity of this formulation.

Finally, the therapeutic potential of pre-PLCaPZ NPs was confirmed in a human prostate cancer xenograft animal model of tumor. We found that, when treated with free ZOL, the inhibition of tumor growth is not significantly different than the control, confirming that ZOL cannot be used as a therapeutic weapon in prostate cancer. On the contrary, a strong reduction of the tumor growth was observed in animals treated with pre-PLCaPZ NPs. Even if a direct bio-distribution study was not performed in the present work, these findings suggest that ZOL loaded into the NPs reaches active concentrations in the tumor tissue. These results encourage further studies to investigate the antitumor activity of ZOL, delivered by pre-PLCaPZ NPs, in other animal models of tumor.

5. Conclusions

We have developed a new self-assembly NP formulation for ZOL delivery. The possibility to prepare NPs before preparation should overcome issues due to drug leakage during storage. Therefore, the formulation here named as pre-PLCaPZ NPs offers good technological characteristics, in terms of homogeneous size distribution and high drug loading. In addition, pre-PLCaPZ NPs assured a strong potentiation of the ZOL toxicity *in vitro* and *in vivo*. Pre-PLCaPZ NPs could be a valid alternative to PEGylated liposomes to deliver ZOL to solid tumors.

Acknowledgments

The authors wish to thank Franco and Gennaro Iamunno for electron microscopy technical assistance. MC and AA received a grant from “Regione Campania”-Laboratori Pubblici “Hauteville”. AA received a grant from MIUR-PRIN 2008. CL received a grant from Italian Association for Cancer Research (AIRC) and Ministero della Salute. MP is recipient of fellowships from the Italian Foundation for Cancer Research (FIRC).

References

Barroug, A., Kuhn, L.T., Gerstenfeld, L.C., Glimcher, M.J., 2004. Interactions of cisplatin with calcium phosphate nanoparticles: in vitro controlled adsorption and release. J. Orthop. Res. 22, 703–708.

Brown, J.E., Webbe, H.N., Coleman, R.E., 2004. The role of bisphosphonates in breast and prostate cancers. Endocr. Relat. Cancer 11, 207–224.

Caraglia, M., Abbruzzese, A., Leardi, A., Pepe, S., Budillon, A., Baldassare, G., Selleri, C., Lorenzo, S.D., Fabbrocini, A., Giuberti, G., Vitale, G., Lupoli, G., Bianco, A.R., Tagliaferri, P., 1999. Interferon-alpha induces apoptosis in human KB cells through a stress-dependent mitogen activated protein kinase pathway that is antagonized by epidermal growth factor. Cell Death Differ. 6, 773–780.

Caraglia, M., D'Alessandro, A.M., Marra, M., Giuberti, G., Vitale, G., Viscomi, C., Colao, A., Prete, S.D., Tagliaferri, P., Tassone, P., Budillon, A., Venuta, S., Abbruzzese, A., 2004. The farnesyl transferase inhibitor R115777 (Zarnestra) synergistically enhances growth inhibition and apoptosis induced on epidermoid cancer cells by Zoledronic acid (Zometa) and Pamidronate. Oncogene 23, 6900–6913.

Caraglia, M., Santini, D., Marra, M., Vincenzi, B., Tonini, G., Budillon, A., 2005. Emerging anti-cancer molecular mechanisms of aminobisphosphonates. Endocr. Relat. Cancer 13, 1–22.

Caraglia, M., Marra, M., Leonetti, C., Meo, G., D'Alessandro, A.M., Baldi, A., Santini, D., Tonini, G., Bertieri, R., Zupi, G., Budillon, A., Abbruzzese, A., 2007. R115777 (Zarnestra)/Zoledronic acid (Zometa) cooperation on inhibition of prostate cancer proliferation is paralleled by Erk/Akt inactivation and reduced Bcl-2 and bad phosphorylation. J. Cell Physiol. 211, 533–543.

Caraglia, M., Marra, M., Naviglio, S., Botti, G., Addeo, R., Abbruzzese, A., 2010. Zoledronic acid: an unending tale for an antiresorptive agent. Expert Opin. Pharmacother. 11, 141–154.

Cheng, X., Kuhn, L., 2007. Chemotherapy drug delivery from calcium phosphate nanoparticles. Int. J. Nanomed. 2, 667–674.

De Rosa, G., De Stefano, M., Ungaro, F., La Rotonda, M.I., 2008. Cold field emission gun-scanning electron microscopy: a new tool for morphological and ultrastructural analysis of liposomes. Int. J. Pharm. 362, 189–192.

Gnant, M., Mlineritsch, B., Schippinger, W., Luschin-Ebengreuth, G., Pöstlberger, S., Menzel, C., Jakesz, R., Seifert, M., Hubalek, M., Bjelic-Radisic, V., Samonigg, H., Tausch, C., Eidtmann, H., Steger, G., Kwasny, W., Dubsky, P., Fridrik, M., Fitzal, F., Stierer, M., Rücklinger, E., Greil, R., 2009. Endocrine therapy plus zoledronic acid in premenopausal breast cancer. N. Engl. J. Med. 360, 679–691.

Gorbunoff, M.J., 1984a. The interaction of proteins with hydroxyapatite. I. Role of protein charge and structure. Anal. Biochem. 136, 425–432.

Gorbunoff, M.J., 1984b. The interaction of proteins with hydroxyapatite. II. Role of acidic and basic groups. Anal. Biochem. 136, 433–439.

He, Q., Mitchell, A.R., Johnson, S.L., Wagner-Bartak, C., Morcol, T., Bell, S.J., 2000. Calcium phosphate nanoparticle adjuvant. Clin. Diagn. Lab. Immunol. 7, 899–903.

Ishida, T., Iden, D.L., Allen, T.M., 1999. A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs. FEBS Lett. 460, 129–133.

Lee, M.V., Fong, E.M., Singer, F.R., Guenette, R.S., 2001. Bisphosphonate treatment inhibits the growth of prostate cancer cells. Cancer Res. 6, 2602–2608.

Li, S.D., Huang, L., 2009. Nanoparticles evading the reticuloendothelial system: role of the supported bilayer. Biochim. Biophys. Acta 1788, 2259–2266.

Lv, H., Zhang, S., Wang, B., Cui, S., Yan, J., 2006. Toxicity of cationic lipids and cationic polymers in gene delivery. J. Control. Release 114, 100–109.

Mackie, P.S., Fisher, J.L., Zhou, H., Choong, P.F., 2001. Bisphosphonates regulate cell growth and gene expression in the UMR 106-101 clonal rat osteosarcoma cell line. Br. J. Cancer 84, 951–958.

Marra, M., Abbruzzese, A., Addeo, R., Del Prete, S., Tassone, P., Tonini, G., Tagliaferri, P., Santini, D., Caraglia, M., 2009. Cutting the limits of aminobisphosphonates: new strategies for the potentiation of their anti-tumour effects. Curr. Cancer Drug Targets 9, 791–800.

Pedraza, C.E., Bassett, D.C., McKee, M.D., Nelea, V., Gbureck, U., Barralet, J.E., 2008. The importance of particle size and DNA condensation salt for calcium phosphate nanoparticle transfection. Biomaterials 29, 3384–3392.

Perouzel, E., Jorgensen, M.R., Keller, M., Miller, A.D., 2003. Synthesis and formulation of neoglycolipids for the functionalization of liposomes and lipoplexes. Bioconjugate Chem. 14, 884–898.

Riebeling, C., Forsea, A.M., Raisova, M., Orfanos, C.E., Geilen, C.C., 2002. The bisphosphonate pamidronate induces apoptosis in human melanoma cells in vitro. Br. J. Cancer 87, 366–371.

Santini, D., Caraglia, M., Vincenzi, B., Holen, I., Scarpa, S., Budillon, A., Tonini, G., 2006. Mechanisms of disease: preclinical reports of antineoplastic synergistic action of bisphosphonates. Nat. Clin. Pract. Oncol. 3, 325–338.

Scheper, M.A., Badros, A., Chaisuparat, R., Cullen, K.J., Meiller, T.F., 2009. Effect of zoledronic acid on oral fibroblasts and epithelial cells: a potential mechanism of bisphosphonate-associated osteonecrosis. Br. J. Haematol. 144, 667–676.

Senaratne, S.G., Pirianov, G., Mansi, J.L., Arnett, T.R., Colston, K.W., 2000. Bisphosphonates induce apoptosis in human breast cancer cell lines. Br. J. Cancer 2, 1459–1468.

Shipman, C.M., Rogers, M.J., Apperley, J.F., Russell, R.G., Croucher, P.I., 1997. Bisphosphonates induce apoptosis in human myeloma cell lines: a novel anti-tumour activity. Br. J. Haematol. 98, 665–672.

Sonnemann, J., Eckervogt, V., Truckenbrod, B., Boos, J., Winkelmann, W., van Valen, F., 2001. The bisphosphonate pamidronate is a potent inhibitor of human osteosarcoma cell growth in vitro. Anticancer Drug 12, 459–465.

Sokolovaa, V.V., Radtkeb, I., Heumannb, R., Epple, M., 2006. Effective transfection of cells with multi-shell calcium phosphate-DNA nanoparticles. Biomaterials 27, 3147–3153.

Spenlehauer, G., Vert, M., Benoit, J.P., Boddaert, A., 1989. In vitro and in vivo degradation of poly(D,L lactide/glycolide) type microspheres made by solvent evaporation method. Biomaterials 10, 557–563.

Uster, P.S., Allen, T.M., Daniel, B.E., Mendez, C.J., Newman, M.S., Zhu, G.Z., 1996. Insertion of poly(ethylene glycol) derivatized phospholipid into pre-formed liposomes results in prolonged in vivo circulation time. FEBS Lett. 386, 243–246.

Van Beek, E., Pieterman, E., Cohen, L., Lowik, C., Papapoulos, S., 1999a. Nitrogen-containing bisphosphonates inhibit isopentenyl pyrophosphate isomerase/farnesyl pyrophosphate synthase activity with relative potencies corresponding to their antiresorptive potencies in vitro and in vivo. Biochem. Biophys. Res. Commun. 255, 491–494.

Van Beek, E., Pieterman, E., Cohen, L., Lowik, C., Papapoulos, S., 1999b. Farnesyl pyrophosphate synthase is the molecular target of nitrogen-containing bisphosphonates. Biochem. Biochem. Biophys. Res. Commun. 264, 108–111.