Stealth liposomes for the delivery of zoledronic acid into tumors enhance the anticancer activity of the drug

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Abstract

Zoledronic acid (ZOL) is a third generation aminobisphosphonate, commonly used for the treatment of bone metastases. Several studies have shown a direct *in vitro* antitumor activity of ZOL but a clear evidence of clinical activity is still lacking. Unfortunately, the use of ZOL as an anticancer agent in extraskeletal tissues is limited probably because it is rapidly removed from the blood and tends to accumulate in the bone. On these bases, we developed stealth liposomes encapsulating ZOL (Lipo-ZOL) to improve the pharmacokinetic profile of the drug. Compared to free ZOL, Lipo-ZOL induced a stronger inhibition of growth in two different cancer cell lines H460 and CG5. Moreover, Lipo-ZOL also significantly caused a larger inhibition of tumor growth and increased the overall survival in murine models of human lung and breast cancer, in comparison with free ZOL. These results suggest the use of Lipo-ZOL as a potential anticancer agent in patients with neoplastic disease.

Introduction

Zoledronic acid (ZOL) is a potent aminobiphosphonate able to inhibit bone resorption by inducing apoptosis in mature osteoclasts.[1] In addition to the well known effects on bone, several studies have demonstrated that ZOL induced apoptosis in various types of cancer by inhibiting the farnesyl pirophosphate synthase (FPPS), the upstream enzyme involved in the cholesterol synthesis and isoprenylation processes.[2-4] Prenylation of small GTPases, including Ras proteins, plays a key role for the correct localization to the inner surface of the cell membrane and the activation of signal transduction pathways involved in cell proliferation and transformation.[1]

Preclinical models of both solid[2,3] and haematological cancer[4] have demonstrated the *in vitro* antitumor activity of ZOL but no clear evidence of its clinical activity has been reported.[1] The most limiting factor of *in vivo* ZOL antitumor activity is its unfavorable pharmacokinetic profile.[5,6] In fact, it is not able to reach active concentrations in tumor site because it accumulates almost exclusively in the bone.[5,6] Nanotechnologies can help to overcome the limitations of ZOL pharmaco-distribution.[7-11] First generation liposomes have already been used to improve the pharmaco-distribution of aminobisphosphonates but the recognition and interaction with the immune system made these agents not suitable for use in anticancer therapy.[12,13] On the other hand, stealth liposomes (pegylated liposomes or second-generation liposomes) are able to evade the immune system and represent a new potential therapeutic agent.[7] They are characterized by favorable pharmacokinetic properties and specific accumulation in tumor tissues.[7] Previously, we developed stealth liposomes encapsulating ZOL (Lipo-ZOL) and self-assembling PEGylated nanoparticles (NPs) consisting of calcium/phosphate NPs and cationic liposomes encapsulating ZOL.[7-10] Both the developed delivery systems showed promising anticancer activity *in vitro* and *in vivo*.[7-10] In fact, we found that stealth Lipo-ZOL induced a significant tumor growth inhibition in different cancer models such as prostate cancer or multiple myeloma compared to free ZOL and improved ZOL pharmacokinetics.[7-10] In particular, LIPOs showed the highest cytotoxicity compared to free ZOL and NPs on prostate adenocarcinoma cells, as evaluated by time-lapse video microscopy.[10]

Moreover, we previously demonstrated that Lipo-ZOL was able to reach active concentrations in CNS. When BBB permeability was altered such as in pathological states or chronic neuropathic pain, Lipo-ZOL was able to cross BBB and deliver ZOL in the brain or spinal cord by reducing neuropathic pain.[11]

In this work, we evaluated the *in vitro* and *in vivo* antitumor activity of ZOL encapsulated in liposomes on breast and lung cancer cell lines.

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Materials and Methods

Materials

Unless otherwise stated, all chemicals were from Sigma-Aldrich (Saint Louis, MO, USA). Tissue culture plasticware was from Becton Dickinson (Lincoln Park, NJ, USA). ZOL was kindly provided by Novartis (Basilea, Switzerland).

Preparation of stealth liposomes encapsulating zoledronic acid

The stealth liposomes encapsulating ZOL (Lipo-ZOL) were prepared by a modified reverse-phase evaporation technique. The lipid mixture composed of EPC/Chol/DSPE-PEG 2000 (1:0.32:0.30 weight ratio) was dissolved in a mixture chloroform/methanol (2:1 v/v). The organic solution was added to a 50 mL round-bottom flask, and the solvent was removed under reduced pressure by a rotary evaporator under nitrogen atmosphere. The resulting lipid film was dissolved in 3 mL diethyl ether and the solution was emulsified, by sonication for 30 min in a bath-type sonicator (Branson 3510, Danbury, USA), with 1 mL of ammonium chloride buffer at pH 9,5 containing 75 mM ZOL and 58 mM lactose in presence of glass beads (Sigma). The

resulting emulsion was then placed on the rotary evaporator (Laborota 4010 digital, Heidolph, Schwabach, Germany) and the organic solvent was removed under reduced pressure at 30°C in nitrogen atmosphere. When viscous gel was obtained, the vacuum was broken and the gel was vortexed for about 1 min. Then, the dispersion was placed at rotary evaporator under vacuum for about 15 min. 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 membrane (Nucleopore Track Membrane 25 mm, Whatman, Brentford, UK) with 0.4 µm pore size. Not encapsulated ZOL was removed and liposomes prepared and stored as previously described.[7-10]

Cell lines and MTT viability assay

Breast cancer cells CG5 and lung cancer cells H-460 were provided by ATCC and grown in medium as suggested by ATCC in a humidified atmosphere of 95% air/5% CO_2 at 37°C. Evaluation of the proliferation of human cancer cell lines was performed in the presence of free ZOL or Lipo-ZOL by MTT assay as previously described.[7] For each liposome formulation, the freeze-dried powder was rapidly dispersed in PBS before use.

In vivo experiments

CD-1 female or male nude (nu/nu) mice, 6–8 weeks old and weighing 22–24 g, were purchased from Charles River Laboratories (Calco, Italy). All animal procedures were in compliance with the national and international directives (D.L. March 4, 2014, no. 26; directive 2010/63/EU of the European Parliament and of the council; Guide for the Care and Use of Laboratory Animals, United States National Research Council, 2011).

Female nude mice were injected intramuscularly (*i.m.*) into the hind leg muscles with $3x10^6$ CG5 breast cancer cells per mouse. Male nude mice were injected intramuscularly into the hind leg muscles with $2x10^6$ H-460 lung cancer cells per mouse.

After five days (when a tumor mass of about 300 mg was evident) the treatment was initiated. The following groups were evaluated: untreated; free ZOL or Lipo-ZOL. Mice were treated intravenously (*i.v.*) with 20 µg of free ZOL or Lipo-ZOL 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. Each experimental group included six mice. Therapeutic efficacy of treatment were assessed by the following endpoints: a) percent tumor weight inhibition (TWI%) calculated as [1-(mean tumor weight of treated mice/ mean tumor weight of controls)]x 100).

Statistical analysis.

The Student's t-test (unpaired, two-tailed) was used for comparing statistical differences. Differences were considered statistically significant when $P<0.05$.

Results

In vitro antitumor activity of zoledronic acid-containing Lipo-ZOL in different cancer cell lines

We have evaluated the anti-proliferative effects of free ZOL and Lipo-ZOL on two different human cancer cell lines with MTT viability assay. Breast cancer cells CG5 and lung cancer cells H-460 were treated for 72 h and then the viability was evaluated. Table 1 shows the IC50 (50% inhibitory concentration) values of free ZOL or ZOL-encapsulated in liposomes 72 hours after addition to the cells. We observed that the encapsulation of ZOL into Lipo increased the cytotoxic activity of ZOL by reducing the IC50 (Table 1, Figure 1). Free ZOL induced a 50% growth inhibition at a concentration of 65 µM and 74 µM in H-460 and CG5 respectively, and this effect was enhanced when ZOL was encapsulated into Lipos. In fact, Lipo-ZOL showed an IC50 equal to 11.8 µM in H-460 and 7.5 µM in CG5, that was significantly lower than that one of free drug (Table 1, Figure 1).

In all the cases, plain Lipos did not induce a significant growth inhibition demonstrating a very low cytotoxicity (Figure 1). The values of the potentiation factor (PF) reported in Table 1 demonstrate that LipoZOL potentiated growth inhibition induced by free ZOL, reaching a potentiation factor (PF) significantly higher than 1.0 in both cancer cell lines. In details, PF was 5.5 and 9.8 for H-460 and CG5, respectively. In conclusion, the encapsulation of ZOL in liposomes potentiated the effects of the drug on growth inhibition.

In vivo effects of lipo-formulations

In order to evaluate the *in vivo* effects of Lipo-ZOL, we injected intramuscularly $3x10^6$ breast cancer cells CG5 or $2x10^6$ lung cancer cells H-460 into nude mice.

After five days, when the tumor mass became palpable, the mice were divided into three groups: untreated mice, mice treated with free ZOL and with Lipo-ZOL.

In Figure 2 are reported the growth curves of CG5 (A) and H-460 (B)tumors. In CG5 tumors Lipo-ZOL treatment produced, at nadir of the effect, a significant ($P=0.0003$ *vs* untreated) tumor weight inhibition (TWI) of 47%, while free ZOL resulted in a not particularly marked tumor growth inhibition (TWI 23%)($P= 0.0017$ *vs* untreated). The good therapeutic efficacy of Lipo-ZOL is also demonstrated by the significant ($P= 0.0002$) delay of tumor growth (12 days) suggesting the Lipo-ZOL treatment produces the stabilization of the disease.

In H-460 tumors, Lipo-ZOL seems to be less efficacy than in CG5 experiments. In fact, this treatment produced a significant tumor weight inhibition of 37% ($P= 0.004$ *vs* untreated) at the end of the first week of treatment, but after this promising initial response we observed a rapid regrowth of tumor. Finally it is interesting to note that all treatments were well tolerated by the animals, as no deaths due to toxicity of the treatments and weight loss was observed in animals.

Discussion

ZOL is a third generation aminobisphosphonate, commonly used for the treatment of bone metastases.[1] Several studies have shown that ZOL was able to inhibit cell proliferation and induce apoptosis on osteoclasts and tumor cells but a clear evidence of its clinical activity is still lacking.[1-4] *In vivo* antitumor activity of ZOL is limited

Table 1. IC:50 and potentiation factor values of Lipo, Lipo zoledronic acid and free zoledronic acid after 72 h of treatment.

Cell line	IC:50 µM LipoZOL	IC:50 µM Blank Lipo	IC:50 µM ZOL	PF
H-460	11.8	>120	65	5.5
CG5	7.5	>120	74	9.8

ZOL, zoledronic acid; PF, potentiation factor. In the table are reported the IC50 values of ZOL, as free or encapsulated in Lipo ZOL. The concentrations are expressed in M. The PF values (mean +/- SD from at least three separate experiments performed in quadruplicates) define the specific contribute of ZOL-containing liposomes evaluated as the ratio of the IC50 of encapsulated ZOL to the IC50 of free ZOL. Statistical analysis: LipoZOL vs free ZOL $P<0.01$.

probably because it is rapidly removed from the blood and tends to accumulate in the bone.[1,5,6] First generation liposomes have already been used to deliver aminobisphosphonatesbut they were not able to evade immune system.[10-12] In this study, we used PEGylated liposomes in order to avoid opsonization and to reach tumor site in active concentrations. In order to optimize ZOL encapsulation into liposomes, the drug was solubilized in an alkaline buffer (pH 8.5) and then, a modified reverse-phase evaporation method was performed to physically entrap ZOL into Lipo-ZOL.[7-11] The combination of reverse-phase evaporation method and an alkaline buffer to solubilize ZOL guaranteed a good encapsulation efficiency; moreover, the conjugation of PEG chains to the liposome surface improved physical stability.[7-11]

PEGylated liposomes encapsulating ZOL had a mean size of about 200 and 240 nm, that was compatible with gaps present in the endothelia of tumor.[7-11]

We found that Lipo-ZOL strongly potentiated the inhibition of cell growth in comparison with ZOL, reaching a PF significantly higher than 1.0 in both the cell lines assessed. Moreover, IC50s of Lipo-ZOL were significantly lower than those of free ZOL in both cell lines. Free ZOL induced a 50% growth inhibition at a concentration of 65 μM and 74 μM in H-460 and CG5 respectively and this effect was enhanced when ZOL was encapsulated into LIPOs (11.8 μM in H-460 and 7.5 μM in CG5).The most interesting aspect was to test *in vivo* antitumor activity of Lipo-ZOL since ZOL activity is very limited in *in vivo* models. In our *in vivo* experiments, Lipo-ZOL induced a significant decrease in tumor size of CG5 cell xenografts (47%) in comparison with free ZOL (23%) (P= 0.0017 *vs* untreated). The good therapeutic efficacy of Lipo-ZOL is also demonstrated by the significant (P= 0.0002) delay of tumor growth (12 days) suggesting the Lipo-ZOL treatment produces the stabilization of the disease. As evaluated *in vitro* also *in vivo*, the encapsulation of ZOL in liposomes potentiates the anticancer activity of the drug. On the other hand, PF values for H-460 was significantly lower than for CG5 and also in H-460 xenografts, Lipo-ZOL seemed to be lesser efficacy than in CG5 models. In fact, this treatment produced a significant tumor weight inhibition of 37% (P=0.004 *vs*

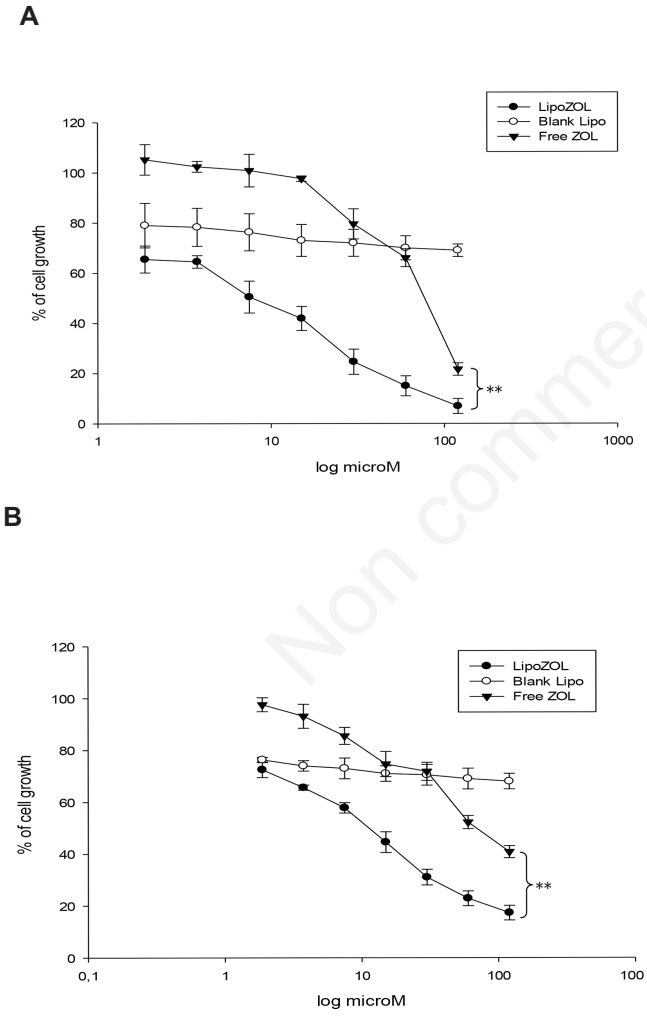

Figure 1. Evaluation of cell growth inhibition in breast (CG5) (A) and lung (H-460) (B) cancer cell lines after 72 h of treatment with Lipo, Lipo-ZOL and free ZOL (A–B). After treatment the viability was evaluated with MTT viability assay. The figure shows representative experiments performed in triplicate with standard deviations. **P<0.01.

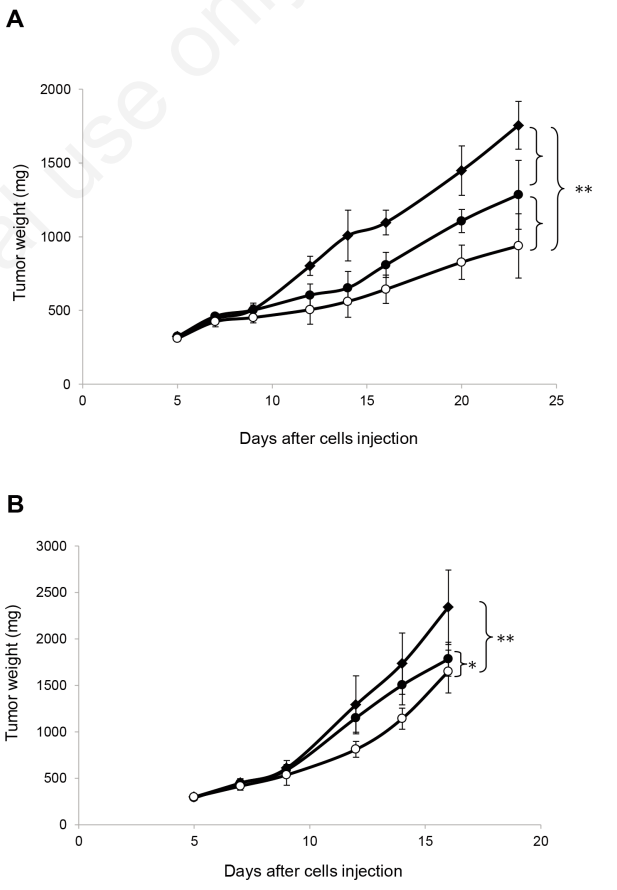

Figure 2. *In vivo* effects of Lipo formulations. Immunosuppressed mice were injected intramuscularly into the hind leg muscles of mice at 3x10⁶ CG5 breast cancer (A) or 2x10⁶ H-460 lung cancer (B) cells/mouse. After five days (when a tumor mass of about 300 mg was evident) the treatment was initiated. The following groups were evaluated: ◆ untreated; • free Zoledronic Acid; o Lipo-ZOL. Mice were treated intravenously 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×b²/2, where a and b are the long and short diameter of the tumor, respectively. *P<0.05; **P<0.01.

untreated) at the end of the first week of treatment, but after this promising initial response we observed a rapid regrowth of tumor.

These *in vivo* experiments suggest that Lipo-ZOL is more potent than free ZOL also in H-460 but it is less efficacious than in CG5. Zoledronic acid has potent antitumor effects that are strictly related to its dosing schedule.[14,15] The change of scheduling of treatment and combination with standard chemotherapeutics used in lung cancer therapy could improve the antitumor efficacy of Lipo-ZOL in these tumors.

Conclusions

In conclusion, we have showed that the encapsulation of ZOL into liposomes potentiated its antitumor activity both *in vitro* and *in vivo*, probably by improving the bioavailability of ZOL and increasing the active drug concentration that reaches its intracellular targets.

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