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In vitro assessment of liposomal neridronate on MDA-MB-231 human breast cancer cells

Imène Chebbi[a], Evelyne Migianu-Griffoni[b], Odile Sainte-Catherine[b], Marc Lecouvey[b], Olivier Seksek[a,*]

[a] *ANBioPhy, CNRS FRE 3207, Université Pierre & Marie Curie, Génopole Campus 1, Evry, France*
[b] *CSPBAT, Equipe C2B, CNRS FRE 3043, Université Paris 13, Bobigny, France*

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ABSTRACT

Bisphosphonates have been used for decades in the standard therapy of bone-related diseases, including bone metastasis of various malignancies, and they might as well be toxic on early cancer cells themselves. In order to allow a better delivery of neridronate (a N-containing bisphosphonate with relatively poor activity), liposomes were evaluated *in vitro* on cancer cell lines (MDA-MB-231, U87-MG and Caco2). After chemical synthesis, this water-soluble molecule was encapsulated into liposomes containing DOPC:DOPG:Chol (72:27:1 molar ratio). The influence of neridronate (free or liposomal) on cell viability or proliferation after treatment was evaluated using the MTT method, as well as cell migration and invasion assays; these techniques showed a drastic improvement of the action of neridronate on MDA-MB-231 cells with an EC_{50} 50 times lower when neridronate was encapsulated. Internalization of liposomes was followed by flow cytometry and fluorescence microscopy, demonstrating internalization via the endocytic pathway. Furthermore, since overexpression of matrix metalloproteinases (particularly MMP-2 and MMP-9) has been correlated to poor prognosis in many cancer types, detection of MMP expression is a satisfactory indication of the therapy efficiency and was then performed on treated cells. On MDA-MB-231 cells, MPPs expression was also significantly reduced by neridronate while entrapped in liposomes.

1. Introduction

Bisphosphonates (BPs) are a family of bone-targeting agents used for decades in the standard therapy of bone-related diseases such as low bone mass, osteoporosis, Paget's disease, multiple myeloma (Shaw and Bishop, 2005). Yet, they are also commonly administered in the treatment of bone metastasis of various malignancies (Layman et al., 2007; Wu et al., 2007). Functionally, they mainly inhibit osteoclastic action on bone resorption after rapidly getting inside the bones and binding to hydroxyapatite crystals (Rogers, 2003). Chemically, they are synthetic analogues of inorganic pyrophosphate, with the presence of a carbon rather than the central oxygen atom of the molecule, resulting in a P–C–P chain; this carbon atom bears two phosphonate functions and two side groups. Importantly, the chemical nature of the side groups strongly influences the pharmacodynamics and the mode of action/potency of these drugs, which can be categorized in two classes: the non-nitrogen-containing and the nitrogen-containing bisphosphonates (Neves et al., 2002). Indeed, apoptosis induction would be the mode of action of the first ones after metabolization into cytotoxic agents, whereas N-containing derivatives would primarily inhibit enzymes of the mevalonate pathway involved in regulatory processes and then altering the osteoclast function (Merrell et al., 2007). An evaluation of the relative potency of BPs can be found in the literature, giving the N-containing compounds as more efficient than the non-N-containing BPs (Zacharis and Tzanavaras, 2008). Moreover, aside the direct action on bone function and bone cells, in particular in case of osteotropic metastasis of breast tumors, *in vitro* studies have also shown that BPs might directly inhibit the proliferation and induce the cell death of the cancer cells themselves (Fromigue et al., 2000; Senaratne et al., 2000; Denoyelle et al., 2003; Muller et al., 2005; Journe et al., 2008; Monkkonen et al., 2008). Finally, it has also been shown that BPs could inhibit tumoral angiogenesis (Ledoux et al., 2006).

On the other hand, while BPs have been used extensively, giving very valuable results on the treatment of various bone conditions, they have also shown a non-negligible number of side effects that could prevent their use (Arum, 2008). Indeed, for the orally administered N-containing compounds, the most common

* Corresponding author. Tel.: +33 1 69 87 43 55; fax: +33 1 69 87 43 60.
E-mail address: olivier.seksek@upmc.fr (O. Seksek).

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drawback is the gastro-intestinal toxicity leading to esophagus inflammation, stomach irritation, abdominal pain or dyspepsia. Concerning the intravenous utilization of BPs, acute phase reactions occur fairly often, including bone pain, myalgias and fevers or even rare ophthalmologic side effects (Tanvetyanon and Stiff, 2006). Furthermore, monitoring of renal function is required, since renal toxicity has been reported, especially with the more efficient BP, zoledronate (Aapro et al., 2008). Osteonecrosis of the jaw has also been observed in patients treated with BPs, with an incidence of up to 10% in patients undergoing cancer IV therapy (Drake et al., 2008).

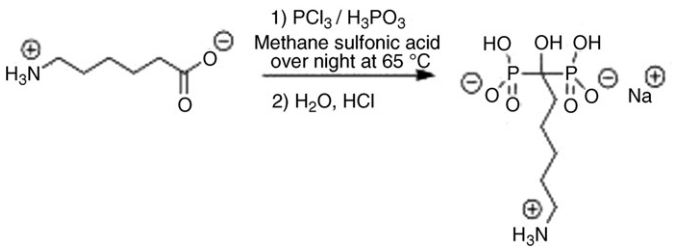
For these reasons and since BPs are fairly potent therapeutical agents while they own an intrinsic toxicity probably dose-dependent, one can consider developing new galenic formulations for these compounds in order to allow a better delivery and less toxicity. For this purpose, liposomes seem to be a good candidate to assess, since they have also been developed and successfully used for decades to deliver therapeutical drugs (Felnerova et al., 2004; Torchilin, 2005; Samad et al., 2007; Fenske and Cullis, 2008). Many articles can be found in the literature to describe these delivery systems, but briefly: liposomes are bubbly self-closed lipid vesicles formed by one or several concentric lipid bilayers with an aqueous phase inside or between the lipid bilayers; they can easily be prepared and their diameter range varies from 100 nm up to 5000 nm; their surface can be grafted with certain polymers or biomolecules in order to confer them specific properties. More importantly, liposomes are biocompatible and they can entrap hydrophilic (in the internal water compartment) or hydrophobic (in the bilayers) pharmaceutical agents, protecting them from external effects and allowing to locally deliver concentrated drugs. In the case of BPs, little has been tested, but it has been shown that liposomes can be effective carriers to deliver these drugs to macrophages, cells that do not internalize the free compound (Van Rooijen and van Kesteren-Hendrikx, 2002). Indeed, this technique even allows selective depletion of macrophages from tissues, since only liposomal BPs would penetrate and kill these specific phagocytic cells, while once liberated the free BP would not have any effect on cells (Van Rooijen and Sanders, 1994; Van Rooijen and van Kesteren-Hendrikx, 2003).

Amongst N-containing BPs, the most potent BPs, neridronate (Corrado et al., 2005; Nicolin et al., 2007) is a fairly good choice in order to optimize its cell delivery using liposomal formulation. In fact, this BP is one of the least efficient ones, as compared to zoledronate and risedronate relatively to their ability for cell killing, but it does not bear the consequent toxicity (Pittari et al., 2006; Zacharis and Tzanavaras, 2008). In the present work, neridronate was tested *in vitro* on human breast cancer cells (MDA-MB-231) both in free and in liposomal forms. Cell viability evaluation showed a drastically more effective action of the neridronate while entrapped in liposome. This result was confirmed using cell migration and cell invasion assays. Furthermore, since overexpression of matrix metalloproteinases (particularly MMP-2 and MMP-9) has been correlated to poor prognosis in many cancer types (Sprague et al., 2006), detection of MMP expression is a rather good indication of the therapy efficiency; in the case of liposomal neridronate, MPPs expression in treated cell lines was significantly reduced.

2. Materials and methods

2.1. Neridronate synthesis

Neridronate was synthesized according to the general procedure for linear aliphatic BPs and characterized by elemental analysis, ^{1}H, ^{31}P and ^{13}C NMR. Neridronate was prepared from the corresponding carboxylic acid precursor according to the following reaction (Kieczykowski et al., 1995):



6-Aminohexanoïc acid (20.0 g, 150 mmol) and H_3PO_3 (12.4 g, 150 mmol) were introduced in a three-necked round-bottom flask under inert atmosphere followed by 30 ml of methanesulfonic acid. After heating at 65 °C for 1 h, PCl_3 (3.5 ml, 40 mmol) was added slowly and the reaction allowed to proceed overnight at 65 °C. The resulting yellow viscous reaction mixture was cooled to room temperature, quenched with 500 ml of ice-cold water (Neves et al., 2002). The pH was adjusted to 4.3 with a NaOH aqueous solution (0.5 M) and the obtained white precipitate was collected by filtration. This solid was washed five times with a mixture of methanol/water (95:5), dialyzed for 3 days and freeze-dried to finally obtain neridronate (46 g, 82%). ^{31}P NMR (D_2O, ppm): 19.1 (s). ^{1}H NMR (D_2O, ppm): 1.35–1.38 (m, 2H); 1.61–1.68 (m, 4H); 1.78–1.91 (m, 2H); 2.96 (t, 2H, $^{3}J_{H-H}$ = 7.4 Hz). ^{13}C NMR (D_2O, ppm): 25.4 (C); 28.7 (C); 35.8 (C); 41.7 (C); 76.2 (t, C–OH, $^{1}J_{C-P}$ = 134.2 Hz). IR (KBr; cm^{-1}): ν_{N-H} = 1637; ν_{C-O} = 1472; $\nu_{P=O}$ = 1221; ν_{P-O} = 1049.

According to the solubility of neridronate, the stock solution was prepared at 10 mM in water. Dilutions of the stock solution were conducted with Eagle's medium, and the highest tested concentration corresponded to 1 mM.

2.2. Liposome preparation

All liposome formulations were performed using the extrusion technique (Olson et al., 1979). 1-Oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (Sodium Salt) (DOPG) and cholesterol (CH) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Briefly, lipids were dissolved in chloroform and mixed together at the appropriate molar ratio (DOPC:DOPG:CH; 72:27:1). The chloroform was evaporated under nitrogen and the samples placed in a high vacuum during 1 h. The lipid films were rehydrated using either a solution of neridronate (pH 7.6; 10 mM), a solution containing neridronate:calcein (1:1) or a solution of neridronate:sulforhodamine 101 (1:1), by gentle mixing, and alternatively freezing (in liquid nitrogen) and heating at 50 °C for 10 times. The newly formed multilamellar vesicles (MLVs) were extruded 10 times through polycarbonate membrane filters (Poretics, Livermore, CA, USA) with a decreasing pore size of 400 nm, 200 nm and 100 nm using an extruder device (Northern Lipids Inc., Vancouver, BC, Canada). Final chromatography through SEPHADEX G-25 gel (GE Healthcare, Buckimghamshire, UK) allowed to get rid of the free compounds and to obtain the final liposomal suspension containing the molecules of interest. Size and ξ-potential measurements were achieved by dynamic light scattering and by electrophoretic mobility using a Nano-ZS (Red Badge) ZEN 3600 device (Malvern Instruments, Malvern, UK). Calcein and sulforhodamine 101 (Sigma, St. Louis, MO) were used only for fluorescence imaging and flow cytometry.

Quantification of encapsulated neridronate was determined along with a TBP (tributylphosphate, Sigma, St. Louis, MO) reference by ^{31}P {1H} (80.9 MHz) NMR. A range of concentrations of

free neridronate (19.1 ppm) solutions added with TBP (97 mg in capillary, 0 ppm) was prepared for calibration. Using a solution of glacial acetic acid (pH 4), the neridronate-containing suspension of liposome (lipids 4 ppm) was disrupted to allow the measurement of the free compound concentration by comparison with the reference calibration.

2.3. Cell lines and cell culture

MDA-MB-231, U87-MG and Caco2 cells were obtained from American Type Culture Collection. Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml streptomycin (all obtained from Life Technologies Inc.), at 37 °C in a 5% CO_2 humidified atmosphere. All *in vitro* cell experiments (viability, migration and invasion assays) were carried out at 37 °C in a 5% CO_2 incubator.

2.4. Cell viability

Cell viability was evaluated using the MTT microculture tetrazolium assay (Mosmann, 1983) based on the ability of mitochondrial enzymes to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) into purple formazan crystals. Cells were seeded at a density of 10^4 cells per well in 96-well flat-bottom plates (Falcon, Strasbourg, France) and incubated in completed culture medium for 24 h. Then, medium was removed and replaced by 10% FCS-medium containing increasing concentrations of neridronate from 16 μM to 1 mM and varying concentration of liposomal suspension containing neridronate from 0.27 μM to 17.56 μM. After 72 h incubation (free neridronate) or 24 h, 48 h and 72 h incubation (liposomal neridronate), cells were washed with phosphate buffered saline (PBS, Life Technologies) and incubated with 0.1 ml of MTT (2 mg/ml, Sigma–Aldrich) for additional 4 h at 37 °C. The insoluble product was then dissolved by addition of 100 μl of DMSO (Sigma–Aldrich). The absorbance corresponding to the solubilized formazan pellet (which reflects the relative viable cell number) was measured at 570 nm using a Labsystems Multiskan MS microplate reader. Dose–response curves were obtained for free and liposomal neridronate concentrations, allowing the determination of EC_{50} values, which refer to the concentration inducing a response halfway between the baseline and the maximum plateau reached.

2.5. Flow cytometry and fluorescence microscopy

Cells were seeded on Petri dishes (Ø 30 mm, density 50,000 cells per Petri dish), grown for 24 h and treated for 12 h: (i) with neridronate/calcein liposomal suspensions, (ii) with neridronate/calcein in solutions at the same neridronate concentration and (iii) with free calcein. Cells were washed twice in PBS. Treated and untreated cells were harvested by trypsinization (500 μl trypsin–EDTA). Five minutes before doing flow cytometry experiments, propidium iodide (PI) (1 mg/ml on ethanol, Sigma–Aldrich) was added to cell suspension. Cell suspensions were analyzed with a Beckton Dickinson FACSCalibur 3C (argon laser wavelength at 488 nm) flow cytometer. Ten thousand cells per sample were measured for forward-angle light scattering (FSC) and fluorescence.

For fluorescence microscopy, MDA-MB-231 cells were washed twice in PBS and 2 ml of PBS were added for imaging cells. Microscopic observation was carried out with a Nikon Optiphot-2 on which was added a coaxial–confocal module with Nipkow wheel (Technical Instruments, model K2 BIO). The optimal depth resolution was 0.5 mm. The excitement light source was a high-pressure mercury lamp. Cells were observed in PBS with Zeiss 63× water immersion objective. Appropriate fluorescence emission filters were used for LysoTracker Green® (Invitrogen, Paisley, UK) (FITC filter set: 450–490, FT510, 520–570) and sulforhodamine 101 (rhodamine filter set: BP546, FT590, LP600). Images were collected using a cooled CCD camera (Micromax, Princeton Instruments, Evry, France). Display and analysis were performed with IPLab software (Scanalytics, Fairfax, VA).

2.6. Cell migration and invasion assays

The influence of neridronate on migration of MDA-MB-231 cells was investigated using Boyden invasion chambers with 8 μm pore size filters, as previously described (Muller et al., 2005). 3×10^6 cells were treated with free neridronate and liposomal suspension (neridronate concentration: 50 μM and 1 μM, respectively) for 48 h. MDA-MB-231 untreated cells were added to each insert (10^5) (upper chamber). A strong chemoattractant (10% FCS) for MDA-MB-231 cells was added to lower chamber. After 24 h incubation at 37 °C in a 5% CO_2 incubator, non-migrated cells were removed by scraping and migrated cells were fixed in methanol and stained with hematoxylin. Cells migrating on the lower surface of the filter were counted in 10 fields using an Optika microscope. Results were expressed as percentage, relative to controls normalized to 100%. Experiments were performed in triplicate.

Cell invasion experiments were performed with Boyden chambers as described above. The inserts were coated with Matrigel membrane matrix (Falcon, Becton Dickinson Labware, Bedford, MA, USA). The MDA-MB-231 cells (10^5) were seeded in the upper well of the Boyden chamber, and 10% FCS was added to the lower chamber. After 24 h, cells are counted on the lower surface of the membrane and normalized relative to control experiments.

2.7. Zymography

MDA-MB-231 cells were seeded at a density of 5×10^5/well into six-well tissue culture plates in DMEM containing 10% FCS. Cells were allowed to adhere for 24 h and incubated with different neridronate concentration 500 nM, 250 nM and 125 nM (liposomal suspension and/or free neridronate) for 48 h. Conditioned media were prepared (with above neridronate concentrations) and collected. Cell numbers were normalized by total protein calibration. Conditioned media were mixed with no-reducing Laemmli buffers and applied on zymography gels. This contained 10% SDS-PAGE and 0.1% (w/v) gelatin. After migration at 100 V, gels were washed three times at room temperature with a solution containing 2.5% (v/v) Triton X-100/water and incubated at 37 °C overnight in enzyme buffer solution (50 nM Tris/HCl at pH 7.4, 0.2 nM NaCl, 5 mM $CaCl_2$ and 0.05% Brij 35). This gel was stained for 60 min in a solution containing 0.5% (w/v) R-250 Coomassie blue, 30% methanol (w/v) and 10% acetic acid (v/v) and was uncolored using a mixture of 50% water (v/v), 10% acetic acid (v/v) and 40% methanol (v/v) and then water.

3. Results and discussion

3.1. In vitro cell treatment

In order to evaluate the feasibility and the efficiency of liposomal encapsulation to deliver neridronate on living cells, three human cancer cell lines were chosen: a breast carcinoma (MDA-MB-231), a brain carcinoma (U87-MG) and a colon carcinoma (Caco2). Each of these adherent cell lines was treated either with free neridronate or with neridronate-containing liposomes, in which neridronate concentration was measured by ^{31}P NMR as described. The bilayer lipid composition of the liposomes was optimized using DOPC, DOPG and cholesterol by changing their relative concentrations (data not

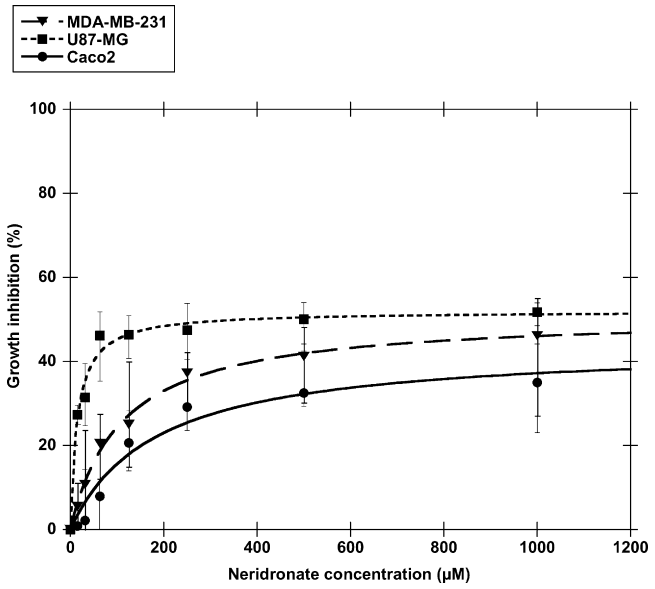


Fig. 1. MTT growth inhibition measurements performed after a 72 h incubation of MDA-MB-231, U87-MG and Caco2 cell lines with free neridronate in solution.

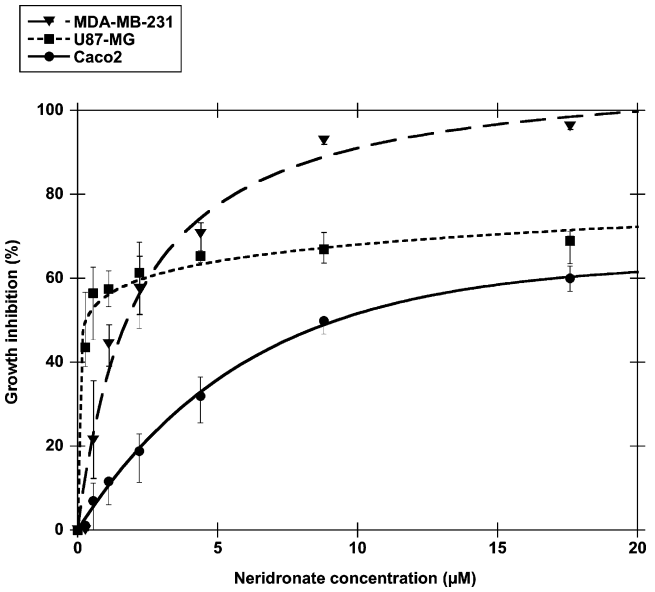


Fig. 2. MTT growth inhibition measurements performed after a 72 h incubation of MDA-MB-231, U87-MG and Caco2 cell lines with neridronate-containing liposomes. Intra-liposomal neridronate concentration was controlled using ^{31}P NMR and a free neridronate reference calibration.

shown). The assay that was retained to determine the best mixture was the MTT method applied to the cell line giving the more significant result (i.e. MDA-MB-231 as this will be stated hereafter). The optimal lipid composition was obtained with a molar ratio of DOPC:DOPG:CH equal to 72:27:1. This result is consistent with 25 mol% of negative lipid (DSPG) used by Monkkonen et al. (2008) to deliver another bisphosphonate (clodronate). Moreover, with the combination of extrusion filters used in this study, the liposome size was around 163 ± 15 nm and ξ-potential was -20 ± 3 mV. In the present work, all data were then obtained with liposomes with the same composition. This composition was also tested for its innocuousness and showed no significant toxicity when the liposomes were empty of any compound. Moreover, liposomes used for the cell treatment were prepared either on the same day of incubation or after a period of up to 2 months after storage at 4 °C; in all cases, results were identical, indicating a good stability of liposomes over time with no liposome bilayer disruption or leak of compound out of the vesicles. Finally, before each experiment, Triton X-100 was added to a small aliquot of liposomes to be used in order to disrupt the lipid membrane and to control the internal compound concentration; in all cases, even after 2 months, the relative leak was negligible. Fig. 1 presents typical dose–response curves of cell growth inhibition versus concentration of free neridronate for the three cell lines after a 72 h incubation time (maximum incubation time). For U87-MG, MDA-MB-231 and Caco2, respectively, the EC_{50} values were 14 μM, 95 μM and 130 μM. For none of them, free neridronate would allow 100% growth inhibition (maximum growth inhibition: 52%, 46% and 37%, respectively for U87-MG, MDA-MB-231 and Caco2). In fact, on these three cell lines, free neridronate was a fairly poor inhibitor of cell growth. In clinical use, free neridronate also showed a low toxicity against non-targeted cells (O'Rourke et al., 1994, Merlotti et al., 2007). Using liposomal neridronate, in the same conditions during 72 h, gave the results showed in Fig. 2. Evidently, the proliferation inhibition was greatly affected since it could reach 100% in the case of MDA-MB-231, and 70% or 62% for U87-MG and Caco2, respectively. Moreover, EC_{50} values were 1.7 μM, 1 μM and 4 μM (MDA-MB-231, U87-MG and Caco2, respectively) and drastically reduced as compared to free neridronate. Significantly enough, in the case of MDA-MB-231, a total growth inhibition (corresponding to a total efficacy of neridronate activity) was obtained while the EC_{50} was divided by more than 50 times. For the two other cell lines, the activity of neridronate was also greatly improved by the delivery system, but the cell proliferation inhibition was still incomplete. Longer incubation times were considered for these cell lines in order to eventually improve the neridronate effect, but after 72 h the reference non-treated cells were overgrown, preventing the measurement of growth rates. In the case of MDA-MB-231, Fig. 3 shows different incubation periods with liposomal neridronate. After 48 h, 100% of growth inhibition was attained, while the EC_{50} was slightly higher (2.4 μM). Since MDA-MB-231 was the cell line giving the more dramatic result in term of difference of efficacy between free and liposomal neridronate, this was the cell line used kept in the pursuit of this study.

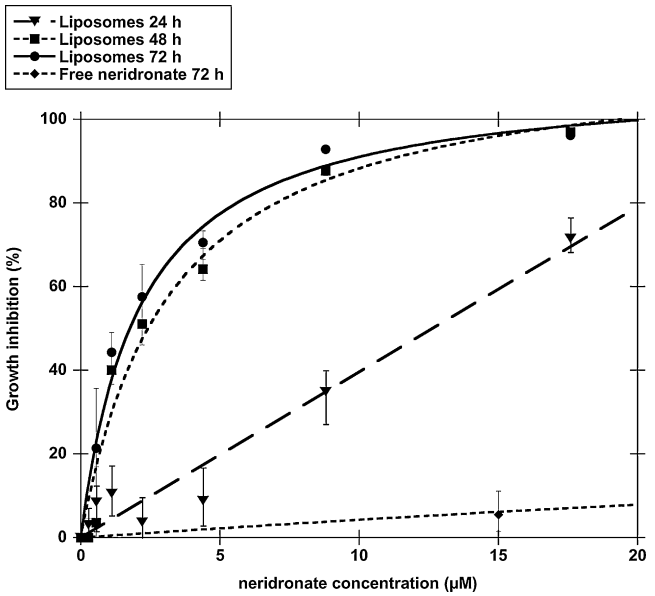


Fig. 3. MTT growth inhibition measurements performed on MDA-MB-231 cells treated with free neridronate in solution or neridronate-containing liposomes at different incubation times.

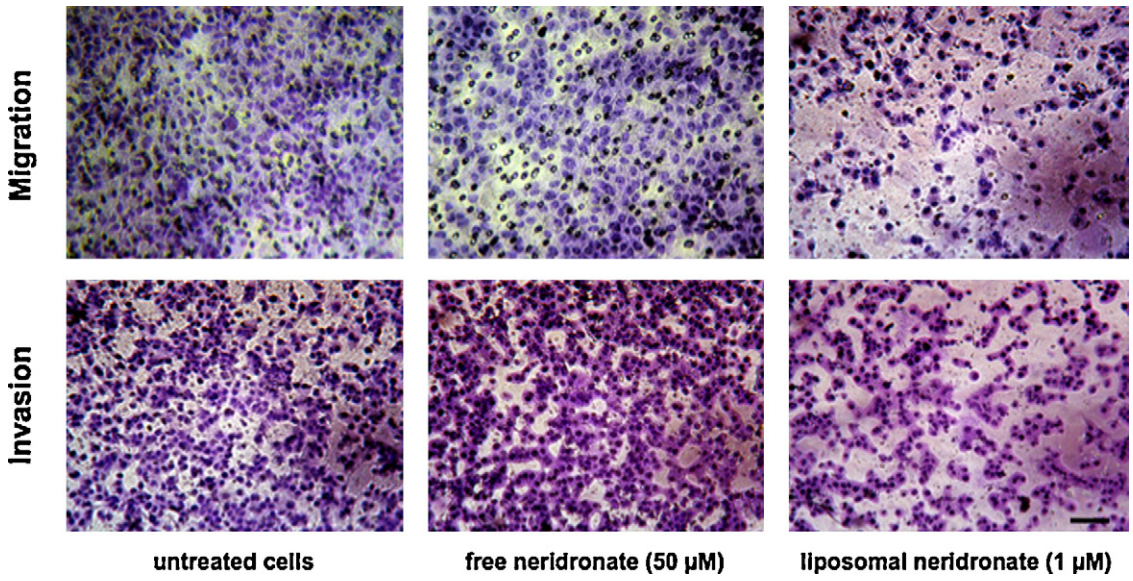


Fig. 4. Migration and invasion assays on MDA-MB-231 cells treated with free neridronate or neridronate-containing liposomes.

To emphasize these first results on the effect of neridronate delivered by liposomes on MDA-MB-231 cells, more qualitative assays such as migration and invasion assays were performed. Indeed, while these assays are fairly correlated to the viability assay (MTT), they allow considering an additional parameter, important in the development of malignancies: the ability of tumor cells to migrate and invade surrounding tissues. Indeed, to establish distant metastases, tumor cells must have the ability to move and cross the basement membrane that underlies epithelia and endothelia and separates them from the stroma. This basement membrane is a thin matrix and malignant cells can produce proteases that degrade this matrix. Of several *in vitro* models for the invasion process, those using Matrigel are the most reliable, reproducible, and representative of *in vivo* invasion (Kleinman and Jacob, 2001; Ribatti et al., 2007). Fig. 4 shows representative experiments performed on MDA-MB-231 cells using neridronate concentrations around halves of the EC_{50} determined by the MTT method. In these cases, the counted measurements of cells were: (i) for the migration assay, with free neridronate (50 μM) and liposomal neridronate (1 μM), 83 ± 2% and 23 ± 3%, respectively; (ii) for the invasion assay, 90 ± 3% and 25 ± 2%, respectively. These results clearly indicated that, even at a lower concentration, liposomal neridronate would have a suppressive effect on tumor cell mobility *in vitro*, whereas free neridronate showed almost no effect.

While these results are obtained on cultured cells, they are a good indication on the potential use of this type of lipid formulation *in vivo*. Indeed, anionic liposomes such as the one used in this study have already shown their effectiveness in animals or even in clinical use. For example, a drug such as the polyene antifungal agent amphotericin B (Veerareddy and Vobalaboina, 2004) can be formulated in a commercially available anionic liposomal form (Ambisome®) containing a mixture of phosphatidylcholine (PC), phosphatidylglycerol (PG) and cholesterol. This permitted to reduce toxicity and to increase tolerance and therapeutic efficacy of the free compound. In the same manner, some anticancer drugs have been delivered *in vivo* via the PC/PG liposomes showing an increased uptake and reduced side toxicity (Waterhouse et al., 2001). As a last example and to some extent, oligonucleotides could also been delivered *in vivo* by these anionic liposomes (Seksek and Bolard, 2004).

3.2. Liposome internalization

Aside the functional effect, the mechanism for which entrapped neridronate would allow a better toxic action than the free neridronate was investigated. Two fluorescence assays were chosen: imaging and flow cytometry; however, since neridronate is not a fluorescent compound, for each assay, a fluorophore was added to neridronate inside the liposomes. Of course, these assays did not directly show the internalization of neridronate but merely allowed to justify an adequate lipid composition for the neridronate-containing liposomes. Fig. 5 shows typical micrographs obtained when MDA-MB-231 cells were treated for 12 h with liposomes containing at the same time in their aqueous phase 10 μM neridronate

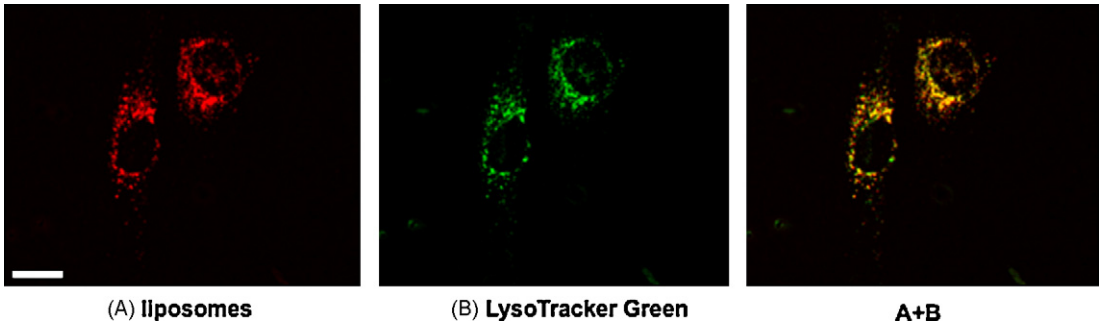


Fig. 5. Neridronate/sulforhodamine 101-containing liposomes colocalization with LysoTracker Green® on MDA-MB-231 cells, visualized by fluorescence imaging.

and 2 mM sulforhodamine 101. Both compounds were clearly remaining inside the liposomes as controlled by fluorescence spectroscopy on the liposomal suspension before and after treatment with a disrupting agent (Triton X-100, 0.1%). The liposome size was around 143 ± 23 nm and ξ-potential was -22 ± 3 mV, values very similar to the ones obtained with liposomes only containing neridronate. Cells treated with this suspension showed an internal punctate pattern clearly similar to the endosomal–lysosomal pathway visualized via the LysoTracker Green® labeling. This result does not come as a surprise since the main mechanism involved in the delivery of therapeutical agents by synthetic liposomes has been evidenced to be the internalization of the liposomes itself followed by the release of their payload. On a more quantitative aspect, cells treated with liposomes containing 10 μM neridronate + 45 mM calcein (both non-permeant compounds remaining inside the liposomes) for 12 h in same conditions than in the previous experiment were investigated by flow cytometry. The liposome size was around 145 ± 23 nm and ξ-potential was -21 ± 3 mV. Fig. 6 shows a representative set of experiments: on the upper histogram, cells treated with liposomes were more labeled ($91 \pm 5\%$ on four experiments) than cells treated with free solutions of neridronate + calcein; on the lower histogram, PI labeling showed that, at this neridronate concentration for 12 h, cells were still viable.

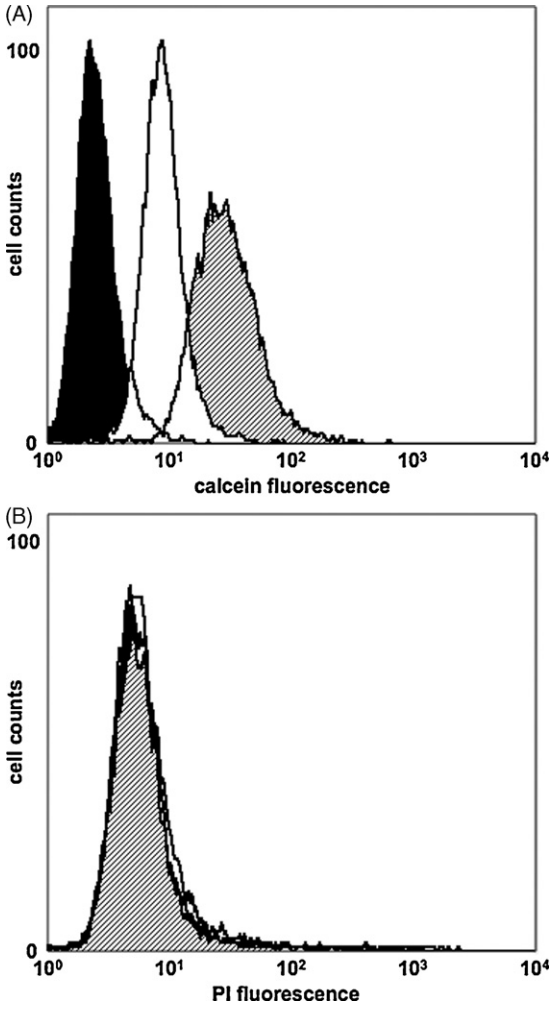


Fig. 6. Internalization of calcein/neridronate-containing liposomes followed by flow cytometry on a population of MDA-MB-231 cells. (A) Calcein fluorescence histogram plot. (B) Propidium iodide histogram plot on the same cell population. For both plots, are represented no treated cells (black histogram), cells treated with free neridronate and calcein (white histogram) and cells treated with liposomes (gray histogram).

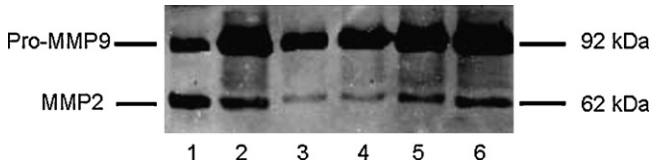


Fig. 7. Zymography gel of MMPs expression by MDA-MB-231 breast carcinoma cells with and without neridronate treatments. Lane 1: HT-1080 cells used as a positive control to identify MMP-9 and MMP-2. Serum-free conditioned media of untreated cells (lane 2, total protein content (TPC) = 2.79 mg), treated with free neridronate at 500 nM (lane 6, TPC = 2.86 mg) and encapsulated neridronate at 500 nM (lane 3, TPC = 2.92 mg), 250 nM (lane 4, TPC = 2.84 mg) and at 125 nM (lane 5, TPC = 2.93 mg).

These latter results are in good correlation with the functional growth inhibitory effect already demonstrated. Moreover, some indication about the mechanism of action might be considered. Indeed, it would seem that cells treated with liposomal neridronate would first internalized the complexes through the endosomal pathway with a relatively short time course. Data shown in this section were obtained after a 12 h incubation period, giving the maximum of fluorescence intensity detectable either by fluorescence microscopy imaging or cytometry. However, other experiments (not shown) with shorter incubation times (from 1 h to 6 h) also displayed some significant internalization. Then, it could be assumed that after a rapid uptake, the drug is released from its liposomal/endosomal compartment, giving only after some time longer than 24 h (probably during some metabolization process) its maximal inhibitory effect.

3.3. Zymography

The potential effects of free and liposomal neridronate pretreatment on MMP-9 and MMP-2 production by MDA-MB-231 were studied by zymography. The serum-free conditioned media from 12-o-tetradecanoylphorbol-13-acetate treated HT-1080 fibrosarcoma cells was used as a positive control to identify MMP-9 and MMP-2 on gelatin zymograms (Fig. 7, lane 1). MDA-MB-231 cells showed a significant expression of MMP-9 and MMP-2 (Fig. 7, lane 2). Neridronate encapsulated in liposomes at different concentrations (500 nM, 250 nM and 125 nM) reduced the MMP-9 activity about 69%, 61% and 50%, respectively (Fig. 7, lanes 3–5). Liposomal suspension containing neridronate inhibited strongly the expression of MMP-9. In contrast, free neridronate (500 nM, Fig. 7, lane 6) did not perform any inhibitory effect.

In addition, free neridronate did not inhibit MMP-2 expression whereas liposomal suspension at different concentrations of neridronate (500 nM, 250 nM and 125 nM) inhibited the MMP-2 expression about 63%, 54% and 32%, respectively.

Liposomal suspension of neridronate inhibited migration and invasion of MDA-MB-231 cell lines. These results were correlated by zymography assay. During the tumor invasion, different proteases as gelatinases MMP-9 and MMP-2, are secreted by carcinoma cells to successively cross-basal lamina, extracellular matrix, blood and/or lymphatic endothelium (Kato et al., 2005). MMP-9 and MMP-2 are the major forms present in extracellular matrix and they are zinc-dependent endopeptidases. In this study, encapsulated neridronate strongly inhibited MMPs secretion. Bisphosphonates have been shown to inhibit enzymes of the mevalonate pathway which are ultimately responsible for events that lead to the post-translational modification of GTP-binding proteins such as Ras, Rap1, Rho and Rab (Rogers, 2003). Ras and Rho signalling has been shown to also mediate MMP-2 and MMP-9 expression (Liu et al., 2000; Hamma-Kourbali et al., 2003). Hence, it could be speculated that neridronate would modulate both MMP-2 and MMP-9 secretion.

4. Conclusion

In this work, neridronate was synthesized using an efficient and one-pot procedure. Then, the best lipid composition of liposomes to encapsulate neridronate was optimized (DOPC:DOPG:Chol (72:27:1 molar ratio)) with a liposome size of 163 ± 15 nm. Action of free neridronate and liposomal neridronate was evaluated on cell viability on three human carcinoma cell lines. Free neridronate was observed as a poor inhibitor of carcinoma cells as expected whereas total inhibition was observed with neridronate entrapped in liposomes after 72 h incubation, particularly on MDA-MB-231 cells. This significant result was confirmed by migration and invasion assays. Indeed, at a lower concentration, liposomal neridronate would have a suppressive effect on tumor cell mobility *in vitro* while free neridronate showed almost no effect. Lastly, zymography study showed that neridronate eventually released from liposomes inhibited the MMPs expression on MDA-MB-231 cells. These converging results are good evidences that liposomal formulation should allow an improved delivery of neridronate on tumor cells. In perspective, this efficient liposomal delivery system would have to be tested *in vivo*, probably after chemical modifications or substitutions to allow a better escape from reticulo-endothelial cells (stealth liposomes with PEG chains) or some targeting towards tumor cells (addition of peptides, dextrans, etc.). This system might also be tested with other bisphosphonates such as risedronate and zoledronate in order to improve their activity.

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