International Journal of Pharmaceutics 379 (2009) 324–327

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Rapid communication

Superparamagnetic nanovector with anti-cancer properties: γFe_2O_3@Zoledronate

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ARTICLE INFO

Article history:
Received 29 January 2009
Received in revised form 31 March 2009
Accepted 5 April 2009
Available online 15 April 2009

Keywords:
Drug nanoparticle
Magnetic nanoparticle
Bisphosphonate
Cancer drugs
Drug targeting
Cell internalization

ABSTRACT

We elaborate a magnetic nanovector to vectorize Zoledronate, an anti-cancer interest molecule of the hydroxmethylenebisphosphonate's family. In fact, Zoledronate is a powerful adjuvant in the treatment of bone diseases such as osteoporosis and Paget's disease. But, recent studies have shown that in addition to anti-osteoclastic properties, it presents antitumour properties notably in the case of breast and prostate cancer. However, these properties cannot be exploited due to their very high affinity to divalent cations and their preferentially accumulation in bone. To overcome this problem, one strategy is the vectorization trough maghemite nanocrystal functionalization. The specific surface coating permits to consider γFe_2O_3@Zoledronate as a drug delivery vehicle for therapeutic activity. The anchoring to the nanoparticle's surface allowed to increase their hydrophobicity and also to change the therapeutic target, increasing the Zoledronate intestinal absorption instead of their accumulation in bone. We show that Zoledronate link the nanoparticle surface through phosphonate groups. The biological *in vitro* tests performed on breast cancer cell line, MDA-MB 231, showed that γFe_2O_3@Zoledronate have antiproliferative activity. In addition, the γFe_2O_3 core could be used as MRI contrast agent for a good therapeutic evaluation.
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Since the last 30 years, Bisphosphonates (BPs) are indispensable for the treatment of both benign and malignant bone disease (Green, 2003). BPs acts as powerful pharmacological agents with the ability to inhibit osteoclast activity, influence cellular processes involved in bone formation and resorption activity, induce osteoclast apoptosis *in vitro* and *in vivo* and reduce biochemical markers of bone resorption (Green and Rogers, 2002).

BPs are stable analogues of the naturally occurring inorganic pyrophosphate in which the phosphoanhydride linkage (P–O–P) has been replaced by a non hydrolysable P–C–P bond. The R_1 and R_2 carbon side chains determine the pharmacological properties of bisphosphonates. Most BPs contain a hydroxyl group at the R_1 position (hydroxyl methylene bisphosphonate, HMBP) that confers high affinity binding to calcium phosphate and is the basis for the bone targeting properties of bisphosphonates. The R_2 side chain is the essential determinant of antiresorptive potency (osteoclasts inhibition). Nonnitrogen-containing compounds are metabolized into cytotoxic analogues of ATP, whereas the more potent nitrogen-containing compounds (N-BPs; e.g., pamidronate, ibandronate, Zoledronate) (Scheme 1) inhibit protein prenylation, thus affecting cell function and survival. Zoledronate is a new potent bisphosphonate of the third generation that is mainly used clinically in prevention of skeletal complications in patients with advanced bone affecting malignant tumours (Russell, 2007).

In addition, BPs also exhibit direct and indirect antitumoural effect against a broad variety of tumour cell lines, such as melanoma, mesothelioma, prostate, breast, lung and myeloma cancer cells *in vitro* (Stresing et al., 2007; Lipton, 2000). The direct growth inhibitory effects have been attributed to cell cycle arrest and/or induction of apoptosis. Numerous studies have also described the ability of BPs to inhibit tumour cell adhesion and invasion but the mechanism is still unclear. In the case of Zoledronate, it has been shown that it act by inhibiting the mevalonate pathway (Rosen et al., 2004). However, the major drawback of this drug resides in their poor bioavailability mainly due to their preferential accumulation in bone (Nancollas et al., 2006). In order to enhance the antitumour activity of BPs, various strategies are used: development of BPs analogues with a lower bone mineral affinity (Boissier et al., 2000), encapsulation in liposomes (Zeisberger et al., 2006). In previous work, we have shown that HMBP groups are highly complexing agent for iron oxide γFe_2O_3 nanocrystal surface (Lalatonne et al., 2008). In this study we used this high iron affinity to coat the nanocrystal surface with Zoledronate (Scheme 1). The antitumoural activity of free Zoledronate and Zoledronate coated nanocrystals

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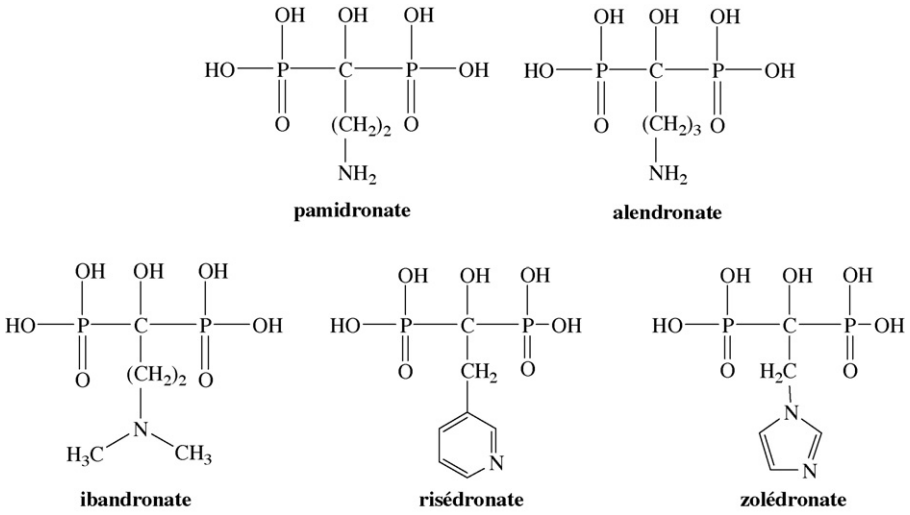
doi:10.1016/j.ijpharm.2009.04.010



Scheme 1. General chemical structure of the most important N-BPs clinically used.

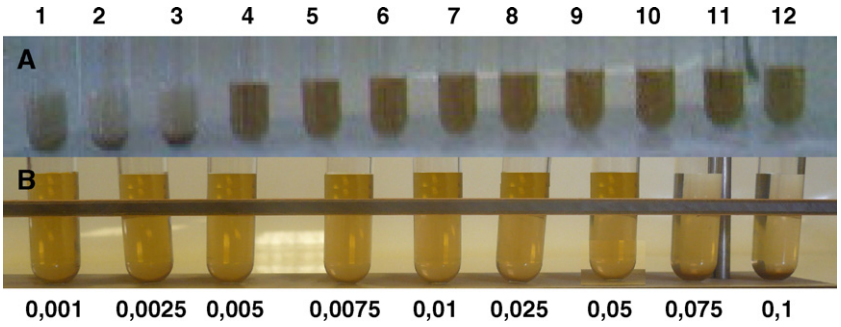


Fig. 1. (A) Behaviour of the γFe_2O_3@Zoledronate solutions, as function of pH. The initial conditions are at pH 7 and iron concentration of 5×10^{-3} mol L^{-1}. The suspension pH is then adjusted with sodium hydroxide (NaOH) or acidic (HCl) solutions. (B) Stability tests of γFe_2O_3@Zoledronate solutions, as function of [NaCl]. The initial conditions are pH 7 and iron concentration of 5×10^{-3} mol L^{-1}.

(γFe_2O_3@Zoledronate) is evaluated *in vitro* and compared using MDA-MB-231 as breast cancer cells models.

Zoledronate or Zoledronic Acid (1-Hydroxy-2-(imidazol-1-yl)ethylidene-1,1-bisphosphonic acid) is synthesized according to the procedure of Takeuchi et al. (1998).

The maghemite γFe_2O_3 nanocrystals (10 nm in diameter, size polydispersity 20%) were synthesized and surface functionalized according to a procedure already described (Lalatonne et al., 2008, 2004). The passivation process is performed in acidic media. γFe_2O_3@Zoledronate were collected under magnetic field and washed three times with ethanol then acidic water to remove free moieties in solution. The powder is easily dispersed in water and is stable over a broad range of pH 3–12 (Fig. 1A) and ionic strength until a concentration in salt [NaCl] = 5×10^{-2} mol L^{-1} at physiological pH (Fig. 1B). Zeta potential measurement indicates an average charge surface of −21.6 mV at physiological pH.

Nanocrystal surface characterization is performed with FTIR and ^{31}P NMR spectroscopies. Comparing the FTIR spectra of γFe_2O_3@Zoledronate nanocrystals (Fig. 2) (red curve) with the free Zoledronate solution (blue curve), large changes are observed within the P–O stretching region (1200–900 cm^{-1}) whereas the imine vibration band is unchanged (1650 cm^{-1}). These results indicate that Zoledronate molecules are grafted onto the nanocrystal surface through the phosphonate groups.

To quantify the average number of molecules per nanocrystal, a calibrate curve is established using free solution of Zoledronate (NMR ^{31}P {1H} (80.9 MHz): 15.36 ppm) at various concentration in presence of an intern reference NaH_2PO_4 (10^{-1} mol L^{-1}; NMR ^{31}P {^{1}H} (80.9 MHz): 0 ppm). After chemical decomposition of the magnetic γFe_2O_3@Zoledronate nanocrystals in acidic medium (nitric acid 65%), the ferrous ions are precipitated by addition of sodium hydroxide NaOH (10^{-1} mol L^{-1}) in order to avoid switching of the ^{31}P NMR signal. The supernatant is analyzed with ^{31}P NMR and the concentration (number of molecules per nanocrystal) of Zoledronate into the sample is deduced from this calibration curve. An average number of 550 ± 50 molecules per nanoparticle is obtained for γFe_2O_3@Zoledronate (corresponding to 0.1 ± 0.01 mg of Zoledronate per mg of γFe_2O_3).

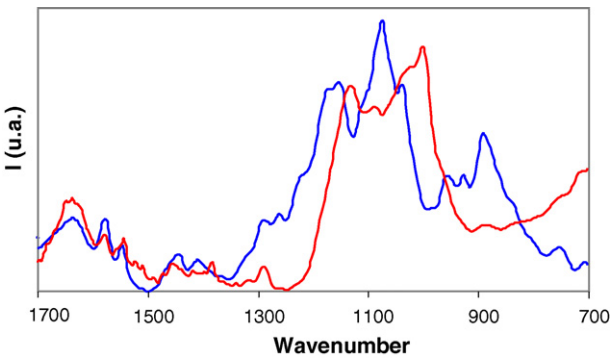
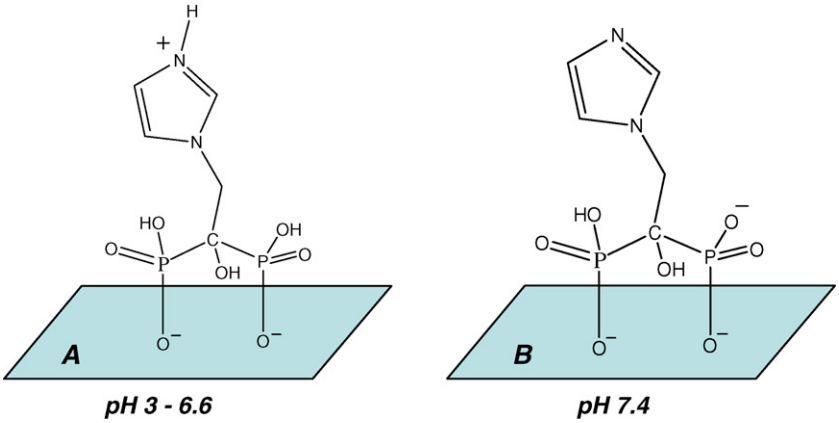


Fig. 2. FTIR spectroscopy in KBr pellets: free Zoledronate molecules (blue curve) and γFe_2O_3@Zoledronate nanocrystals (red curve). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)



Scheme 2. Proposed structure of Zoledronate interacting on γFe_2O_3 nanocrystals surface (A) at pH 3–6.6 and (B) at pH > 7.

Zoledronate is highly charged molecules with 4 pK_a for the two phosphonate groups (~0.8, 2.89, 6.63, and 10.99) and one for the R_2 group similar to H-imidazole (~6.95) (Nancollas et al., 2006). Considering that Zoledronate coordinate to iron surface via two Fe–O–P bonds corresponding to pK_a1 and pK_a2, hence under pH 3 surface coordination is not highly efficient to stabilize nanoparticles (Fig. 1A). Between pH 3 and 6.6, it can be expected that stability is induced by electrostatic repulsion between nanocrystals via the protonated nitrogen of the imidazol (Scheme 2A). At pH 7.4, the H-imidazole is deprotonated and phosphonate protons dissociate leading to increased concentrations of monoprotonated species (Scheme 2B). The average charge surface at physiological pH is then globally negative.

In previous work, we have shown that γFe_2O_3 nanocrystal surface are internalized following the endocytosis pathway and concentrate within intracellular vesicles (endosomes). Free Zoledronate and the γFe_2O_3@Zoledronate nanocrystals were incubated with MDA-MB231 breast cancer cells for various extra cellular Zoledronate concentrations up to 0.1 mmol L^{-1} for 48 h and 72 h. As shown in Fig. 3A, γFe_2O_3@Zoledronate nanocrystals uptake by cells is well visualized by phase contrast optical microscope after Prussian blue staining.

Fig. 3B shows that Zoledronate and γFe_2O_3@Zoledronate induce a concentration related and time dependant reduction in cell viability. For free Zoledronate the IC50 at 48 h is achieved to a concentration of 10 μmol L^{-1} while for γFe_2O_3@Zoledronate it is at 100 μmol L^{-1}. Hence, the activity of free Zoledronate is more important than γFe_2O_3@Zoledronate. However such results indicate that Zoledronate vectorization into cells through nanocrystal surface functionalization can be achieved in order to target selectively malignant cells. In order to increase γFe_2O_3@Zoledronate nanocrystals cell internalization, a magnetic field can be applied during the incubation. Magnetofection is a simple and highly efficient transfection method that uses magnetic fields to concentrate particles into the target cells (Scherer et al., 2002). When a magnetic field is applied, cell viability is considerably reduced in presence of γFe_2O_3@Zoledronate whereas such behaviour is not observed for γFe_2O_3 coated with HMBP-$(CH_2)_3$-COOH molecules with no antitumoural activity (Lalatonne et al., 2008). Hence, at 100 μmol L^{-1} under an applied of magnetic field, proliferation decrease to 75% compared to 40% without magnetic field. In this way, the magnetic force allows a very rapid concentration of the entire applied vector dose onto cells, so that 100% of the cells get in contact with a significant vector dose. The magnetic particles are then concentrated on the target cells by the influence of the external magnetic field generated by magnets. The cellular uptake of the particles is accomplished by endocytosis, natural biological processes. Consequently, membrane architecture and structure stay intact, in contrast to other physical transfection methods that damage the cell membrane.

Hence, results obtained with γFe_2O_3@Zoledronate clearly indicate that inhibitory effect on cells proliferation is induced by drugs after nanocrystal cell internalization. The mechanism of drugs action or drug release is not demonstrated here. However the anchoring to the nanoparticle's surface allowed to increase their hydrophobicity and also to change the therapeutic target,

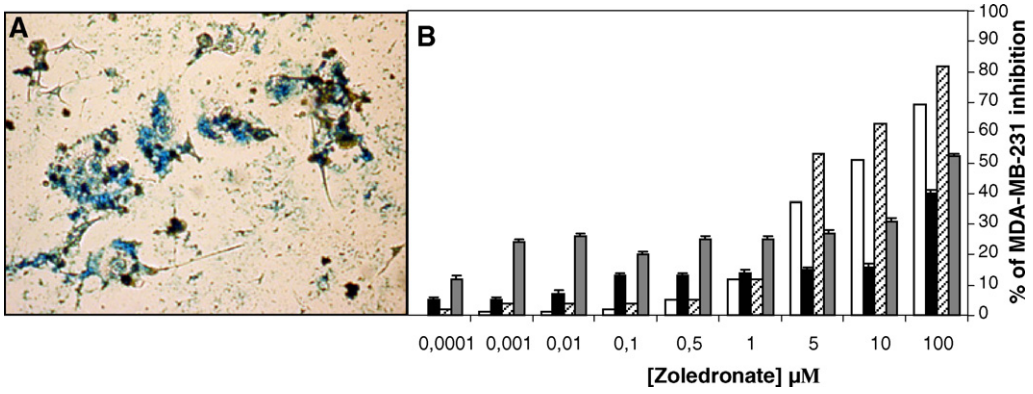


Fig. 3. (A) Optical image of mammal cell (MDA-MB-231) incubated with (B) γFe_2O_3@Zoledronate for 48 h and corresponding to 0.01 mM in Zoledronate, showing nanocrystals incorporation into cells, stained by Prussian blue. (B) Comparative effects on MDA-MB-231 cell proliferation after different time of incubation of free Zoledronate ((□) 48 h, (▨) 72 h) and γFe_2O_3@Zoledronate nanocrystals ((■) 48 h, (▩) 72 h). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

increasing the Zoledronate intestinal absorption instead of their accumulation in bone.

As conclusion, we have coated Zoledronate, an anti-cancer drug, on γFe_2O_3 particles. These new nanohybrid particles are internalized into cells and induce a diminution in cell viability. The activity is slightly less important than free molecules but increase considerably with magnetotransfection. These results allow us to consider these nanohybrid particles as a drug delivery system. Experiments *in vivo* are in progress with and without magnetic field to reduce their affinity with bones and therefore to improve the antitumoural potency of these new γFe_2O_3@Zoledronate nanocrystals. In addition, the γFe_2O_3 core could be used as MRI contrast agent (Lalatonne et al., 2008) for a good therapeutic evaluation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2009.04.010.

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