European Journal of Pharmaceutical Sciences 86 (2016) 58–66

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

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

Uptake of free, calcium-bound and liposomal encapsulated nitrogen containing bisphosphonates by breast cancer cells

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ARTICLE INFO

Article history:
Received 4 January 2016
Received in revised form 24 February 2016
Accepted 26 February 2016
Available online 5 March 2016

Keywords:
Zoledronic acid
Risedronic acid
Drug uptake
Breast cancer

ABSTRACT

Purpose: We have examined the uptake routes by which breast cancer cells internalize different formulations of nitrogen containing bisphosphonates (N-BPs).
Methods: Cell viability was assessed with the tetrazolium colorimetric test (MTT assay) after treatment with different N-BP formulations in the presence or absence of inhibitors for different endocytosis mechanisms. Intracellular formation of isopentenyl pyrophosphate (IPP) and triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester (ApppI), were quantified with mass spectrometry (ES-LTQ-MS) as surrogate markers for N-BP efficacy. Direct quantification intracellular [^{14}C]-labeled zoledronic acid was done with liquid scintillation counting.
Results: The main uptake route for all the different formulations of nitrogen containing bisphosphonates was shown to be dynamin dependent endocytosis, which was significantly enhanced with calcium. This uptake mechanism was mostly caveolin and clathrin independent in MCF7 cells, but more clathrin dependent in T47D cells. Liposome encapsulation of the drug shifted the uptake mechanism to be more dependent on caveolin in both the cell lines. The cytotoxicity of N-BPs and the concentrations of formed intracellular ApppI and IPP were significantly increased by calcium chelation and liposome encapsulation, the latter being the most potent formulation.
Conclusion: Nitrogen containing bisphosphonates require active endocytosis for cellular uptake and in the breast cancer cells the mechanism is uniformly dynamin dependent for all the formulations tested. This differs e.g. from the previous observations on macrophages, which mostly utilize macropinocytosis. Liposomal formulation was found to prolong the duration of the drug effect in cells.

1. Introduction

Bisphosphonates are a class of drugs that are widely used to treat diseases associated with bone loss like osteoporosis, Paget's disease, cancer associated bone loss and bone metastasis (Clézardin, 2011; Daubiné et al., 2007). Bisphosphonates could be divided in two main classes depending on if they carry a nitrogen atom in their structure — nitrogen containing N-BPs (e.g. pamidronate, risedronate, zoledronate) and non-nitrogen containing BPs (clodronate, etidronate). They have different mechanisms of action and they form different intracellular metabolites (Rogers et al., 2000). Non-nitrogen containing BPs are metabolized and they react with ATP to form toxic metabolites – in the case of clodronate – AppCCl$_2$p. AppCCl$_2$p inhibits the mitochondrial adenine nucleotide translocase (ANT) (Mönkkönen et al., 2001). On the other hand the N-BPs are not metabolized but they inhibit an enzyme of the mevalonate pathway — the farnesyl pyrophosphate synthase (FPPS). This leads to intracellular accumulation of isopentenyl pyrophosphate (IPP), a metabolite preceding FPPS. IPP can conjugate with AMP in a reaction catalyzed by the tRNA-synthetase (Mönkkönen et al., 2006) to form a toxic metabolite triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester, ApppI (Jauhiainen et al., 2009; Mönkkönen et al., 2006). ApppI has also been shown to inhibit adenine nucleotide translocase (ANT) in the mitochondria (Mönkkönen et al., 2006, 2008). Further, IPP is a potent phosphoantigen shown to activate the Vγ9Vδ2-T cells, which effect has been explored to eradicate cancer cells (Benzaïd et al., 2011; Clézardin, 2011; Arkko et al., 2015).

Cellular uptake of small molecules is a complex process, which is regulated in a spatiotemporal manner. In general, the uptake can be divided in two main groups: clathrin dependent and clathrin independent endocytosis (Table 1). The clathrin-independent endocytosis can be further categorized into four other sub-classes: caveolin-dependent, Cdc42-dependent, Rho A-dependent and macropinocytosis. Another classification of the endocytosis can be made based on its dependence on dynamin (Table 1).

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http://dx.doi.org/10.1016/j.ejps.2016.02.016
0928-0987/

Table 1
Classification of the endocytosis based on dependency on clathrin or dynamin.

	Clathrin independent endocytosis	Clathrin independent endocytosis			
Dynamin dependent	Clathrin mediated endocytosis	Caveolin mediated endocytosis	Rho-A mediated endocytosis		
Dynamin independent		Cdc42 mediated endocytosis	Flotillin dependent	Arf6-dependent	Macropinocytosis

The significance of the different uptake mechanisms in pharmacology have been demonstrated in several studies (Guillaumond et al., 2015; Ben-Dov and Korenstein, 2015). It has been demonstrated that different pharmaceutical formulations of bisphosphonates have different levels of cytotoxicity and effect on the cell viability (Mönkkönen et al., 1994). For example a liposomal formulation of N-BPs exhibits different pharmacokinetics compared to the free formulation of the same drug (Shmeeda et al., 2013). Further, drugs with different molecular structures are known to explore different uptake routes (Kim et al., 2014; Dolan et al., 2013; Badolo et al., 2010). Bisphosphonates have the specificity to "hook" themselves to the bone hydroxyapatite (Lawson et al., 2010) and to chelate calcium (Freire et al., 2010).

Serum bisphosphonate concentrations between 250 nM and 1.4 μM have been reported after administration of the drug (Skerjanec et al., 2003; Weiss et al., 2008). However, due to the inherent adherence to bone, significantly higher concentrations have been shown to be present in bone where was found 38 μM of zoledronic acid after a 3 year treatment (Weiss et al., 2008; Otto et al., 2010). A recent study demonstrated that tumor macrophages are associated with microcalcifications. Microcalcifications apparently serve as a substrate for the bone "hook" of the bisphosphonates probably easing their uptake by macrophages (Junankar et al., 2015). Further, the tissue tropism of BPs has been altered by packing them to liposomes. The plasma clearance of Zoledronate packed into folate containing liposomes has been shown to be greatly retarded in mice and the drug formulation was effectively internalized by folate receptor expressing mouse lung carcinoma and human head and neck cancer cell lines (Shmeeda et al., 2010). In this study we have explored how three different formulations of N-BPs, plain drug, a formulation complexed with calcium or liposomal encapsulated N-BP are internalized in MCF7 and T47D breast cancer cell lines. Our results demonstrate for the first time, that N-BPs are internalized in the tested breast cancer cell lines by endocytosis which is always dynamin dependent despite the formulation used. This differs from the macropinocytosis mechanism previously proposed for osteoclasts and macrophages (Thompson et al., 2006).

2. Material and Methods

2.1. Materials

Risedronic acid sodium, [1-hydroxy-2-(1H-imidazol-1-yl)ethane-1,1-diyl]bis(phosphonic acid) CID 4194514, was a gift from Prof. Juoko Vepsäläinen and zoledronic acid, [1-hydroxy-2-(1H-imidazol-1-yl)ethane-1,1-diyl]bis(phosphonic acid) CID 68740, and its [^{14}C]-labeled analog were a generous gift from Novartis (Novartis International AG, Switzerland). MEM alpha, Trypsin-EDTA 0.5%, and (streptomycin, penicillin, amphotericin B) were obtained from Life Technologies Europe, Stockholm, Sweden. RPMI-1640, DMSO, chlorpromazine, methyl-beta-cyclodextrin, wortmannin, N, N-dimethylformamide, methanol, and acetonitrile were from Sigma-Aldrich, Steinheim am Albuch, Germany. Dynasore was purchased from Merck Millipore, Germany.

2.2. Cell Cultures

Breast cancer cell lines: MCF-7 (ECACC, Salisbury, UK) was grown in MEM alpha (Gibco, USA) supplemented with 0.01 mg/ml recombinant human insulin (Sigma-Aldrich, Steinheim, Germany), 10% v/v fetal bovine serum, 1% v/v antibiotic–antimycotic (Gibco, USA), and 10 nM 17-beta-estradiol (Sigma-Aldrich, Steinheim, Germany). T47D cell line (ATCC, USA) was adjusted to grow without estrogen or progesterone supplementation in RPMI-1640 with 10% FBS and 1% antibiotic–antimytotic. The cells were grown in a T-75 cm^3 sterile flask (Nunc, Denmark) cell incubator with 95% air, 5% CO_2 at 37 °C.

2.3. Uptake Inhibition Assay

MCF-7 and T47D cell lines were seeded in 96 well plates in 4000 cell/well and allowed to adhere for 12 h at 37 °C. Subsequently cells were pretreated for 15 min. with PBS (control) or one of the following inhibitors: clathrin dependent endocytosis inhibitor chlorpromazine 30 μM, caveolin dependent endocytosis inhibitor methyl-β-cyclodextrin (mβCD) 2 mM, macropinocytosis inhibitor wortmannin 200 nM (all ordered from Sigma-Aldrich, Germany) and 80 μM dynamin inhibitor Dynasore (Millipore, Merck KGaA, Darmstadt, Germany). After that, the media with inhibitors were removed and the cells were treated with N-BP (zoledronic acid or risedronic acid), N-BP in media enriched to 4.5 mM Ca^{2+} or liposomal encapsulated N-BP. The plates were incubated for 72 h at 37 °C, 5% CO_2. At the third day the media with the different drug formulations were removed and 100 μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added. The cells were incubated for 2 h to form formazan crystals and then 100 μl 10% SDS/0.01 M HCl was added and the plates were incubated overnight to dissolve the crystals (Mosmann, 1983; Nargi and Yang, 1993). Colorimetric measurements at 570 nm were performed with an EnVision Multilabel Reader (Perkin-Elmer, USA).

2.4. Nitrogen Containing Bisphosphonate Formulations

2.4.1. Free and Calcium Complexed Formulation

Risedronic or zoledronic acid was dissolved in deionized water and adjusted to pH of 7.2 at temperature of 37 °C and stored as 30 mM stock at −20 °C until use. To produce calcium complexed formulation of N-BPs the medium was enriched to 4.5 mM of Ca^{2+} with sterile $CaCl_2$.

2.4.2. Liposomal Formulation

Risedronic acid (RIS) or zoledronic acid (ZOL) was dissolved in deionized water, pH 7.2 and with final osmolarity range of 285–295 mOsm. Then the solution was filter-sterilized through a syringe filter with a pore size of 0.2 μm (Millipore, Merck GKaA, Germany). The liposomes were prepared by the reverse phase evaporation method