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Low doses amino-bisphosphonates stimulate keratinocytes growth inactivating glucocorticoid receptor

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ABSTRACT

Amino-bisphosphonates (N-BPs) have a wide range of clinical applications to treat bone diseases. Their activity lowered farnesyl pyrophosphate (FPP) endogenous levels by inhibiting FPP synthase. In epithelial cells it has been demonstrated that FPP reduces both cell proliferation and migration activting glucocorticoid receptor. In this study two N-BPs (zoledronate and neridronate) used at low concentrations (100 nM to 10 μM) are able to stimulate human keratinocytes proliferation reducing glucocorticoid receptor activation.

1. Introduction

Bisphosphonates (BPs) are synthetic compounds, characterized by a P–C–P structure, displaying high affinity for bone and antiresorptive activity, accounting for their use in clinical practice since the end of the 1960s for the treatment of bone, tooth and calcium metabolism diseases, along with the oncological treatment and prevention of skeletal complications of multiple myeloma or bone metastases arising from different types of cancer (Fleisch, 1998, 2002; Guise, 2008; Russell et al., 2008). The P–C–P structure allows them to bind divalent ions, such as Ca^{++} (Roelofs et al., 2006; Russell et al., 2008). These P–C–P backbone can undergo a great number of modifications, resulting in extensive alterations in their physicochemical, biological, therapeutic and toxicological characteristics, by changing the two lateral chains (conventionally identified as R1 and R2) on the carbon atom (Fleisch, 1998, 2002). Changes in R1 substituents result in an alteration of BPs Ca^{++} chelating ability, while changes in R2 substituents mainly affect BPs antiresorptive effects. In particular, nitrogen substituents at critical positions in R2 chain are essential in determining drug potency, as nitrogen-containing BPs are up to 100-fold more potent than non amino-containing compounds (Rogers et al., 1997; Russell et al., 2008). As BPs are characterized by a very high chemical stability, they cannot be metabolized by human body: they are rapidly cleared from the circulation (half-life in the range of hours) and excreted unaltered (Magremanne, 2008; Roelofs et al., 2006; Russell et al., 2008).

According to their mechanism of action, BPs can be classified into two distinct groups: non nitrogen-containing (non N-BPs) and nitrogen-containing (N-BPs) compounds. Non N-BPs, resembling inorganic pyrophosphate (PPi), are incorporated into ATP containing compounds, forming non hydrolyzable toxic analogs thus resulting in energy starvation and consequent cell death (Drake et al., 2008; Hasmim et al., 2007; Magremanne, 2008; Rogers et al., 1997). On the other hand, N-BPs, inhibiting farnesyl pyrophosphate (FPP) synthase, interfere with protein prenylation and finally affect the intracellular trafficking of regulatory proteins, resulting in cellular microarchitecture loss and osteoclast apoptosis. Although FPP synthase is a ubiquitous enzyme in mammals, N-BPs seem to induce apoptosis in osteoclasts as a direct consequence of their strong affinity for bone matrix and their local accumulation (Drake et al., 2008; Hasmim et al., 2007; Magremanne, 2008; Rogers et al., 1997).

BPs are widely known as antiresorptive drugs, but they display also anti-angiogenic, anti-proliferative and immunological activities (Green and Guenther, 2011), along with direct BPs apoptotic effects on different tumor cell lines (Coleman, 2011; Green and Guenther, 2011; Guise, 2008).

In a previous study (Renò et al., 2012) we demonstrated that low concentrations (10 nM to 10 μM) of Zoledronate (Zol) and Neridronate (Ner) unexpectedly stimulated keratinocytes (HaCaT cells) proliferation and wound healing in an in vitro model. As it is known that N-BP treatment reduces farnesyl pyrophosphate (FPP) endogenous levels (Fig. 1) and that FPP can act as glucocorticoid receptor agonist (Vukelic et al., 2010), the aim of this study was to investigate the FPP involvement in the observed N-BPs induced increase in cell proliferation.

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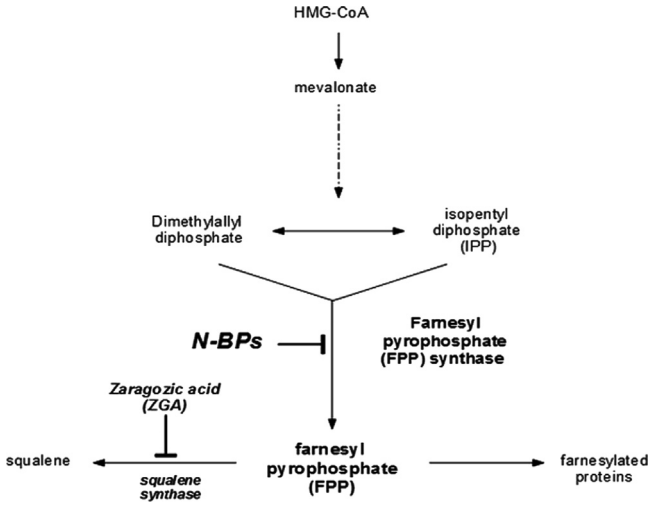

Fig. 1. Diagram of the mevalonate pathway, showing the key reactions inhibited by N-BPs and ZGA.

2. Materials and methods

2.1. Cell culture

Spontaneously immortalized keratinocytes (HaCaT), isolated from human adult skin (Boukamp et al., 1988) were a kind gift of Dr. M. De Andrea from the Microbiology Laboratory, University of Eastern Piedmont "A. Avogadro". Cells were grown in culture flask (75 cm^2) in DMEM medium (Euroclone, Milan, Italy) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Euroclone), penicillin (100 U/mL), streptomycin (100 mg/mL) and L-glutamine (2 mM) (Euroclone) in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.2. Pharmacological cell treatment

HaCaT cells were treated with commercial nitrogen containing bisphosphonate solutions for clinical use (Aclasta, Novartis Pharma GmbH, Nürnberg, Germany, for zoledronic acid (Zol) and Nerixia, Abiogen Pharma S.p.A., Ospedaletto, Italy, for neridronic acid (Ner)). As a further control, HaCaT cells were also treated with commercial non amino bisphosphonate solution for clinical use (Niklod, Savio S.r.L., Ronco Scrivia, Italy, for disodium clodronate). All the aqueous solutions for intravenous or intramuscular administration were diluted to the tested concentrations (100 nM to 10 μM) in DMEM without FBS just before cell treatments. Squalene synthase inhibitor zaragozic acid (ZGA, Sigma Aldrich, Milwaukee, WI, USA) was dissolved in ethanol and diluted to the final concentration (50 μM) (Vukelic et al., 2010) in complete medium. Farnesyl pyrophosphate solution (Sigma Aldrich) was diluted to the final concentration (10 μM) (Vukelic et al., 2010) in complete medium. Control samples (Cnt) were left untreated and incubated in complete DMEM medium.

2.3. Cell proliferation

Aliquots of 5×10^4 cells were seeded in cell culture multiwell plates and allowed to adhere overnight. Unadherent cells were removed by gentle wash in phosphate buffer (PBS, pH=7.4) and drugs were added in fresh medium while fresh medium without drugs was added to control samples. Some samples were pre-treated for 10 min with ZGA before N-BPs addition. After 48 h of incubation cell proliferation was quantified using a fluorimetric method based on the quantification of resazurin reduction by viable cells (TOX-8, Sigma Aldrich). Briefly, 25 μl of dye were dissolved in 0.5 ml complete cell growth medium and after 4 h the optical density was measured at 620 nm. Cell proliferation was expressed as optical density (O.D.) variation ± standard deviation (S.D.). At the end of the experiment cells samples were fixed with a 3.7% formaldehyde, 3% sucrose solution in PBS at 4 °C and then stained with 0.1% toluidine blue solution. Stained samples were observed using a Leica ICC50HD (Leica Microsystems, Wetzlar GmbH, Wetzlar, Germany) optical microscope at 10 × magnification.

2.4. Immunofluorescence

Aliquot of 5×10^4 HaCaT cells were dropped onto glass coverslips and allowed to adhere overnight.

Unadherent cells were removed by gentle wash in phosphate buffer (PBS; pH=7.4) and drugs were added in fresh medium while fresh medium without drugs was added to control samples. Some samples were pre-treated for 10 min with ZGA or FPP before N-BPs addition. After 24 h of incubation cells were fixed in acetone–methanol (1:1) solution for 2 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min and incubated overnight at 4 °C with a FITC conjugated anti-Ser(P)^{211} glucocorticoid receptor antibody (Biorbyt, Cambridge, UK, 1:200 in 2.5% FBS in PBS) (Vukelic et al., 2010). Cell nuclea were stained with Hoechst 33342 solution (2 μg/ml) and samples were observed under UV (Hoechst) and blue (FITC) light using a Leica DM500 microscope (Leica Microsystems, Wetzlar GmbH, Wetzlar, Germany) at 20 × magnification.

2.5. Statistical analysis

Unpaired Student's *t*-tests were done for statistical analysis. Probability values of $p < 0.05$ were considered statistically significant. Data were expressed as mean values ± standard deviation (S.D.).

3. Results

3.1. Cell proliferation

The effects of the N-BPs Zoledronate and Neridronate, along with the non N-BP Clodronate, on cell proliferation were analyzed measuring the increase of fluorescence values due to the bioreduction of resazurine dye by viable cells using the commercial Tox-8 assay. As shown in Fig. 2A, after 48 h of incubation, Zol low concentrations (100 nM and 1 μM) induced a significant increase in cell proliferation ($p < 0.05$ compared to control samples). On the other hand 10 μM Zol was not able to stimulate HaCaT cell proliferation that occurred at a slightly lower level than in control samples. The Zol effect on HaCaT proliferation was also evident from sample cell density observation (Fig. 2B). The other N-BP used, Ner, had an effect on keratynocytes proliferation similar to the one observed for Zol (Fig. 2C) with the only difference that at the highest concentration tested (10 μM) it still stimulated cell proliferation ($p < 0.05$ compared to control samples). Again, the proliferation stimulation induced by Ner was also evident observing sample cell density (Fig. 2D). In order to verify that the observed effect on cell proliferation was specifically due to N-BPs action, in some experiment clodronate, a non N-BP compound was used and it did not altered cellular proliferation (data not shown). To test the involvement of FPP levels on the observed N-BPs induced keratinocytes proliferation, zaragozic acid (ZGA, 50 μM) was used to inhibit the squalene synthase activity (Fig. 1), therefore increasing the intracellular level of FPP. After 48 h of incubation, ZGA did not alter significantly basal cell proliferation (Fig. 2A and C), while was able to reduce Zol induced proliferation (Fig. 2A,