Pulse treatment with zoledronic acid causes sustained commitment of bone marrow derived mesenchymal stem cells for osteogenic differentiation

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A B S T R A C T

The aminobisphosphonate zoledronic acid (ZA) is a bone seeking specific inhibitor of protein farnesylation and geranylgeranylation, which causes inhibition of osteoclast function and apoptosis. It is widely used as an osteoclast targeted antiresorptive treatment of metastatic bone disease, Paget’s disease and osteoporosis. Mesenchymal stem cells (MSC) and osteoblast precursors can also be targets of bisphosphonates, but the clinical relevance of these effects is under debate. We show here that ZA in vitro causes inhibition of proliferation and induction of apoptosis in hMSC, when applied in concentrations of 20 and 50 μM for more than 24 h which can be rescued by treatment with 10 μM geranylgeranyl pyrophosphate (GGPP). However, pulse stimulation for 3 and 6 h with these concentrations and subsequent culture for up to 2 weeks under osteogenic conditions exerts sustained regulation of osteogenic marker genes in hMSC. The effect on gene regulation translates into marked enhancement of mineralization, as shown by alizarin red and alkaline phosphatase staining after 4 weeks of osteogenic culture. ZA, when applied as a pulse stimulus, might therefore also stimulate osteogenic differentiation in vivo, since μM plasma concentrations can be achieved by intravenous application of 5 mg in patients. These data set the stage for the future dissection of the effects of ZA and other aminobisphosphonates on cells beyond osteoclasts, with respect to cell differentiation in benign metabolic and to antitumor efficacy in metastatic bone diseases, as well as adverse events due to putative substance accumulation in bone during long-term treatment.

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Introduction

Aminobisphosphonates (ABP) are bone-seeking pyrophosphate derived compounds, which are rapidly adsorbed to bone hydroxyapatite. Osteoclasts incorporate bisphosphonates by phagocytosis and pinocytosis and are thus primary targets within the bone [33]. The principal targets of ABP are the enzymes farnesyl and geranylgeranyl diphosphate synthases, which are part of the mevalonate pathway. ABP bind to its active centers with nM affinity, inhibit enzyme activities and may cause substrate and upstream accumulation of isoprenoid pyrophosphate lipids. Effective enzyme inhibition disrupts the prenylation of target proteins like the GTP binding protein Ras and Rho, thereby influencing their subcellular distribution. This together with downstream events can cause caspase-associated apoptosis in osteoclasts and probably any other cell types in vitro as shown for mesenchymal precursor cells, osteoblasts and also tumor cells using μM concentrations. [5,6,26,33,35,45]. The in vitro proapoptotic and antiangiogenic effects of ABP on tumor cells is discussed as a basis of their putative antitumoral activity in metastatic bone disease [7,9,13,15,46,47]. Recently it has been reported for the ABP zoledronic acid (ZA) that treatment of cells produced a new endogenous ATP analog (triphosphoric acid 1-adenosin-50-yl ester 3-(3-methylbut-3-enyl) ester, abbreviated to ‘APPPI’), which inhibited the mitochondrial ADP/ATP translocase and caused apoptosis in osteoclasts [21]. So far, the production of toxic, apoptosis inducing ATP analogues was only reported for early bisphosphonates like clodronate [10]. Thus ZA can act both via the inhibition of the protein prenylation and by the blockade of mitochondrial ADP/ATP translocase.

ZA, one of the most potent ABP, is approved for metastatic bone disease including malignant hypercalcemia and Paget’s disease of bone and also for osteoporosis in North America and the EU [2,13,29,30]. Apoptosis induction upon exposure to μM concentrations of ZA has also recently been demonstrated in osteoblasts in vitro [24]. However, the inhibition of cell viability by ZA could be reduced by using calcium phosphate bound ZA [41]. If apoptosis induction and/or impairment of cell specific functions by ABP in osteoblasts was clinically relevant, this could hardly be reconciled with their well established beneficial effects on fragility fractures in osteoporosis [18,36,37]. Additionally, antiangiogenic properties of accumulated BP in the bone could also contribute to the pathogenesis of osteonecrosis of the jaw (ONJ), which is a multifaceted complication in patients on long-term ABP treatment, suffering from malignant diseases and often
impaired immunocompetence [8]. Osteoblast apoptosis or ONJ however are definitely unwarranted in general but especially in treatment modalities for benign metabolic bone diseases like osteoporosis. Intravenous ZA, 4 mg every 3–4 weeks over years for treatment of metastatic bone disease, was comparably frequently associated with the occurrence of ONJ, while ZA 5 mg i.v. once a year has very recently been shown to substantially reduce fracture risk in osteoporotic women in a controlled trial over 3 years, without any increased risk for ONJ [2]. Thus the in vitro data shown for osteoblasts have to be reconciled with the excellent performance of this compound in osteoporosis treatment in vivo. It is unknown if, by local accumulation or during peak serum concentrations after single infusions of ABP, permanent μM concentrations can be achieved in vivo to induce osteoblast apoptosis. The in vivo systemic exposure of the organism during a single ZA infusion to high concentrations — if correctly deducible from pharmacokinetic data — is considerably short and may last for 3–6 h, where peak concentrations in the serum reach 1–2 μM after an infusion of 4 mg in 100 ml over 15 min [4]. As a working hypothesis, the double edged effects could be a matter of dose and time of exposure.

We show here that a 3 to 6 h exposure to μM concentrations of ZA, as well as permanent exposure to 10 and 100 nM enhance osteogenic differentiation of hMSC in vitro, while a 2 to 3 d exposure to >5 μM ZA as well as permanent exposure to concentrations >200 nM impair hMSC proliferation and induce apoptosis. This might be of clinical relevance to better define the dosing and the upper threshold of BP accumulation in the bone microenvironment, when constantly high local BP concentrations start to impair osteogenic and angiogenic effects in bone, e.g. in bone regeneration. Our data may help to characterize the molecular and pharmacological attitudes associated with ZA therapy in the two settings of low to moderate dose in extended time frames (osteoporosis) on one hand, and high dose in short time intervals (metastatic bone disease) with potential long-term accumulation in bone.

Materials and methods

Cell culture

Media for cell culture and fetal calf serum (FCS) were obtained from PAA Laboratories (Linz, Austria, www.paa.at). Primary human mesenchymal stem cells (hMSC) were isolated from bone marrow from different donors and cultivated up to four weeks by a standardized protocol [23]. Bone marrows were recovered after informed consent from the explanted femoral heads of patients undergoing elective hip arthroplasty. The procedure was approved by the local Ethics Committee of the University of Würzburg. Briefly, bone marrow preparations were washed with Dulbecco’s modified Eagle’s medium, (DMEM/F12) supplemented with 10% FCS, penicillin/streptomycin, and 50 μg/ml ascorbate (Sigma-Aldrich GmbH, Munich, Germany www.sigmaaldrich.com), and centrifuged at 1200 rpm for 5 min. The pellet was reconstituted in medium and washed four times, and the supernatants of the washing steps containing the released cells were collected. Cells were centrifuged and cultivated at a density of 3×10^5 cells per 150 cm² culture flask. Adherent cells were washed after 2 days and cultivated until confluence. Cells were grown at 37 °C in a humidified atmosphere consisting of 5% CO₂ and 95% air.

Cell proliferation and apoptosis assays

For determination of long-term effects of ZA on proliferation and apoptosis, hMSC were seeded on a 96-well plate with a density of 1000 cells/well and were stimulated with 1, 10, 100, and 500 nM and 1, 5, 20 and 50 μM ZA for 24, 48 and 72 h. To block apoptotic effects of 50 μM ZA on hMSC, cells were cotreated with 40 μM farnesyl pyrophosphate (FPP) and 10 μM geranylgeranyl pyrophosphate (GGPP) (Sigma-Aldrich GmbH) for 72 h. Proliferation and apoptosis rates were measured by the CellTiter-Glo Luminescence Cell Viability Assay (proliferation) and the Caspase-Glo 3/7 Assay (apoptosis) (both Promega GmbH, Mannheim, Germany, www.promega.com) according to the manufacturer’s instructions. Luminescence was measured with an Orion II Luminometer (Berthold Detection Systems, Pforzheim, Germany, www.berthold-ds.com). Significances were calculated with ANOVA by comparison of the untreated control to the stimulated values.

Osteogenic differentiation of hMSC

For osteogenic differentiation, hMSC were cultivated in 6-well plates until confluence. Cells were pre-treated with 5, 20 and 50 μM ZA for 3 and 6 h, respectively, and incubated after a medium change in osteogenic differentiation medium (DMEM high glucose, 50 μg/ml ascorbate, 10 mM β-glycerophosphate, 100 nM dexamethasone (Sigma Aldrich GmbH)) for 2 weeks (RNA isolation) and 4 weeks (analysis of mineralization). To block ZA effects, cells were cotreated with 10 μM GGPP for the whole differentiation period. Additionally, cells were cultivated in osteogenic differentiation medium for 4 weeks, containing 10 and 100 nM ZA for chronic treatment. Mineralized extracellular matrix was detected by Alizarin red staining (Alizarin Red S, Chroma-Gesellschaft Schmid & Co., Stuttgart, Germany) as described previously [39,40]. Cytoplasmic ALP was stained using the Alkaline Phosphatase, Leukocyte Kit 86-C (Sigma Aldrich GmbH) according to the manufacturer’s instructions. ALP staining and mineralization was quantified with the AutoMess tool of the Axiosvision Software (Carl Zeiss Microimaging GmbH, Göttingen, Germany, www.zeiss.de). Significances after osteogenic differentiation were calculated with ANOVA by comparison of the untreated controls (c) to the stimulated values (5, 20, 50 μM, 10, 100 nM).

RT-PCR

Total RNA was isolated from hMSC treated as described above and differentiated towards the osteogenic phenotype for 2 weeks, using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany, www.macherey-nagel.de) according to the manufacturer’s instructions. Two micrograms of total RNA were reverse-transcribed with MMLV reverse transcriptase (Promega GmbH) in a volume of 20 μl. For amplification of osteogenic markers (alkaline phosphatase (AP), osteopontin (OP), osteocalcin (OC), collagen 1A2 (COL1A2) [23] and the wnt signalling inhibitor dickkopf 1 (DKK1) [141]) 1 μl of cDNA was used as a template in a volume of 50 μl. Taq DNA polymerase was obtained from Pqlab GmbH (Erlangen, Germany, www.pqlab.de). PCR conditions were as follows: 30 sec at 94 °C, 30 sec at annealing temperature, 60 sec at 72 °C; 40 cycles. The sequences of the primers used, annealing temperatures, MgCl₂ concentrations, and the sizes of the PCR products are listed in Table 1. PCR bands were semiquantitatively analysed by densitometry measurements using the Bio1D software (Vilber Lourmat GmbH, Eberhardzell, Germany, www.vilber.de).

Results

Effects of permanent incubation with ZA on hMSC proliferation and apoptosis

MSC preparations were subjected to various concentrations of ZA (1, 10, 100, and 500 nM, 1, 5, 20 and 50 μM) for various time points. A significant inhibition of proliferation after treatment with 20 and 50 μM ZA was noted after 24 h, which could be enhanced after 48 and 72 h (Fig. 1A, dark grey bars). 20 and 50 μM ZA reduced the rate of apoptosis of hMSC after 24 h. From 48 h of treatment, apoptosis was...
significantly enhanced using 20 and 50 μM ZA (Fig. 1A, light grey bars). Treatment with 5 μM ZA had no effect on proliferation and apoptosis rates of hMSC at any time point up to 72 h, as well as ZA concentrations between 1 nM and 1 μM (data not shown). Costimulation of cells with 10 μM GGPP could rescue the ZA (50 μM) induced induction of apoptosis and the inhibition of proliferation, respectively. In contrast, costimulation with 40 μM FPP could partly rescue the ZA induced apoptosis but had no effect on ZA induced proliferation inhibition. There was no effect of GGPP or FPP alone on proliferation or apoptosis (Fig. 1B). Methanol, the solvent for GGPP and FPP, showed no effect on proliferation or apoptosis (Fig. 1B). Effects of pulse treatment with ZA on the regulation of osteogenic markers in hMSC after osteogenic differentiation

Four independent preparations of MSC exposed to different concentrations (5, 20, 50 μM) of ZA for 3 h were cultured in osteogenic differentiation medium for 2 weeks and analyzed for dose-dependent regulation of osteogenic markers by RT-PCR. Densitometric analysis of the DNA bands revealed a dose-dependent and significant down-regulation of osteocalcin (OC), osteopontin (OP) and collagen type I (Col1A2) expression, an unchanged expression of alkaline phosphatase (ALP) and a variable expression of dickkopf 1 (DKK1) e.g. upregulation in individual donors without overall significance. A representative experiment is shown in Fig. 2A, the semiquantitative analysis by densitometry is shown in Fig. 2B. When we tried to block the ZA induced effects on osteogenic differentiation with GGPP we found a massive effect of the GGPP solvent methanol on the transcription of osteogenic markers like OC (up to 2.4 fold induction), OP (up to 2.9 fold induction) and DKK1 (up to 3.2 fold induction) after 2 weeks of osteogenic differentiation (data not shown). Under these circumstances GGPP rescue experiments were not realizable. Effects of pulse treatment with high ZA concentrations on the osteogenic mineralization potential of hMSC

MSC exposed to different concentrations (5, 20, 50 μM) of ZA for 3 or 6 h were cultured in osteogenic differentiation medium for 4 weeks. The degree of mineralization, analyzed by Alizarin red staining (Figs. 3A, B and 4A), as well as ALP expression (Fig. 4B), analyzed after substrate staining, were quantified on digital images. After stimulation of hMSC with different doses of ZA for 3 and 6 h, respectively and a subsequent cultivation period of 4 weeks in osteogenic medium,

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### Table 1

<table>
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<tr>
<th>Primer name</th>
<th>Sequence 5′→3′</th>
<th>Product size</th>
<th>Annealing temp.</th>
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<td>ALP sense</td>
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<td>453 bp</td>
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<td>Col1A2 sense</td>
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<td>460 bp</td>
<td>52 °C</td>
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<tr>
<td>Col1A2 antisense</td>
<td>TAACAAGGCTCCAAGCTGGA</td>
<td></td>
<td></td>
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<tr>
<td>DKK1 sense</td>
<td>ATGAGGCTGCTCGGCGCNG</td>
<td>800 bp</td>
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<td>EF1α sense</td>
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<td>OC sense</td>
<td>GCTGAGACCGCTCAAGCTTC</td>
<td>293 bp</td>
<td>60 °C</td>
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<td>OP antisense</td>
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The sequences of the primers are shown with the corresponding product sizes, annealing temperatures and concentrations of MgCl₂. (DKK1: dickkopf 1; Col1A2: collagen 1A2; OC: osteocalcin; OP: osteopontin; ALP: alkaline phosphatase; EF1α: elongation factor 1 α).
mineralization was significantly enhanced in samples treated with 20 and 50 μM ZA, compared to untreated cells. This indicates that even pulse treatment with ZA results in a sustained long term effect and robustly enhances in vitro osteogenic differentiation and mineralization. Pretreatment with 5 μM ZA had no significant effect on the degree of mineralization in osteogenic differentiation of hMSC. The fact that methanol as a solvent for GGPP caused striking effects on regulation of osteogenic genes (see above) did not allow for long term mineralization rescue experiments using GGPP (in contrast to apoptosis and proliferation experiments where methanol had no influence and the regulation of genes relevant in mineralization is of minor relevance).

**Effects of permanent nM ZA concentrations on the osteogenic mineralization potential of hMSC**

Chronic exposure of hMSC to low concentrations (10 and 100 nM) of ZA during osteogenic differentiation resulted in enhanced mineralization after 4 weeks. Mineralization was significantly enhanced by using 100 nM ZA (p < 0.005) and slightly enhanced by using 10 nM ZA (Fig. 4C). ALP staining showed a significant effect of permanent ZA treatment with 10 and 100 nM ZA, respectively (Fig. 4D). Permanent treatment with 500 nM or 1 μM ZA resulted in the detachment of the cells from the culture flask surface by two weeks time (data not shown).

**Discussion**

Bisphosphonates are successful drugs for treatment of metabolic bone diseases. Their mechanism of action is carefully characterized and their profile of side effects has been very benign [18,28,36]. However, the pharmacokinetic and pharmacologic attitudes of various BP have recently been described to vary considerably, in terms of binding to the target enzymes, binding to hydroxypatite (HA) and dissociation from HA in an acidic milieu [16,22]. Moreover, the pharmacological profiles of substance application are also different between permanent oral, intermittent oral and intermittent intravenous applications in time frames up to 12 months. These differences are now more being attributed to clinically relevant issues between various BP. In patients treated for 5 years with oral alendronate, bone metabolism remained largely unchanged in the lower normal range for 5 years of follow up, which may reflect the long half-life of ABP displaying high affinity to HA [3,18]. Similarly, the effect of a single infusion of 5 mg of ZA in patients suffering from osteoporosis lasted

![Fig. 3. Osteogenic differentiation of hMSC for 4 weeks after 3 h incubation with 5, 20, and 50 μM of ZA. Mineralization was visualized by alizarin red staining. Photographs of (A) 9.6 cm dishes and (B) microscopy magnifications of a representative experiment are shown. The bar represents 200 μm (n. d. not differentiated; C: differentiated control).](image-url)
for at least 12 months and the same infusion regimen produced long-term remission in Paget’s disease of bone, which was superior to high-dose oral risedronate. The latter displays a comparably lower affinity to HA, an increased off-rate and a shorter half-life in bone when compared with ZA [22,30,31]. The clinical data are substantiated by preclinical studies, where a single i.v. dose of ZA protected ovariectomized rats from bone loss for 32 weeks [12]. These observations indicate that there is indeed BP accumulation in bone, causing sustained pharmacological effects upon activation of bone remodeling or changes in local pH with consecutive diffusion of HA-immobilized BP.

Zoledronic acid is intravenously applied as a single shot infusion within 20–30 min, which leads to transient μM concentrations in the circulation. Recent data indicate that this regimen leads to enhanced bone mass and mineral apposition rate and reduces vertebral fracture risk up to 70% [2,27]. It is generally believed that the main mechanism of action is antiresorptive through osteoclast inhibition. It is well accepted that osteoclasts and other phagocytosing cells can internalize bisphosphonates. Cellular uptake by specific pumps or channels has been discussed but never finally demonstrated [11,32,42]. BP uptake by non-phagocytosing cells can also be envisaged through pinocytosis, but may require higher concentrations of BP in the microenvironment.

The data reported here again confirm that hMSC can internalize the compounds. The concentrations needed to induce apoptosis and inhibit cell proliferation in hMSC are higher compared to osteoclasts [34]. We co-stimulated with geranylgeranyl pyrophosphate (GGPP) and farnesy1 pyrophosphate (FPP) to find out, if the cellular effects of ZA demonstrated in hMSC are also due to the inhibition of protein prenylation or by the accumulation of APPPI. We achieved a rescue of ZA induced apoptosis and ZA induced inhibition of proliferation after GGPP treatment, as it was reported for osteoclasts [5]. Thus the mechanism of ZA action in hMSC with respect to apoptosis induction seems to be dependent on ZA effects on the mevalonate pathway and consecutive protein prenylation. When we tried to block the ZA induced impact on osteogenic differentiation with GGPP we found a massive effect of the GGPP solvent methanol on the transcription of osteogenic markers like OC, OP and DKK1 after 2 weeks of osteogenic differentiation. Since GGPP is only available on the market with the solvent methanol we felt that we could not reliably set up experiments to rescue the ZA induced osteogenesis and mineralization effects. However, given that GGPP could rescue the ZA effects on apoptosis and proliferation probably allows for the assumption that the mevalonate pathway is the main target of ZA in osteogenic cells.

Inhibition of the mevalonate pathway in embryonic stem cells by simvastatin was reported to block self renewal through impaired prenylation of Rho small GTP-binding protein, which lead to alteration of its association to membrane structures. Applying geranylgeranyl pyrophosphate could rescue the expression of pluripotency factors like Oct4, Nanog and Rex-1. This indicates that the principal activity of the mevalonate pathway is important for the

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Fig. 4. Mineralization and ALP staining. Induction of mineralization (A) after 3 h (light grey bars) and 6 h (dark grey bars) and ALP staining (B) of ZA pulse pre-treatment followed by 4 weeks of osteogenic differentiation (c = differentiated control). Data are expressed as means of 9 (3 h treatment) and 3 (6 h treatment) and 3 (ALP staining) independent experiments ± SEM. Induction of mineralization (C) and ALP staining (D) after permanent treatment with ZA during a 4 weeks period of osteogenic differentiation (c = differentiated control). Data are expressed as means of 3 independent experiments ± SEM, using different preparations of hMSC. In each case, 3 to 5 pictures of each staining were analyzed with the AutMess tool of the Axiovision software (Carl Zeiss Gmbih). Significances were calculated by ANOVA (⁎: p < 0.05; ⁎⁎: p < 0.005) by comparison of the differentiated controls (c) to the stimulated values (5, 20, 50 μM, 10, 100 nM) after osteogenic differentiation.
fate decision of pluripotent ES cells in that inhibition of this pathway and depletion of CGPP pools alters stemness and promotes commitment [17]. Our data might indicate that in the setting of multipotent hMSC very similar effects on commitment and differentiation are exerted by blocking the mevalonate pathway using ZA. The sustained effects on cell differentiation and maturation exerted by pulse exposure to ZA followed by enhanced morphogenic signals in osteogenic medium are compatible with an effect on cell biology in terms of commitment.

We realized that there were long lasting effects of ZA stimulation on the regulation of osteogenic marker genes. OC and OP were down-regulated in a dose-dependent manner, as it was reported for late stages during osteogenic differentiation [19,20]. The upregulation of DKK1 in individual donors would be coincident with several publications which reported a down-regulation of Wnt signalling in maturing osteoblasts to enable the formation of a mineralized bone matrix [43,44]. The phenomenon could also reflect the beginning of osteoblast-osteocyte transition where DKK1 was reported to be upregulated [25]. Our data show that osteogenic differentiation of hMSC can be remarkably enhanced by pulse stimulation with μM concentrations of ZA. These findings are consistent with the clinical situation, where a significant increase of mineral apposition rate was found in patients treated with 5 mg ZA once yearly [27].

Our data and an increasing body of evidence from the literature show that the mevalonate pathway modulates stemness versus morphogenesis or apoptosis in mesenchymal cells. Hence the degree of intracellular accumulation of BP determines if cells undergo morphogenic commitment or apoptosis induction. Micro-environment exposure of cells to BP is either fed by intravenous or oral pharmacological applications or by continuous release of bone accumulated BP. Focal high bone turnover lesions like chronic osteomyelitis or bone metastases do enrich BP in the surrounding environment exposure of cells to BP is either fed by intravenous or oral pharmacological applications or by continuous release of bone accumulated BP. Focal high bone turnover lesions like chronic osteomyelitis or bone metastases do enrich BP in the surrounding bone especially under long term frequent dosing regimens. Only under those circumstances may it be envisaged that BP concentrations in the microenvironment exceed μM concentrations for longer periods of time and thus propagate apoptosis of mesenchymal, endothelial or tumor cells. Such effects may be effective in tumor therapy but may also contribute to ONJ pathogenesis together with infection, local acidification and antiangiogenic effects in a skeletal site exposed to mastication strain and a high rate of microdamage [1,8,38]. Short term exposure to μM BP in intravenous osteoporosis therapy according to our data should be morphogenic and thus beneficial.

In summary we show here that μM concentrations of ZA enhance osteogenic differentiation of hMSC in vitro, but also induce hMSC apoptosis, depending on the concentration and time of exposure. Short term μM ZA exposure enhances osteogenic differentiation and in vitro mineralization of hMSC and shows sustained effects on cell biology, possibly resembling the in vivo situation of intravenous ZA infusions. These data contribute to the dissection of beneficial versus adverse effects of BP at the level of molecular and cell biology and may help to translate these findings into the in vivo situation. Further research is needed to work out an upper limit of BP accumulation in bone to avoid serious adverse events in benign clinical settings, and to clarify if in metastatic diseases apoptosis induction can be developed into intelligent combination strategies of anti-tumor therapy.

Conflict of interest
All authors have no conflicts of interest except: Franz Jakob receives honoraria for lectures and consulting from Novartis, Procter & Gamble, Servier, Lilly, MSD, and Roche.

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References


