Differential Response of MC3T3-E1 and Human Mesenchymal Stem Cells to Inositol Hexakisphosphate

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Key Words
Inositol hexakisphosphate • Phytic acid • Osteoblast • Human umbilical cord mesenchymal stem cells (hUC-MSCs) • Mineralization • Gene expression • IP6 kinase inhibitor

Abstract
Background: Inositol hexakisphosphate (IP6) has been found to have an important role in biomineralization. Methods: Because the complete mechanism of action of IP6 on osteoblasts is not fully understood and its potential use in the primary prevention of osteoporosis, we examined the direct effect of IP6 on cell viability and differentiation of MC3T3-E1 cells and on differentiation of human umbilical cord mesenchymal stem cells (hUC-MSCs). Results: We show that IP6 has different effects depending on the origin of the cell target. Thus, while IP6 decreased gene expression of osteoblast markers and mineralization in MC3T3-E1 cells without negatively affecting cell viability and ALP activity, an increase in gene expression of ALP was observed in hUC-MSCs committed to the osteoblastic lineage. This increasing effect of IP6 on ALP mRNA expression levels was reversed by the addition of a selective inhibitor of IP6 kinase, suggesting that the effect of IP6 might be due through its pyrophosphorylated derivatives. Besides, Rankl mRNA levels were decreased after IP6 treatment in MC3T3-E1 cells, pointing to a paracrine effect on osteoclasts. Conclusion: Our results indicate that IP6 has different effects on osteoblast differentiation depending on the cell type and origin. However, further studies are needed to examine the net effect of IP6 on bone formation and its potential as novel antiosteoporosis drug.

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**Introduction**

Maintenance of bone mass is a dynamic process regulated by two cellular mechanisms, bone formation and bone resorption. Thus, while osteoblasts control bone formation, the osteoclasts are responsible for bone matrix resorption, and these functions are tightly regulated via reciprocal crosstalk to maintain a constant bone mass. Disruption of this strict balance leads to systemic skeletal diseases like osteoporosis. The therapeutic options for the treatment of osteoporosis have so far comprised mostly antiresorptive drugs (biphosphonates [1], replacement therapy with estrogen [2], calcitonin [3]), treatments that stimulate bone formation (selective estrogen receptor modulator (SERM) [4], PTH derivatives (i.e. recombinant teriparatide) [5]) or the recently approved monoclonal antibody, denosumab [6].

Most of the current therapies present limitations, however, in particular the fact that they lead to a low turnover state where bone formation decreases with the concomitant decrease in bone-remodeling activity. One additional issue remains in the fact that the current gold-standard treatment, the use of biphosphonates, show several side effects such as gastrointestinal intolerability and mandibular osteonecrosis [7-9], which make them not appropriate for certain populations. Given the limitations of current antiosteoporosis drugs, a search for new therapeutics has focused in the last few years on the identification of novel antiresorptive drugs that prevent the decrease in bone turnover and on bone anabolics, which increase bone formation directly without affecting bone resorption [10].

In our previous studies, we have observed that inositol hexakisphosphate (IP6, phytic acid) has a direct effect on osteoclasts, inhibiting RANKL-induced osteoclastogenesis of RAW 264.7 precursor cells and human primary osteoclasts [11]. IP6 is found in high amounts in plant seeds, being their major phosphate store [12, 13] and it has also been shown to be widely distributed in animal cells and tissues [14-16]. Intake of IP6 has been shown to correlate with an increase on bone mineral density (BMD) [17] and with a reduced BMD loss due to estrogen deficiency in an osteoporosis animal model [18]. In fact, IP6 has been proposed to exhibit similar effects to those of biphosphonates (BP) on bone resorption and to be of use in the primary prevention of osteoporosis [18]. A recent study by Addison et al. [19] showed that IP6 inhibits mineralization of MC3T3-E1 osteoblast cultures, increases gene expression of the mineralization inhibitor osteopontin, but does not impair the ability of osteoblasts to synthesize a collagenous matrix, express alkaline phosphatase or differentiate to produce specific bone matrix proteins. However, further investigation is needed to fully understand the direct effect of IP6 on osteoblasts and the net effect on bone formation.

The aim of the present study was to investigate the direct effect of IP6 on cell viability and differentiation of the MC3T3-E1 cells and to confirm the effect on differentiation of human umbilical cord mesenchymal stem cells (hUC-MSCs) stimulated to the osteogenic lineage. Moreover, since it has been hypothesized that the pyrophosphorylated derivatives of IP6 (IP7 and IP8), may be responsible for many of the signaling-related functions previously attributed to IP6 [20], and to give some insight into the mechanism of action of IP6, we have used a selective IP6 kinase inhibitor.

**Materials and Methods**

**Cell culture**

The mouse osteoblastic cell line MC3T3-E1 (DSMZ, Braunschweig, Germany) was maintained in α-minimum essential media (Gibco, Grand Island, NY, US) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH) and 1%-penicillin–streptomycin (Sigma, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 5% CO2. All experiments were performed after 13-15 passages of the MC3T3-E1 cells. Cells were seeded in 24-well plates at a plating density of 20,000 cells. Cell differentiation and mineralization were initiated 48h after plating by replacing the growth media with fresh differentiating media, supplemented with 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate and 200 nM hydrocortisone (Sigma, St. Louis, MO,
USA). Cell culture media, with or without IP6 (Azopharma Product Development Group, Miramar, FL, USA), was refreshed every 48 hours over a 14-day period.

Human umbilical cord derived mesenchymal stem cells (hUC-MSCs) were isolated from umbilical cords obtained in the process of human umbilical cord blood donation under the Concordia Cord Blood Donation Program. The samples were obtained after informed consent and with the approval of the Ethical Committee of Balearic Islands (CEIC-IB). hUC-MSCs were isolated as previously described [21].

hUC-MSCs from two different donors (six replicates each one) were seeded in 24-well plates and grown to confluence in growth media consisting of DMEM-LG supplemented with penicillin (50 IU/ml), streptomycin (50 µg streptomycin/ml) and 20% FBS (HyClone). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. At confluence, cells were grown in differentiation media consisting of growth media supplemented with hydrocortisone (200 nM), ascorbic acid (50µg/ml) and β-glycerophosphate (10 mM).

Cell culture media, with or without 4µM IP6, and with or without 10µM TNP (N₂-(m-(trifluoromethy)lbenzyl) N₆-(p-nitrobenzyl)purine) (Sigma, St Louis, MO, USA), a selective inhibitor of IP6Ks [22], was refreshed twice a week over a 14-day period.

Cytotoxicity assay
Culture media from MC3T3-E1 cells was collected after 24h of treatment to test cytotoxicity (LDH activity). LDH cytotoxicity assays were performed according to the manufacturer’s protocol (Cytotoxicity Detection Kit (LDH), Roche Diagnostics, Switzerland). This colorimetric assay quantifies activity of LDH released from the cytosol of damaged cells into the supernatant and thus serves as an index of cell death. Results were presented relative to the LDH activity in the media of control cells (100% of cell viability) and of cells treated with 1% Triton X-100 (0% cell viability) using the equation: Cell viability (%) = (IP6-treated cells - Control cells) / (Triton-treated cells- control cells) x 100.

Propidium Iodide staining
Cell death was assessed in MC3T3-E1 cells by the uptake of the fluorescent exclusion dye Propidium Iodide (PI). PI is impermeable to cells with intact plasma membranes, but when a cell's integrity becomes compromised, it enters the cells and stains the nucleus. 2x10⁴ cells were seeded on coverslips in 24 well-plates. At confluence, cells were treated with IP6 (100 µM), either in growth or in differentiation media. After 14 days of culture, cells were incubated with PI 5µg/ml (Sigma, St Louis, US) for 30 minutes at 37°C and then fixed with 4% paraformaldehyde solution (Sigma, St Louis, US), and permeabilized with 0.1% Triton X-100 (Sigma, St Louis, US) for 15 minutes at room temperature. Nuclei were stained with Fluoroshield with DAPI (Sigma, St Louis, US). Apoptotic cells were counted with a fluorescence microscope.

Assay for alkaline phosphatase (ALP) activity
ALP activity was determined from cell monolayers after 14 days for MC3T3-E1. Cells were washed twice in PBS, solubilized with 0.1 % Triton X-100. Then, samples were incubated with an assay mixture of p-Nitrophenyl Phosphate. Cleavage of p-Nitrophenyl Phosphate (pNPP) (Sigma, Saint Louis, Missouri, US) in a soluble yellow end product which absorbs at 405nm was used to assess ALP activity. In parallel to the samples, a standard curve with calf intestinal alkaline phosphatase (CIAP) (Promega, Madison, US) was constructed. Protein concentration was determined with a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) following the manufacturer’s kit instructions.

Determination of calcium accumulation
Total calcium content was quantified on day 14 in MC3T3-E1 cells by washing cells twice with PBS, digesting in 0.5 N HCL overnight, followed by centrifugation at 500 x g for 2 minutes for the subsequent determination of Ca²⁺ content in the supernatant using the Calcium (CPC) Liquicolor Kit (Stanbio Laboratory, Boerne USA). In parallel to the samples, a standard curve with known concentrations of calcium was constructed.

RNA isolation and real-time RT-PCR analysis
Total RNA was isolated with Tripure (Roche Diagnostics), following the instructions of the manufacturer. RNA was quantified using a spectrophotometer set at 260 nm (Nanodrop, Thermo Fisher Scientific Inc, US).
The same amount of total RNA (1µg) from each sample was reverse transcribed to cDNA at 37°C for 60 minutes in a final volume of 20µl, using High Capacity RNA to cDNA kit (Applied Biosystems, USA). Each cDNA was diluted 1/5 and aliquots, to avoid freezing and thawing cycles, were stored at -20°C until the PCR reactions were carried out. The specific primer sequences were designed using the NCBI primer designing tool and are given in Table 1.

Real-time PCR was performed for two reference genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin (β-Actin), and for several target genes: alkaline phosphatase (ALP), bone sialoprotein (BSP), collagen type I (COL1A), osteocalcin (OC), osteopontin (OPN), osteoprotegerin (OPG), osterix (OSX), receptor activator for nuclear factor kappaB ligand (RANKL) and runt-related transcription factor 2 (RUNX2).

### Table 1. Primers used in the real-time RT-PCR analysis. Murine (m) and human (h) sequences are shown 5' to 3'.

| Gene (mouse) | ACCESSION Nr. | Sequences |
|--------------|---------------|-----------|
| mALP         | NM_007431     | Forward: 5'-AACCCAGACACAGCATTCC-3'  
Reverse: 5'-GAGAGCGAAGGTTCAGCAG-3' |
| mBSP         | NM_008318     | Forward: 5'-GAAATGAGAGGAGGATAG-3'  
Reverse: 5'-ACCCGAGTGTGGAAAGTG-3' |
| mGAPDH       | NM_009084     | Forward: 5'-ACCCAGAGACCTGATGATG-3'  
Reverse: 5'-CACTTGGGGGTAGGAACAC-3' |
| mOC          | NM_007541     | Forward: 5'-CCGGAGCAGTGTGAGTCA-3'  
Reverse: 5'-TAGATGCTTTGTTGAGGTC-3' |
| mOPG         | NM_008764     | Forward: 5'-AGACGACGTTAGTGTGAC-3'  
Reverse: 5'-AACACAGCCATGACCTTC-3' |
| mOPN         | NM_009263     | Forward: 5'-TCCTGCGGACCATTCTGCG-3'  
Reverse: 5'-GTCATTTTACCGGGAGGGA-3' |
| mOSX         | NM_130458     | Forward: 5'-ACTGGCTAGTGTGATCG-3'  
Reverse: 5'-GGTAGGAGGTGTTGTTAAGG-3' |
| mRANKL       | NM_011613     | Forward: 5'-GCGCACAGCTTCTCTAC-3'  
Reverse: 5'-TGACTTTATGGGAAACCAG-3' |

| Gene (human) | ACCESSION Nr. | Sequences |
|--------------|---------------|-----------|
| Hβ-Actin     | NM_001101     | Forward: 5'-CTGGAACGATGAGTGACA-3'  
Reverse: 5'-AAGGAGCTTCTGTAACAA-3' |
| hALP         | NM_000478     | Forward: 5'-CCGCTATCTGTCCTGGCC-3'  
Reverse: 5'-GGTGCGCTGCGACATGTCAG-3' |
| hCOL1A       | NM_000088     | Forward: 5'-CTGACGACAGGAGCCAGAGG-3'  
Reverse: 5'-GCGAGGGCTGCGGTTCTTCCAC-3' |
| hGAPDH       | NM_002046     | Forward: 5'-TGCACACAACTGCTTACG-3'  
Reverse: 5'-GGCATGGACTGTTGGTCATG-3' |

Real-time PCR was performed in the Lightcycler 480® (Roche Diagnostics, Mannheim, Germany) using SYBR green detection. Each reaction contained 7 µl Lightcycler-FastStart DNA MasterPLUS SYBR Green I (containing Fast Start Taq polymerase, reaction buffer, dNTPs mix, SYBRGreen I dye and MgCl2), 0.5µM of each, the sense and the antisense specific primers and 3µl of the cDNA dilution in a final volume of 10 µl. The amplification program consisted of a preincubation step for denaturation of the template cDNA (5min 95°C), followed by 45 cycles consisting of a denaturation step (10s 95°C), an annealing step for mouse primers (10s 60°C for GAPDH, OC and BSP; 62°C for osteopontin, 65°C for ALP and 68°C for Osterix) and for human primers (10s 60°C for GAPDH, β-Actin, COL1A and 5s 68°C for ALP) and an extension step (10s...
72°C). After each cycle, fluorescence was measured at 72°C. A negative control without cDNA template was run in each assay.

To allow relative quantification after PCR, standard curves were constructed from standard reactions for each target and reference genes. The crossing point readings for each of the unknown samples were used to calculate the amount of either the target or reference relative to a standard curve, using the Second Derivative Maximum Method. Relative mRNA levels were calculated as the ratio of relative concentration for the target genes in the same sample using the Advanced Relative Quantification Method provided by the LightCycler 480 analysis software version 1.5 (Roche Diagnostics, Mannheim, Germany).

Statistical analysis of data
The data were presented as mean values ± SEM. Differences between groups were assessed by Student’s t test or Mann-Whitney test, depending on their normal distribution, using the statistical software package SPSS® program for Windows, version 17.0 (SPSS, Chicago, IL, US). Results were considered statistically significant at the p-values ≤0.05.

Results
Effect of IP6 on cell viability and apoptosis of MC3T3-E1 cells
Cell viability was first determined after 24h in MC3T3-E1 cells cultured in growth media with increasing doses of IP6, in the concentration range 0.1-100 µM. We found that only cells treated for 24h with the dose of 100 µM IP6 showed statistically lower cell viability than control cells (Fig. 1).

Next, apoptosis and cell morphology were evaluated after long term treatment with IP6 (14 days) in MC3T3-E1 cells cultured in growth and in differentiation media (Fig. 2). As shown in Figure 2A, treatment with 100 µM IP6 induced a higher degree of apoptosis of MC3T3-E1 cells in both, growth and differentiation media, with significant results for cells treated with IP6 in differentiation media. An image of the cells cultured in growth and differentiation media and treated with 100 µM IP6 for 14 days can be seen in Figure 2B. In agreement with the apoptosis results, the morphology of cells treated with 100 µM of IP6 in differentiation media indicated a negative effect on cell viability after long term incubation. Thus, 100 µM IP6-treated cells and cultured with the differentiation supplements lost round-shaped morphology typical of mature osteoblasts, displaying a fibroblast-like morphology with unordered cellular arrangement. No morphological differences were observed for the cells treated with IP6 for 14 days in the concentration range of 0.1-10 µM (data not shown).
Effect of IP6 on gene expression of osteoblast differentiation markers in MC3T3-E1 cells

By real-time RT-PCR, we studied the effect of IP6 on several osteoblast markers associated to differentiation and mineralization. Osterix (OSX) is a zinc finger transcription factor expressed in osteoblasts, involved in the differentiation step from preosteoblasts to fully functional osteoblasts [23]. Treatment of MC3T3-E1 cells with 4 µM of IP6 in growth media induced Osx mRNA levels (Fig. 3A). However, with the highest IP6 dose of 100µM, Osx mRNA levels were decreased in both growth and differentiation media. We also studied Alp mRNA levels in cells cultured with IP6 (Fig. 3B). Alp mRNA levels were significantly decreased with all the doses of IP6 in cells cultured in differentiation media, while no significant changes were found in MC3T3-E1 cells treated with IP6 and cultured in growth media. Bone sialoprotein (Bsp) mRNA levels significantly increased with the dose of 4 µM of IP6 in growth media, while decreased significantly with 4 µM, 10 µM and 100µM of IP6 in differentiation media (Fig. 3C). Cells treated with IP6 and cultured in growth media showed lower mRNA levels of osteocalcin compared to untreated cells, with statistical significance for the doses of 1µM, 4µM, 10 µM and 100µM of IP6 (Fig. 3D). Treatment with IP6 did not induce differences on Oc mRNA levels on cells cultured on differentiation media. Osteopontin (OPN) is an extracellular glycoprotein found in bone and binds to multiple organic or mineral ligands such as CD44, integrin receptor and hydroxyapatite [24-26], inhibiting extracellular matrix mineralization. We found no important changes in Opn mRNA levels either in growth or differentiation media, only 100 µM of IP6 in growth media decreased significantly Opn mRNA levels (Fig. 3E).

Osteoblast function is linked to osteoclast activity via osteoblast production of factors that either stimulate (RANKL) or inhibit (OPG) osteoclast differentiation, being their source primarily from osteoblastic cells [27]. As shown in Figure 3F, IP6 decreased in a dose-dependent manner Rankl mRNA levels in cells cultured in differentiation media, with significant down-regulation for the doses of 10 µM and 100µM. In growth media, 100 µM of
IP6 also decreased Rankl mRNA levels. Opg mRNA levels did not show important differences among the groups treated with IP6 (data not shown), only higher Opg mRNA levels were observed under osteogenic conditions in all the groups.

**Effect of IP6 on ALP activity and mineralization of MC3T3-E1 cells**

The appearance of ALP activity is represented as an early phenotypic marker for differentiated osteoblasts, as ALP is known to be critically involved in the initiation of mineralization. ALP activity was measured on the cell monolayer after 14 days of cell culture. As seen in Figure 4A, ALP activity was maintained or slightly increased with IP6, either in growth or differentiation media, in the concentration range of 0.1-10 µM, while a significant decrease in ALP activity was found on MC3T3-E1 cells treated with 100 µM of IP6 and cultured for 14 days in differentiation media.

The effects of IP6 on extracellular matrix mineralization were tested by measuring the calcium deposition after 14 days, under both growing and differentiating conditions. As shown in Figure 4B, cells cultured with lower doses of IP6 (1-10 µM) in differentiation media showed a decrease in calcium deposition, whereas higher calcium depositions were found in cells treated with 100 µM of IP6 in both conditions, probably due to IP6 co-precipitation with calcium ions.

**Effect of IP6 and TNP on gene expression of osteoblast marker genes in hUC-MSCs**

Our next goal was to investigate whether IP6 had the same effects on the osteoblast differentiation of more primitive cells and to give some insight into the mechanism of action of IP6 using the selective IP6K inhibitor TNP [22]. Therefore, human umbilical cordon
Mesenchymal stem cells (hUC-MSCs) were stimulated to the osteogenic lineage and treated with 4 µM of IP6 for 14 days with or without 10 µM TNP. Gene expression of COL1A and ALP was analysed (Fig. 5). COL1A, which is related to the proliferation period and its gene expression decreases when differentiation starts, was downregulated with IP6 compared to control, although differences in gene expression did not reach statistical significance because of the variability between the donors. Moreover, IP6 treatment significantly induced higher mRNA levels of ALP compared to the control group. The addition of TNP reversed the effect of IP6 on ALP mRNA expression levels, giving some insights into the mechanism of action of IP6.
Discussion

New bone formation is the main function of osteoblasts, as they synthesize and regulate the deposition and mineralization of the extracellular matrix of bone. MC3T3-E1 is an osteoblast-like cell line that has been reported to keep their ability to differentiate into osteoblasts, providing very useful information about regulation of osteoblast differentiation [28]. Human mesenchymal stem cells (hMSCs) are multipotent cells that can differentiate into multiple lineages such as osteoblasts, and have been used in tissue-engineering strategies with potential in clinical applications [29]. The present study demonstrates that IP6 impairs the differentiation and mineralization of MC3T3-E1 cells while increases gene expression of the osteoblast differentiation marker ALP in hUC-MSCs. The difference found in the effect of IP6 on MC3T3-E1 and hUC-MSCs could be related to the developmental stage at the time of isolation. MC3T3-E1 cells are isolated from calvaria of C57BL/6 newborn mouse, while hUC-MSCs were isolated from umbilical cord of two different donors, reflecting a more primitive and earlier stage. The combination of both types of approaches (primary cells vs. cell lines) is important in the in vitro assessment of new potential treatments.

In osteoporosis, the equilibrium between the synthesis of new bone by osteoblasts and resorption by osteoclasts is disrupted in favor of a higher resorption rate [30]. Bisphosphonates (BPs) are so far the most commonly used antiresorptive drugs in osteoporosis therapy [31, 32]. Some reports have shown that BPs not only act on osteoclasts but also affect other bone-related cells, such as osteoblasts, osteocytes, and macrophages [33, 34]. IP6 has been proposed to be of use in the primary prevention of osteoporosis [18]. In vivo, the adequate consumption of IP6 may play an important role in the prevention of bone mineral density loss in postmenopausal women [35] and in animal models [18]. The direct effect of IP6 on bone cells, osteoblasts and osteoclasts, has not been studied deeply. To the best of our knowledge, there is just one in vitro study analyzing the effects of IP6 on the differentiation and mineralization of MC3T3-E1 cells [19]. Addison and coworkers showed in this study that IP6 inhibits mineralization but does not impair the ability of osteoblasts to synthesize a collagenous matrix, express alkaline phosphatase or differentiate to produce specific bone matrix proteins. However, here we show that differentiation and mineralization was decreased by IP6 treatment using the same cell line. The main difference between this and our study is that the former only studied the effects of a dose of 4 µM IP6, while we have used, in most of the analysis performed in the present study, and in osteogenic and non-osteogenic conditions, a wider concentration range (0.1-100 µM) of IP6, which covers physiological and pharmaceutical doses. Thus, as it has been observed in the treatment of osteoblasts with BPs [36, 37], the effects of IP6 on osteoblasts is dose dependent. As regards to cell viability, only the highest dose of IP6 used was found to be toxic. Thus, we can demonstrate that the effects observed of IP6 on osteoblasts are not due to toxic effects.

To determine the effect of IP6 on osteoblast differentiation, we determined ALP activity, as it has been reported to be an essential enzyme for mineralization [38]. No changes in ALP activity were found after 14 days of treatment with IP6, just a significant decrease was found with 100 µM of IP6 under differentiating conditions, which could be related with the higher cytotoxicity found. In the study performed by Addison and coworkers [19], a slightly elevated ALP activity was found with 4 µM of IP6; however, we did not observe such results.

While IP6 in the dosage range of 0.1-10 µM decreased calcium deposition in differentiation media, there was a significant increase in the calcium content for the dose of 100 µM in both growth and differentiation media. Given the higher cytotoxicity found and the morphological aspect of the cells treated with this higher dose, it is possible that this increase in calcium content does not reflect an increase in extracellular matrix mineralization and, rather, is due to a precipitation of IP6 in the cell monolayer as calcium phytate.

With regards to osteoblast differentiation, an array of different marker genes was studied in MC3T3-E1. In MC3T3-E1 cells we observed a decrease in gene expression of osteocalcin and bone sialoprotein, while little or no effects were found on osterix and osteopontin, respectively. Addison et al. [19] showed that mRNA levels of osteocalcin and bone sialoprotein
did not change with the treatment of 4 µM of IP6. However, our study demonstrates that the same markers were downregulated with 4 µM of IP6 in differentiation conditions. The same authors indicated that IP6 upregulated osteopontin, an important inhibitor of osteoblast mineralization. In our study, osteopontin mRNA levels were decreased by IP6 only with the dose of 100 µM. The discrepancy between both studies might be attributed to the use of a different method for the determination of mRNA levels. Thus, while Addison’s study used a traditional RT-PCR, we used real-time RT-PCR, which is more accurate in the determination of gene expression changes. In the present work, Alp mRNA levels decreased with IP6 treatment in osteogenic conditions although ALP activity only decreased significantly with 100 µM of IP6. Complex biological processes, such as transcriptional and post-transcriptional splicing, translational modifications, and protein complex formation, could affect the correlation between mRNA levels and protein expression and activity.

Another interesting observation of the present study was the effect found on Rankl gene expression with IP6, as the OPG/RANKL ratio has a key role in the control of bone resorption and a direct effect of IP6 has already been found in vitro with osteoclasts [11]. This would point towards an indirect effect of IP6 on osteoclastogenesis. Interestingly, physiological bone intracellular concentrations of IP6 have been reported to be in the range from 1.5 to 3 µM and to be from a dietary origin [16], which corresponds to the doses where we have observed a biological effect on both osteoclasts [11] and osteoblasts.

Moreover, we found a differential response of MC3T3-E1 cells and hUC-MSCs to IP6. Our results have shown that 4 µM IP6 treatment increased gene expression of the osteoblast differentiation marker ALP in hUC-MSCs, when these cells were under osteogenic conditions. Contradictory results have been found on the effects of BPs on MSCs differentiation towards osteoblasts. Risedronate, a nitrogen-containing BP, has been reported to suppress osteoblast differentiation in cloned hMSCs [39], although it has been reported that the same BP and zoledronate stimulate osteoblast-specific gene expression and ALP activity in bone marrow stromal cells [40] and alendronate enhances the osteogenesis of bone marrow mesenchymal stem cells [40-42].

It is important to mention that IP6 is the precursor of inositol pyrophosphates, like IP7 and IP8, molecules that contain one or two high-energy pyrophosphate bonds [43, 44]. Pyrophosphates are present in all eukaryotic cells and regulate numerous processes including chemotaxis, telomere length, endocytic trafficking, exocytosis, and apoptosis (reviewed by Chakraborty et al. [45]). Although the levels of inositol pyrophosphate are relatively low, less than 5% of IP6 [46] in mammalian cells, it has been estimated a rapid turnover, converting up to 50% of IP6 pool every hour to its pyrophosphorylated derivatives [43], pointing to the hypothesis that IP7 and IP8 may be responsible for many of the signaling-related functions previously attributed to IP6 [20]. Therefore, we cannot exclude the possibility that the effects found in the present study could be mediated through the synthesis and action of inositol pyrophosphates IP7 and/or IP8 from IP6. In fact, the present study shows that inhibition of the conversion of IP6 into inositol pyrophosphates using the selective IP6K inhibitor TNP [22], reversed the increasing effect of IP6 on ALP mRNA levels in hUC-MSCs. TNP treatment alone showed a significant decrease of COL1A mRNA levels, which can be explained by a reduction on basal IP7/IP8 levels, as previously reported in other cell types [22], suggesting that basal levels of pyrophosphates are required for osteoblast differentiation.

In conclusion, our results indicate that IP6 has different effects on osteoblast differentiation depending on the cell type and origin. The present observations are physiologically relevant, considering that the doses used in this study are in the range with those found in mammalian cells [15, 47-49], opening the possibility to the future use of IP6 in the treatment of different bone diseases without adverse effects as current treatments, as IP6 has already shown its positive use in the prevention of bone loss in animal and human studies [18, 35, 50]. However, further studies are needed to examine the net effect of IP6 on bone formation and its potential as novel antiosteoporosis drug.
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Conflict of interest statement

All authors declare that they are inventors of a pending patent application based on some aspects of this work.

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