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Evaluation of Osteolforte on Bone Marrow Stromal Cells (BMSC): A Study on Cell Viability, Osteogenic Differentiation, and Gene Expression

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Evaluation of Osteolforte on Bone Marrow Stromal Cells (BMSC): A Study on Cell Viability, Osteogenic Differentiation, and Gene Expression

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Abstract: Osteolforte, a compound with potential bone-regenerative properties, was investigated for its effects on human bone marrow stromal cells (BMSCs). The study aimed to evaluate its impact on cell viability, osteogenic differentiation, and gene expression using multiple assays, including MTT, Alizarin Red S staining, Real-Time PCR, and Western Blot. Results demonstrated that Osteolforte significantly enhanced osteogenic differentiation in BMSCs. Increased mineralization was observed through Alizarin Red S staining indicating higher calcium deposition. Gene expression analysis revealed upregulation of key osteogenic markers, including RUNX2, COL1, and BMP2, suggesting that Osteolforte promotes osteoblastic activity. However, an interesting dose-dependent decrease in OPN expression was noted, raising questions about its specific role in bone formation. The increased expression of RUNX2, a master regulator of osteoblast differentiation, alongside COL1 (collagen type I), a major bone matrix protein, supports the compound's osteogenic potential. Additionally, the upregulation of BMP2, a critical bone morphogenetic protein, further highlights its role in stimulating bone formation. The observed reduction in OPN (osteopontin) expression suggests that Osteolforte may modulate late-stage osteogenic differentiation differently from conventional inducers, warranting further investigation. Overall, these findings indicate that Osteolforte enhances early-stage osteogenesis and mineralization in BMSCs, making it a promising candidate for bone regeneration. However, the dose-dependent effects on OPN necessitate further studies to fully elucidate its mechanism of action and optimize its therapeutic potential. Future research should focus on in vivo validation and long-term safety assessments to establish Osteolforte as a viable treatment for bone defects and osteoporosis.

Keywords: Osteolforte; human bone marrow stromal cells (BMSCs); osteoblast; cell signaling

1. Introduction

Bone regeneration is a crucial aspect of regenerative medicine, focusing on restoring bone integrity and function following injuries, fractures, or degenerative conditions such as osteoporosis. The ability to promote osteogenesis—the formation of new bone tissue—is a primary goal in tissue engineering and regenerative medicine. Various strategies have been employed to enhance bone repair, including the use of biomaterials, growth factors, and stem cell-based therapies. Among these, small-molecule compounds that stimulate osteogenic differentiation in bone marrow stromal cells (BMSCs) represent a promising avenue for bone tissue engineering [1]. BMSCs are multipotent progenitor cells capable of differentiating into various mesenchymal lineages, including osteoblasts, chondrocytes, and adipocytes. Their osteogenic differentiation is tightly regulated by numerous signaling pathways and transcription factors, such as runt-related transcription factor-2 (RUNX-2), bone morphogenetic proteins (BMPs), and collagen type I (COL1), which play key roles in early osteoblastic commitment and matrix mineralization [2]. Understanding the mechanisms that drive

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BMSC differentiation into osteoblasts is essential for developing novel therapeutic agents for bone regeneration.

Osteolforte is a newly identified compound with potential osteoinductive properties. Preliminary studies suggest that it enhances osteogenic differentiation, making it a candidate for bone tissue engineering applications. However, its specific mechanisms of action remain largely unexplored. Investigating its effects on BMSC viability, osteogenic differentiation, and gene expression could provide valuable insights into its therapeutic potential and pave the way for future clinical applications [3].

Bone is a dynamic tissue that undergoes continuous remodeling, balancing bone formation by osteoblasts and resorption by osteoclasts. In conditions such as osteoporosis, fractures, or bone defects resulting from trauma, infection, or tumor resection, this balance is disrupted, leading to impaired bone healing [4]. Traditional treatments, including bone grafting and synthetic scaffolds, have limitations such as donor site morbidity, immune rejection, and insufficient integration with host tissue [5]. Hence, alternative strategies that harness the regenerative potential of stem cells and bioactive molecules are gaining traction in the field.

BMSCs are one of the most extensively studied cell sources for bone regeneration due to their ability to differentiate into osteoblasts under appropriate stimuli. [6]. However, using recombinant proteins and gene therapy approaches comes with safety and cost concerns, highlighting the need for small-molecule compounds that can effectively promote osteogenesis without these drawbacks [7]. The osteogenic differentiation of BMSCs is governed by several key markers and signaling pathways. RUNX2, a transcription factor, is considered the master regulator of osteogenesis, as it controls the expression of essential bone matrix proteins and promotes the transition from mesenchymal stem cells to pre-osteoblasts [6]. Another critical marker is COL1, which constitutes a major component of the extracellular matrix in bone and provides structural support for mineral deposition. Additionally, BMP2 plays a vital role in initiating osteoblast differentiation by activating downstream signaling pathways, including the Smad pathway, which drives the expression of osteogenic genes [8]. Interestingly, osteopontin (OPN), a glycoprotein involved in bone remodeling, has a complex role in osteogenesis. While it contributes to cell adhesion and mineralization, excessive OPN expression has been linked to inhibitory effects on late-stage osteoblast differentiation [9]. Investigating the effects of Osteolforte on these molecular markers can provide insights into its osteoinductive potential and reveal any unique regulatory mechanisms it may exert on bone formation. Small-molecule compounds with osteogenic properties have garnered attention due to their ability to target intracellular pathways and enhance osteogenesis efficiently. Unlike recombinant proteins and cell-based therapies, these compounds offer advantages such as ease of administration, cost-effectiveness, and better stability [7,10]. Osteolforte has emerged as a promising candidate, showing potential in preclinical evaluations. The current study aims to evaluate the impact of Osteolforte on BMSC viability, differentiation, and gene expression. By employing multiple experimental approaches such as MTT assays for cell viability, Alizarin Red S staining for mineralization, and gene/protein expression studies using Real-Time PCR and Western Blot, this study seeks to provide comprehensive insights into the osteogenic potential of Osteolforte. The results will contribute to the growing field of bone tissue engineering and help determine whether Osteolforte can serve as a viable therapeutic agent for bone regeneration.

2. Materials and Methods

2.1. Cell culture and Treatments

Human Bone marrow stromal cells (BMSCs, cat no.) were cultured and treated with Osteolforte (Gagopa corp) at concentrations of 0.01, 0.1, and 1 μg/mL. The cells were incubated for specified durations before assessment. hBMSCs were purchased from Lonza (PT-2501, Basel, Switzerland). The cells were isolated from human posterior iliac crest, with three different primary cells being obtained from three different individuals. The cells were characterized by flow cytometry using the CD105+,

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CD166+, CD29+, CD90+, CD73+, CD133-, CD34-, and CD45- surface antigens. The cells were maintained in StemMACS Media XF containing 1X anti-anti at 37 °C in a 5% CO_2 incubator.

2.2. Chemicals and Reagents

The 1,1-diphenyl-2-picrylhydrazyl radical; 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The cell counting kit -8 (CCK-8) assay was obtained from Abbkine (CA, USA). The dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St Louis, MO, USA). The α-MEM and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA), The antibiotic–antimycotic solution and the phosphate-buffered saline (PBS) were purchased from Biowest (Nuaillé, France). The RIPA lysis buffer was obtained from iNtRON Biotechnology (Gyeonggi, Korea).

2.3. Osteoforte Preparation

Osteolforte (As & Co Pharm Group LLC, Yekaterinburg, Russia; Patent No. N2527042) is a food additive developed to prevent osteoporosis. It was created through technology transfer from Dr. Sergei in Russia at Gagopa Healing Foods. Unlike conventional calcium supplements, Osteolforte does not cause stomach discomfort or constipation while effectively increasing bone density. This is because the key know-how lies in the ratio of components in the calcium complex formulation, including magnesium, vitamin D3, boron, zinc, selenium, vitamin B6, and calcium. hBMSCs ere cultured and treated with Osteolforte (Gagopa corp) at concentrations of 0.01, 0.1, and 1 μg/mL. The cells were incubated for specified durations before assessment.

2.4. Cell Proliferation Assay

The in vitro proliferation of hBMSCs was determined using Cell counting kit-8 (CCK-8, CCK-3000, Dongin Biotech, Seoul, Korea) assay. The hBMSCs were seeded into 48-well culture plates (30048, SPL, Gyeonggi-do, Korea) at a density of 1×10^4 cells/well and cultured in StemMACS MSC expansion Media XF (130-101-375, Miltenyi Biote, Bergisch Gladbach, Germany) containing 1X anti-anti (L0010-020, Biowest, Nuaillé, France), and *Osteolforte*.

The cells were cultured for 4 days at 37 °C in a 5% CO_2 incubator. Following this, CCK-8 solution was added and the cells were incubated at 37 °C in a 5% CO_2 incubator for 2 h. Absorbance was measured using an ELX800 spectrophotometer (BioTek, Winooski, VT, USA) at 450 nm. Each experiment was performed in triplicates.

2.5. Osteogenic Differentiation

Osteogenic differentiation was induced by culturing cells for 7–14 days in the osteogenic medium StemMACS™ OsteoDiff Medium, human (130-091-678, Miltenyi Biote, Bergisch Gladbach, Germany). Calcification of the extracellular matrix was estimated using 2% Alizarin Red S (ARS) solution (pH 4.3, A-5533, Sigma-Aldrich) for 15 min. To obtain quantitative data, 200 μL of 10% (w/v) cetylpyridinium chloride (CPC, C-0732, Sigma-Aldrich) and 10 mM sodium phosphate solution (pH 7.0) were added to the dishes containing the staining solution. The absorbance of the extracted dye was measured at a wavelength of 570 nm.

2.6. Osteolimage Mineralization Assay

OsteoImage™ Mineralization Assay (Lonza, USA) was performed according to the manufacturer's protocol. Briefly, cells were cultured in osteogenic differentiation medium for the indicated time points. At the end of each differentiation period, culture plates were brought to room temperature and media were aspirated. Cells were washed with PBS and fixed with 70% ethanol for 20 minutes. Following fixation, cells were rinsed twice with 1× Wash Buffer (prepared by diluting 10× Wash Buffer in deionized water) and stained with OsteoImage™ Staining Reagent diluted 1:100 in Staining Reagent Dilution Buffer. The staining reaction was carried out at room temperature,

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protected from light, for 30 minutes. After incubation, cells were washed three times with 1× Wash Buffer, with each wash lasting approximately 5 minutes. Fluorescent signals, corresponding to hydroxyapatite deposition, were measured using a microplate reader at 492 nm excitation and 520 nm emission. Mineralization levels were quantified as relative fluorescence units (RFU), reflecting the extent of calcium phosphate deposition.

2.7. Real-Time Polymerase Chain Reaction Analysis

Total RNA was isolated using TRIzol reagent (17061, iNtRON Biotechnology Inc., Seongnam, Korea) according to the manufacturer's instructions and reverse-transcribed into complementary (cDNA) using a First Strand cDNA Synthesis Kit (K-2041, Bioneer, Daejeon, Korea). The primer sequences used are shown in Table 1. Quantitative reverse transcriptase polymerase chain reaction (PCR) was performed using TOPreal™ qPCR 2X PreMIX (RT-500M, SYBR Green with low ROX, Daejeon, Korea) on an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA). Data analysis was performed using ΔΔCt method, and the experiments were repeated three times.

Table 1. Real-time PCR primer sequences.

Name		Sequences (5′ → 3′)
ALP	F	GACCTCCTCGGAAGACACTC
	R	TGAAGGGCTTCTTGTCTGTG
RUNX-2	F	GGTTAATCTCCGCAGGTCACT
	R	CACTGTGCTGAAGAGGCTGTT
OC	F	GCAGCGAGGTAGTGAAGAGAC
	R	AGCAGAGCGACACCCTAGA
OPN	F	CAAGACAGTGCCCAAGATAC
	R	TTCCCTCATCGTCCAACT
BMP2	F	ACC CGC TGT CTT CTA GCG T
	R	CTC AGG ACC TCG TCA GAG GG
COL1	F	CAG CCG CTT CAC CTA CAG C
	R	TTT TGT ATT CAA TCA CTG TCT TGC C
β-actin	F	GGCACCCAGCACAATGAAG
	R	TGCGGTGGACGATGGAGG

PCR, polymerase chain reaction.

2.8. Western Blot Analysis

Radioimmunoprecipitation assay (RIPA) buffer (Sigma, St Louis, MO, USA) with a protease inhibitor cocktail (PIC, Roche, Indianapolis, IN, USA) and phosphatase inhibitor was used for cell lysis. The isolated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to PVDF (Millipore, Bedford, MA, USA). The blots were probed with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. The antibodies used in this study were obtained from the following sources: anti-ALP (B-10, sc-365765) and anti-β-actin (sc-47778) from Santa Cruz Biotechnology (MA, USA); anti-RUNX2 (D1L7F, #12556) from Cell Signaling Technology (Danvers, MA, USA); anti-OPN (Osteopontin, PA5-16821) and anti-OC (Osteocalcin, #33-5400) from Invitrogen (Carlsbad, CA, USA). The antibodies were detected using an ECL detection kit (Pierce Biotechnology, Rockford, IL, USA) and visualized using an LAS 4000 Luminoimage Analyzer (Fujifilm, Tokyo, Japan). The protein levels were quantified using the National Institutes of Health ImageJ software (Bethesda, MD, USA). A list of primary and secondary antibodies used for the Western blot analysis is shown in Table 2.

2.9. Statistical Analysis

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The results are expressed as the mean ± standard error of the mean (SEM) values for more than three independent experiments. Statistical significance was determined between the treatment groups and the positive and negative controls. The *P*-value was calculated using Student's *t-test*. Each experiment was repeated at least three times to yield comparable results. Values of $^{*}P < 0.05$, $^{**}P < 0.02$, and $^{***}P < 0.01$ were considered significant.

3. Results

3.1. Effect of Osteolforte on Cell Viability and Osteogenic Differentiation in hBMSCs

To investigate both the cytotoxicity and osteogenic potential of Osteolforte in human bone marrow-derived mesenchymal stem cells (hBMSCs), a two-step in vitro experiment was conducted using concentrations of 0.01, 0.1, and 1 μg/mL.

First, potential cytotoxic effects were assessed using the CCK-8 assay. hBMSCs were treated with each concentration of Osteolforte for 24 hours, and cell viability was subsequently measured. As shown in **Figure 1A**, there was no significant decrease in cell viability at any concentration when compared to the untreated control group. In all treated groups, viability remained above 90%, indicating that Osteolforte does not exert cytotoxic effects on hBMSCs within this concentration range. These results confirm the safety of Osteolforte treatment and justify its use for further differentiation studies.

Subsequently, the osteogenic potential of Osteolforte was examined by culturing hBMSCs in osteogenic induction medium supplemented with 0.01, 0.1, or 1 μg/mL of the compound for 10 days. Osteogenic differentiation was evaluated by Alizarin Red S (ARS) staining, which detects calcium-rich deposits as a marker of extracellular matrix mineralization. As shown in **Figure 1B**, Osteolforte treatment led to a notable, concentration-dependent increase in ARS staining, with more intense red staining observed at higher doses. Quantitative analysis of ARS staining was conducted by dye extraction with 10% cetylpyridinium chloride (CPC), and the absorbance was measured at 570 nm (**Figure 1C**). All concentrations of Osteolforte significantly increased mineral deposition compared to the control, with the most substantial effect observed at 1 μg/mL ($p < 0.01$).

Together, these findings indicate that Osteolforte is both non-cytotoxic and capable of significantly enhancing osteogenic differentiation in hBMSCs. The observed dose-dependent increase in calcium deposition supports its potential application as a bioactive agent for promoting bone regeneration in regenerative medicine strategies.

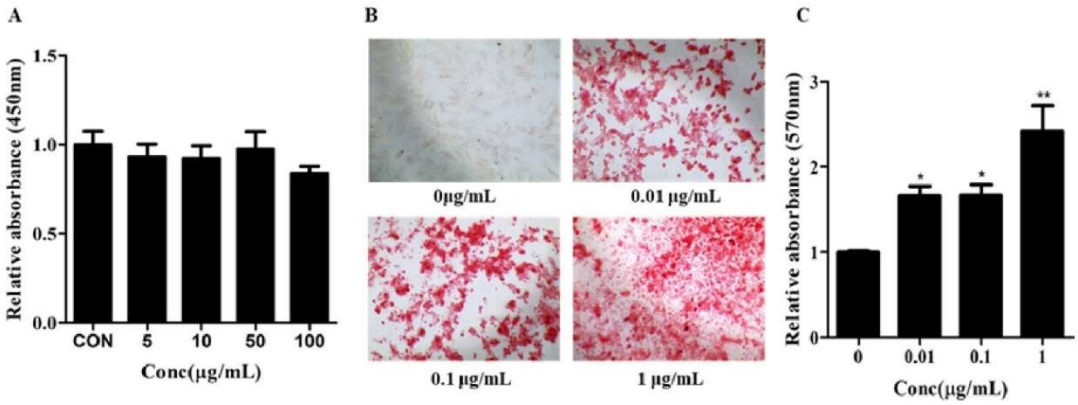

Figure 1. Effects of Osteolforte on cell viability and osteogenic differentiation of BMSCs. (A) Cell viability assessed by CCK-8 assay after treatment with Osteolforte at various concentrations (5–100 μg/mL) for 48 h. No significant cytotoxicity was observed across tested concentrations. (B) Representative images of Alizarin Red S staining showing calcium deposition in BMSCs treated with Osteolforte at 0, 0.01, 0.1, and 1 μg/mL for 14 days. The intensity of red staining indicates the extent of osteogenic differentiation. (C) Quantification of Alizarin Red

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S staining by measuring absorbance at 570 nm. Osteolforte significantly enhanced mineralization in a dose-dependent manner. Data are presented as mean ± SEM (n = 3). *p < 0.05, **p < 0.01 vs. control (0 μg/mL).

3.2. Effect of Osteolforte on Osteolimage Mineralization, Real-Time PCR and Western Blot Analysis During Osteogenic Differentiation in hBMSCs.

To further evaluate the osteoinductive potential of Osteolforte, we assessed early osteogenic activity and the expression of osteogenic markers at both mRNA and protein levels in hBMSCs.

First, ALP activity, an early marker of osteogenic differentiation, was measured on day 7 using a fluorometric assay. As shown in **Figure 2A**, Osteolforte treatment significantly increased ALP activity in a dose-dependent manner. Even at the lowest concentration (0.01 μg/mL), ALP activity was markedly elevated compared to control (***p < 0.001), with the highest activity observed at 1 μg/mL, suggesting enhanced early osteogenic commitment.

Next, quantitative RT-PCR analysis was conducted to examine the expression of key osteogenic genes. As shown in **Figure 2B**, treatment with Osteolforte significantly upregulated the mRNA levels of RUNX-2, ALP, OC (osteocalcin), OPN (osteopontin), BMP-2, and COL-1. Notably, RUNX-2 and OC expression increased up to 4- to 9-fold at 0.1 μg/mL, while BMP-2 and COL-1 also showed significant upregulation (**p < 0.01). These results indicate that Osteolforte activates multiple signaling pathways and transcriptional programs involved in osteoblast differentiation and matrix formation.

Western blot analysis confirmed the upregulation of osteogenic marker proteins. As shown in **Figure 2C**, Osteolforte treatment led to increased expression of RUNX-2, ALP, and OC proteins, particularly at 0.1 μg/mL. Densitometric analysis revealed a significant increase in RUNX-2 and OC levels (*p < 0.05 and **p < 0.01, respectively), consistent with the qPCR results. ALP protein expression was also elevated, albeit to a lesser extent.

Collectively, these data demonstrate that Osteolforte not only enhances early osteogenic marker activity but also promotes the transcription and translation of key regulators of osteogenesis in hBMSCs, further supporting its potential as an effective pro-osteogenic agent.

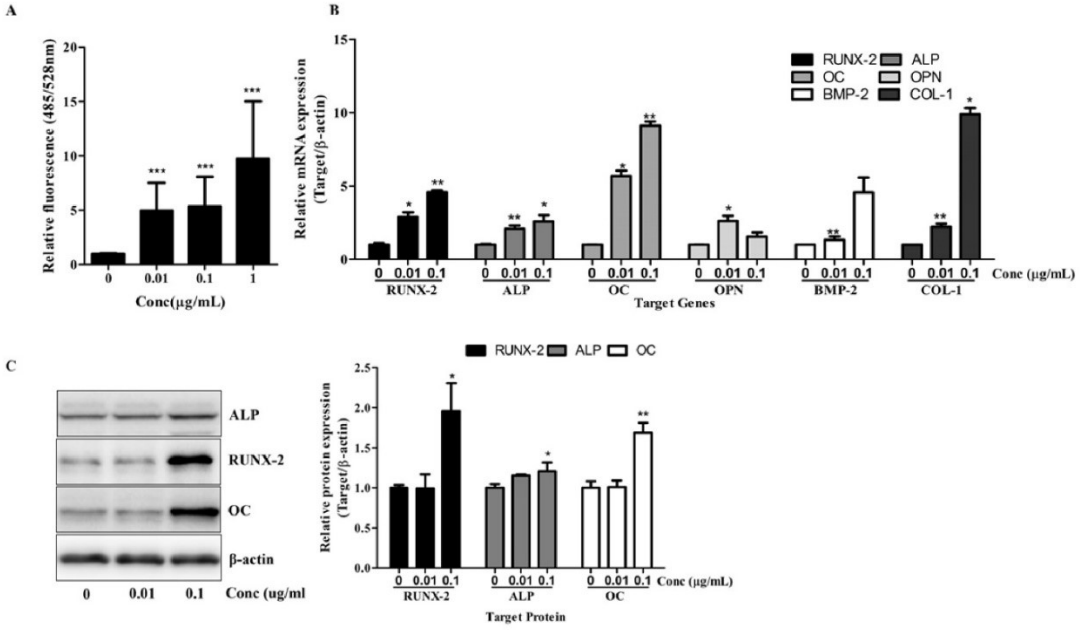

Figure 2. Osteolforte enhances early osteogenic activity and upregulates osteogenic gene and protein expression in hBMSCs. (A) ALP activity was quantified using a fluorometric assay (excitation/emission: 485/528 nm) after 7 days of osteogenic induction in the presence of 0.01, 0.1, or 1 μg/mL Osteolforte. (B) Relative mRNA expression of osteogenic markers (RUNX-2, ALP, OC, OPN, BMP-2, and COL-1) was analyzed by qRT-PCR after 7 days of treatment. Expression levels were normalized to β-actin. (C) Western blot analysis of RUNX-2, ALP,

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and OC protein levels after 7 days of treatment; β-actin was used as a loading control. Densitometric quantification of protein expression is shown in the graph on the right. Data are presented as mean ± SEM (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 vs. control.

4. Discussion

Bone Marrow-drived Mesenchymal stem cells (BMSCs) have emerged as powerful biological agents in tissue regeneration due to their multi-lineage differentiation potential, immunomodulatory capacity, and trophic effects[11].

This study demonstrates that Osteolforte exerts potent pro-osteogenic effects on human bone marrow-derived mesenchymal stem cells (hBMSCs) without inducing cytotoxicity, thereby supporting its potential as a promising therapeutic agent for bone regeneration.

Our initial cytotoxicity assessment using the CCK-8 assay confirmed the biocompatibility of Osteolforte at all tested concentrations (0.01–1 μg/mL). Cell viability remained above 90% across all groups, indicating that Osteolforte does not adversely affect hBMSC survival in vitro. This finding is consistent with prior reports emphasizing the importance of maintaining cellular integrity when evaluating new osteoinductive compounds.

The osteogenic potential of Osteolforte was demonstrated through multiple complementary assays. Alizarin Red S staining revealed a dose-dependent increase in calcium deposition, with significant mineralization observed at even the lowest dose. This suggests that Osteolforte actively promotes extracellular matrix mineralization, a hallmark of osteoblast maturation. Furthermore, ALP activity, a critical early indicator of osteoblast differentiation [12], was significantly upregulated by Osteolforte treatment. The observed elevation in ALP even at low concentrations highlights the compound's ability to initiate osteogenic commitment at early stages of differentiation.

At the molecular level, Osteolforte markedly upregulated the transcription of key osteogenic markers, including RUNX-2, ALP, OC, OPN, BMP-2, and COL-1 [13]. RUNX-2, as a regulator of osteogenesis, showed up to a nine-fold increase, which was further supported by enhanced protein expression in Western blot analysis. These results suggest that Osteolforte activates multiple osteogenic signaling pathways, promoting both matrix maturation and mineralization. Notably, the protein expression levels of OC and ALP also increased in a pattern consistent with the gene expression data, validating the translational relevance of the observed transcriptional changes.

The ability of Osteolforte to enhance osteogenic differentiation at both early and late stages positions it as a viable adjunct for regenerative therapies, particularly in clinical contexts involving large bone defects or compromised bone healing. Moreover, the concentration-dependent responses observed throughout our assays indicate a tunable therapeutic window, which is advantageous for dose optimization in future in vivo and clinical studies.

Nonetheless, the current study is limited to in vitro observations. While hBMSCs are a well-established model for studying bone biology, further validation in animal models and human clinical settings is necessary to assess the pharmacokinetics, bioavailability, and long-term safety of Osteolforte.

In conclusion, our findings highlight Osteolforte as a non-cytotoxic, osteoinductive compound that promotes osteogenic differentiation of hBMSCs through upregulation of key genetic and protein markers. These results lay the groundwork for further preclinical development of Osteolforte as a candidate agent for bone tissue engineering and regenerative medicine.

5. Conclusions

Bone regeneration remains a key challenge in regenerative medicine, necessitating novel approaches to enhance osteogenesis. Osteolforte, a newly identified compound, shows promise in promoting BMSC differentiation into osteoblasts, but its precise mechanisms need further elucidation. By investigating its effects on critical osteogenic markers such as RUNX2, COL1, BMP2, and OPN, this study aims to establish its potential as a bone-regenerative agent. The findings will not

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only advance our understanding of Osteolforte's biological activity but also pave the way for future translational research and clinical applications in bone repair and regeneration.

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Abbreviations

hBMSCs	human bone marrow stromal cells
GSK-3β	glycogen synthase kinase 3 beta
HLA	human leukocyte antigen
DMSO	Dimethyl sulfoxide
CCK-8	Cell counting kit-8
ARS	Alizarin Red S
CPC	Cetylpyridinium chloride
PCR	polymerase chain reactin
SEM	standard error of the mean

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