

Activation of the AKT/GSK-3 β / β -Catenin Axis Underlies the Pro-Osteogenic Effects of Penicopeptide A from Deep-Sea-Derived Fungusa

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ABSTRACT

Despite their pharmacological promise, marine-derived compounds remain largely unexplored in the context of osteoporosis therapeutics. To address this gap, the present investigation evaluated whether primary isolates sourced from the deep-marine fungus *Penicillium solitum* MCCC 3A00215 could stimulate osteogenesis utilizing both living subjects and controlled laboratory environments. Initial assessments revealed that bone marrow mesenchymal stem cells (BMSCs) exhibited increased osteoblastic mineralization when exposed to penicopeptide A (PPA), with the effect scaling alongside dosage; consequently, this naturally occurring peptide was advanced for comprehensive evaluation. Subsequent analyses demonstrated PPA's capacity to markedly drive BMSC lineage commitment toward osteogenesis and suppress adipocytic development, all without altering chondrogenic pathways. At the molecular level, PPA engages in direct binding with both AKT and GSK-3 β , triggering their phosphorylation and ultimately driving the intracellular buildup of β -catenin. The clinical applicability of PPA was further validated using a systemic skeletal depletion model in ovariectomized female mice, where therapeutic intervention successfully preserved trabecular thickness and overall skeletal volume. Ultimately, these combined cellular and organismal findings establish PPA as a potent driver of osteoblast-mediated skeletal regeneration through the modulation of AKT, GSK-3 β , and β -catenin signaling, thereby highlighting its translational promise as a preventative intervention for osteoporosis.

Keywords: Osteoporosis, Osteoblasts, Penicopeptide A, Marine natural product, AKT/GSK-3 β / β -catenin

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Introduction

The escalating incidence rates of both osteoporosis and obesity over the past several decades have elevated these conditions to the forefront of global public health crises [1]. Recent evidence supports the paradigm that excess marrow adiposity drives osteoporotic pathology—a clinical presentation frequently noted in postmenopausal populations and individuals suffering from systemic obesity [2, 3]. Because bone marrow mesenchymal stem cells (BMSCs) serve as the common progenitor for both lipid-regulating adipocytes and bone-regulating osteoblasts, their cellular differentiation pathways operate in direct competition. Consequently, structural skeletal degradation typically presents alongside elevated intramedullary lipid accumulation [4-6]. Dysregulation in lipid homeostasis inevitably triggers erratic skeletal remodeling, precipitating various metabolic bone morbidities. Parallel to this, conditions such as osteoarthritis and generalized osteopenia are frequently comorbid with metabolic disorders like hepatic steatosis and hyperlipidemia. This body of literature underscores the profound biological crossover between adipose and skeletal regulatory systems. Thus, engineering the targeted osteogenic lineage commitment of BMSCs to facilitate skeletal repair stands as a biologically sound strategy for thwarting senile osteoporosis [7].

Naturally derived marine metabolites (MNPs) exhibit formidable pharmacological activities across a spectrum of pathologies, with particularly notable success in infectious and oncological contexts [8, 9]. At present, the Food and Drug Administration has granted clinical approval to several MNP-derived therapeutics, such as trabectedin, cytarabine, and ziconotide. Despite the broader utility of MNPs in pharmaceutical pipelines, researchers have documented only a scarce handful of osteogenesis-inducing marine compounds, including majusculamide A and B, phorboketal A, hymenialdisine, and phorbasone A and B. Broadly speaking, the discovery of naturally sourced osteoblast stimulants lags significantly behind the identification of compounds that merely inhibit osteoclastic activity [10]. Consequently, isolating low-molecular-weight marine bioactives capable of steering BMSC differentiation emerges as an innovative frontier in anti-osteoporotic drug discovery.

Previous investigations by our group yielded 18 novel tetracyclic steroids featuring a bicyclo[4.4.1] structural motif, isolated from the deep-ocean fungus *Rhizopus* sp. W23, which displayed robust anti-osteoporotic characteristics. Notably, neocyclocitrinol C heavily favored osteoblastic BMSC differentiation at the expense of adipogenesis, thereby reinforcing the viability of MNPs as primary candidates for skeletal therapeutics [11]. In separate work, our team successfully purified 25 distinct secondary metabolites from the deep-sea strain *Penicillium solitum* MCCC 3A00215 [12]. Pushing this investigative trajectory forward, the current manuscript evaluates these specific molecules for osteoprotective efficacy. We utilized both MC3T3-E1 osteoblastic cells and BMSCs in vitro, paired with an in vivo ovariectomized (OVX) murine model, to definitively ascertain their capacity to neutralize skeletal deterioration following estrogen withdrawal.

Materials and Methods

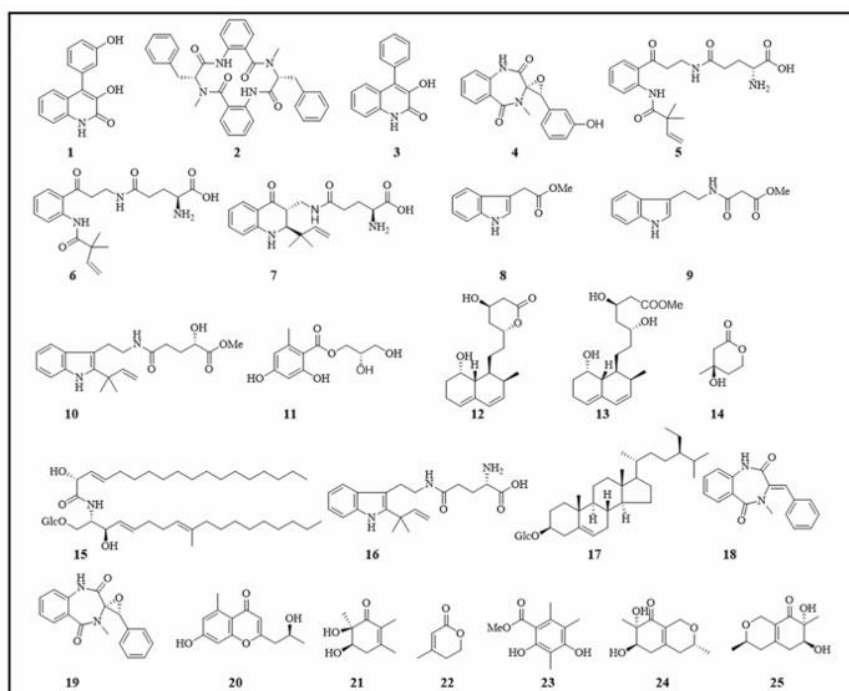
Reagents and antibodies

Cell culture essentials, specifically penicillin/streptomycin, fetal bovine serum (FBS), and alpha-modified minimal essential medium (α -MEM), were procured from Gibco (Carlsbad, CA, USA). Reagents including oil red O, Alizarin red S (A5533), DMSO (34869), and β -glycerophosphate (S0942) were supplied by Sigma (Saint Louis, Missouri, USA), while Yeasen (Shanghai, China) served as the source for ascorbic acid (60374ES60). For immunodetection, Cell Signaling Technology (Boston, USA) provided primary antibodies targeting GAPDH (D4C6R, 1:1000), phospho-AKT (Ser473) (D9E, 1:2000), AKT (C67E7, 1:1000), phospho-GSK-3 β (Ser9) (D3A4, 1:1000), GSK-3 β (D5C5Z, 1:1000), and β -catenin (15B8, 1:1000). Abcam (Cambridge, UK) was the vendor for the OCN primary antibody (ab93876, 1:200). HRP-conjugated mouse and rabbit secondary antibodies were exclusively sourced from LABLEAD (S0100 and S0101; 1:5000, Beijing, China). Thermo Fisher Scientific's Invitrogen brand (Waltham, MA, USA) delivered DAPI (0.1 μ g/mL, 1:10000), Alexa Fluor 488 donkey anti-rabbit (H+L) (A21206, 1:400 dilution), and Alexa Fluor 594 donkey anti-mouse IgG (H+L) (A11032, 1:400 dilution). Molecular biology kits, notably the ChamQ Universal SYBR qPCR Master Mix (Q71102), TRIzol (R40101AA), and HiScript II Q RT SuperMix (R22301), were supplied by Vazyme (Nanjing, China). Cell viability was assessed using a Cell Counting Kit-8 (CCK-8, CT01A) acquired from Cellcook (Guangzhou, China). Sangon Biotech (Shanghai, China) was utilized for both free fatty acid (FFA, D799794–0100) and micromethod triglyceride (TG, D799796–0100) assay kits. Origene (Maryland, USA) synthesized all RT-qPCR primer sequences. Sinopharm (Beijing, USA) provided all standard organic solvents, including ethanol, isopropanol, and chloroform. Notably, all antibodies deployed throughout the study were of monoclonal origin. Finally, mouse GSK-3 β protein (HY-P73090, New Jersey, USA) and Akt1 (NM_009652) mouse recombinant protein (TP507696, Maryland, USA) were purchased from MCE and Origene, respectively.

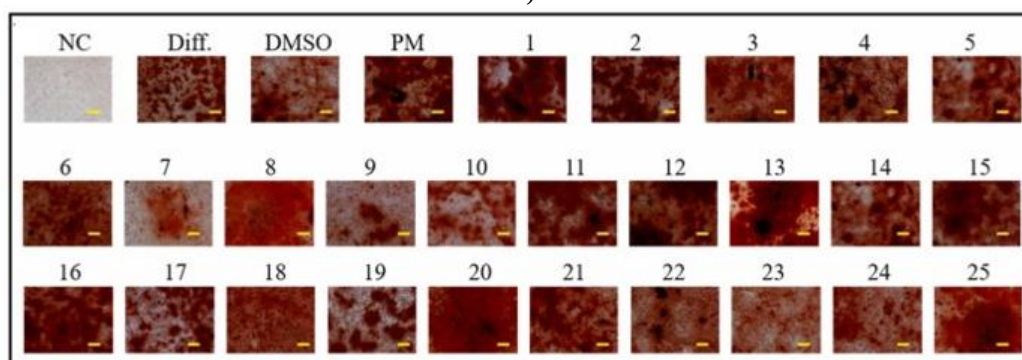
Compound source and identification

The entire set of 25 evaluated compounds (1–25) was extracted from the deep-sea fungus *Penicillium solitum* MCCC 3A00215 [12]. Fungal cultivation was performed on PDA plates at 28 °C for 3 d. The seed inoculum was grown for 1 day, utilizing 1 L flasks that each held 400 mL of PDB medium. For large-scale fermentation, 48 Erlenmeyer flasks (1 L) were utilized, with each containing 80 g of oats and 120 mL of tap water supplemented with 3% marine salt. Following a 30-day incubation period at 25 °C, the fermentation broth underwent triplicate extraction using ethyl acetate. The resulting filtrate was concentrated under reduced pressure to yield 200 g of crude extract. This extract was then separated via silica gel column chromatography, employing successive elution with petroleum ether, CH₂Cl₂, and ethyl acetate. The CH₂Cl₂ fraction of the crude extract was further divided into six subfractions utilizing medium-pressure liquid chromatography (460 mm $\times$ 36 mm) via a gradient of

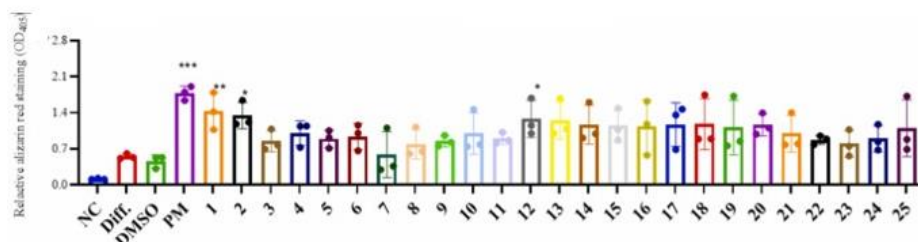
petroleum ether and ethyl acetate (5:1 $\rightarrow$ 1:5). Subfractions B-F underwent subsequent purification using Sephadex LH-20 column chromatography (1.5 m $\times$ 3 cm; CH₂Cl₂-MeOH, 1:1). This was followed by semipreparative high-performance liquid chromatography eluting with MeOH-H₂O (40% $\rightarrow$ 100%), ultimately yielding compound 2 (280 mg). The remaining fractions were subjected to iterative separation techniques involving silica gel, ODS, and Sephadex LH-20 column chromatography, alongside semipreparative high-performance liquid chromatography, to isolate the additional compounds. The chemical structures of all isolated compounds were elucidated by comparing their nuclear magnetic resonance (NMR) and mass spectrometry (MS) profiles with existing literature data, as depicted in **Figure 1a**.



a)



b)



c)

Figure 1. Initial evaluation of the osteogenic mineralization-inducing potential of compounds (1–25) derived from the marine fungus *Penicillium solitum* MCCC 3A00215 in BMSCs. (a) Chemical structures of compounds 1–25 isolated from *Penicillium solitum* MCCC 3A00215. (b) Alizarin Red S staining assay.

BMSCs were treated with compounds 1–25 (20 μ M) and incubated for 14 days. Experimental conditions including basic culture medium, osteogenic differentiation medium (50 μ g/mL ascorbic acid and 5 mM β -glycerophosphate), DMSO (0.1%), and purmorphamine (1 μ M) served as the blank control, differentiation, solvent, and positive control cohorts, respectively. Scale bar = 100 μ M. (c) Optical density (OD)-based quantitative analysis of Alizarin Red S staining. To evaluate BMSC mineralization activity, 10% acetic acid was introduced into each well, followed by absorbance measurement at 405 nm using a spectrophotometer (BioTek Epoch2). Relative staining intensity was determined using the formula (OD_{sample}-OD_{blank})/OD_{DMSO}-OD_{blank}). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to the DMSO treatment group. NC, negative control; Diff, Differentiation; PM, Purmorphamine. (n = 3).

Penicopeptide A (PPA, 2) was isolated as a colorless oil. Its identity was confirmed by matching its NMR (Bruker 400 MHz spectrometer) and HRESIMS (Waters Q-TOF spectrometer, Xevo G2) spectra with established literature references. HRESIMS m/z 583.2324 [M + Na]⁺ (calc. for C₃₄H₃₂N₄O₄Na, 583.2321); ¹H NMR (400 MHz, CD₃OD) δ 2.62 (1 H, m), 2.74 (1 H, m), 2.86 (3 H, s), 3.02 (3 H, s), 3.19 (1 H, m), 3.36 (1 H, m), 4.33 (1 H, m), 4.40 (1 H, m), 6.96 – 7.03 (3 H, m), 7.01 (1 H, m), 7.15 – 7.22 (7 H, m), 7.28 (1 H, t, J = 7.6 Hz), 7.42 (1 H, t, J = 7.4 Hz), 7.53 (1 H, t, J = 7.4 Hz), 7.81 (1 H, d, J = 7.6 Hz), 7.96 (1 H, d, J = 7.6 Hz); ¹³C NMR (100 MHz, CD₃OD) δ 29.6, 32.8, 35.1, 39.8, 57.7, 69.4, 121.5, 121.9, 125.7, 125.8, 127.6, 127.7, 128.1, 128.2, 129.5, 129.7, 129.9, 131.8, 132.3, 133.6, 134.0, 136.8, 137.0, 137.8, 138.0, 168.0, 170.4, 171.0, 172.1.

Cell sourcing and cultivation

Primary bone mesenchymal stem cells (BMSCs) and bone marrow monocytes (BMMs) were harvested by flushing the femurs of 3-week-old C57BL/6J mice. In detail, the subjects were first euthanized via cervical dislocation. The extracted femurs were subsequently sanitized with 75% alcohol before excising the surrounding soft tissues. Utilizing a sterile 1 mL syringe, an appropriate culture medium was drawn and injected into the medullary cavity via the femoral head, effectively flushing the bone marrow cells into a sterile culture dish. This flushing procedure was performed three times. Following thorough homogenization with pipette tips, the resulting cell suspension was maintained in an adequate volume of complete α -MEM (supplemented with 10% FBS and 1% penicillin/streptomycin) under standard incubator conditions of 37 °C and a CO₂ concentration of 5%. Additionally, BMMs were procured from the femurs of six-week-old female C57BL/6J mice. To successfully derive the BMMs, these cells were cultivated for 5 days in complete α -MEM enriched with 30 ng/mL M-CSF at 37 °C in a 5% CO₂ atmosphere.

The MC3T3-E1 preosteoblast cell line (clone 4; CRL-2593, sourced from the American Type Culture Collection, Rockville, MD, USA) was generously provided by the laboratory of Zhang Bing. Both BMSCs and MC3T3-E1 cells were maintained in complete α -MEM (Gibco) formulated with 10% FBS (Gibco) and 1% penicillin/streptomycin (BI, Beit Haemek, Israel). The current study utilized BMSCs at passage 2 and MC3T3-E1 cells at passage 5.

Assay for osteogenic mineralization

Following enzymatic digestion, BMSCs were seeded into 96-well culture plates at a cellular density of 2×10^4 cells/well and incubated overnight. To induce osteogenic differentiation, the culture medium was supplemented with ascorbic acid (50 μ g/mL) and β -glycerophosphate (5 mM), and the cellular differentiation process was carried out over 14 days. Calcification conditions within the cultures were evaluated utilizing Alizarin red staining. Specifically, cells underwent fixation using 4% neutral-buffered formalin for 30 min, followed by a 2-min incubation with 2% Alizarin red S at room temperature. For quantification, 10% acetic acid was dispensed into each well, and the optical absorbance was subsequently recorded at 405 nm using a spectrophotometer (BioTek Epoch2). The half-maximal effective concentration (EC₅₀) was computed utilizing Prism software 9.5. The percentage of mineralization promotion was determined using the formula: Mineralization promotion (%) = (OD_{sample}-OD_{DMSO})/OD_{DMSO} $\times$ 100%.

The preosteoblast MC3T3-E1 cells were plated in 96-well plates at a density of 5×10^3 cells/well. Following a 21-day treatment in a medium containing 50 μ g/mL ascorbic acid and 5 mM β -glycerophosphate, with or without the test compounds, the cultures were incubated with 2% Alizarin red S for staining and quantification according to the aforementioned protocol.

Purmorphamine, acting as a smoothened/Smo receptor agonist with an established EC₅₀ of 1 μ M, was deployed as the positive control. Its selection was based on its capacity to upregulate the expression of osteogenic mineralization markers via the activation of both the Hedgehog and Smad signaling pathways [13-15].

Alkaline phosphatase (ALP) assay

MC3T3-E1 preosteoblasts were seeded into 96-well culture plates at a concentration of 5×10^3 cells per well. These cells were subsequently cultured for 7 days in the presence of 50 μ g/mL ascorbic acid and 5 mM β -glycerophosphate, either supplemented with the test compounds or left untreated. Following this incubation, the cultures were exposed to a 10-fold dilution of alamarBlue solution for a 4-hour period. Absorbance was then recorded at 570 nm, after which the cells underwent three separate washes. Subsequently, the cells were treated with a specialized reaction solution comprising the phosphatase substrate nitrophenol phosphate (pNPP), 6.5 mM Na₂CO₃, 18.5 mM NaHCO₃, and 2 mM MgCl₂ (Sigma Aldrich). The activity of ALP was determined at a pH of 10.2 utilizing pNPP as the enzymatic substrate, which involved measuring the optical density of the resulting yellow product at 405 nm. The data were calculated using the following formula:

$$\text{ALP activity} = (\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}})_{405 \text{ nm}} / (\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}})_{570 \text{ nm}} \times 1000.$$

Cytotoxicity assay

An in vitro cytotoxicity assessment was performed utilizing the CCK-8 protocol, in alignment with previously established procedures [11]. In detail, bone marrow mesenchymal stem cells (BMSCs) were maintained in complete α -MEM (Gibco, USA) enriched with 10% FBS (Gibco, USA) and 1% penicillin/streptomycin. The BMSCs were plated at a density of 2×10^4 cells per well in 96-well plates and allowed to adhere overnight, prior to treatment with the experimental samples. Following a 48-hour cultivation period, 20 μ L of CCK-8 reagent (Cellcook, CT01A, China) was introduced to each well, and the plates were incubated at 37 °C for a further 2 hours. All optical density (OD) measurements were acquired at 450 nm utilizing a BioTek Epoch2 microplate spectrophotometer. Cell proliferation was quantified using the equation:

$$\text{Growth rate (\%)} = (\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}) \times 100\%.$$

Additionally, MC3T3-E1 preosteoblasts were seeded into 96-well plates at a concentration of 5000 cells per well. With the exception of this initial plating density, all subsequent operating procedures and analytical parameters remained identical to the aforementioned protocol.

Adipogenic differentiation

Following enzymatic digestion, BMSCs were seeded into 96-well culture plates at 6×10^4 cells per well and incubated overnight. To induce adipogenesis, the cells were maintained in a basal differentiation medium supplemented with 0.5 μ g/mL insulin, 5 μ M dexamethasone, 1 μ M rosiglitazone, and 0.5 mM isobutylmethylxanthine. Upon six days of adipogenic induction, the majority of the progenitor cells had successfully matured into adipocytes. The cultures were then rinsed with PBS, fixed utilizing 4% formaldehyde, and stained for 1 hour at ambient temperature using a filtered Oil Red O in 60% isopropanol solution. The positively stained red adipocytes were subsequently documented via light microscopy (Olympus CKX3-SLP, Japan) [16].

Chondrogenic differentiation

Digested BMSCs were transferred into 15 mL centrifuge tubes at a concentration of 6×10^5 cells per tube and centrifuged at 1200 rpm for 3 minutes. After cautiously discarding the supernatant, 0.5 mL of culture medium was applied to the tube's cap, and the cells were maintained in an incubator at 37 °C with 5% CO₂. Twenty-four hours later, the base of the tube was gently manipulated to detach the cellular pellet, which was then entirely submerged in a chondrogenic induction medium (consisting of 1% insulin–transferrin–selenium solution, 10 ng/mL TGF- β 3, 100 nM dexamethasone, 40 μ g/mL proline, and 50 μ g/mL l-ascorbic acid 2-phosphate). Cultivation proceeded at 37 °C and 5% CO₂ for a duration of 21 days, with the fresh induction medium being replenished generally every 2 days.

The cell pellets were rinsed twice in PBS and subjected to overnight fixation in 4% neutral formaldehyde. Following fixation, the samples were directly embedded in optimal cutting temperature (OCT) compound (Sakura) and sectioned sagittally at a thickness of 10 μ m utilizing a cryostat (Leica). These tissue slices underwent staining with an Alcian blue dye solution for 30 minutes. Following a 2-minute wash in tap water and a single

rinse in distilled water, the stained chondrogenic samples were examined and photographed under a microscope. Chondrogenesis efficacy was subsequently quantified using ImageJ software.

Quantitative polymerase chain reaction (RT-qPCR)

BMSCs were cultivated in 6-well plates using complete α -MEM supplemented with 50 μ M L-ascorbic acid and 5 mM β -glycerophosphate, either in the presence or absence of 20 μ M PPA. Total cellular RNA was extracted utilizing the TRIzol reagent (Vazyme, R40101AA) on the fifth day of culture. For downstream analysis, 1000 nanograms of this extracted RNA served as the template to synthesize complementary DNA (cDNA) via the HiScript II Q RT Super Mix (Vazyme, R22301). The resulting cDNA was subsequently utilized to assess target gene expression levels through RT-qPCR, employing the Cham Q Universal SYBR qPCR Master Mix (Vazyme, Q71102). Data normalization was achieved utilizing the $2^{-\Delta\Delta CT}$ method. The precise SYBR Green primer sequences (designed based on murine sequences) are detailed in **Table 1**.

Table 1. Primer sequences utilized for RT-qPCR analysis

Genes	Forward (5'→3')	Reverse (5'→3')
<i>Runx2</i>	CCTGAACCTCTGCACCAAGTCCT	TCATCTGGCTCAGATAGGAGGG
<i>Ibsp</i>	AATGGAGACGGCGATAGTTCCG	GGAAAGTGTGGAGTTCTCTGCC
<i>Bglap</i>	GCCAATGGCAAAGATGACCACAG	TGCGTGGAGTATCCATCTCTGC
<i>HIVEP3</i>	CTGGTTCCATCCAACCTCCCGAA	CCTCTCTTGAAGTGGGAGTAC
<i>HPRT</i>	CTGGTGAAAAGGACCTCTCGAAG	CCAGTTTCACTAATGACACAAACG

RNA sequencing (RNA-seq) and data processing

The RNA-seq assays, alongside the subsequent high-throughput sequencing and bioinformatic data analyses, were executed by Seqhealth Technology Co., Ltd. (Wuhan, China). Extraction of total RNA was completed utilizing a TRIzol kit (Promega, USA) in accordance with the provided commercial protocol. The purity of the RNA was assessed by evaluating the A260/A280 absorbance ratio via a NanoDrop One spectrophotometer (Thermo Fisher Scientific Inc.). Furthermore, RNA integrity was verified through electrophoretic separation on a 1.5% agarose gel. Samples meeting quality standards were ultimately quantified utilizing a Qubit 3.0 fluorometer paired with the Qubit™ RNA Broad Range Assay Kit (Life Technologies, Q10210).

Following rigorous quality control, 1 μ g of total RNA from each sample was directed toward library construction. To identify differential expression, the DESeq2 Bioconductor software package was applied, employing a statistical model founded upon the negative binomial distribution. Statistical significance for these differentially expressed genes (DEGs) was defined by a p value cutoff of 0.05 coupled with a minimum fold-change of 2. Functional annotation was performed using Gene Ontology (GO) Seq (v1.34.1), which pinpointed enriched GO terms among the target genes that exhibited a significant padj of less than 0.05. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database repository encompasses comprehensive data regarding genomes, biological pathways, diseases, drugs, and chemical substances (<https://www.genome.jp/kegg/>). Proprietary analytical scripts were utilized to map and enrich the significant DEGs within these KEGG pathways.

Western blotting

Western blot analysis was executed in accordance with previously detailed methodologies [17]. BMSCs were harvested and subsequently seeded into 6-well plates at a concentration of 5×10^5 cells per well. While maintained under inductive conditions (supplemented with 50 μ g/mL ascorbic acid and 5 mM β -glycerophosphate), the cells were exposed to either 20 μ M PPA or 0.1% DMSO (serving as the control group) for a duration of 5 days. Following this incubation, cellular lysates were prepared using RIPA buffer (Solarbic, R0010) fortified with both a protease inhibitor cocktail (MCE, HY-K0010) and a phosphatase inhibitor cocktail II (MCE, HY-K0022). These lysates were then subjected to centrifugation at 12,000g for 30 minutes at 4 °C.

Equivalent protein quantities (30 μ g) were resolved via a 10% SDS PAGE gel (initially at 70 v for 25 min, followed by 110 v for 90 min). The separated proteins were subsequently electrotransferred onto a PVDF membrane (Millipore, IPVH00010) using a constant current of 250 mA for 90 minutes. The membranes underwent a blocking step using 5% nonfat blocking grade milk prior to an overnight incubation at 4 °C with the specified primary antibodies. The following day, the blots were exposed to respective HRP-conjugated secondary antibodies (either rabbit or mouse; S0101 and S0100; 1:5000 dilution, LABELAD, Beijing, China) for 2 hours at ambient temperature. Visualization of the immunoblots was achieved utilizing an ultrasensitive ECL Plus

chemiluminescence reagent kit (P0018S, Beyotime, Shanghai, China), and band densities were quantitatively evaluated via ImageJ software. All targeted expression levels were normalized using GAPDH as an internal control.

Immunofluorescence staining

BMSCs were cultured within glass-bottom cell culture dishes (NEST, 801001–1, 20 mm, TC) and subjected to a 5-day incubation with PPA. After undergoing two rinses with ice-cold PBS, the cells were fixed in 4% paraformaldehyde for 30 minutes. Cellular permeabilization was then accomplished by applying 0.3% Triton X-100 in PBS for 15 minutes. Following three sequential washes, the specimens were blocked using 5% bovine serum albumin for 60 minutes and subsequently incubated with primary antibodies (mouse anti- β -catenin, 1:3000 dilution; rabbit anti-OCN, 1:200 dilution) overnight at 4 °C. Upon further washing, the cells were treated with secondary antibodies (Alexa Fluor 594 anti-rabbit, 1:200 dilution; and Alexa Fluor 488 anti-mouse, 1:200 dilution) for 2 hours at room temperature under dark conditions. Nuclear counterstaining was executed using DAPI (0.1 μ g/mL, 1:10000 dilution) for 15 minutes. A final series of three PBS washes was conducted before examining the cells via a fluorescence microscope (Olympus, APEXVIEW APX100, Japan).

Regarding the immunofluorescence analysis of bone tissue, freshly harvested bone was immediately placed into an ice-cold 4% paraformaldehyde solution for overnight fixation. Decalcification of the samples was achieved over a 6-week period utilizing 0.5 M EDTA, which was exchanged daily at 4 °C under continuous agitation. The decalcified tissues were subsequently embedded within OCT compound (Sakura) and sectioned sagittally at a 40- μ m thickness employing a cryostat (Leica). The ensuing immunofluorescence staining and evaluations were executed in alignment with previously reported methods [17].

In brief, the tissue slices were permeabilized with 0.3% Triton X-100 for 15 minutes. This was followed by a 30-minute blocking phase at room temperature using 5% donkey serum. The sections were then subjected to an overnight incubation at 4 °C with antibodies targeting β -catenin (1:3000 dilution) and GSK-3 β (1:800 dilution). Detection was facilitated by corresponding species-appropriate Alexa Fluor-coupled secondary antibodies. Nuclear material was counterstained utilizing DAPI for 30 minutes. Imaging of the prepared samples was conducted using either an Olympus APX100 confocal microscope or a Leica confocal microscope (Leica, TCS SP8 DLS, Germany). Finally, quantitative fluorescence analysis was performed utilizing ImageJ software.

Computational molecular docking

Three-dimensional crystal coordinates for the target receptor proteins, specifically GSK-3 β (PDB ID: 5OY4) and AKT (PDB ID: 4RDJ), were retrieved from the Protein Data Bank (PDB). Molecular docking simulations were executed using the Glide module within the Schrödinger software suite (Schrödinger Maestro, version 11.1.011; Macromodel version 3.7.011, Release 2017-1; Windows-x64 platform).

Initial receptor grid generation was performed to define the localized binding domain. Small-molecule ligands were prepared under the OPLS3e force field, with plausible ionization and tautomeric states simulated across a physiological pH range of 7.0 $\pm$ 2.0. The macromolecular structures were prepared and refined via restrained minimization applying the OPLS3e force field. Binding region grids were established using the Glide® receptor grid generation tool, specifying a search space bounding box length of 20 Å. The coordinates for the grid box center were aligned with the centroid of the co-crystallized ligand obtained from the original PDB file, while all alternative configurations were maintained at default settings.

Semiflexible docking simulations were conducted utilizing the standard precision (SP) mode of Glide. Protomeric state penalties for the ligands were incorporated into the empirical scoring function via the Epik module, with all other operational constraints assigned to default specifications. The molecular docking program was subsequently initiated within the Schrödinger Glide module by inputting the pre-calculated receptor grid and the optimized small-molecule ligand structures according to the defined parameters. Binding affinities are expressed as docking scores in units of kcal/mol, where increasingly negative values correspond to superior thermodynamic binding stability. Visual representations of the optimal docking configurations were generated and exported utilizing PyMOL software.

Surface plasmon resonance (SPR) analysis

Chemically derivatized, label-free photocrosslinker sensor chips were obtained from Betterways Inc. (China). Microarray microspotting was conducted using a BioDot™ AD-1520 Array Printer (BIODOT Inc., USA) to

deposit both the experimental samples and corresponding control agents onto the chip interface. Individual candidate molecules were printed at a precise volume of 1.875 nL per spot, generating four spatially isolated replicates of each test sample across a single substrate chip. Following array printing, microfluidic flow cell chambers were mounted onto the chip surface, and the assembled chips were sequentially loaded into the SPR apparatus for continuous monitoring.

To verify the assay's capacity to resolve small molecule-protein binding events on the photocrosslinker SPR substrate, rapamycin and biotin served as internal validation controls; specifically, kinetic constant assessments were performed for the interaction of FKBP12 with rapamycin, as well as streptavidin with biotin, immediately following sample evaluation. Furthermore, the vehicle solutions for the small molecules (DMSO) and the target proteins (PBS, pH = 7.0) were systematically evaluated independently to serve as baseline reference blanks and background noise corrections.

Real-time association and accumulation profiles of the target proteins were monitored employing a bScreen LB 991 Label-free Microarray System (Berthold Technologies, Germany). For the SPR runs, the chip face was initially equilibrated via three consecutive cycles with HBS-EP running buffer (composed of 10 mM HEPES, pH 7.0, 150 mM NaCl, 3 mM EDTA, and 0.005% [v/v] surfactant P20) at a hydrodynamic flow rate of 2 μ L/s for 40 s per cycle, followed by a single priming step with the working running buffer (1 $\times$ PBS containing 5% DMSO) at 2 μ L/s for 40 s. Recombinant murine AKT protein (Origene, TP507696, USA, Maryland) and GSK-3 β protein (MCE, HY-P73090, USA, New Jersey) were individually prepared in the running buffer across a serial concentration range (10 nM, 40 nM, 160 nM, 640 nM, and 2560 nM). These protein solutions were introduced during the association phase over a 600 s interval at a constant flow rate of 0.5 μ L/s, followed immediately by a dissociation phase during which the analyte-free running buffer was passed through the system for 360 s at the same flow velocity of 0.5 μ L/s. Upon completion of each individual association-dissociation phase, the sensor matrix was regenerated to remove residual complexes by applying a brief pulse of 10 mM glycine-HCl (pH 2.5) at a delivery rate of 2 μ L/min for 300 s.

Ovariectomy (OVX)-induced osteoporosis mouse model

A mouse model of postmenopausal osteoporosis was established via bilateral ovariectomy following a standard methodology reported previously [18]. Female C57BL/6J mice (n = 24, weighing ~20 g, 10 weeks of age; procured from Gempharmatech Co., Ltd) were selected for model development and randomly assigned into three experimental cohorts (with n = 8 per group): a sham-operated control group (undergoing no ovariectomy), an OVX group receiving the vehicle control, and an OVX group treated with 10 mg/kg PPA.

Following the induction of anesthesia using inhaled isoflurane, bilateral ovariectomies were performed to elicit systemic osteopenic changes according to established surgical protocols. Animals assigned to the sham and vehicle-treated OVX cohorts received intravenous tail vein injections of physiological saline, whereas the OVX + PPA treatment group was administered 10 mg/kg of PPA intravenously every alternate day. Experimental endpoints were reached at week 6, at which time all animals were humanely euthanized. For bone formation tracking, calcein (20 mg/kg) was administered via subcutaneous injection to all subjects at 8 days and 1 day prior to sacrifice.

Micro-computed tomography (μ CT) and bone histomorphometry evaluation

Right-side femoral specimens were isolated and fixed overnight in a 4% paraformaldehyde solution, followed by preservation in 75% ethanol. High-resolution μ CT scanning of both the distal metaphysis and mid-diaphysis segments was conducted using a SkyScan1272 system (United States) in accordance with a previous methodology [17]. Three-dimensional tomographic reconstructions were generated from raw projection data via the NRecon software interface, followed by structural quantification using the cTAn software package. Structural metrics determined included bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), alongside cross-sectional cortical thickness (Cs.th) or total cortical bone volume.

Subsequent to μ CT imaging, the femoral samples and lumbar vertebral bodies were subjected to chemical decalcification in a 10% EDTA solution at ambient temperature for 6 weeks. Following complete demineralization, the tissues were embedded in polymethylmethacrylate resin to facilitate histomorphometric examination. Slices measuring 7 μ m in thickness were cut and prepared for toluidine blue staining, Von Kossa mineralization staining, and the visualization of fluorochrome calcein double-labeling. Concurrently, 5 μ m sections were processed for tartrate-resistant acid phosphatase (TRAP) activity staining. Histomorphometric

parameters—comprising osteoblast perimeter fraction (Ob.S/BS), osteoclast density per bone perimeter (N.Oc./B.Pm), fractional mineralizing surface (MS/BS), mineral apposition rate (MAR), and normalized bone formation rate (BFR/BS)—were quantified utilizing the OsteoMeasure analysis platform (OsteoMetrics, USA).

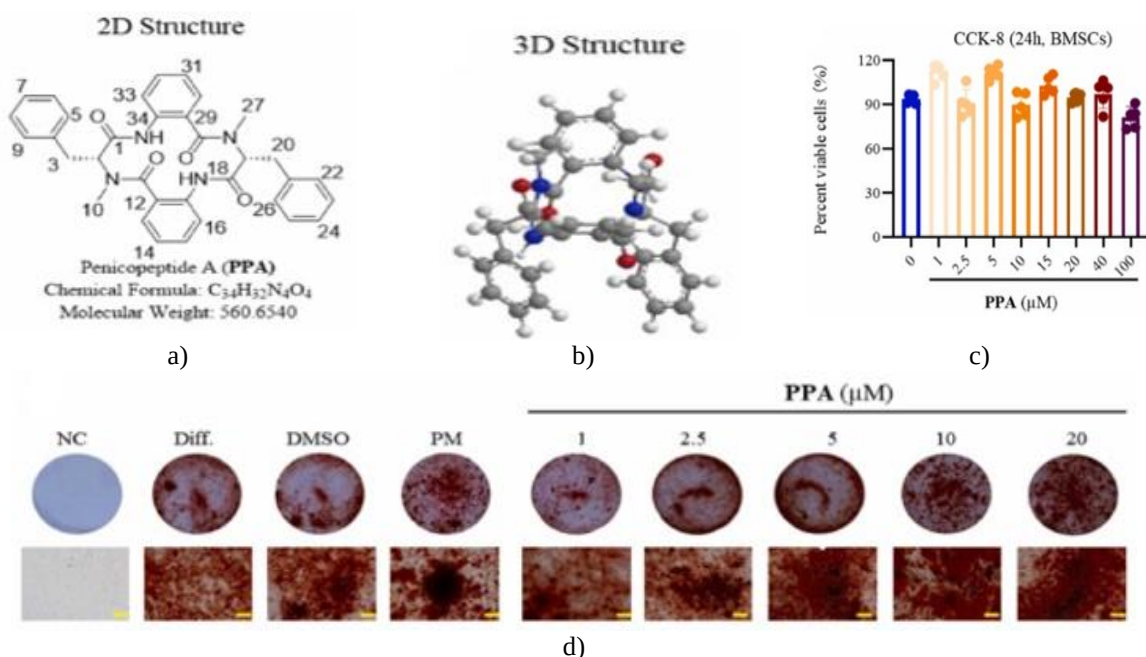
Statistical evaluation

All experimental assays detailed within this study were performed in at least three independent biological replicates. Quantitative values are represented as the mean $\pm$ standard deviation (SD). GraphPad Prism software (version 8.0) was used to perform statistical analyses. Variations between two distinct cohorts were evaluated via Student's *t*-test, whereas multi-group differences were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test for comparisons against a singular control pool or Tukey's post-hoc test for all pairwise evaluations. Statistical significance thresholds across all tests were set *a priori*, with **p* < 0.05, *p* < 0.01, ****p* < 0.001, and *****p* < 0.0001 serving as indicators of significant variance.

Results and Discussion

Screening of compounds for the ability to induce osteogenic mineralization

Twenty-five compounds (1–25) obtained from the marine fungus **Penicillium solitum** MCCC 3A00215 underwent screening to assess their capacity to drive osteogenic mineralization in bone marrow mesenchymal stem cells (BMSCs). At 20 μ M, compounds 1, 2, and 12 produced a clear stimulatory response, markedly elevating the number of mineralized nodules relative to untreated cultures. This enhancement translated into more than a 1.5-fold increase in quantitative osteogenic activity compared with the control (**Figure 1**). Consequently, these three compounds were selected for EC₅₀ determination in mineralization assays, yielding values of 4.408 μ M (compound 1), 20.310 μ M (compound 2), and 21.43 μ M (compound 12). Owing to its abundant yield (280 mg for PPA, compound 2), combined with its unique structural features and previously undocumented role in bone metabolism, PPA was prioritized for comprehensive studies on its anti-osteoporosis potential and mechanistic actions. The planar and spatial chemical structures of PPA appear in **Figures 2a** and **2b**.



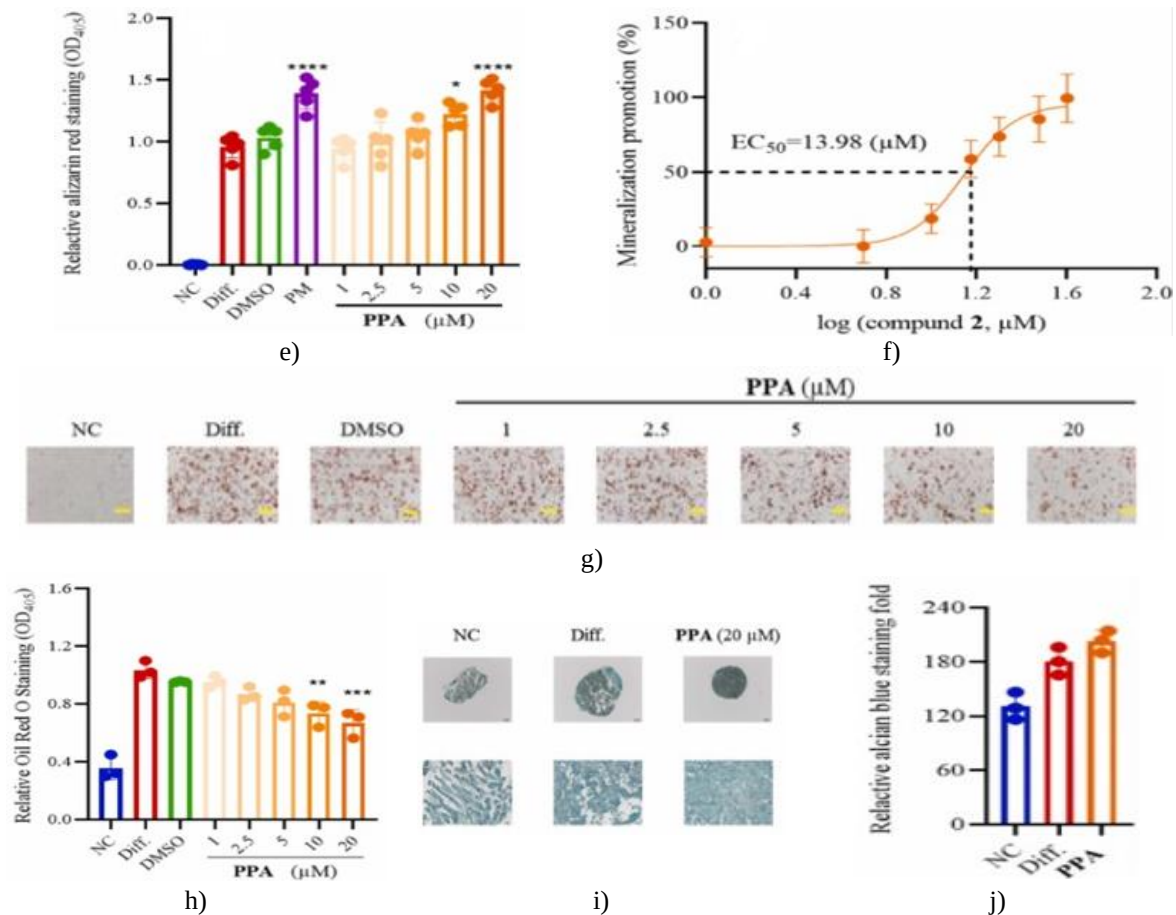


Figure 2. PPA stimulates osteoblastic mineralization and inhibits adipogenic differentiation in BMSCs. (a, b) 2D and 3D chemical structures of PPA. (c) BMSC viability determined by CCK-8 assay after 48 h exposure to PPA (0–100 μM). Stock solutions of PPA were prepared in DMSO and diluted into culture medium; 0.1% DMSO functioned as the solvent control. (n = 5) (d) Alizarin Red S staining of BMSCs cultured for 14 days under osteogenic conditions (50 μg/mL ascorbic acid, 5 mM β -glycerophosphate) with PPA at 1, 2.5, 5, 10, or 20 μM. Controls included normal culture medium (negative control, NC), full osteogenic differentiation medium (Diff.), 0.1% DMSO (solvent), and purmorphamine (1 μM, positive control). Scale bar = 100 μM. (n = 5) (e-f) Quantitative measurement of Alizarin Red S staining and corresponding EC₅₀ calculation. (g) Oil Red O staining of BMSCs subjected to adipogenic induction (0.5 μg/mL insulin, 5 μM dexamethasone, 1 μM rosiglitazone, 0.5 mM isobutylmethylxanthine) plus PPA (1–20 μM) for 6 days. Scale bar = 100 μM. (n = 3) (h) Isopropanol-extracted Oil Red O quantification. (n = 3) (i) Alcian Blue staining following 21-day chondrogenic induction (1% insulin transferrin-selenium, 10 ng/mL TGF- β 3, 100 nM dexamethasone, 40 μg/mL proline, 50 μg/mL l-ascorbic acid 2-phosphate) in the presence of 20 μM PPA. Scale bars = 100 μM and 20 μM. (j) ImageJ-based quantification of Alcian Blue staining. (n = 3) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus DMSO group. NC, negative control; Diff, Differentiation; PM, Purmorphamine.

PPA stimulates osteogenic mineralization in BMSCs without cytotoxicity

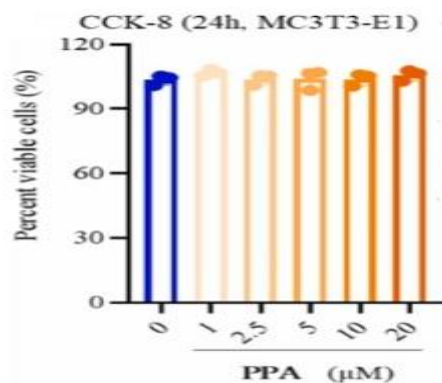
Owing to their multipotent nature, BMSCs can give rise to osteoblasts, chondroblasts, and adipocytes [19]. Osteogenic differentiation of MSCs therefore holds therapeutic value for mitigating age-related bone loss [7]. To explore this potential, PPA was tested for its direct influence on osteoblastic lineage commitment in BMSCs. Initial viability assays confirmed that PPA exerted no cytotoxic impact across a broad concentration range (0–100 μM) (**Figure 2c**). Subsequent experiments applied PPA at 1, 2.5, 5, 10, 20, 30, and 40 μM under osteogenic conditions, revealing robust, concentration-dependent promotion of mineral deposition (**Figures 2d and 2f**).

PPA suppresses adipogenic differentiation of BMSCs with no apparent effect on the chondrogenic differentiation of BMSCs

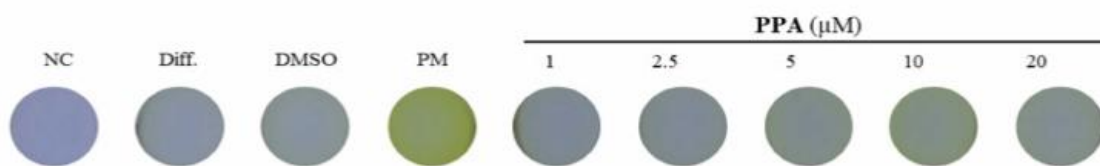
Adipogenesis in BMSCs was monitored through Oil Red O staining. Exposure to 10 or 20 μ M PPA significantly lowered the proportion of lipid-accumulating cells, demonstrating clear dose-dependent inhibition of adipogenic differentiation (**Figures 2g and 2h**). In parallel, chondrogenic differentiation was examined using Alcian Blue staining. While induction medium triggered the expected change in cell morphology, PPA treatment produced no measurable alteration in chondrogenic outcomes (**Figures 2i and 2j**).

PPA promotes osteogenic differentiation and mineralization in MC3T3-E1 cells

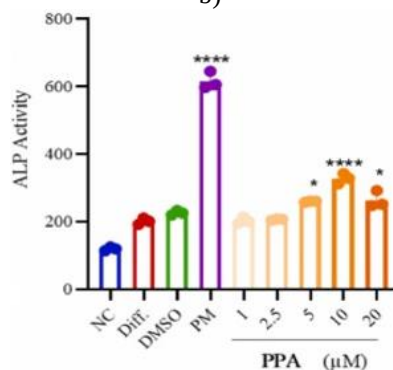
The osteogenic efficacy of PPA was further corroborated in the MC3T3-E1 pre-osteoblastic cell line by evaluating ALP enzymatic activity and extracellular matrix mineralization. PPA showed no adverse effect on MC3T3-E1 cell viability (**Figure 3a**). Treatment resulted in elevated ALP activity starting at 5 μ M (**Figures 3b and 3c**) and enhanced mineralized nodule formation at 10 and 20 μ M (**Figures 3d and 3e**). At 20 μ M, mineralization levels reached approximately three times those observed in the solvent-treated group. These observations, together with the BMSC data, establish PPA as a potent osteogenic agent in multiple relevant cell models.



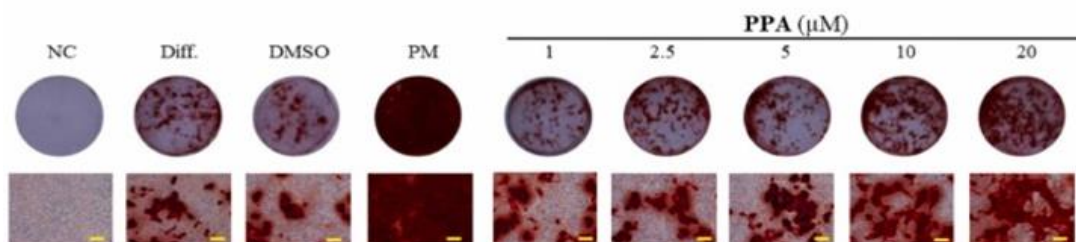
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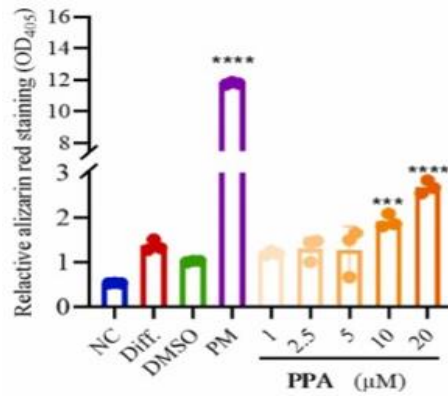
b)



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d)

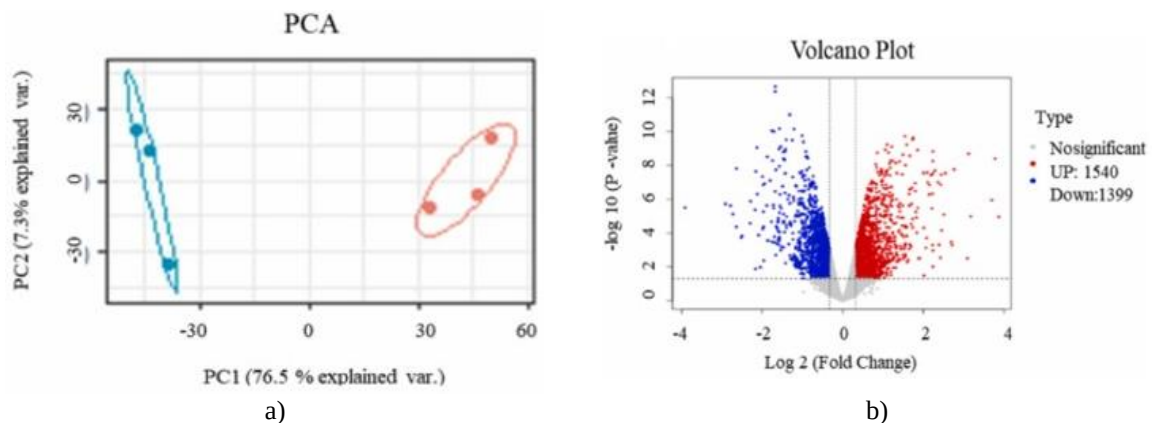


e)

Figure 3. PPA promotes osteogenic differentiation and mineralization in MC3T3-E1 cells. (a) Cytotoxicity profile of PPA on MC3T3-E1 cells assessed by CCK-8 assay after 48 h treatment (1–20 μ M). (b) ALP activity measured in MC3T3-E1 cells cultured for 7 days under osteogenic conditions with various PPA concentrations. (c) Quantitative ALP data. (d) Alizarin Red S staining of MC3T3-E1 cells after 6 days of osteogenic induction plus PPA treatment. Scale bar = 100 μ M. (e) Quantification of Alizarin Red S staining. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus DMSO group. NC, negative control; Diff, Differentiation; PM, Purmorphamine. (n = 3).

PPA induces osteogenesis via PI3K-AKT signaling

Researchers employed RNA-sequencing (RNA-seq) to uncover the molecular pathways responsible for PPA's osteogenic effects in BMSCs. Principal component analysis (PCA) of the transcriptomic data confirmed tight clustering within groups and distinct separation between control and PPA-treated samples (**Figure 4a**). According to volcano plot analysis, PPA treatment led to the upregulation of 1540 genes by more than 2-fold and the downregulation of 1399 genes compared with controls. Heatmap visualization and volcano plots together illustrated widespread transcriptional reprogramming between the two groups (**Figures 4b and 4c**). Subsequent Gene Ontology (GO) enrichment analysis of the differentially expressed genes uncovered 1281 significantly enriched biological processes ($p < 0.05$), with prominent terms related to ossification regulation, bone mineralization, bone morphogenesis, biomineralization, and osteoblast differentiation (**Figure 4d**). KEGG pathway mapping further linked these genes to multiple signaling networks, including PI3K-AKT, MAPK, calcium, tumor necrosis factor (TNF), and transforming growth factor beta (TGF- β) pathways. Given that PI3K-AKT, MAPK, and calcium signaling are established contributors to osteoblast development, and considering its superior enrichment score and statistical significance, the PI3K-AKT pathway was identified as the most probable route through which PPA drives osteogenesis (**Figure 4e**).



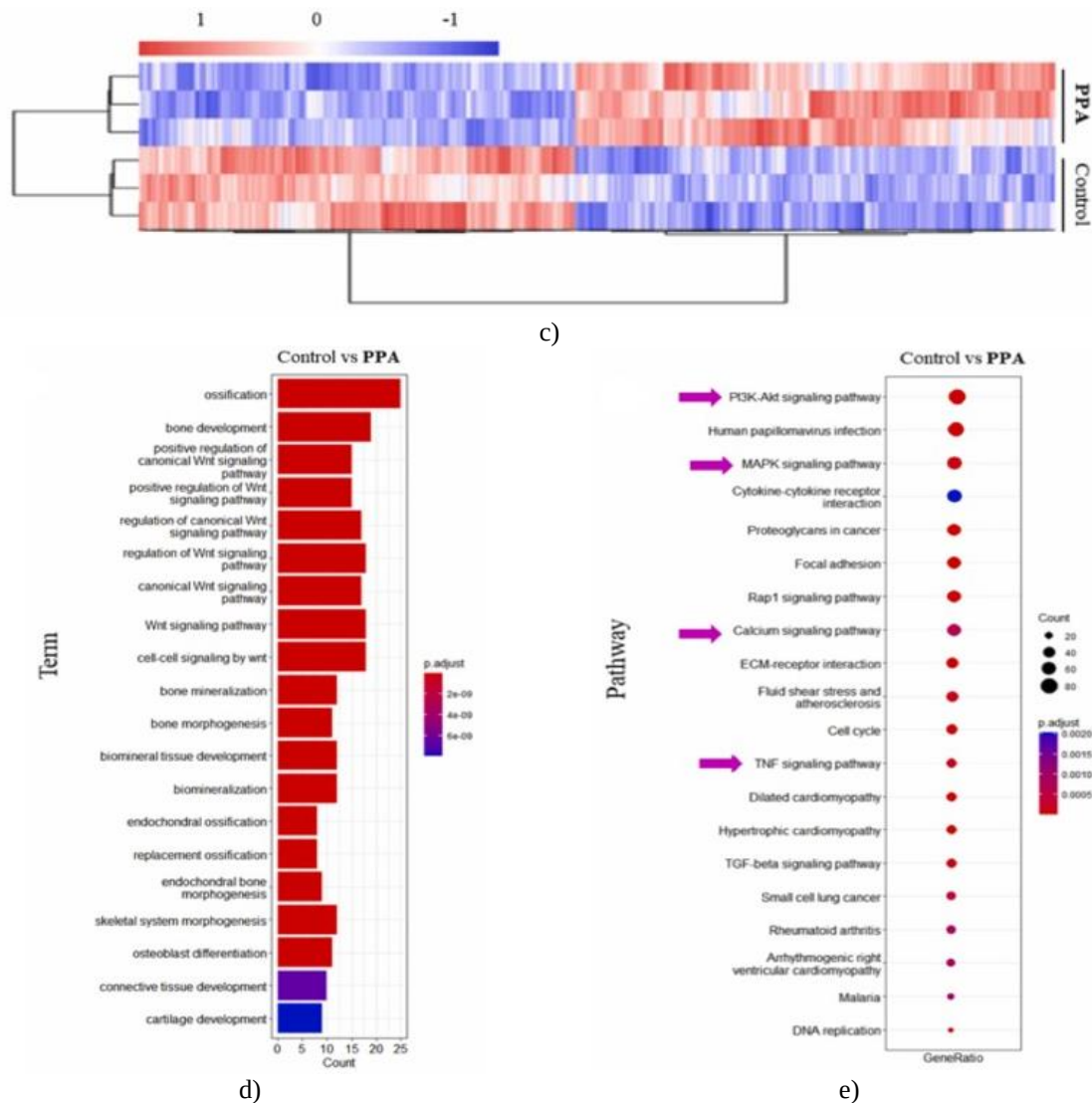


Figure 4. RNA-sequencing to explore the mechanism of PPA-induced osteogenesis in BMSCs. BMSCs were cultured under osteogenic induction conditions (50 μ g/mL ascorbic acid and 5 mM β -glycerolphosphate) and exposed to 20 μ M PPA for 5 days, using 0.1% DMSO as the vehicle control. (a) PCA plot illustrating gene expression variation among different samples. (b) Volcano plot highlighting differentially expressed genes in control versus PPA-treated groups, with selected osteoblastogenesis-related genes annotated by log2-fold change and p-value. (c) Heatmap displaying patterns of differentially expressed genes in the PPA-treated group relative to controls. (d) GO enrichment results for selected differentially expressed genes. (e) KEGG pathway enrichment analysis for all differentially expressed genes.

PPA stimulates expression of osteoblast-specific genes and proteins by activating the AKT/GSK-3 β / β -catenin signaling pathway

Further investigation into the signaling events triggered by PPA focused on the AKT/GSK-3 β / β -catenin axis. Western blotting was utilized to quantify total and phosphorylated forms of key proteins in this pathway. After 5 days of PPA treatment, levels of p-AKT and p-GSK-3 β increased significantly while total AKT and GSK-3 β protein amounts remained stable. β -catenin protein expression was also substantially elevated (**Figures 5a and 5c**). By phosphorylating AKT, the pathway prevents GSK-3 β from targeting β -catenin for degradation, leading to its stabilization and accumulation; this process is additionally supported by p-GSK-3 β activity [18, 20].

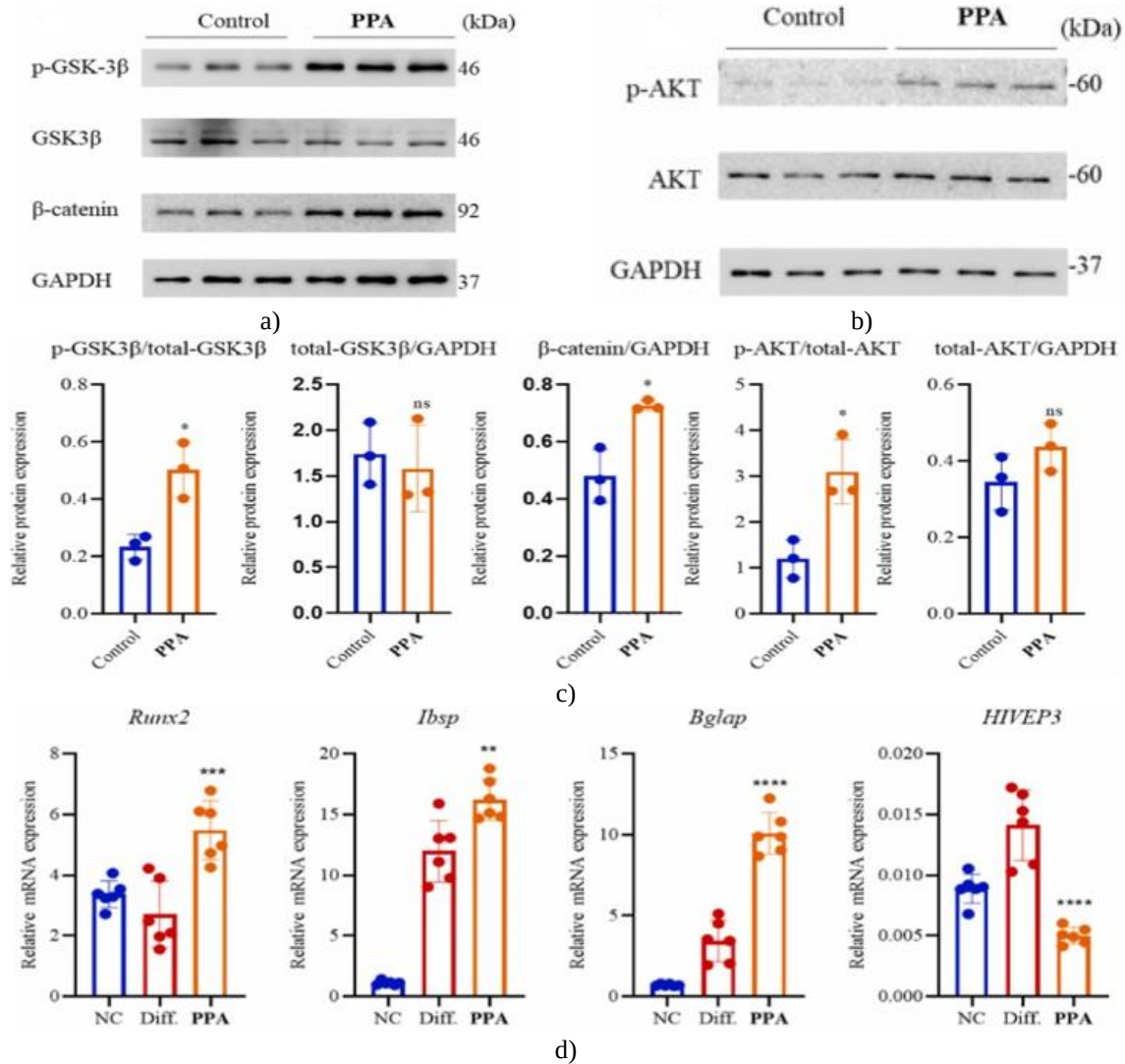


Figure 5. PPA stimulates expression of osteoblast-specific genes and proteins. BMSCs were subjected to osteogenic induction (50 μ g/mL ascorbic acid and 5 mM β -glycerophosphate) and treated with 20 μ M PPA for 5 days. (a) Representative western blot images for GSK-3 β , p-GSK-3 β , and β -catenin (normalized to GAPDH; n = 3). (b) Representative western blot images for AKT and p-AKT (normalized to GAPDH; n = 3). (c) Densitometric quantification showing fold changes in AKT, p-AKT, GSK-3 β , p-GSK-3 β , and β -catenin.

Relative mRNA expression (normalized to Hprt) of osteogenic genes in BMSCs after 5 days of PPA exposure. (d) PPA-mediated induction of osteogenic marker genes Runx2, Ibsp, and Bglap, along with suppression of the negative regulator HIVEP3. NC, negative control; Diff, Differentiation. * $p < 0.05$,

** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs the DMSO group. (n = 6).

To confirm pathway activation, immunofluorescence staining assessed β -catenin subcellular localization together with osteocalcin (OCN) expression. PPA treatment produced a notable rise in both OCN abundance and nuclear accumulation of β -catenin compared with vehicle-treated cells (**Figure 6a**). These molecular changes appear to drive increased transcription of positive osteogenic regulators Runx2, Ibsp, and Bglap while reducing expression of the inhibitory gene HIVEP3 (**Figure 5d**), ultimately supporting enhanced differentiation and mineralization. In vivo validation in OVX mouse femur sections similarly revealed elevated β -catenin levels following PPA administration, with minimal impact on GSK-3 β expression, consistent with the cell-based observations (**Figure 6b**).

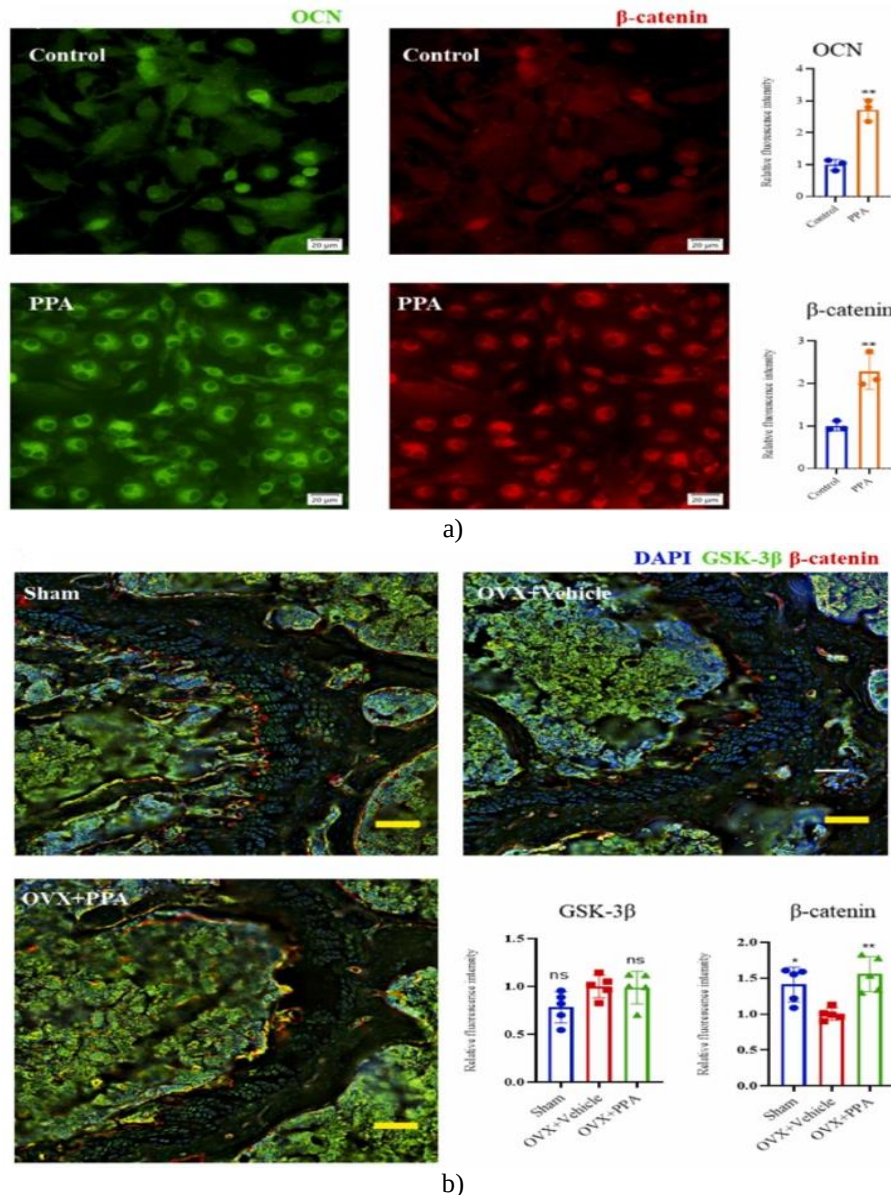


Figure 6. PPA promoted expression of OCN and β -catenin. (a) Representative immunofluorescence images and quantitative analysis of OCN and β -catenin in BMSCs after 5 days of 20 μ M PPA treatment under osteogenic conditions. All cultures contained a final DMSO concentration of 0.1%. Scale bar = 20 μ m. (n = 3) ** p < 0.01 vs the control group. (b) Representative immunofluorescence images and quantification of GSK-3 β and β -catenin in femoral sections from sham-operated, OVX, and PPA-treated OVX mice (10 mg/kg). Following fixation in 4% paraformaldehyde, decalcification with 0.5 M EDTA at 37 $^{\circ}$ C for 6 weeks, and paraffin embedding, 40- μ m sections were immunostained for GSK-3 β (green) and β -catenin (red) with DAPI counterstaining (blue). Scale bars = 50 μ m. (n = 5) * p < 0.05, ** p < 0.01 vs the OVX+Vehicle group.

PPA interacts with AKT and GSK-3 β

Possible direct binding interactions of PPA with AKT (PDB: 4DRJ) and GSK-3 β (PDB: 5OY4) were evaluated using molecular docking simulations in Schrödinger software. Residues positioned within 5 $\text{\AA}$ of the docked ligand were highlighted for analysis. PPA was found to fit comfortably into the active site pocket of 4DRJ (**Figures 7a and 7b**). Close examination showed that binding is stabilized by a network of hydrogen bonds and π - π stacking interactions with residues TYR113, SER118, and LYS121. The calculated docking score for PPA with 4DRJ (-5.500) surpassed that of the native ligand (-4.109) (**Figures 7c and 7d**). In the case of 5OY4, PPA engaged the active pocket primarily through π -cation interactions with LYS183 (**Figures 7e and 7f**), yielding a docking score of -5.507 kcal/mol (**Figures 7g and 7h**).

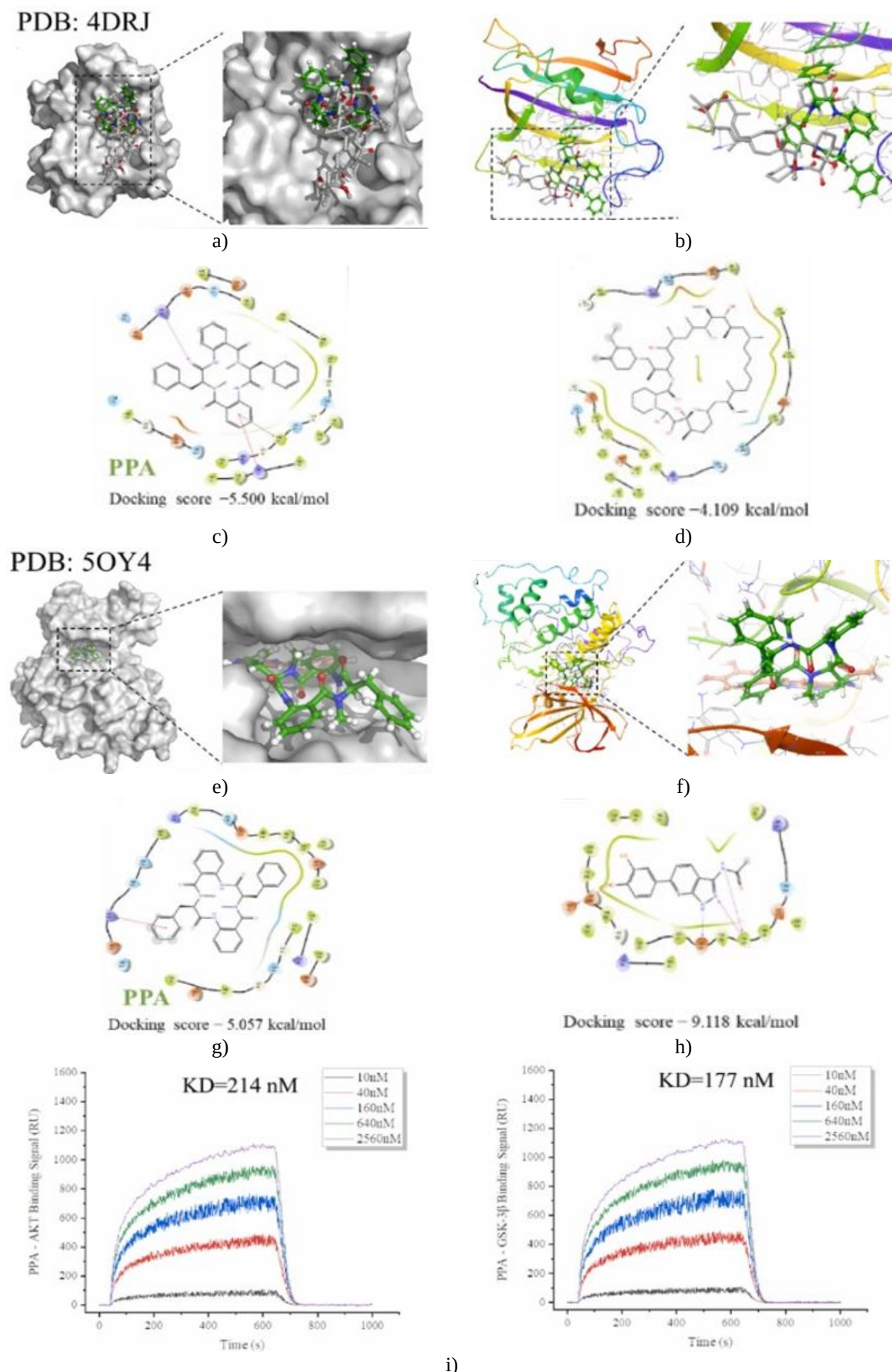


Figure 7. Molecular docking of PPA toward AKT (4DRJ) and GSK-3 β (5OY4). Interpretation of absolute docking scores: > 4.25 but < 5 suggests potential binding; ≥ 5 but < 7 indicates good binding activity; ≥ 7

denotes strong binding activity. In 3D views, the native ligand of 4DRJ (grey), native ligand of 5OY4 (faded red-orange), and PPA (green) are rendered as stick models, with nitrogen atoms in dark blue and oxygen atoms in red. (a) 3D surface representation comparing binding poses of PPA and the native ligand in 4DRJ.

(b) 3D cartoon representation comparing binding poses of PPA and the native ligand in 4DRJ. (c) 2D diagram of PPA interactions with 4DRJ (purple arrows denote H-bonds; green threads indicate π - π stacking).

(d) 2D diagram of native ligand interactions with 4DRJ. (e) 3D surface representation comparing binding poses of PPA and the native ligand in 5OY4. (f) 3D cartoon representation comparing binding poses of PPA and the native ligand in 5OY4.

(g) 2D diagram of PPA interactions with 5OY4 (red thread represents π -cation bonding). (h) 2D diagram of native ligand interactions with 5OY4 (purple arrows denote H-bonds). (i)

SPR results for PPA binding to AKT and GSK-3 β .

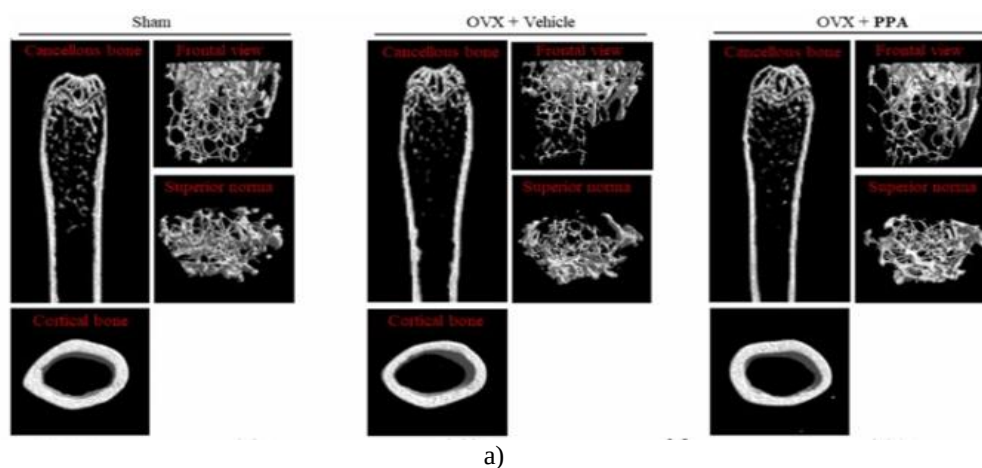
Surface plasmon resonance (SPR) assays were subsequently performed to experimentally confirm the predicted interactions and determine binding kinetics. Concentration-dependent sensorgrams demonstrated direct physical association. PPA bound to AKT with rapid on/off kinetics and a K_D value of 214 nM. Comparable fast association and dissociation behavior was observed with GSK-3 β , yielding a K_D of 177 nM (**Figure 7i**). These results collectively establish that PPA physically interacts with both AKT and GSK-3 β at physiologically relevant affinities.

PPA ameliorates OVX-induced bone loss

The potential of PPA to counteract osteoporosis was examined in an ovariectomized (OVX) mouse model. Mice in the treatment arm received intravenous injections of 10 mg/kg PPA (OVX+PPA group), while controls were given 0.25% DMSO-NS (OVX + Vehicle group) every two days for six weeks before euthanasia. Long-term PPA administration did not affect body weight and elicited no apparent toxicological responses.

Micro-computed tomography analysis demonstrated that PPA effectively protected against OVX-mediated bone deterioration in the femur (**Figure 8a**). Detailed morphometric evaluation (**Figures 8b and 8e**) revealed that, in comparison with sham-operated animals, the OVX + Vehicle group displayed substantial declines of roughly 32.6% in BV/TV, 37.2% in Tb.N, and 6.1% in Tb.Th, together with a 15.6% elevation in Tb/Sp. These changes verified the successful induction of severe bone loss, confirming the reliability of the experimental model. In contrast, PPA supplementation in OVX mice led to notable recovery in BV/TV and Tb.N values relative to vehicle-treated OVX animals (**Figure 8b**). Supporting the micro-CT findings, Von Kossa staining confirmed reduced bone resorption following PPA therapy. Moreover, Toluidine blue and TRAP staining of femoral and vertebral sections showed that OVX + Vehicle treatment sharply diminished Ob.S/BS while increasing N.Oc.S/B.Pm; PPA intervention largely reversed these imbalances (**Figures 8c and 8g**).

Dynamic bone histomorphometry using dual calcein labeling further highlighted PPA's anabolic influence. The parameters MS/BS, MAR, and BFR/BS rose by 24.8%, 34.1%, and 36.1%, respectively, in the PPA group compared with OVX + Vehicle controls, reflecting enhanced bone-forming activity (**Figure 8h**). Overall, these data indicate that PPA augments bone mass in adult mice by stimulating osteoblast differentiation and promoting bone formation *in vivo*.



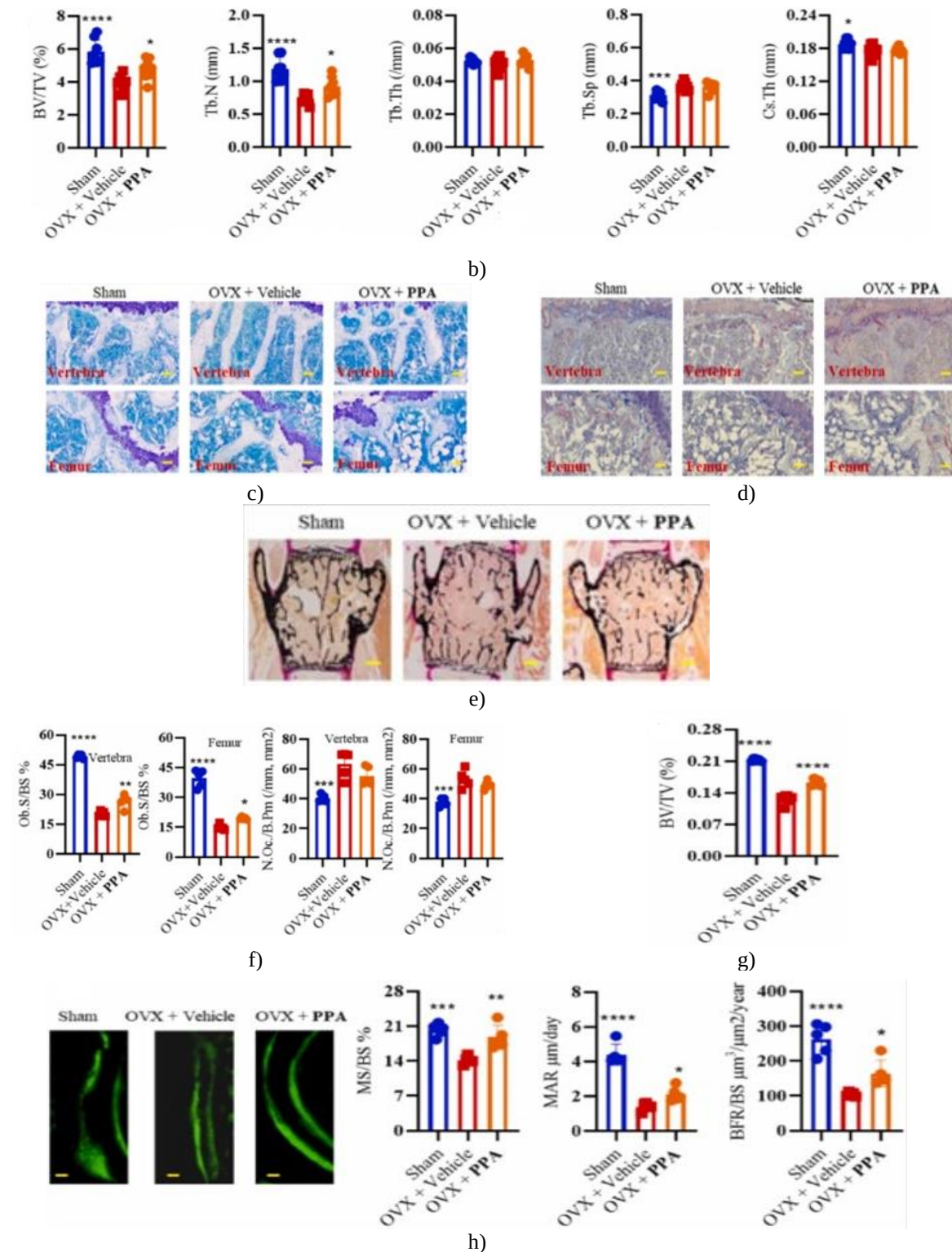


Figure 8. PPA ameliorates OVX-induced bone loss in vivo. (a) Representative three-dimensional micro-CT reconstructions of femoral trabecular architecture in sham, vehicle-treated, and PPA-treated (10 mg/kg) mice (n = 8). (b) Quantitative trabecular and cortical bone parameters including BV/TV, Tb.Sp, Tb.N, Tb.Th, and Cs.Th. (c-e) Typical histological sections of decalcified bone stained with Toluidine blue (c), TRAP (d), and Von Kossa (e). Scale bar = 100 μ M (c, d) and 250 μ M (e). (f-g) Static histomorphometric indices: Ob.S/BS, N.Oc./B.Pm, and BV/TV. (h) Fluorescence micrographs of double calcein labeling in vertebrae and corresponding dynamic parameters: MS/BS, MAR, and BFR/BS. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs the OVX + Vehicle group. BV/TV, bone volume/total volume; Tb.Sp, trabecular separation; Tb.N, trabecular number; Tb.Th, trabecular thickness; Cs.Th, cortical width; Ob.S/BS, osteoblast

surface per bone surface; N.Oc./B.Pm, osteoclast number/bone surface; MS/BS, mineralizing surface/bone surface; MAR, mineral apposition rate; BFR/BS, bone formation rate/bone surface.

Marine species offer a rich reservoir of bioactive secondary metabolites characterized by remarkable structural variety. These natural products have served as important leads for developing therapies against a wide range of conditions, with particular success in oncology. Despite their potential, marine natural products (MNPs) face significant hurdles in pharmaceutical development, including difficulties associated with high-throughput screening, compound isolation, structural elucidation, and lead optimization. These obstacles largely explain the decreased interest in MNPs by the pharmaceutical sector for new drug discovery since the 1990s [9]. PPA was originally obtained from *Penicillium commune* at a yield of 40.7 mg [21]. Five years subsequently, our laboratory successfully isolated a considerably larger amount of PPA (280 mg) from the marine-derived fungus *Penicillium solitum* MCCC 3A00215 [12]. This elevated production level from *Penicillium solitum* underscores the compound's viability as a lead candidate for therapeutic development. In the current work, an initial evaluation of 25 metabolites isolated from *Penicillium solitum* MCCC 3A00215 revealed that PPA strongly induced osteoblast mineralization in bone marrow mesenchymal stem cells (BMSCs) in a concentration-dependent manner. Building upon these observations, the present investigation systematically assessed the anti-osteoporotic properties and molecular mechanisms of PPA using both *in vitro* and *in vivo* models to evaluate its potential as a therapeutic agent for osteoporosis.

Osteoporosis is a widespread skeletal disorder that necessitates effective strategies to counteract progressive bone loss, microstructural degradation, and heightened fracture risk [22]. Bone marrow mesenchymal stem cells (BMSCs) are pivotal regulators of bone homeostasis and repair, being recruited to injury sites where they support regenerative processes [23]. With advancing age, the pool of BMSCs capable of committing to the osteoblastic lineage diminishes, resulting in compromised bone formation and the emergence of osteoporosis. Consequently, agents capable of steering BMSC differentiation toward osteoblasts hold substantial promise for bone repair and osteoporosis management [7]. Supporting this concept, multiple studies have shown that boosting the osteogenic capacity of BMSCs in models of aging or estrogen deficiency enhances bone formation and restores skeletal integrity [24, 25]. Nevertheless, only a small number of MNPs have demonstrated positive effects on osteoblastic cells, thereby constraining their advancement toward clinical use in osteoporosis therapy [26, 27]. In the present study, PPA significantly enhanced extracellular matrix mineralization in both BMSCs and MC3T3-E1 pre-osteoblasts in a dose-dependent manner. It also suppressed adipogenic differentiation of BMSCs while leaving chondrogenic differentiation largely unaffected. Transcriptional analysis showed that PPA upregulated key osteogenic markers (*Runx2*, *Ibsp*, and *Bglap*) and concurrently downregulated adipogenic regulators (*Pref1*, *Zfp423*, and *Fabp4*) in BMSCs. These findings indicate that PPA effectively shifts BMSC lineage commitment in favor of osteogenesis. Moreover, PPA increased alkaline phosphatase (ALP) activity during the early differentiation phase and promoted calcium deposition at later stages (**Figures 3b and 3e**). Collectively, these results establish that PPA triggers the molecular cascade leading to osteoblast differentiation in both primary BMSCs and MC3T3-E1 cells.

Transcriptomic profiling via RNA-seq identified extensive alterations in gene expression patterns in PPA-treated BMSCs. Pathway enrichment analysis highlighted significant involvement of the PI3K-AKT, MAPK, calcium, TNF, and TGF- β signaling cascades. GSK-3 β functions as a central regulator within the β -catenin destruction complex and participates in the control of several upstream pathways, notably the AKT cascade. Phosphorylation of GSK-3 β at Ser9 by activated AKT prevents its kinase activity, allowing β -catenin to accumulate and translocate to the nucleus, where it drives osteogenic gene expression and bone regeneration [20]. In this study, western blotting and immunofluorescence assays confirmed the engagement of the AKT/GSK-3 β axis, demonstrating that PPA treatment elevated protein levels of β -catenin, phosphorylated GSK-3 β , and phosphorylated AKT (**Figures 5 and 6**). Complementary molecular docking simulations were conducted to examine direct interactions between PPA and these target proteins. The results indicated stable binding of PPA within the ligand-binding sites of both AKT and GSK-3 β , yielding docking energies of -5.500 kcal/mol and -5.057 kcal/mol, respectively, with compatible binding orientations (**Figure 7**). By engaging the AKT/GSK-3 β complex, PPA modulates downstream gene networks through the AKT/GSK-3 β / β -catenin pathway, ultimately potentiating osteoblast differentiation and matrix mineralization (**Figure 9**).

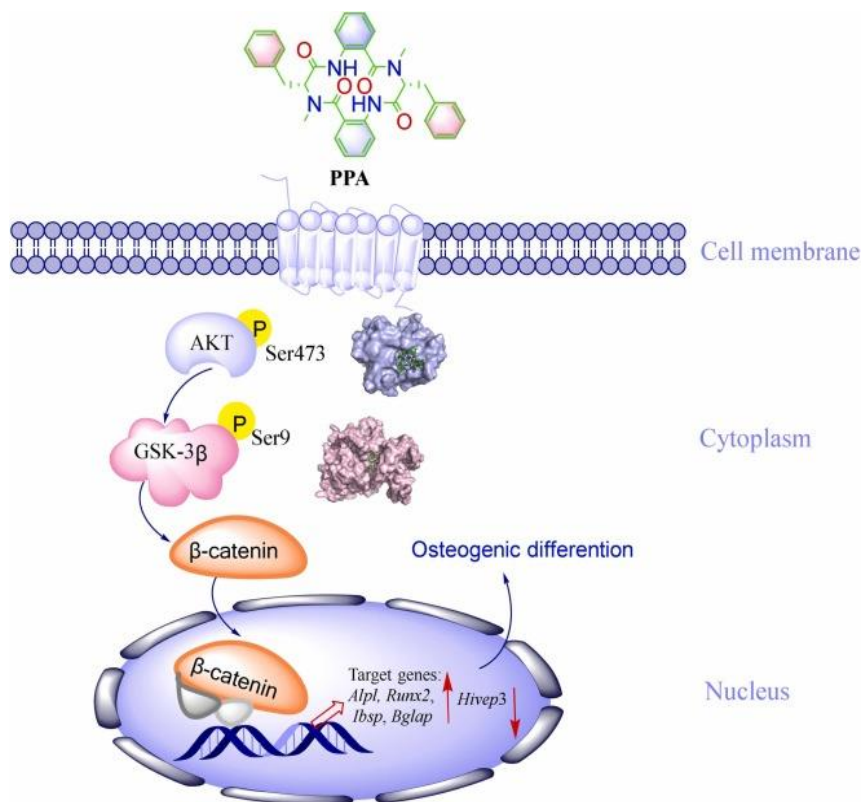


Figure 9. Schematic illustration of the proposed mechanism underlying PPA-mediated promotion of osteoblastogenesis and bone synthesis through activation of the AKT/GSK-3 β / β -catenin signaling cascade.

Conclusion

Taken together, the present findings demonstrate that the marine-derived peptide PPA stimulates osteoblast differentiation and mineralization through activation of the AKT/GSK-3 β / β -catenin signaling pathway *in vitro*. This is accompanied by increased expression of *Alpl*, *Runx2*, *Ibsp*, and *Bglap*, together with reduced expression of *HIVEP3*. The study also elucidates a previously unrecognized mechanism whereby PPA influences osteoclast activity via direct interaction with AKT and GSK-3 β . *In vivo* experiments further confirmed the therapeutic benefit of PPA by significantly attenuating systemic bone loss in the ovariectomy (OVX) model. As a natural product isolated from the deep-sea fungus *Penicillium solitum*, PPA emerges as a compelling candidate for the treatment of osteolytic disorders, with particular relevance to osteoporosis.

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Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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