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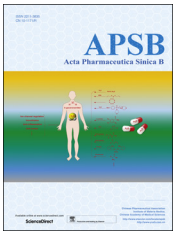

ORIGINAL ARTICLE

# Calycosin-7-*O*-*β*-D-glucopyranoside stimulates osteoblast differentiation through regulating the BMP/WNT signaling pathways

**Jing Jian, Lijuan Sun*, Xun Cheng, Xiaofang Hu, Jichao Liang, Yong Chen****

*Hubei Province Key Laboratory of Biotechnology of Chinese Traditional Medicine, Hubei Collaborative Innovation Center for Green Transformation of Bio-Resources, Hubei University, Wuhan 430062, China*

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**KEY WORDS**

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ST2 cells

**Abstract** The isoflavone calycosin-7-*O*-*β*-D-glucopyranoside (CG) is a principal constituent of *Astragalus membranaceus* (AR) and has been reported to inhibit osteoclast development *in vitro* and bone loss *in vivo*. The aim of this study was to investigate the osteogenic effects of CG and its underlying mechanism in ST2 cells. The results show that exposure of cells to CG in osteogenic differentiation medium increases ALP activity, osteocalcin (*Ocal*) mRNA expression and the osteoblastic mineralization process. Mechanistically, CG treatment increased the expression of bone morphogenetic protein 2 (BMP-2), p-Smad 1/5/8, *β*-catenin and Runx2, all of which are regulators of the BMP- or wingless-type MMTV integration site family (WNT)/*β*-catenin-signaling pathways. Moreover, the osteogenic effects of CG were inhibited by Noggin and DKK-1 which are classical inhibitors of the BMP and WNT/*β*-catenin-signaling pathways, respectively. Taken together, the results indicate that CG promotes the osteoblastic

*Abbreviations:* ALP, alkaline phosphatase; AR, *Astragalus membranaceus*; BMP, bone morphogenetic protein; CG, calycosin-7-*O*-*β*-D-glucopyranoside; DKK-1, dickkopf-1; ECL, enhanced chemiluminescence; FGF, fibroblast growth factor; HAase, hyaluronidase; IGF1, insulin-like growth factor 1; MAPK, mitogen-activated protein kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; OBM, osteogenic differentiation medium; Ocal, osteocalcin; OPN, osteopontin; OVX, ovariectomized; PVDF, polyvinylidine fluoride; TGF-*β*, transforming growth factor *β*; WNT, wingless-type MMTV integration site family

*Corresponding author. Tel.: +86 27 88663882.
**Corresponding author. Tel.: +86 27 88668070.
E-mail addresses: lijuansun1212@163.com (Lijuan Sun), cy101610@qq.com (Yong Chen).

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differentiation of ST2 cells through regulating the BMP/WNT signaling pathways. On this basis, CG may be a useful lead compound for improving the treatment of bone-decreasing diseases and enhancing bone regeneration.



## 1. Introduction

Regulation of bone mass is controlled by continuous bone remodeling through osteoblastic bone formation and resorption. Disorders of bone remodeling are implicated in a variety of diseases such as osteoporosis, hypercalcemia and rheumatoid arthritis as well as tumor metastasis into bone[1]. Understanding osteoblastic differentiation is therefore crucial to improving the treatment of such disorders.

Osteoblasts are the main bone-forming cells arising from mesenchymal stem cells. They produce alkaline phosphatase (ALP) and bone matrix proteins such as osteocalcin (Ocal) and osteopontin (OPN) which act to induce osteoblastic mineralization[2]. Osteoblast differentiation is regulated by various signaling pathways involving bone morphogenetic proteins (BMPs), wingless-type MMTV integration site family (WNT)/*β*-catenin proteins, transforming growth factor-*β* (TGF-*β*), insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF), and mitogen-activated protein kinase (MAPK)[3–5]. Of these pathways, the one involving BMPs is key in skeletal development, maintaining adult bone homeostasis and stimulating bone formation in fracture healing[6]. Activation of the WNT/*β*-catenin signaling pathway is essential for proper bone development[7] and, in cooperation with the BMP signaling pathway, regulates osteoblast differentiation and bone formation[8].

*Astragalus membranaceus* (AR) is one of the most important medicinal plants in traditional Chinese medicine. In recent years, it has received considerable attention because of its immunostimulant effects[9], antibacterial and antiviral properties, hepatoprotective and antiinflammatory activity and beneficial cardiovascular effects[10]. Of particular interest is the observation that AR inhibits osteoclast development *in vitro* and bone loss *in vivo* in ovariectomized (OVX) rats[11]. Moreover, AR combined with calcium has been shown to significantly improve bone mineral density, biomechanical strength, and ash weight of the femur and tibia of OVX rats[12]. However, the main osteogenically active components of AR remain to be identified.

The isoflavone calycosin-7-*O*-*β*-D-glucopyranoside (CG, Fig. 1) is a principal constituent of AR. It is a strong inhibitor of hyaluronidase (HAase)[13] and of matrix degradation caused by IL-1*β* or HAase in human articular cartilage explant and chondrocytes[14]. Since it is known that isoflavones are active in preventing osteoporosis[15,16], it is reasonable to hypothesize that CG may exhibit osteogenic effects. Accordingly this study aimed to investigate the osteogenic effects of CG and its role in the osteogenic differentiation of bone marrow stromal cell.

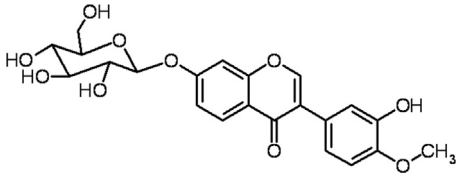


**Figure 1** Chemical structure of calycosin-7-*O*-*β*-D-glucopyranoside (CG).

## 2. Materials and methods

### 2.1. Cell culture

Bone marrow stromal ST2 cells were seeded at a density of $1 \times 10^5$ cells/mL and cultured in regular growth culture medium containing *α*-minimum essential medium supplemented with 15% fetal bovine serum (Biochrom, Australia), 100 units/mL penicillin (Gibco, Australia) and 100 mg/L streptomycin (Gibco) in a humidified atmosphere of 5% $CO_2$ at 37 °C. At 80% confluence, the cells were cultured in osteogenic differentiation medium (OBM) which consisted of the above culture medium containing 10 nmol/L dexamethasone (Sigma-Aldrich, USA), 10 mmol/L *β*-glycerophosphate (Sigma-Aldrich), 50 μg/mL ascorbic acid (Sinopharm Chemical Reagent, China) and various concentrations of CG (98.3%, National Institutes for Food and Drug Control, CAS 20633-67-4, China) added as a solution in dimethyl sulfoxide (Sigma-Aldrich, final concentration 0.1%).

### 2.2. Cell viability assay

Cell viability was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) assay. Briefly, cells ($1 \times 10^4$ cells/well in 96-well plates) were maintained in OBM at 37 °C for 24 h. Cells were then treated with CG (4, 8, 16 and 32 μmol/L) in OBM for 1, 3 and 6 days at which times 20 μL MTT (5 mg/mL) was added to each well and samples incubated in the dark at 37 °C. After 4 h, medium was discarded and the precipitated formazan dissolved in DMSO (150 μL/well). Absorbance was measured with a microplate

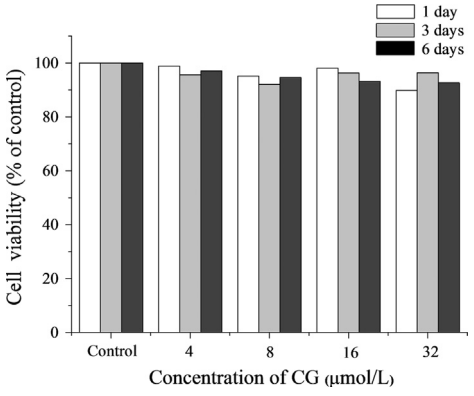


**Figure 2** Effect of CG on the proliferation of ST2 cells. Cells were seeded in 96-well plates for 24 h and then treated with different concentrations of CG for 1, 3 and 6 days. Cellular proliferation was determined using the MTT assay. Results are expressed as means ± SD ($n = 3$).