Vitisin B stimulates osteoblastogenesis via estrogen receptor-mediated activation of non-genomic Src and MAPKs pathway

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Background/Purpose: Vitisin B is a major component existed in Vitis thunbergii, a herbal medicine used in Taiwan for treatment of inflammatory bone diseases. We recently reported that vitisin B stimulated differentiation in primary cultured osteoblasts and treatment with vitisin B-enriched preparation obviously ameliorated ovariectomy-induced bone loss in mice. This study further delineated the action mechanism(s) that how vitisin B stimulates osteoblastogenesis by using MC3T3-E1 osteoblasts. Methods: Cell differentiation and mineralization were identified by alkaline phosphatase (ALP) activity and Alizarin red S staining, respectively. RT-PCR and western blot were used to analyze the expression of osteoblast-associated genes and signal molecules. The transcriptional activity of estrogen receptor (ER) was also assessed. Results: Vitisin B significantly increased ALP activity, bone mineralization, mRNA expression of osteoids (type I collagen, bone sialprotein and osteocalcin) and bone-characteristic transcription factors (runt-related transcription factor-2 and osterix) through ER since such responsiveness were obviously repressed by ER antagonist ICI182,780. Unlike 17β-estradiol (E2), vitisin B failed to stimulate either ERα- or ERβ-mediated transcriptional activity. Nevertheless, vitisin B rapidly induced ERα and Src phosphorylations within 5 min and evoked late phosphorylations of p38 and ERK after 15-30 min stimulation through ER. Furthermore, p38 inhibitor SB203580 and MEK inhibitor PD98059 significantly inhibited vitisin B induced differentiation. Src inhibitor PP2 significantly repressed vitisin B-induced activation of MAPK and final mineralization suggesting that Src might play an important role. Conclusions: Vitisin B
might act through ER-mediated activation of Src and downstream MAPK to stimulate osteoblastogenesis which contributed to its beneficial effect in prevent bone loss.

**Key words:** estrogen receptor; osteoblast; Src kinase; vitisin B

**Introduction**

Osteoporosis is a reduction in bone mass due to an imbalance between bone resorption and bone formation. It can be treated by either anti-osteoporotic drugs (anti-resorptives), anabolic drugs (bone formation), or both [1,2]. Many plant-derived compounds have the potential to counteract the deleterious effects of estrogen deficiency on bone. Members of this class are the so-called phytoestrogens. Estrogen receptors (ERs) stimulate classical effects by acting as nuclear transcription factors as well as non-classical effects by activating distinct cytoplasmic protein kinase cascades. Multiple lines of evidence suggest that activation of the tyrosine kinase c-Src represents one of the initial steps in ER-α-mediated cell signaling [3]. Numerous studies have demonstrated that estrogens induce rapid and transient activation of the c-Src phosphorylation cascade. Activation of this cascade triggers vital cellular functions including cell proliferation and differentiation. The essential role of c-Src in the non-classical action of steroid receptors was demonstrated in experiments with embryonic fibroblasts derived from c-Src−/− mice [4].

Vitisin B is a dominant stilbene contained in the root of *Vitis thunbergii* Sieb. & Zucc. (*Vitis ficifolia* Bge. Vitaceae), a well-known herbal remedy traditionally used in Taiwan for diarrhea, jaundice, hepatitis, fracture and injury [5]. Accumulating use experience suggests an alcoholic drench of the roots of *V. thunbergii* also helps cure bone fractures, contusions and prevents bone loss, suggesting that this herb might either stimulate new bone formation or inhibit bone resorption. We recently reported that orally administered with (+)-vitisin B-enriched preparation (VtR) significantly ameliorated the deterioration of bone mineral density in ovariectomized mice and found that vitisin B could stimulate osteoblast function in cultured bone marrow cells [6]. Considering that vitisin B might display protective effect against bone loss, this study aimed to clarify how vitisin B stimulates osteoblastogenesis.

**Materials and methods**

**1. Chemicals and antibodies**

Fetal bovine serum (FBS), charcoal-stripped FBS (sFBS), α-modified minimum essential medium (α-MEM), TRIzol reagent, penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA). 17β-Estradiol (E2), ICI 182,780 (Fulvestrant, (7a,17b)-7-[9-[(4,4,5,5,5pentafluoropentyl)Sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol), ascorbic acid, 3-[(4,4-dimethyl-thiazol-2yl)-2,5-
diphenyltetrazolium bromide (MTT), Alizarin Red S and \( p \)-nitrophenyl phosphate (\( p \)-NPP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PP2 (AG 1879, 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo [3,4-d]pyrimidine) was purchased from Merck (Darmstadt, Germany). \( \beta \)-glycerophosphate was obtained from Wako Pure Chemicals (Osaka, Japan). Antibodies against total and phosphorylated c-Src were purchased from Cell Signaling Technology (Beverly, MA, USA). The other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) Western blotting detection reagent was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

2. Extraction and isolation of vitisin B

*Vitis thunbergii* was purchased in Taipei, Taiwan, in July 1998 and identified by Mr. Jun-Chih Ou, a taxonomist retiring from our institute. A voucher specimen (NRICM-98-010) is deposited at the Herbarium of National Research Institute of Chinese Medicine. Detailed information for the HPLC analysis and the structure identification of (+)-vitisin B (purity 99.4%) was described in our previous work [7, 8].

3. Cell culture, differentiation and mineralization

Murine osteoblastic MC3T3-E1 cell exhibited high levels of known osteoblast markers. This provides a suitable model to analyze osteoblast function and related signaling pathway since osteoblastogenic process can be easily and quickly assessed. Cells were maintained in \( \alpha \)-MEM as described previously [9]. For differentiation or mineralization, cells seeded in 96- or 24-well plates were cultured to reach confluence. After that, the medium was replaced to phenol red-free MEM\( \alpha \) supplemented with 1% sFBS, 50 \( \mu \)g/mL ascorbic acid and 10 mM\( \beta \)-glycerophosphate (define as differentiation medium) for 24 h. After that, cells were stimulated with vitisin B, E2 or vehicle for different time periods to induce differentiation (3-8 days) or mineralization (7-28 days). Osteoblastic differentiation was assessed by measuring the alkaline phosphatase (ALP) activity and represented either as \( \mu \)M \( p \)-NPP/ mg protein/hour or indicated as \% of control (maximum response is defined as 100%) [10]. The calcified bone nodules (mineralization) appeared bright red in color by Alizarin red S staining was observed under light microscope and photographed. Following, calcium precipitate was extracted by 0.02N sodium hydroxide and the dissolved product was determined by measuring the absorbance at 548 nm [10].

4. RNA preparation and reverse transcriptase polymerase chain reaction (RT-PCR)

The total cellular RNA was isolated using TRIzol reagent and RT-PCR was performed using RevertAid™ First Strand cDNA Synthesis Kit and Go Taq® Flexi DNA Polymerase according to the manufacturer’s instruction. Mouse-specific primer sequences for sense or antisense strand of osteocalcin (OCN), bone sialoprotein (BSP), type 1 collagen (Col-1), runt-
related transcription factor-2 (Runx2), osterix and GAPDH were described in our previous study [11, 12].

5. Transient transfection and ER-mediated luciferase activity assay

The cells were transfected by Lipofectamine™ 2000 reagent. ER-α, ER-β and estrogen response element (ERE)-containing luciferase reporter plasmid vERETkluc were purchased from Clontech (Mountain View, CA, USA). 0.4 μg ER-α or ER-β plasmid, 0.4μg vERETkluc, together with 0.1 μg internal control reporter plasmid pRL-TK, a Renilla luciferase control vector, was cotransfected into the cells in triplicate. Five hours after transfection, cells were treated with vehicle, E2 or vitisin B for 24 h. After treatment, the cells were lysed and the luciferase activity was measured using the Dual Luciferase Reporter assay System and the signal was detected by TD-20/20 Luminometer (Turner Design, Sunnyvale, CA, USA). The estrogen promoter activity was expressed as firefly luciferase values normalized to pRL-TK Renilla luciferase values.

6. Immunoblotting

Treated cells were harvested and lysed with lysis buffer [11, 12]. Equal amounts of proteins were separated by SDS-PAGE and transblotted onto PVDF membranes (Immobilin-P, Millipore Corp., Danvers, MA, USA). After blocking the non-specific binding sites, the blots was probed with first antibodies and followed by incubation with secondary antibodies. The antigen-antibody complexes were then detected with ECL reagent.

7. Statistical analysis

The results are expressed as mean ± Sp.E. Statistical analysis was performed with an analysis of variance followed by the one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons. A value of p<0.05 is accepted as statistically significant difference.

Results

1. Effects of vitisin B on osteoblastic differentiation and mineralization

This study aimed to clarify whether vitisin B stimulated MC3T3-E1 differentiation as did in primary cultured osteoblasts. Vitisin B significantly stimulated ALP activity in MC3T3-E1 cells in time-dependent (Fig. 1A) and concentration-dependent (Fig. 1B) manner but without detectable effect on cell proliferation or viability (data not shown). About 2-folds and 2.2-folds induction was reached after cultured with 10-20 μM vitisin B and 100 nM E2 for 8 days, respectively. Because vitisin B-evoked osteogenic effects in MC3T3-E1 cells were equal potent as did in primary cultures [6], MC3T3-E1 cell was thus used for mineralization assay and the others.

As shown in Figs. 2A & 2B, addition of vitisin B (0.5-20μM) time-dependently and concentration-dependently enhanced mineralization when compared with control (differentiation medium alone). Significant enhancement in mineralization was occurred up to 1 μM of vitisin B and a plateau effect (~4-fold) noted at 10-20 μM. 100 nM of E2 also
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(A) Vitisin B stimulated the increase in ALP activity in time- and concentration-dependent manner.
(B) Osteogenic effect of vitisin B was compared with 17β-estradiol (E2, 100 nM). Each value is the mean ± SE of six independent experiments. * p<0.05, ** p<0.01 and *** p<0.001, compared with cell incubated with differentiation medium alone.

Figure 1   Effect of vitisin B on ALP activity in MC3T3-E1 cells.

(A) Vitisin B stimulated the formation of calcified nodules by approximately 4-fold when compared to the control. The mRNA expressions of osteoid (bone matrix) (Fig. 3A) and osteoblast-characteristic
transcription factors (Fig. 3B) were also concentration-dependently increased by vitisin B (1, 5, 10 and 15μM) and E2 (1, 10 and 100 nM) stimulation, respectively.

2. The roles of estrogen receptor (ER) in vitisin B-induced osteoblastogenesis

Estrogen receptor (ER) plays an important role in regulation of osteoblastogenesis. The result shown in Fig. 4A indicated that vitisin B (10 μM) or E2 (100 nM) induced increase in ALP activity were concentration-dependently abolished by ICI-182,780. Furthermore, the stimulatory effects of vitisin B on mineralization (Fig. 4B) and mRNA expression of bone-characteristic genes (Fig. 4C) were also significantly reduced by ICI-182,780.

3. Effects of vitisin B on ER-mediated luciferase activities

100 nM of E2 significantly increased the ERE-dependent luciferase activities via ERα or ERβ (Fig. 5A). In contrast, vitisin B failed to induce ERE-dependent luciferase activities via ERs. Western blot analysis reconfirmed that vitisin B did not induce ERα nor ERβ nuclear translocation indicating that vitisin B-evoked bone anabolic action was not via the activation
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Figure 4  Vitisin B-evoked increase in (A) ALP activity, (B) mineralization and (C) mRNA expression of bone-characteristic genes (Col-1: type 1 collagen; BSP, bone sialoprotein; OCN, osteocalcin; Runx2, runt-related transcription factor-2; Osx, osterix) were significantly repressed by ICI-182,780 treatment. Each value is the mean ± SE of five independent experiments. * p<0.05, ** p<0.01 and *** p<0.001, compared with cell incubated with vitisin B alone or 17β-estradiol (E2) alone.

4. Effects of vitisin B on ERα phosphorylation
   Because vitisin B failed to activate ERE-dependent transcription via ERs, we hypothesized that it might alternatively activate ER-mediated non-genomic signaling pathway to induce osteoblastogenesis. Results showed that
cells stimulated with vitisin B (10 μM) or E2 (100 nM) for a short time rapidly increased the expression ratio of phospho-ERα to ERα (pERα/ERα) suggesting that vitisin B and E2 could activate ERα by phosphorylation. As shown in Fig. 6A (left), E2 (100 nM) increased pERα/ERα within 5 min and remained up-regulated for 15 min treatment. Similarly, vitisin B (10 μM) was able to significantly increase pERα/ERα at 1-5 min and the phosphorylation was sustained.
Vitisin B stimulates osteoblastogenesis throughout around 10-15 min of incubation. The concentration-response for vitisin B and E2 on ERα phosphorylation were shown in the right of Fig. 6A.

5. ER-mediated early phosphorylation of c-Src is essential for vitisin B-induced

Figure 6  Vitisin B and 17β-estradiol (E2) both evoked time-related and concentration-related phosphorylations of ERα (A) and Src (B) in MC3T3-E1 cells and vitisin B-evoked such phosphorylations were significantly inhibited in the presence of ICI-182,780 (C). Proteins extracted from vitisin B- and E2-stimulated cells were analyzed by western blotting and probed with antibodies against ERα, phospho-ERα, Src, phospho-Src in the absence or presence of ICI-182,780, respectively.
osteoblastogenesis

Increasing evidence indicates that c-Src might exert key functions in ERα mediated non-classical effect of estrogens [3]. Whether E2 or vitisin B act through c-Src pathway to induce osteoblastogenesis was studied by evaluate the phosphorylation (activation) of c-Src. Results showed in Fig. 6B demonstrated that E2 (100 nM) and vitisin B (10μM) both increased tyrosine phosphorylation of c-Src (Tyr 416) within 5 min stimulation. The concentration-response of vitisin B and E2 on Src phosphorylation were shown in the right of Fig. 6B. Interestingly, vitisin B induced c-Src phosphorylation was repressed by ICI 182,780 treatment (Fig. 6C). This result indicated that vitisin B-induced activation of c-Src is ER-dependent. To further clarify whether Src really participating in vitisin B-induced osteoblastogenesis or not, a widely used, specific inhibitor for all members of Src families PP2 was applied. As shown in Fig 7, PP2 obviously and concentration-dependently repressed not only the

![Figure 7](image)

**Figure 7** Vitisin B-evoked increase in mRNA expression of bone-characteristic genes (BSP, bone sialprotein; Col-1: type 1 collagen; Runx2, runt-related transcription factor-2; Osx, osterix) and mineralization were significantly repressed by Src kinase inhibitor PP2. Each value is the mean ± SE of five to six independent experiments. * p<0.01 and ** p<0.001, compared with cell incubated with vitisin B alone.
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expression of osteoblast-characteristic genes but also the mineralization evoked by vitisin B.

6. Vitisin B-evoked activation of MAPKs is mediated by ER/Src and is necessary for vitisin B-induced osteoblast differentiation

Several studies indicated that MAPKs were involved in regulation of osteoblastic differentiation and maturation [13, 14]. We also like to know whether vitisin B evoked differentiation and maturation was mediated by MAPKs or not. Results showed in Fig. 8A indicated that treatment with vitisin B significantly increased the phosphorylation of p38 and ERK, but not JNK (data not shown).

Overall, the activated pattern for MAPKs was relative late than ERα or Src activation. As shown in Fig. 8A, the time-dependent activation of p38 and ERK was obviously detected at 15 min and further up-regulated after 30 min stimulation and such phosphorylations were obviously repressed by ICI-182,780 and PP2 treatment (Fig. 8B), respectively. These results suggested that ER and Src are upstream components in the activation of MAPKs by vitisin B. Subsequent functional study was designed to further explore whether

Figure 8  Vitisin B activated p38 MAPK and ERK phosphorylations in time- and concentration-dependent manner (A) and such phosphorylations were significantly repressed by ICI-182,780 and PP2 (B). In (A), proteins extracted from vitisin B-stimulated cells were analyzed by western blotting and probed with antibodies against p38, phospho-p38, ERK, phospho-ERK and actin. In (B), transblotted membranes were reacted with antibodies against p38, phospho-p38, ERK, phospho-ERK, phosphor-Src and actin in the absence or presence of ICI-182,780 or PP2, respectively.
p38 MAPK or ERK signaling is necessary for vitisin B-induced osteoblast differentiation by using individual inhibitor (SB203580 and PD98059). As shown in Fig. 9, vitisin B-evoked increase in ALP activity was repressed by about 23%, 62%, 88% and 97% in the presence of 0.5, 1, 5 and 10 μM SB203580 and similar inhibitory pattern was observed by PD98059 treatment.

Discussion

The present study clearly demonstrated that vitisin B mimicked E2 in stimulating osteoblast differentiation, expression of bone-characteristic genes and induced mineralization via ER in MC3T3-E1 cells, suggesting that vitisin B could exert estrogen-like effects in

Figure 9 Vitisin B-stimulated ALP activity was significantly abolished by SB203580 and PD98059 treatment, respectively. Each value is the mean ± SE of four to five independent experiments. * p<0.05 and ** p<0.01, compared with cell incubated with vitisin B alone.
promoting osteoblastic functions. Evidence indicates that estrogen-mediated responsiveness is mediated by a complex interface of direct control of gene expression (so-called “genomic action”) and by regulation of cell signaling/phosphorylation cascades (referred to as the “non-genomic”, or “extranuclear” action) [15-17]. ERs are the members of the superfamily of ligand-regulated nuclear transcription factors. ERα and ERβ have been identified in cultured rat osteoblasts and estrogen was shown to stimulate the differentiation of osteoblasts [18]. When bound to E2, E2 stimulated ERα translocation to the nucleus and activate the expression of genes that have EREs in their promoter regions [19]. Homozygous deletion of the ERα gene in mice results in decreased longitudinal bone growth, decreased cortical bone density, as well as decreased bone formation, suggesting that ERα is of critical importance in promoting overall bone growth [20, 21]. Unlike E2, the transfection study indicated that vitisin B failed to induce ERE-dependent luciferase activities via either ERα or ERβ. Results obtained from western blotting also revealed that vitisin B did not increase the nuclear translocation of ERs. However, vitisin B could activate ERα phosphorylation (but not ERβ) in MC3T3-E1 cells within 5 min of incubation as E2 did. ERα can activate protein-protein signaling cascade in the cytoplasm and to regulation of genes expression in which instead of ERα entering the nucleus to activate transcription [15]. Thus, we hypothesized that vitisin B would act through so-called “non-genomic action” to induce osteoblastogenesis.

c-Src is known to play a pivotal role in osteoblast differentiation, and c-Src null primary osteoblasts appear to undergo ‘premature’ differentiation. Multiple lines of evidence suggest that activation of the tyrosine kinase c-Src, represents one of the initial steps in ER-mediated cell-signaling [3]. In non-genomic pathway, binding of E2 to membrane ER rapidly activates c-Src kinase. Src undergoes an intermolecular autophosphorylation at tyrosine 416, and its phosphorylation promotes kinase activity [22]. The present results indicated that vitisin B induced phosphorylation of c-Src and such phosphorylation was concentration-dependently repressed by ICI-182,780 treatment. Recently, c-Src was observed to serve as a transducer of signaling events taking place in osteoblast cells in response to resveratrol and diosgenin. [23, 24] Furthermore, the c-Src inhibitor dasatinib decreases osteoblastic differentiation in human bone marrow-derived mesenchymal stromal cells, primary mouse osteoblasts and MC3T3-E1 cells [25, 26]. We also examine whether c-Src signaling mediated the osteogenesis by vitisin B in MC3T3-E1. Pharmacologic blockade of c-Src activity by PP2 significantly blunted vitisin B-induced expressions of bone-specific genes and transcription factors and final mineralization. The present results indicated that ER-mediated c-Src phosphorylation is required for vitisin B-induced osteoblastic differentiation and maturation in MC3T3-E1 cells.

Several studies demonstrated that Src/MAPKs influenced osteoblast differentiation and
maturation. The essential role of Src kinase in the non-genomic action of steroid receptors was demonstrated in experiments with embryonic fibroblasts derived from Src$^{−/−}$ mice. These cells did not show rapid activation of the MAP kinase pathway in response to androgen receptor and ERα activation, whereas wild-type Src$^{+/+}$ cells did [4]. Suzuki et al. (1999) showed that ERKs play an important role in cell replication, whereas p38 MAPK participates in the regulation of ALP expression during osteoblast differentiation [27]. Liao et al. found that genistein induced the expression of ALP, BSP and OCN through activation of p38 MAPK pathway [28]. Tang et al. reported that imperatorin and bergapten enhance ALP activity, type I collagen synthesis and mineralization in rat via the p38- and ERK-dependent signaling pathway [29]. The present results showed that treated the cells with vitisin B induced the phosphorylation of p38 MAPK and ERK. Furthermore, inhibition of p38 MAPK and ERK activation by either SB203580 or PD98059 effectively decreased vitisin B-induced ALP activity. Collectively, the present findings suggested that activation of p38 MAPK and ERK were involved in vitisin B regulated osteoblastic differentiation.

We recently reported that orally administered with VtR significantly ameliorated the deterioration of bone mineral density in ovariectomized mice [6]. The present study further clarified the (+)-vitisin B could stimulate osteoblastogenesis via ER-mediated activation of non-genomic Src and MAPKs pathway. In conclusion, our findings provide the evidence to support the use of VtR as a potential botanic remedy for osteoporosis therapy.

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Vitisin B 經由雌激素受體調節 Src 激酶及下游 MAPK 之非基因型訊息路徑而刺激骨質新生

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Vitisin B 是小本山葡萄主要成分之一，民間常使用該本土植物於發炎性骨疾，宣稱可強壯筋骨。我們先前發現餵食富含 vitisin B 的提取物可明顯改善卵巢切除鼠的骨質流失現象，現在進一步利用 MC3T3-E1 造骨細胞，研究 vitisin B 對骨質新生的影響。結果發現與骨分化 / 成熟有關的礦解酶活性及礦化程度，均因處理 vitisin B 而明顯提高。vitisin B 也使骨基質以及特有轉錄因子的表現量增加，且上述促進作用皆會被雌激素受體 (ER) 拮抗劑所抑制。不像典型的雌激素作用劑 17-estradiol，vitisin B 不會刺激 ER 或 ER 調控的轉錄活性，但反而可快速磷酸化 ER 受體及 Src 激酶，繼而磷酸化 p38 及 ERK 這二個 MAPK，這些作用也都會被 ER 拮抗劑所抑制。進一步發現 p38 及 ERK 抑制劑會干擾 vitisin B 誘導的造骨細胞分化，而 Src 抑制劑也會阻斷 vitisin B 誘導的 MAPK 活化與礦化。綜上，本研究發現 vitisin B 具改善骨質流失的功效，其機轉可能是經由雌激素受體去活化細胞內非基因路徑的 Src 激酶及下游的 MAPK 訊息分子，進一步啟動造骨細胞特有的轉錄因子活化，繼而促進骨基質生成及礦化。

關鍵字：雌激素受體、造骨細胞、Src 激酶、vitisin B

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