Platonin inhibited PDGF-BB-induced proliferation of rat vascular smooth muscle cells via JNK1/2-dependent signaling

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Aim: To examine the inhibitory actions of the immunoregulator platonin against proliferation of rat vascular smooth muscle cells (VSMCs).

Methods: VSMCs were prepared from the thoracic aortas of male Wistar rats. Cell proliferation was examined using MTT assays. Cell cycles were analyzed using flow cytometry. c-Jun N-terminal kinase (JNK)1/2, extracellular signal-regulated kinase (ERK)1/2, AKT, and c-Jun phosphorylation or p27 expression were detected using immunoblotting.

Results: Pretreatment with platonin (1–5 μmol/L) significantly suppressed VSMC proliferation stimulated by PDGF-BB (10 ng/mL) or 10% fetal bovine serum (FBS), and arrested cell cycle progression in the S and G2/M phases. The same concentrations of platonin significantly inhibited the phosphorylation of JNK1/2 but not ERK1/2 or AKT in VSMCs stimulated by PDGF-BB. Furthermore, platonin also attenuated c-Jun phosphorylation and markedly reversed the down-regulation of p27 expression after PDGF-BB stimulation.

Conclusion: Platonin inhibited VSMC proliferation, possibly via inhibiting phosphorylation of JNK1/2 and c-Jun, and reversal of p27 down-regulation, thereby leading to cell cycle arrest at the S and G2/M phases. Thus, platonin may represent a novel approach for lowering the risk of abnormal VSMC proliferation and related vascular diseases.

Keywords: platonin; cell cycle; JNK1/2; PDGF-BB; p27; vascular smooth muscle cells

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Introduction

Abnormal proliferation of vascular smooth muscle cells (VSMCs) is implicated in the pathogenesis of several diseases, including atherosclerosis, restenosis after angioplasty, transplant vasculopathy, and failure of vein graft bypasses[1]. Numerous growth factors and cytokines are reported to be released in human vascular lesions by dysfunctional endothelial cells, inflammatory cells, platelets, and VSMCs, and these mediate chemotraction, cell migration, proliferation, apoptosis, and matrix modulation[2]. Basic fibroblast growth factor initiates medial proliferation of VSMCs, whereas platelet-derived growth factor (PDGF) induces subsequent migration of VSMCs toward the intima. Intimal proliferation and matrix accumulation have been reported to occur under the influence of PDGF, transforming growth factor-β, angiotensin II, epidermal growth factor, and insulin-like growth factor 1[3]. Although all of these factors may play roles in driving cellular events that lead to vascular proliferative diseases, PDGF is considered to be the main cause[4].

PDGF is a peptide growth factor that provides signals for the proliferation of target cells. PDGF isoforms consist of different combinations of two polypeptide chains (the A- and B-chains), including PDGF-AA, -AB, and -BB. α- and β-receptors of PDGF have specific affinities for their isoforms, a preference of the β-receptor for the PDGF-B chain, for example[5]. PDGF receptor (PDGFR)-β expression increases in atherosclerotic lesions and is primarily limited to VSMCs[6]. PDGF-BB propagates mitogenic signals through the autophosphorylation of the PDGFR-β tyrosine residues. Tyrosine-phosphorylated PDGFR-β interacts with several other cytoplasmic proteins
that constitute Src homology 2 (SH2) domains, including phospholipase Cγ (PLCγ), ras guanine 5'-triphosphatase-activating protein, phosphatidylinositol 3-kinase (PI-3K), and tyrosine phosphatase SHP-2. These signaling molecules mediate cellular activities, including proliferation, migration, and differentiation in response to PDGF in VSMCs\(^7\). Furthermore, it is well known that PDGF transmits its signal into the intracellular space through the activation of AKT and mitogen-activated protein kinases (MAPKs)\(^9\).

Platonin (4,4′,4″-thrimethyl-3,3′,3″-triheptyl-7-[2″-thiazolyl]-2,2″-trimethinethiazolocyanine-3-3″-diiodide) (Figure 1), a cyanine photosensitizing dye, is an immunomodulator\(^9, 10\) currently in use as an effective medicine for rheumatoid arthritis\(^10, 11\). Administration of platonin is known to inhibit the up-regulation of inflammatory molecules, including interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and inducible nitric oxide synthase (iNOS) in endotoxin-activated macrophages\(^12, 13\). Furthermore, platonin also reduces circulatory failure and mortality in septic rats\(^14\). The anti-inflammatory mechanisms of platonin may be due to the suppression of MAPKs, nuclear factor (NF)-κB, and activator protein (AP)-1\(^15\). Recently, platonin was also reported to be capable of inhibiting cell growth and inducing extensive autophagy-associated cell death in leukemic cells\(^16\).

![Figure 1](image_url). Chemical structure of platonin (4,4′,4″-thrimethyl-3,3′,3″-triheptyl-7-[2″-thiazolyl]-2,2″-trimethinethiazolocyanine-3-3″-diiodide).

Considering the pivotal roles of VSMC proliferation in the development of atherosclerosis and restenosis, this study was designed to examine the action mechanisms of platonin in inhibiting VSMC proliferation stimulated by PDGF-BB.

### Materials and methods

**Materials**

Platonin was synthesized by Kankohsha (Osaka, Japan) and obtained from Gwo Chiang Pharmaceuticals (Tainan, Taiwan, China). Male Wistar rats were purchased from BioLASCO (Taipei, Taiwan, China). Dulbecco’s modified Eagle’s medium (DMEM), trypsin (0.25%), L-glutamine, penicillin/streptomycin, and fetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, MD, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sp600125 (an inhibitor of JNK1/2 phosphorylation) were from Sigma-Aldrich (St Louis, MO, USA). Recombinant PDGF-BB was purchased from PeproTech (Rocky Hill, NJ, USA). The anti-phospho-

**VSMC isolation and culture**

All animal experiments were carried out according to the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, USA, 1996). VSMCs were enzymatically dispersed from the thoracic aortas of male Wistar rats (250–300 g). The thoracic aorta was removed and stripped of the endothelium and adventitia. VSMCs were obtained using a combination of collagenase and elastase digestion\(^17\). The cells were grown in DMEM supplemented with 20 mmol/L HEPES, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2 mmol/L glutamate at 37°C in a humidified atmosphere of 5% CO\(_2\). VSMCs at passage 4–8 were used in all experiments. Primary cultured rat aortic VSMCs showed the “hills and valleys” pattern, and the expression of α-smooth muscle actin was confirmed (data not shown).

**Proliferation assays**

VSMCs (2×10\(^4\) cells/well) were seeded on 24-well plates and cultured in DMEM containing 10% FBS for 24 h. The medium was then replaced with serum-free medium for 24 h. Serum-starved VSMCs were pretreated with platonin (1–5 μmol/L), sp600125 (5 and 10 μmol/L) or an isovolumetric solvent control (PBS) for 20 min and then stimulated with PDGF-BB (10 ng/mL) or 10% FBS for 48 h. The cell number was measured using a colorimetric assay based on the ability of mitochondria in viable cells to reduce the MTT as previously described\(^18\). The cell number index was calculated as the absorbance of treated cells/control cells×100%.

**Cell cycle analysis**

For cell cycle analysis, starved VSMCs (2×10\(^5\) cells/dish) were pretreated with platonin (2 and 5 μmol/L), sp600125 (5 and 10 μmol/L) or PBS for 20 min and then stimulated with PDGF-BB (10 ng/mL) for 24 h. After 24 h, cells were detached from the plate using trypsin, washed with PBS, and fixed in 70% ethanol for 30 min. Cells were then washed with PBS and resuspended in a solution containing RNase (50 μg/mL), propidium iodide (PI; 80 μg/mL) and Triton-X-100 (0.2%). Samples were incubated for 20 min and subjected to flow cytometric...
Immunoblotting
Immunoblotting analysis was performed to determine the expression of proteins in VSMCs as described previously[10]. Serum-starved VSMCs (2×10^5 cells/dish) were treated with platonin (1–5 µmol/L) or PBS for 20 min, followed by the addition of PDGF-BB (10 ng/mL) or 10% FBS for the indicated times. After treatment, proteins were extracted with lysis buffer. The lysates were centrifuged, the supernatant protein (50 µg) was collected and subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were electrophoretically transferred onto 0.45-µm polyvinylidene difluoride (PVDF) membranes. The blots were blocked with TBST (10 mmol/L Tris-base, 100 mmol/L NaCl, and 0.01% Tween 20) containing 5% bovine serum albumin (BSA) for 1 h and were then probed with various primary antibodies. The membranes were incubated with HRP-linked anti-mouse IgG or anti-rabbit IgG (diluted 1:3000 in TBST) for 1 h. Immunoreactive bands were detected by an enhanced chemiluminescence (ECL) system. The bar graph depicts the ratios of quantitative results obtained by scanning reactive bands and quantifying the optical density using videodensitometry (Bio-profil; Biolight Windows Application V2000.01; Vilber Lourmat, France).

Confocal microscopy
Confocal microscopy was used to evaluate the expression of phospho-JNK1/2 in VSMCs. VSMCs (1×10^5 cells/cover slip) were placed on cover slips and allowed to adhere in a cell culture incubator overnight and then were starved for 24 h. VSMCs were treated as per the design of the experiment and were then fixed with 4% paraformaldehyde for 30 min and permeabilized with 80% methanol for 15 min. After incubation with 3% skimmed milk in PBS for 60 min, the preparation was incubated for 1 h with a primary Ab (1:80). Cells were then washed three times with PBS and exposed to the secondary Ab [FITC-conjugated anti-rabbit immunoglobulin G (IgG) at 1:100, 1% BSA/PBS] for 60 min. The slides were prepared with a mounting buffer (Vector Laboratories, Burlingame, CA, USA) under a glass cover slip on a Leica TCS SP5 Confocal Spectral Microscope Imaging System using an argon/krypton laser (Mannheim, Germany).

Statistical analysis
The experimental results are expressed as mean±SEM and are accompanied by the number of observations. The data were assessed by analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared using the Newman-Keuls method. A P value of <0.05 was considered statistically significant.

Results
Effects of platonin on VSMC proliferation stimulated by PDGF-BB or FBS
Figure 2 (panels A and B) shows that VSMC proliferation induced by PDGF-BB (10 ng/mL) or 10% FBS increased by approximately 89% and 94%, respectively. Furthermore, pretreatment with platonin inhibited cell proliferation after both PDGF-BB (66.3%, 96.6%, and 122.4%, respectively) and FBS (57.4%, 67.3%, and 84.3%, respectively) stimulation in a concentration-dependent (1, 2, and 5 µmol/L) manner, indicating that the inhibitory effects of platonin on VSMC proliferation are not specific to PDGF-BB. Morphological analysis also showed a similar effect as exhibited in the MTT assay of PDGF-BB-stimulated VSMCs (Figure 2C). These results suggest that platonin inhibited both PDGF-BB- and FBS-induced VSMC proliferation in a concentration-dependent manner.

Effects of platonin on cell cycle progression in PDGF-BB-stimulated VSMCs
To investigate the effect of platonin on cell cycle progression in VSMCs, the DNA content was analyzed using PI staining. After stimulation with PDGF-BB (10 ng/mL), the percentage of cells in S (6.7%±0.2% to 9.3%±0.8%, P<0.05; n=5) and G2/M phases (19.9%±1.0% to 23.1%±0.8%, P<0.05; n=5) increased, while the proportion of cells in the G0/G1 phase was reduced (70.6%±1.3% to 64.4%±1.9%, P<0.05; n=5) (Table 1). Platonin (5 µmol/L) treatment resulted in an accumulation of cells in the S and G2/M phases, and a reduction was noted in the G0/G1 phase compared to the PBS-treated group (S phase, 9.3%±0.8% vs 14.2%±0.9%, P<0.01; n=5; G2/M phase, 23.1%±0.8% vs 26.7%±0.9%, P<0.05; n=5; G0/G1 phase, 64.4%±1.9% vs 56.9%±1.1%, P<0.01; n=5) (Table 1). These results indicate that platonin was effective in arresting the cell cycle in the S and G2/M phases in PDGF-BB-stimulated VSMCs.

Table 1. Effects of platonin on cell cycle progression in platelet-derived growth factor (PDGF)-BB (10 ng/mL)-stimulated vascular smooth muscle cells (VSMCs).

| Condition | Sub-G1 (%) | G0/G1 (%) | S (%) | G2/M (%) |
|-----------|------------|-----------|-------|----------|
| Resting   | 1.6±0.5    | 70.6±1.3  | 6.7±0.2 | 19.9±1.0  |
| PBS+PDGF-BB | 1.8±0.3    | 64.4±1.9  | 9.3±0.8 | 23.1±0.8  |
| Platonin (2 µmol/L) +PDGF-BB | 1.5±0.3    | 62.6±1.7  | 9.5±1.0 | 26.3±1.1  |
| Platonin (5 µmol/L) +PDGF-BB | 2.2±0.4    | 56.9±1.1  | 14.2±0.9 | 26.7±0.9  |

The cell cycle distribution was assessed by flow cytometry as described in “Materials and methods”. Data are presented as the mean±SEM. n=5, *P<0.05, compared to the resting group. †P<0.05, ‡P<0.01, compared to the PBS+PDGF-BB group.

The effects of platonin on AKT, ERK1/2, and JNK1/2 phosphorylation in PDGF-BB-stimulated VSMCs
The PDGF-BB-induced activation of several signaling proteins, including AKT (Figure 3), ERK1/2 (Figure 4A), and JNK1/2 (Figure 4B), was detected to unravel the mechanisms of platonin in VSMC proliferation. In the present study, phospho-
rylation of JNK1/2, but not ERK1/2 or AKT, stimulated by PDGF-BB (10 ng/mL) for 10 min was markedly inhibited by platonin (2 and 5 µmol/L) (Figures 3, 4). Confocal microscopy provided further evidence of the inhibitory effect of platonin on JNK1/2 phosphorylation in VSMCs. As shown in Figure 4C, minimal expression of phospho-JNK1/2 was detected in resting cells (Figure 4Ca) and was more pronounced in the PDGF-BB (10 ng/mL)-treated cells (Figure 4Cb). However, pretreatment with platonin (2 µmol/L) resulted in a significantly decreased expression of phospho-JNK1/2 (Figure 4Cc) in PDGF-BB-stimulated cells. These findings are consistent with the results described in Figure 4B. In addition, as shown in Figure 4D, treatment with platonin (2 and 5 µmol/L) significantly inhibited JNK1/2 phosphorylation in activated VSMCs but not in resting cells. This result indicates that platonin may inhibit JNK1/2 phosphorylation in activated VSMCs but not in resting cells. These results suggest that the JNK1/2 signaling pathway may play an important role in platonin-mediated inhibition of PDGF-BB-stimulated VSMC proliferation.

**The effects of platonin on c-Jun phosphorylation and p27 expression in PDGF-BB-stimulated VSMCs**

Several lines of evidence indicate that the JNK-mediated phosphorylation of c-Jun is necessary for cell proliferation[20, 21]. Zhan et al[22] also reported that the pivotal role of c-Jun in PDGF-BB-induced VSMC proliferation is mediated by the down-regulation of p27 expression, an inhibitor of cyclin-dependent kinase (CDK). Based on the above results demonstrating that platonin’s inhibition of cell proliferation may interfere with JNK1/2 phosphorylation, we sought to examine...
whether platonin interferes with c-Jun phosphorylation and p27 expression. As shown in Figure 5A, PDGF-BB (10 ng/mL) induced expression of phospho-c-Jun in VSMCs after 30 min of stimulation. Figure 5B shows that c-Jun phosphorylation was markedly inhibited by platonin (1–5 µmol/L) in a concentration-dependent manner. p27 protein was robustly expressed in resting cells, whereas PDGF-BB (10 ng/mL) treatment for 24 h caused significant down-regulation of p27 expression. Platonin (2 and 5 µmol/L) markedly reversed this effect (Figure 5C).

The correlation between the JNK1/2 phosphorylation and cell proliferation in activated VSMCs

As shown in Figure 6A, pretreatment with sp600125 (5 and 10 µmol/L) markedly suppressed cell proliferation in PDGF-BB-stimulated VSMCs. In addition, sp600125 (10 µmol/L) treatment in PDGF-BB-stimulated cells resulted in an increase of cells in the G2/M phase and a reduction in the G0/G1 phase compared to the DMSO-treated group (G2/M phase, 21.7%±0.7% vs 32.2%±2.4%, P<0.01, n=3; G0/G1 phase, 66.8%±2.9% vs 56.6%±1.5%, P<0.05, n=3) (Figure 6B). These results further demonstrate that changes in JNK1/2 phosphorylation status play a pivotal role in the regulation of cell proliferation in activated VSMCs.

Discussion

This study demonstrates that platonin, a trithiazole pentamethine cyanine, inhibits PDGF-BB-stimulated VSMC prolifera-
tion by suppressing JNK-dependent signals, resulting in cell cycle arrest in the S and G_2/M phases. VSMC proliferation plays an important role in the pathophysiological course of atherosclerosis and restenosis after balloon angioplasty. Therefore, the modulation of VSMC proliferation has important therapeutic implications\[1\]. In the present study, we found that platonin inhibited cell proliferation in PDGF-BB-stimulated VSMCs. This result suggests that platonin could be a potential agent for treating VSMC proliferation-related diseases.

VSMCs proliferate via a mitotic process determined by the progression of the cell cycle. The cell cycle can be divided into two distinct phases: the synthesis (S) phase, in which DNA is replicated, and the mitosis (M) phase, in which cell division occurs. In animal cells, the components required for these phases are regulated by extracellular growth factors, and they are found mainly in the two gap phases, G_1 (between M and

Figure 6. The effects of sp600125 on cell proliferation and cell cycle progression in PDGF-BB-stimulated VSMCs. VSMCs were incubated with PBS (resting) or were pretreated with either sp600125 (5 and 10 µmol/L) or an isovolumetric solvent control (0.1% DMSO), followed by the addition of PDGF-BB (10 ng/mL) to stimulate (A) cell proliferation by MTT assay and (B) cell cycle progression by flow cytometry, as described in our methods. The data are presented as the means±SEM. \( n = 3 \). \( a \) \( P < 0.05 \), \( b \) \( P < 0.01 \) compared to the resting group. \( c \) \( P < 0.05 \), \( d \) \( P < 0.01 \) compared to the 0.1% DMSO group.
S) and G₂ (between S and M)[29]. Platonin has been reported to induce significant G₀/G₁ arrest of a panel of human leukemic cell lines, including U937, HL-60, K562, NB4, and THP-1[16].

In this study, we also found that the loss of the proliferative capacity of VSMCs that had been treated with platonin is associated with cells that have been arrested in the S and G₂/M phases. This phenomenon indicates that platonin may have different effects on the cell cycle in different cell types.

PDGF-BB is considered to be the most important chemoattractant for VSMCs, and it activates multiple signaling pathways, including PI-3K/AKT and MAPKs[30]. In the present study, we found that platonin inhibited the phosphorylation of JNK1/2, but not AKT or ERK1/2, in PDGF-BB-stimulated VSMCs. The JNK protein kinases include at least three subtypes: JNK1, JNK2, and JNK3. JNK1 and 2 are ubiquitously expressed, and the expression of JNK3 is restricted to the brain, heart, and testes[29]. Extracellular stimuli lead to the activation of MAPK kinase kinases (MAPKKKs) that subsequently activate MAPK kinases 4 and 7 (MKK4 and MKK7), both of which phosphorylate JNKs. Activated JNKs result in the phosphorylation of many transcription factors, including the c-Jun component of the activator protein (AP)-1 transcription family[26, 27]. c-Jun is known to be required for PDGF-induced VSMC migration and proliferation, and JNK knockdown can attenuate cell migration and proliferation in PDGF-stimulated VSMCs[20, 21]. Furthermore, dominant-negative c-Jun lacking the transactivation domain of wild-type c-Jun (Ad-DN-c-Jun), which specifically blocks AP-1 transcriptional activity, significantly inhibited PDGF-BB-induced VSMC proliferation and suppressed PDGF-BB-induced down-regulation of CDK p27[23]. In this study, platonin inhibited JNK1/2 and c-Jun phosphorylation and reversed the down-regulation of p27 expression in VSMCs stimulated by PDGF-BB. These results indicate that JNK1/2-dependent signals may play important roles in the platonin-mediated inhibition of VSMC proliferation.

Furthermore, JNK, c-Jun, and p27 activation have also been reported to regulate S and G₂/M phases of cell-cycle progression[28-31]. These studies are consistent with our finding that the platonin treatment arrested cell cycle progression in the S and G₂/M phases, which may have resulted from the suppression of JNK1/2-dependent signals.

In conclusion, this study demonstrates that platonin inhibits VSMC proliferation; this inhibition may involve the inhibition of both JNK1/2 phosphorylation and c-Jun activation and the reversal of down-regulated p27 expression, thereby leading to cell cycle arrest in the S and G₂/M phases. We suggest that platonin may be a potential therapeutic agent for treating diseases related to VSMC proliferation.

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Author contribution
Yi CHANG and Chang-Chih CHEN performed the research and wrote the manuscript; Yih-Huei UEN designed and performed the research; Song-Chow LIN, Shiao-Yun TSENG, and Yi-Hsuan WANG performed some of the experiments and analyzed data; and Joen-Rong SHEU and Cheng-Ying HSIEH designed the research.

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