Celecoxib derivative OSU-03012 inhibits the proliferation and activation of hepatic stellate cells by inducing cell senescence

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Abstract. Liver fibrosis may lead to portal hypertension, liver failure or hepatocellular carcinoma, and predominantly results from the proliferation and activation of hepatic stellate cells. OSU-03012, a non-cyclooxygenase-inhibiting celecoxib derivative, has been previously demonstrated to promote apoptosis in certain cell types, however, its function in hepatic fibrosis remains unclear. In the current study, the inhibitory effect of OSU-03012 on the proliferation of the LX2 human hepatic stellate cell line was evaluated by cell counting kit-8 assay. Reverse transcription-quantitative polymerase chain reaction was performed in order to examine the expression of α-smooth muscle actin and type I collagen, which are representative of LX2 cell activation. The senescence of LX2 cells was measured by senescence-associated β-galactosidase staining, and the cell cycle and apoptosis levels were assessed by flow cytometry. The impact of senescence-associated signaling on protein expression was assessed by western blot analysis. OSU-03012 was observed to inhibit cell proliferation and prevent the secretion of profibrotic factors in LX2 cells in a dose-dependent manner. Furthermore, the results demonstrated that OSU-03012 inhibited the proliferation and activation of LX2 via the induction of cell senescence at the G_1 phase, rather than via cell apoptosis. The induction of senescence may be via the upregulation of p16, p21 and p27. In conclusion, the current study provided insight into the pharmacological mechanisms of OSU-03012 in preventing the proliferation and activation of hepatic stellate cells through cell senescence. The current study supports the theory that OSU-03012 is a novel agent for potential use against liver fibrosis.

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Introduction

Liver fibrosis is a serious health problem worldwide, which induces portal hypertension, liver cirrhosis and liver failure, and increases the risk of hepatocellular carcinoma (1). The hepatic stellate cell (HSC) is a key cell type contributing to liver fibrosis, and transdifferentiates to become activated under conditions of liver damage, including hepatitis viral infection, alcohol abuse, nonalcoholic steatohepatitis or fatty liver disease (1). These activated HSCs secrete certain profibrogenic cytokines, such as transforming growth factor-β (TGF-β), and promote the development of liver fibrosis (2). Therefore, inhibition of the activation of HSCs is hypothesized to be an effective strategy to prevent the development of liver fibrosis.

Cell senescence involves normal cells losing the ability to proliferate, despite the presence of sufficient space, nutrients and growth factors in the medium (3). Senescent HSCs have been demonstrated to exhibit a gene expression profile consistent with cell cycle exit, reduced secretion of extracellular matrix (ECM) components, enhanced secretion of ECM-degrading enzymes and enhanced immune surveillance (4), which indicate its value in protecting against liver fibrosis and cancer.

OSU-03012, a derivative of celecoxib lacking cyclooxygenase-2 inhibitory activity, is able to inhibit the activity of phosphomositide-dependent kinase-1 (PDK1) and induce cell death in various types of cancer cell, including hepatocellular carcinoma (5), primary chronic lymphocytic leukemia (6), glioblastoma (7,8), breast cancer (9) and pancreatic cancer (10) cell lines. In addition to the inhibition of PDK1/protein kinase B (AKT) signaling, previous studies have suggested that OSU-03012 may be a multitargeted inhibitor in a cell type-dependent manner (6,7,11). However, the effect of this compound in hepatic fibrosis remains to be fully elucidated. Thus, in the current study, the ability of OSU-03012 to inhibit activated HSCs was investigated in order to verify its potential as a drug against liver fibrosis. Mechanisms underlying this inhibitory effect were also investigated.

Materials and methods

Cell lines and reagents. The LX2 human HSC cell line originated from the American Type Culture Collection (Manassas, VA, USA) was purchased from Shanghai Fuxiang Biotechnology Co., Ltd. (Shanghai, China). It was cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM-h) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Carlsbad, CA, USA) and maintained in a 5% CO_2 atmosphere at 37˚C. OSU-03012 was purchased from Selleck Chemicals (Houston, TX, USA) and was dissolved in dimethyl sulfoxide (DMSO; Sangon Biotech, Co., Ltd., Shanghai, China) at a concentration of 50 μM as stock solution for further use. The rabbit anti-human antibodies against P15INK4B (#4822; polyclonal), p16INK4A (#4824; polyclonal), p21Cip1 (#9932; monoclonal), P27Kip1 (#9932; monoclonal), AKT (#9272; polyclonal), p-AKT (activated AKT; #9275; polyclonal) and β-actin (#4967; polyclonal) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA; 1:1,000 dilution) and diluted with Primary Antibody Dilution Buffer (Beyotime Institute of Biotechnology).

Cell proliferation assay. The cell counting kit-8 (CCK-8) assay (Beyotime Institute of Biotechnology, Shanghai, China) was used to evaluate cell proliferation. LX2 cells were seeded in 96-well plates at a density of $2x10^3$ cells/well in DMEM-h supplemented with 10% FBS. Following a resting period of 24 h, cells were washed with phosphate-buffered saline (PBS; Medicago AB, Uppsala, Sweden) and exposed to either DMSO alone or a series of dilutions of OSU-03012 in DMSO (1, 5 or 15 μM). Subsequent to incubation for another 24, 48 or 72 h, the medium was replaced with fresh DMEM-h. A total of 10 μl CCK-8 was added to each well and the cells were incubated in the presence of CCK-8 for 3 h. The absorbencies were determined with a DTX 800 Multimode Detector (Beckman Coulter, Brea, CA, USA) at a wavelength of 450 nm. The absorbance values were normalized by subtracting blank values obtained from untreated cells.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following a resting period of 0, 24, 48 or 72 h subsequent to the addition of 1 μM OSU-03012, the total RNA in the LX2 cells was extracted using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. The RNA concentrations were assessed by spectrophotometry at a wavelength of 260 nm using a Nano-100 spectrophotometer (Hangzhou Allsheng Instruments Co., Ltd., Hangzhou, China). RNA was reverse-transcribed using the PrimeScript RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) and qPCR was performed using the 7500 Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA). The PCR primer sequences used were as follows: Type I collagen, F 5'-TGA CGA GAC CAA GAA CTG-3' and R 5'-CCA TCC AAA CCA CTG AAA-3'; α-smooth muscle actin (α-SMA), F 5'-TTC GTT ACT ACT GCT GAG CGT GAG A-3' and R 5'-AAA GAT GGC TGG AAG AGG GTC-3'; internal reference gene GAPDH, F 5'-ACC ACA GTC CAT GCC ATC AC-3' and R 5'-TCC ACC ACC CTG TTG CTG T-3'. The PCR cycling conditions were as follows: 95˚C for 30 sec, 40 cycles of 95˚C for 5 sec and 60˚C for 34 sec.

Apoptosis detection. Approximately $5x10^5$ LX2 cells were seeded onto 10-cm dishes and incubated at 37˚C overnight. The cells were treated with either DMSO alone or 1 μM OSU-03012 in DMSO for 48 or 72 h, and then suspended by treatment with trypsin 0.25%-EDTA (Biosera, Boussens, France) and fixed with 70% ice-cold ethanol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) overnight at 4˚C. Subsequent to washing the cells with PBS and resuspending in the binding buffer (Beyotime Institute of Biotechnology) containing Annexin V-fluorescein isothiocyanate and propidium iodide (PI) according to the manufacturer's instructions, apoptosis was assessed by flow cytometry using a FACScan cytometer (Beckman, Pasadena, CA, USA).

Senescence-associated β-galactosidase (SA-β-Gal) staining. SA-β-Gal staining was performed using the Senescence-Associated β-Galactosidase Staining kit (Beyotime Institute of Biotechnology). Approximately $3x10^5$ LX2 cells were seeded onto 6-cm dishes and incubated at 37˚C overnight. The cells were treated with 1 μM OSU-03012 in DMSO or DMSO alone for 48 h or 72 h. The cells were then washed three times with PBS and fixed with 4% paraformaldehyde (Beyotime Institute of Biotechnology) for 15 min at room temperature. Next, the cells were incubated overnight at 37˚C in darkness with the working solution containing 0.05 mg/ml X-gal (Beyotime Institute of Biotechnology) and viewed under an optical microscope (TE2000-S; Nikon Corp., Tokyo, Japan).

Cell cycle analysis. Approximately $5x10^5$ LX2 cells were seeded onto 10-cm dishes and incubated at 37˚C overnight. The cells were treated with 1 μM OSU-03012 in DMSO or DMSO alone for 48 h or 72 h and then suspended by treatment with trypsin and fixed with 70% ice-cold ethanol overnight at 4˚C. The cells were then stained with PI in the presence of RNase A (Beyotime Institute of Biotechnology). The DNA content was analyzed by flow cytometry (FACScan cytometer; Beckman) and data were analyzed using Modfit LT software version 3.2 (Verity Software House, Inc., Topsham, ME, USA).

Western blot analysis. Following the above treatments, the LX2 cells were lysed in protein extraction buffer (Beyotime Institute of Biotechnology) followed by incubation at 95˚C for 5 min. Samples were separated using an SDS-PAGE system (MINI-P TET SYS/PPAC BASIC; Bio-Rad Laboratories, Inc., Hercules, CA, USA), transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories), blocked with 5% nonfat milk in Tris-buffered saline/Tween-20 (Shanghai BioScience Co., Ltd., Shanghai, China) for 1 h and probed with the antibodies at 4˚C overnight. The membranes were then incubated with corresponding horseradish peroxidase (HRP)-conjugated polyclonal goat anti-rabbit secondary antibody (#HA1001-100; 1:2000; Hangzhou Huaan Biotechnology Co., Ltd., Hangzhou, China) for 1 h at room temperature. The immunoblots were visualized using Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA, USA).

Statistical analysis. All data were collected from a minimum of three independent replicates. Analysis was performed using SPSS software, version 11.0 (SPSS, Inc., Chicago, IL, USA).

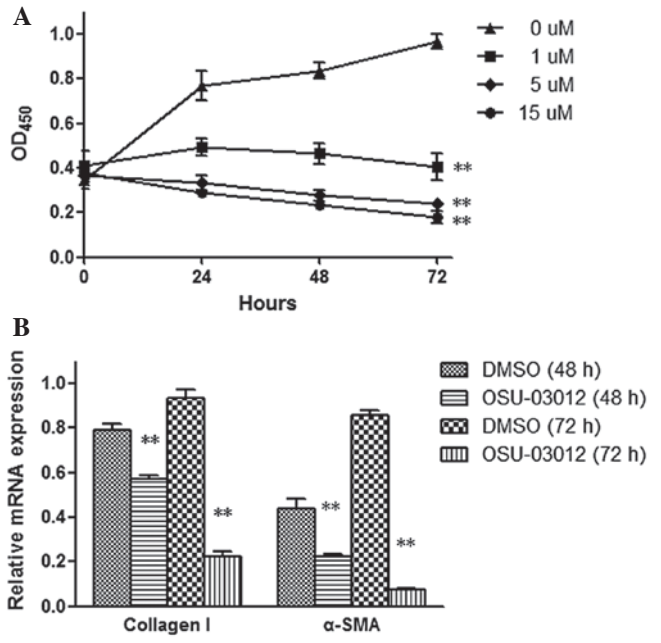

Figure 1. OSU-03012-mediated inhibition of LX2 cell growth and activation. (A) Cell viability assessed by CCK-8 assay (measured as OD_{450}) following treatment with OSU-03012. **P<0.01 vs. 0 μM. (B) The mRNA levels of type I collagen and α-SMA were examined. At 48 and 72 h, respectively, compared with DMSO, the type I collagen levels of OSU-03012-treated LX2 cells were 26.6 and 76.1% lower, while the levels of α-SMA were 48.8 and 95.2% lower. **P<0.01 vs. DMSO. CCK-8, cell counting kit-8, OD, optical density; α-SMA, α-smooth muscle actin; DMSO, dimethyl sulfoxide.

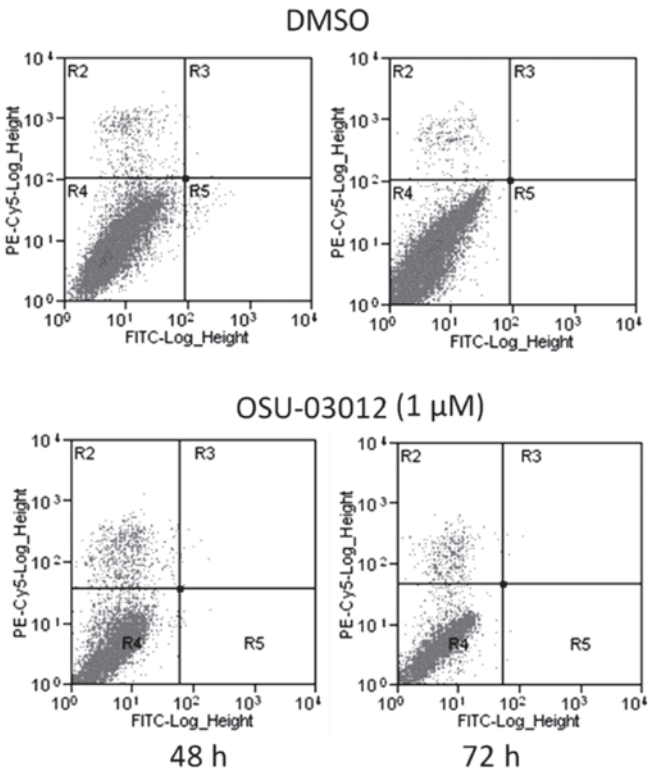

Figure 2. OSU-03012 did not have an apoptotic effect on LX2 cells. In the flow cytometric diagrams of cells treated with annexin V-FITC (x-axis) and PI (y-axis), the points in the lower left/lower right/upper right corner represent normal/early apoptotic/late apoptotic cells (or necrotic), respectively. FITC, fluorescein isothiocyanate, PI, propidium iodide; DMSO, dimethyl sulfoxide.

Comparisons between groups were performed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

OSU-03012 inhibits the growth and activation of LX2 cells. To examine whether OSU-03012 has an inhibitory effect on LX2 cell proliferation, LX2 cells were treated with a range of doses (0-15 μM) of OSU-03012 and assessed for viability using the CCK-8 assay. As demonstrated in Fig. 1A, the growth of cells was suppressed by OSU-03012 in a dose-dependent manner, indicating that OSU-03012 may inhibit LX2 cell proliferation.

Activated HSCs express type I collagen and α-SMA, which primarily contribute to liver fibrogenesis. To investigate the effect of OSU-03012 on the activation of HSCs, the mRNA levels of the hepatic profibrotic factors type I collagen and α-SMA were analyzed using RT-qPCR. Following 48 or 72 h of exposure, a reduction in the mRNA levels of type I collagen and α-SMA was observed in OSU-03012 cells, as presented in Fig. 1B. These results demonstrate the inhibitory effect of OSU-03012 on the activation of HSCs, indicating its potential function against hepatic fibrosis.

OSU-03012 induces senescence, not apoptosis, in LX2 cells. It was reported that the inhibitory effect of OSU-03012 on cell proliferation was mediated via the induction of cell apoptosis in certain cancer cells (6-11). To verify this hypothesis, the ability of OSU-03012 to induce apoptosis in LX2 cells was investigated by flow cytometric analysis. As demonstrated in Fig. 2, apoptosis was not induced in LX2 cells treated with OSU-03012, compared with those treated with DMSO. These results indicated that OSU-03012 did not inhibit the growth of LX2 cells via the induction of apoptosis.

Cell senescence involves normal cells losing the ability to proliferate, thus it was hypothesized that the inhibition of LX2 cells by OSU-03012 is mediated by senescence. LX2 cells were exposed to OSU-03012 and assessed for senescence using the SA-β-Gal assay 48 h later. The cells treated with OSU-03012 displayed a large increase in SA-β-Gal activity (P<0.02; Fig. 3) compared with the control (DMSO). Senescence-associated morphological alterations were observed in the OSU-03012-treated group, with the cells frequently becoming flat with pyknosis of the nucleus. These results demonstrate that OSU-03012 may induce senescence in LX2 cells.

OSU-03012 induces G_1 phase arrest in LX2 cells. Cell senescence is a stable form of cell cycle arrest in mitotic cells, usually at the G_1 phase. However, certain cells become senescent at the G_2 or S phase (3). Thus, the influence of OSU-03012 on the cell cycle of LX2 cells was investigated. The cells treated with OSU-03012 for 48 or 72 h were stained with PI and then subjected to flow cytometry. As demonstrated in Fig. 4, LX2 cells treated with OSU-03012 exhibited a significant ~20x increase in G_1-arrested cells compared with those treated with DMSO (P<0.05). These

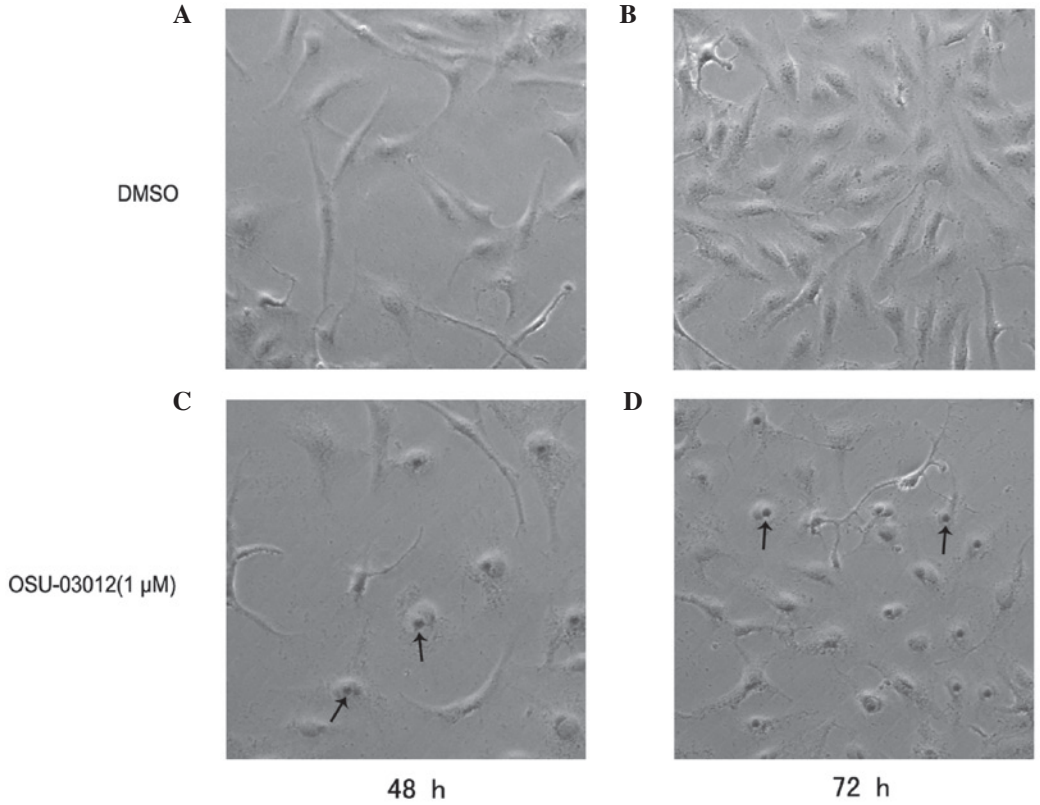

Figure 3. OSU-03012 induced senescence in LX2 cells. Cell senescence was determined by senescence-associated β-galactosidase analysis. The arrows demonstrate the flattening of the adherent cells with pyknosis of the nucleus, a typical morphological alteration associated with cell senescence. DMSO-treated for (A) 48 h or (B) 72 h. OSU-03012-treated for (C) 48 h or (D) 72 h (magnification, x100). DMSO, dimethyl sulfoxide.

results suggest that OSU-03012 may induce G_1 phase arrest in HSCs.

OSU-03012 induces senescence in LX2 cells via the upregulation of p16, p21 and p27. There are several pathways known to be associated with cell senescence. P53 promotes senescence by inhibiting cyclin-dependent kinases 2 and 4 by transactivating p21 or p16 (INK4a) (3,12). In addition, p15 and p27 have been identified to be associated with cell senescence (13,14). Therefore, to elucidate the mechanism of OSU-03012 in the induction of senescence in LX2 cells, total proteins were extracted from cells treated with 1 μM OSU-03012 or DMSO, for 48 or 72 h and analyzed by western blot analysis with the antibodies against p15, p16, p21 and p27. As demonstrated in Fig. 5, at 48 and 72 h, OSU-03012 significantly increased the protein levels of p16, p21 and p27, compared with DMSO-treated cells ($P<0.01$). No significant difference was observed in the levels of p15 between OSU-03012- and DMSO-treated cells at 48 or 72 h.

Furthermore, the PDK1/AKT signaling pathway, an important senescence regulator, has been suggested to be involved in the inhibition of proliferation in certain cells by OSU-03012 (15,16), but its role in LX2 cells remains unclear. To investigate whether OSU-03012 acts via the inhibition of the PDK1/AKT signaling pathway in LX2 cells, the levels of AKT and p-AKT were also measured in the western blot analysis. As demonstrated in Fig. 5, the level of p-AKT was reduced following treatment with OSU-03012 ($P<0.01$). These results suggest that OSU-03012 induced senescence of LX2 cells via p16, p21, p27 and the PDK1/AKT signaling pathway.

Discussion

Liver fibrosis is a common developmental stage in the majority of chronic liver diseases and may result in liver cirrhosis, failure and in severe cases, hepatocellular carcinoma. At present, there are no effective antifibrotic agents on the market, however a previous study hypothesized that targeting the senescence of activated HSCs may be a novel strategy to inhibit the development of liver fibrosis (4). In the current study OSU-03012, a novel celecoxib derivative, was demonstrated to induce cell senescence in activated HSCs and thus, inhibit cell proliferation and prevent the secretion of profibrotic factors. The results indicated OSU-03012 as a potential agent against liver fibrosis.

HSCs become activated following injury and are crucial in the pathogenesis of liver fibrosis via the excessive accumulation of α-SMA and ECM proteins, including type I, III and IV collagen. The rates of synthesis and degradation of the ECM determines the severity of liver fibrosis. Type I collagen constitutes a high proportion of the ECM in hepatic fibrosis, while α-SMA is also commonly known as a marker of fibrosis. In the current study, the mRNA levels of type I collagen and α-SMA were observed to be significantly reduced following treatment with OSU-03012, indicating the role of OSU-03012 in the inactivation of HSCs and reduction of ECM.

Apoptosis is a process of programmed cell death, in which the chromatin condenses, DNA becomes fragmented and is

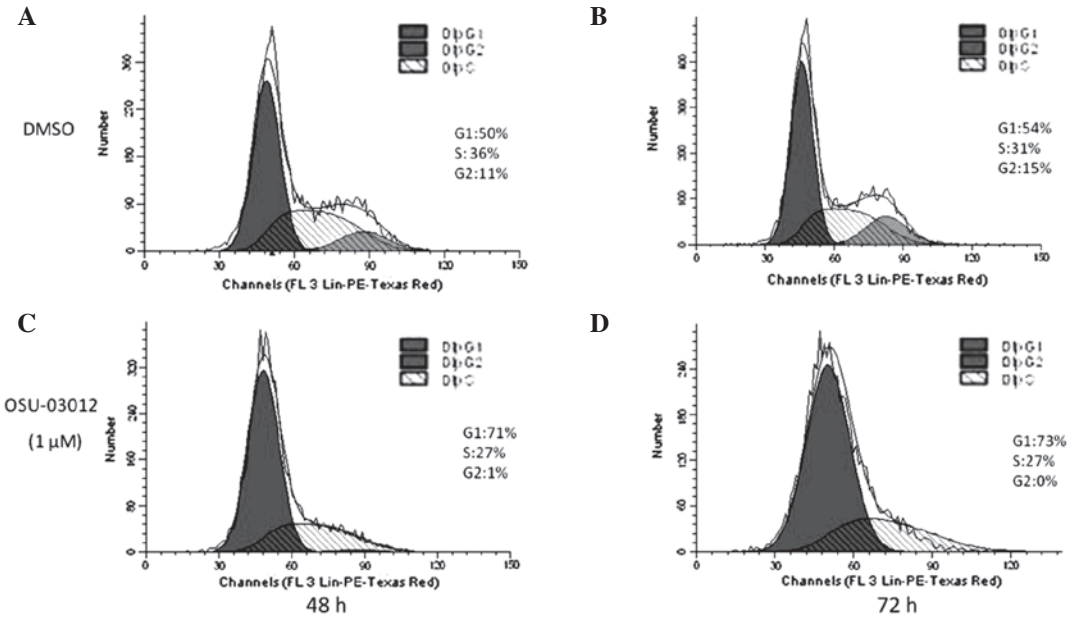

Figure 4. OSU-03012 induced arrest of LX2 cells in the G_1 phase. Flow cytometry was used to detect the cell cycle distribution. DMSO-treated for (A) 48 h or (B) 72 h. OSU-03012-treated for (C) 48 h or (D) 72 h. DMSO, dimethyl sulfoxide.

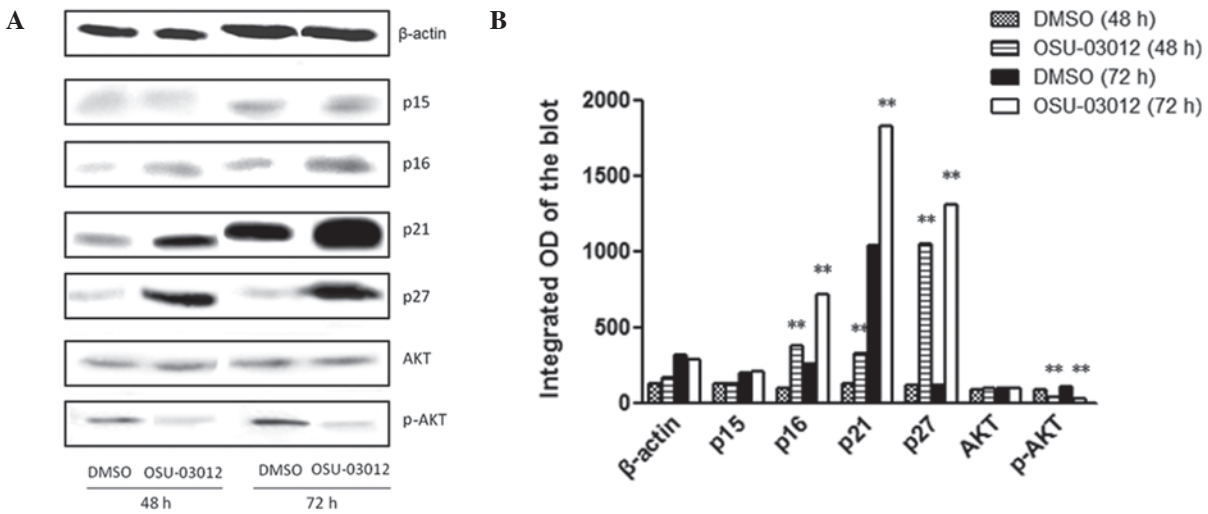

Figure 5. OSU-03012 upregulated the expression of p16, p21 and p27. (A) Western blot analysis of the proteins extracted from LX2 cells treated with 1 μM OSU-03012 or DMSO, for 48 or 72 h. (B) Densitometric analysis of the blots. **P<0.01 vs. DMSO. DMSO, dimethyl sulfoxide; AKT, protein kinase B; p-AKT, activated AKT; OD, optical density.

phagocytosed by the macrophages without inflammation. It was reported that OSU-03012 may induce the apoptosis of non-small cell lung cancer (14) and breast cancer (17,5) cells. However, a previous study observed that OSU-03012 was not able to induce apoptosis in liver cancer cells (5), which is in agreement with the observations in the present study in LX2 cells. It is hypothesized that the different effects of OSU-03012 on apoptosis are due to the varied cell types used. The underlying molecular mechanism remains to be fully elucidated.

Cell senescence may induce irreversible arrest of cell growth, which may be a novel strategy for inhibiting cell proliferation. The hallmark of cell senescence is an inability to progress through the cell cycle, which cannot be activated by any known physiological stimulation (3). The results of the current study demonstrate that OSU-03012 induced cell cycle arrest at the G_1 phase, which suppressed cell proliferation. Further investigation to fully elucidate the effects of OSU-03012 treatment on the cell cycle are necessary.

Additionally, the current study demonstrated that OSU-03012 induced senescence in LX2 cells via the activation of p16, p21 and p27. Previous studies have identified that OSU-03012 inhibits the PDK1/AKT signaling pathway (18) and that p-AKT (activated AKT) is one of the important pathway molecules responsible for regulating cell senescence (19). AKT is able to downregulate p27 via the phosphorylation and inhibition of the FOXO transcription factors (20) and downregulate p21 via the activation of MDM2 (5,21). Therefore, it was hypothesized in the present study that the upregulation of p21 and p27 by OSU-03012 was via the inhibition of the PDK1/AKT signaling pathway, which thus induced cell

senescence. The current study demonstrated the complexity of the underlying mechanism of cell senescence induced by OSU-03012, which requires further investigation.

In conclusion, the present study identified the inhibitory effect of OSU-03012 on the proliferation and activation of HSCs, which was mediated via cell senescence. The results of the current study support the use of OSU-03012 against liver fibrosis, however, further studies are required to confirm its effects *in vivo* in an animal model of hepatic fibrosis.

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