Abstract. Background: Inhibition of cell-cycle progression is a target for the treatment of cancer. 3-Oxoolean-12-en-27-oic acid (3-OOLA) has shown significant anticancer activity towards diverse cancer cells, but has not been investigated for non-small cell lung carcinoma (NSCLC) cells. In this study, we investigated the antiproliferative effect of 3-OOLA in NSCLC cell lines and its underlying mechanism. Materials and Methods: The MTT assay, bromodeoxyuridine (BrdU) incorporation assay, and flow cytometry were used for cell proliferation studies, and annexin V staining for apoptotic effects. Western blot analysis was used to evaluate expression of cell-cycle regulatory proteins, such as cyclins and cyclin-dependent kinases (CDKs). Results: 3-OOLA caused G0/G1 phase cell-cycle arrest without inducing apoptosis in NSCLC cells, and Western blot analyses demonstrated down-regulation of cyclin D1, cyclin E and phosphorylated Rb. Conclusion: 3-OOLA inhibits cell proliferation of NSCLC cells by inducing cell-cycle arrest at G0/G1 through down-regulation of cyclin D1 and cyclin E.

Lung cancer is a leading cause of cancer death in the US and other countries (1). Many therapies for lung cancer such as radiotherapy, docetaxel, the combination of carboplatin and paclitaxel, and others have been developed (2-4); however, drug development for lung cancer is still challenging. Natural products have been shown to be an excellent and reliable source for the development of new drugs (5). Many natural products have pharmacological applications, in particular their potential for use in cancer chemotherapy (6). Indeed, amongst the well-known anticancer drugs are colchicine, etoposide, and taxol (7).

Triterpenes represent a varied class of natural products. Triterpene carboxylic acids, such as oleanolic acid, ursolic acid, and betulinic acid, are among the most widely distributed triterpene series and are reported to exhibit several types of biological activity, including antitumor activity (8). Ursolic acid and oleanolic acid, for example, have been found to inhibit tumor cell proliferation, growth and metastasis (9-11). Oleanane-type triterpenoid with a carboxyl group at C-27 is present in a limited number of natural resources (12). We have previously reported the isolation and structure elucidation of olean-12-en-27-oic acid derivatives from *Aceriphyllum rossii* (Saxifragaceae), which a perennial herb that grows on damp rocks along valleys in the central northern part of Korea (13, 14). Olean-12-en-27-oic acid derivatives such as 3-OOLA (Figure 1) have various pharmacological activities. These include inhibitory activity to the protein tyrosine phosphatase 1B (15), anti-complementary activity (16), and antitumor activity against various human cancer cell lines (12-14, 17). Recently, olean-12-en-27-oic acid derivatives have been reported to induce apoptosis in human promyelocytic leukemia HL-60 cells (12) and human cervical squamous carcinoma HeLa cells (17). However, the antitumor activity of 3-OOLA is largely unknown.

In this study, we investigated the anti-proliferative effects of 3-OOLA on human NSCLC cells and its underlying mechanism. Our result demonstrated that 3-OOLA inhibited growth of human NSCLC cell lines by inducing cell-cycle arrest at G0/G1 phase. Induction of cell-cycle arrest was associated with down-regulation of cyclin D1, cyclin E and phosphorylated Rb.

Materials and Methods

Cell culture. A549, NCI-H1299, and NCI-H1703 human NSCLC cell lines were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were maintained in RPMI-1640 medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and 10%
heat-inactivated fetal bovine serum in a humidified atmosphere containing 5% CO₂.

Chemicals and antibodies. 3-OOLA used for this study was isolated from A. rossii as previously described (13, 14). The purity of this compound was determined to be more than 97% by high performance liquid chromatography analysis. Etoposide was obtained from Calbiochem (San Diego, CA, USA). 3-OOLA and etoposide were solubilized in 100% dimethyl sulfoxide (DMSO) and used at a final concentration of less than 0.05% DMSO. Antibodies for phospho-specific Rb (Ser780) and Rb were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies for cyclin-dependent kinase 2 (CDK2), CDK4, cyclin D1, cyclin E, p21, and p27 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for α-tubulin was from Sigma (St. Louis, MO, USA).

Cell viability. The cytotoxic activity of 3-OOLA was determined by MTT 3 (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based colorimetric assay (18). MTT was purchased from Sigma. In brief, 1×10⁴ cells per well were seeded in 96-well plates and allowed to grow on the plate for 24 h. The tested compounds were added to the wells at the indicated concentrations. After incubation for an additional 48 h, MTT solution (5 mg/ml) was added and plates further incubated for 4 h. The experiment was performed in triplicate and cell viability was presented as a percentage that of the control. The concentrations needed to reduce cell density by 50% (IC₅₀ values) were calculated using non-linear regression analysis (percentage survival versus concentration).

Cell proliferation. Incorporation of the thymidine analog, bromodeoxyuridine (BrdU), was measured to determine the effect of 3-OOLA on DNA synthesis using BrdU proliferation assay kit according to the manufacturer’s instructions (Millipore, Billerica, MA, USA). In brief, both the vehicle and 3-OOLA-treated cells were labeled with BrdU (10 μM) for 4 h prior to incubation with anti-BrdU-peroxidase for 1 h. The immune complex was detected following the addition of trimethyl benzidine substrate and measured at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader. The number of proliferating cells is represented by the level of BrdU incorporation which directly correlates to the BrdU incorporation. The IC₅₀ value for 3-OOLA against A549, NCI-H1299, and NCI-H1703 (Figure 1B). Cells were treated with different concentrations of 3-OOLA (1-30 μM) for 48 h and the viability was determined by MTT assay. 3-OOLA effectively inhibited the growth of NSCLC cells in a dose-dependent manner. The IC₅₀ value for 3-OOLA against A549, NCI-H1299, and NCI-H1703 cells was 8.6, 12.5, and 24.1 μM, respectively, while the IC₅₀ value for etoposide was 23.5, 32.7, and 6.4 μM, respectively, under the same condition.

We further studied the antiproliferative effect of 3-OOLA in A549 cells, since A549 cells were the most sensitive to 3-OOLA among these NSCLC cell lines. A549 cells were treated with different concentrations of 3-OOLA for 24, 48, and 72 h, and cell viability was determined (Figure 2A). 3-OOLA induced a marked dose- and time-dependent inhibition of A549 cell proliferation. Furthermore, 3-OOLA inhibited DNA synthesis in a dose-dependent manner as evident by the observed decrease in the incorporation of BrdU into DNA in the 3-OOLA-treated cells compared to the controls (Figure 2B).

3-OOLA does not induce apoptosis. To examine whether the 3-OOLA-mediated inhibition of cell proliferation was associated with the induction of apoptosis, we measured the binding of annexin V-FITC in A549 cells treated with 3-OOLA for 48 h (Figure 3). A small percentage of untreated A549 cells bound annexin V-FITC (<5%). Following treatment of 3-OOLA at 3, 10, and 30 μM, the percentage...
of annexin V-FITC-binding cells did not significantly increase, suggesting that 3-OOLA-mediated inhibition of cell proliferation was not associated with the induction of apoptosis.

3-OOLA induces cell-cycle arrest at G₀/G₁ phase. We next asked the question of whether the growth inhibitory effect of 3-OOLA is associated with an alteration in cell-cycle progression of NSCLC cells, and found that indeed 3-OOLA induced a prominent G₀/G₁ arrest in the cell-cycle progression of A549 cells (Figure 4). Compared with vehicle-treated control A549 cells, 3-OOLA treatment increased G₀/G₁ phase and reduced S phase cells in a concentration- and time-dependent manner. 3-OOLA treatment at 3, 10, and 30 μM for 48 h increased cells in G₀/G₁ phase 65.8% to 70.3%, 74.5%, and 82.1%, respectively.

3-OOLA down-regulates cyclin D1 and cyclin E, and up-
regulates p27kip1 expression. We analyzed the effect of 3-OOLA on the expression of cell-cycle regulatory molecules which could be involved in the strong G0/G1 arrest. Western blot analysis showed that 3-OOLA reduced the expression of cyclin D1 and cyclin E expression and increased CDK inhibitor p27kip1 expression in a concentration- and time-dependent manner (Figure 5A and Figure 5B). The expression level of p21waf1, another CDK inhibitor, was rather reduced by 3-OOLA treatment compared to the vehicle-treated control. However, the expression levels of
CDK2 and CDK4 were not significantly modulated by the treatment of 3-OOLA.

We next determined whether the down-regulation of cyclin D1 and cyclin E, and the up-regulation of p27Kip1 was accompanied by a decrease in Rb phosphorylation, contributing to the growth-inhibitory effect of 3-OOLA. Western blot analysis showed a strong decrease in the phosphorylation of Rb at Ser780 site by the treatment of 3-OOLA in a concentration- and time-dependent manner (Figure 5C and Figure 5D). However, the total Rb levels remained almost unchanged.

To address the issue whether 3-OOLA-induced cell-cycle arrest was primarily caused by the down-regulation of cyclin D1 and cyclin E expression or the up-regulation of p27Kip1 expression, A549 cells were treated with 30 μM of 3-OOLA for 3, 6, and 9 h. At the end of each time point, the expression levels of cyclin D1, cyclin E, p27Kip1, and phosphorylated Rb were determined by Western blot analysis (Figure 6). 3-OOLA gradually reduced the expression levels of cyclin D1, cyclin E and phosphorylated Rb from 3 h treatment, whereas the expression level of p27Kip1 was not increased, but rather decreased until 12 h.
treatment, suggesting that down-regulation of cyclin D1 and cyclin E could be primarily responsible for 3-OOLA-induced cell-cycle arrest at G0/G1 phase.

**Discussion**

The fresh young leaves and stems of *A. rossii* have been used as a nutritious food in Korea. Recently, several oleanane-type triterpenoid compounds from *A. rossii* were reported to potently inhibit the growth of several cultured human cancer cells. In particular, olean-12-en-27-oic acid derivatives, including 3-OOLA, were shown to have potent cytotoxic activity against human cancer cell lines (13, 14). Since lung cancer is the leading cause of cancer deaths worldwide and NSCLC accounts for more than 80% of all lung cancer cases (21), we investigated the antiproliferative effects of 3-OOLA on human NSCLC cells. We showed that 3-OOLA exhibited the potent antiproliferative effects on NSCLC cells by arresting the cell-cycle at the G0/G1 phase in a concentration- and time-dependent manner. Apoptosis was not detected in 3-OOLA-treated cells up to 30 μM as assessed by annexin V-FITC staining, suggesting that the antiproliferative mechanism of 3-OOLA was not related to the induction of apoptosis.

One of the characteristics of cancer cells is their uncontrolled growth by mutation or deregulation of cell-cycle regulators such as cyclins, CDKs, or CDK inhibitors (22-25). Thus, inhibition of cell-cycle progression might be an appropriate target for the treatment of cancer (22-24). In fact, several drugs targeting the cell-cycle have entered clinical trials (25). It has been well known that cell-cycle control is a highly regulated process that involves a complex cascade of cellular events including activation of cyclins and CDKs (24). CDK4/cyclin D complex promotes the progression through G1 phase into S phase. CDK2 is associated with entry of cells into S phase by binding cyclin E and makes a complex with cyclin A through S phase. In addition, the activity of CDK/cyclin complexes is negatively regulated by binding to CDK inhibitors, which are largely classified into the INK4 family (p16, p15, p18, and p19) and the Cip/Kip family (p21, p27, and p57) (26). We showed that 3-OOLA significantly down-regulated the expression of cyclin D1 and cyclin E before p27Kip induction. Consistent with the down-regulation of cyclin D1 and cyclin E, we were able to demonstrate that the expression level of phosphorylated Rb was also significantly reduced by the treatment of 3-OOLA. In contrast, the levels of CDK2 and CDK4 expression were not affected by the treatment with 3-OOLA. Therefore, 3-OOLA-mediated cell-cycle arrest at G0/G1 phase could be related to the inhibition of the formation of CDK/cyclin complexes by the suppression of cyclin D1 and cyclin E expression, and the subsequent suppression of CDK activity. The mechanisms by which olean-12-en-27-oic acid derivatives inhibit the expression of cyclin D1 and cyclin E remain to be elucidated.

In summary, these results suggest that one of the antiproliferative mechanisms of 3-OOLA in NSCLC cells is to induce cell-cycle arrest at the G0/G1 phase by down-regulating the expression of cyclin D1 and cyclin E. This study also presents an additional biological activity of 3-OOLA and points to the therapeutic potential of 3-OOLA in the development of cancer chemotherapeutic agents derived from natural products.

**Acknowledgements**

This work was supported by grants from the National Research Foundation of Korea funded by the Korea government [MEST] (Regional Research Universities Program/Medical & Biomaterial Research Center) and NRF-2009-0084675).
References


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Received January 26, 2011
Revised May 13, 2011
Accepted May 17, 2011