Acteoside inhibits human promyelocytic HL-60 leukemia cell proliferation via inducing cell cycle arrest at G0/G1 phase and differentiation into monocyte

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We investigated the in vitro effects of acteoside on the proliferation, cell cycle regulation and differentiation of HL-60 human promyelocytic leukemia cells. Acteoside inhibited the proliferation of HL-60 cells in a concentration- and time-dependent manner with an IC50, ~30 μM. DNA flow cytometric analysis indicated that acteoside blocked cell cycle progression at the G1 phase in HL-60 human promyelocytic leukemia cells. Among the G1 phase cell cycle-related proteins, the levels of cyclin-dependent protein kinase (CDK)2, CDK6, cyclin D1, cyclin D2, cyclin D3 and cyclin E were reduced by acteoside, whereas the steady-state level of CDK4 was unaffected. The protein and mRNA levels of CDK inhibitors (cyclin-dependent kinase inhibitors), such as p21CIP1/WAF1 and p27KIP1, were gradually increased after acteoside treatment in a time-dependent manner. In addition, acteoside markedly enhanced the binding of p21CIP1/WAF1 and p27KIP1 to CDK4 and CDK6, resulting in the reduction of CDK2, CDK4 and CDK6 activities. Moreover, the hypophosphorylated form of retinoblastoma increased, leading to the enhanced binding of protein retinoblastoma (pRb) and E2F1. Our results further suggest that acteoside is a potent inducer of differentiation of HL-60 cells based on biochemical activities and the expression level of CD14 cell surface antigen. In conclusion, the onset of acteoside-induced G0/G1 arrest of HL-60 cells prior to the differentiation appears to be tightly linked to up-regulation of the p21CIP1/WAF1 and p27KIP1 levels and decreases in the CDK2, CDK4 and CDK6 activities. These findings, for the first time, reveal the mechanism underlying the anti-proliferative effect of acteoside on human promyelocytic HL-60 cells.

Introduction

Cancers are characterized by three major clonal cellular disorders, cell differentiation arrest (i.e. the presence of immature cells), an inhibition of apoptosis (the accumulation of cells) and accelerated multiplication (proliferation). Cell proliferation in vitro and in vivo can be regulated by the coupling of growth arrest and cell differentiation (1,2). It is not surprising that the de-regulation of the cell cycle is one of the most frequent alterations during tumor development (3). Therefore, blockade of the cell cycle is regarded as an effective strategy for eliminating cancer cells (4,5).

Cell cycle progression is a highly ordered and tightly regulated process that involves multiple checkpoints, at which extracellular growth signals, cell size and DNA integrity are assessed. The cyclin-dependent protein kinases (CDKs) are the major regulators of the cell cycle, and these are regulated by multiple mechanisms (6,7). CDKs bind to and are activated by various cyclin regulatory subunits, and the synthesis and the ubiquitin-mediated destruction of cyclins are closely associated with cell cycle progression, and confine the activities of CDKs to appropriate cell cycle compartments. Also, CDKs are phosphorylated by cyclin-activating kinase, a phosphorylation event that allows subsequent CDK phosphorylations by a variety of protein kinases, which may either activate or inhibit CDK activity (8).

Finally, the activities of cyclin–CDK complexes are carefully regulated by two families of CDK inhibitors. While members of the INK4 family (p16INK4a, p15INK4b, p18INK4c and p19INK4D) interact specifically with CDK4 and CDK6, the CIP/KIP family (p21CIP1/WAF1, p27KIP1 and p57KIP2) inhibit a broader spectrum of CDKs. These cyclin-dependent kinase inhibitors (CKIs), especially p21CIP1/WAF1 and p27KIP1, which bind to cyclin–CDK complexes, render these complexes inactive (9). This multiplicity of regulatory mechanisms allows cell cycle progression to be responsive to a variety array of external and internal factors, and prevents cell cycle progression during periods when DNA damage or other cellular conditions would make such progression harmful to the cell. CKIs that act as brakes to stop cell cycle progression in response to regulatory signals are important negative regulators (10).

The transition from the G1 to the S phase is initiated by the expression of D-type cyclins and by their assembly into kinase complexes with CDK4 and CDK6 (11). The most recognized function of cyclin D-dependent kinases is the inactivation of tumor suppressor protein Rb (retinoblastoma). The initial phosphorylation of Rb by cyclin D–CDK4 complexes in mid-G1 negates the ability of Rb to repress transcription and results in increased cyclin E expression, the regulatory partner for CDK2. Subsequently, cyclin E–CDK2 complexes phosphorylate pRb on additional sites, causing the release of free E2F and the activated transcription of genes required for S-phase entry, including cyclin A.

The growth and differentiation of hematopoietic cells are regulated by a number of cytokines, in vitro and in vivo. HL-60, a human promyelocytic cell line, has been extensively used as an in vitro model for studying the effects of factors that regulate growth and differentiation of hematopoietic cells in general and of myeloid leukemia cells in particular (12). These cells proliferate as promyelocytes, yet retain the capacity to undergo terminal myeloid or monocytic differentiation in response to various inducing agents. In the presence of all-trans-retinoic acid, HL-60 cells undergo differentiation to granulocytes, whereas 1α,25(OH)2-vitamin D3 and 12-O-tetradecanoylphorbol-13-acetate induce differentiation into monocytes/macrophages (13). Transforming growth factor-β (TGF-β), known to be a negative regulator of growth at all stages of hematopoiesis (14,15), induces differentiation of HL-60 cells to promonocytes, and has been shown to act synergistically with vitamin D3, tumor necrosis factor or the combination of all-trans-retinoic acid plus tumor necrosis factor to induce monocytic differentiation of several other myeloid leukemia cell lines (16,17). Other studies showing that induction of terminal
Acteoside induces differentiation via cell cycle arrest

differentiation by retinoids and vitamin D3 requires TGF-β1 as an autocrine mediator suggest that endogenous TGF-β1 plays a critical role in the differentiation of leukemia cells (18,19).

Various plants used in traditional medicines contain significant amounts of phenylpropanoids (20). For example, acteoside [2-(3,4-dihydroxyphenethyl)-1-O-α-L-rhamnopyranosyl-(1→3)-β-D-(4-O-cafeyl)-glucopyranoside] is a phenylpropanoid glycoside that is widely distributed in plants (21). Several studies have shown that acteoside has various biological activities such as antioxidant activity (22,23), ability to modulate nitric oxide production (24,25) and cytotoxicity against various tumor cells (26–28). However, the mechanisms by which acteoside induces cell cycle arrest and differentiation in cancer cells have not been established. Thus, as a part of our screening program to evaluate the chemopreventive potential effect of natural compounds, we examined the cytotoxic effects of acteoside and of its related phenylpropanoids on the various tumor cells. The cytotoxicity data obtained indicates that acteoside was the most effective among the phenylpropanoids examined. Here, we report the effects of acteoside on the proliferation, cell cycle regulation and differentiation of human leukemia HL-60 cells.

Materials and methods

Materials

The acteoside and other phenylglycosides used in this study were isolated from the stems of Cleandrodon trichotomum (Verbenaceae) and Fraxinus sieboldi-ana var. angustata (Oleaceae) and structural identities were determined spectr-rometrically (1H and 13C Nuclear Magnetic Resonance (NMR), Infrared (IR), Mass (MS)) as described previously (29,30). These compounds isolated were checked by high-performance liquid chromatography and were found to be >98% pure.

Cell culture and MTT assay

3LL Lewis mouse lung carcinoma, U-937 human histiocytic lymphoma, HL-60 human promyelocytic leukemia, SNU-C5 human colon cancer and HepG2 human hepatoma cell lines were obtained from the Korean Cell Line Bank. The cells were cultured in RPMI 1640 medium (Life technologies, Grand Island, NY) with 10% fetal bovine serum in a 37°C humidified atmosphere of 5% CO2. Cells were seeded at a concentration of 2×104 per ml and were maintained for logarithmic growth by passaging them every 2–4 days and incubated for 1–4 days with acteoside at various concentrations. Acteoside dissolved in DMSO was added to the medium in serial dilution (the final concentration of 100 μM leupeptin and 1 mM phenylmethylsulfonyl fluoride), and released from the beads by boiling in 2× SDS sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 2% glycerol and 0.02% bromophenolblue) for 5 min; the reaction mixture was then resolved by a 12% SDS–polyacrylamide gel electrophoresis gel, transferred onto a nitrocellulose membrane by electroblotting and probed with antibodies. The blot was developed using an enhanced chemiluminescence kit (Amersham Bioscience, Uppsala, Sweden).

Immunoprecipitation

Samples of the total protein (100 μg) were incubated with the anti-CDK2, anti-CDK4 and anti-CDK6 polyclonal antibodies as described above. Fifty microliter of protein A-Sepharose CL–4B (Amersham Bioscience) prepared at 6 mg/ml in 0.1 M potassium phosphate buffer (pH 8.0) was added to each sample and incubated for 18 h at 4°C. Immunocomplexes were then recovered by centrifugation for 30 s at 14 000g and washed three times in a lysis buffer. The immunocomplexes were then re-suspended and washed three times with a kinase buffer (50 mM Tris–HCl, pH 7.4, 1 mM DTT, 10 mM MgCl2, 2.5 mM ethylenediaminetetraacetic acid, 10 mM β-glycerophosphate and 0.5 μg/ml of oligo (dT)12 (Takara Biomedicals, Shiga, Japan), 1 mM DTT and 10 mM MgCl2 for CDK2). The kinase reactions were carried out in a final volume of 40 μl containing 25 μM ATP, 25 μM [γ-32P]ATP, 2 μg histone H1 for CDK2 or 1 μg glutathione S-transferase (GST)-Rb for CDK4 and CDK6. The reactions were performed for 20 min at 30°C and quenched by adding an equal volume of a 2× SDS loading buffer. After boiling for 10 min, the reaction products were separated by 12% SDS-polyacrylamide gel electrophoresis gel and the phosphorylated proteins were detected by autoradiography.

Reverse transcription–polymerase chain reaction of p21CIP1/WAF1 and p27KIP1

The total cellular RNA was isolated using Easy Blue® kits according to the manufacturer’s instructions (NIRON Biotechnology, Seoul, Korea). From each sample, 1 μg of RNA was reverse transcribed using a MuLV reverse trans-criptase (Takara Biomedicals, Shiga, Japan), 1 mM deoxyribonucleoside tri-phosphates and 0.5 μg/ml of oligo (dT)12 (Takara Biomedicals, Shiga, Japan). The polymerase chain reaction (PCR) analyses were then performed on the aliquots of the cDNA preparations to detect the p21, p27 and β-actin gene expression using a thermal cycler (Perkin Elmer Cetus, Foster City, CA). The reactions were carried out in a volume of 25 μl containing (final concentration) 2 U of Taq DNA polymerase, 0.2 mM deoxyribonucleoside triphosphates, ×10 reaction buffer and 100 pmol of 5’ and 3’ primers. For p21 amplification, the PCR primers were 5’ to 3’ AGAGAGGCCCCTAGTAGACATAC and ACCAG-TGGGGAGGAGGAGTGAC (Bioneer). The denaturation cycle 95°C: 5 min was followed to 35 cycles at 95°C: 45 s, 60°C: 45 s, 72°C: 1 min and an elongation cycle 72°C: 5 min. For p27 amplification, the PCR primers were 5’ to 3’ CCCGGGAGTCTTGAGGAGCAC and AGAAGAATGCGGGGTTTG- GAG (Bioneer). The denaturation cycle 95°C: 5 min was followed to 35 cycles at 95°C: 45 s, 60°C: 45 s, 72°C: 1 min and an elongation cycle 72°C: 5 min. For β-actin amplification, the PCR primers were 5’ to 3’ GATATGCGGCCGCTGTC- GTCCGAG and CAGGAAAAGGGACTGAC (Bioneer). The denaturation cycle 95°C: 5 min was followed to 35 cycles at 95°C: 45 s, 60°C: 45 s, 72°C: 1 min and an elongation cycle 72°C: 5 min. The PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining and Ultraviolet irradiation.

Electrophoretic mobility shift assays

Cells were incubated with or without 30 μM acteoside, and the cells (1×107) were washed twice with ice-cold PBS and pelleted. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM

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Table I. Cytotoxic activity of different phenylglycosides on cancer cell growth in vitro

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC50 (µM)</th>
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<tbody>
<tr>
<td></td>
<td>HL-60</td>
</tr>
<tr>
<td>Acteoside</td>
<td>38.3 ± 1.3</td>
</tr>
<tr>
<td>Isoacteoside</td>
<td>45.6 ± 1.4</td>
</tr>
<tr>
<td>Martynoside</td>
<td>&gt;150</td>
</tr>
<tr>
<td>Isoacteoside</td>
<td>&gt;150</td>
</tr>
<tr>
<td>Calceolaroside A (5)</td>
<td>91.2 ± 6.4</td>
</tr>
<tr>
<td>Calceolaroside B</td>
<td>109.3 ± 5.8</td>
</tr>
<tr>
<td>Leucosceptoside A</td>
<td>85.9 ± 4.9</td>
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</tbody>
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*IC50 is defined as the concentration that results in a 50% decrease in the number of cells compared with that of the control cultures in the absence of an inhibitor. The values represent the means ± SDs of three independent experiments.
Effect of acteoside on the expressions of cell cycle regulatory proteins in HL-60 cells

It is well known that CDK2, CDK4/6, cyclin D and cyclin E cooperate to promote G1-phase progression. We first determined whether acteoside affected the expression of these G1-related proteins in cells treated with 30 μM acteoside for 96 h. Under this condition, acteoside down-regulated CDK2, CDK6, cyclin D1, cyclin D2, cyclin D3 and cyclin E protein levels, whereas CDK4 was unaffected (Figure 4). CKIs are well known to interfere with cell cycle progression to cause phase-specific cycle arrest (32,33). These kinase inhibitors perturb the phosphorylation process by interacting directly with their target proteins, i.e. cyclins or CDKs. The protein levels of certain CKI family members, crucially required for the regulation of G1-phase progression, were determined by western blot analysis (Figure 5). The related CDK inhibitor p21CIP1/WAF1 became detectable after 24 h and increased further at 96 h. The related CDK inhibitor p27KIP1 was also increased ~7-fold at 72 h. In accordance with observed protein levels, the mRNA levels of p21CIP1/WAF1 and p27KIP1 were also found to increase in a time-dependent manner, as evaluated by the semi-quantitative reverse transcriptase–PCR (Figure 5B). Therefore, these results indicate that the inhibitory effect of acteoside on cell proliferation is a result of the induction of the G1 phase arrest of the HL-60 cell cycle through changes in the expressions of G1 phase regulatory proteins.

Effect of acteoside on the p21CIP1/WAF1 and p27KIP1 levels of the CDK immune complex and on the CDK-associated kinase activity

Next, we questioned whether or not acteoside-induced p21CIP1/WAF1 and p27KIP1 would be detected in the complexes with the CDKs. Thus, CDK2, CDK4 and CDK6 complexes were immunoprecipitated from HL-60 cells, which were either treated or not treated with acteoside, and the levels of coimmunoprecipitated p21CIP1/WAF1 and p27KIP1 in each immune complex were determined by western blot analysis using anti-p21 or anti-p27 antibodies. As shown in Figure 6A, the levels of p21CIP1/WAF1 and p27KIP1 in the CDK4 and CDK6 immune complexes of acteoside-treated cells were distinctly higher than in those of the untreated cells. However, there was essentially no difference in the p21CIP1/WAF1 and p27KIP1 levels of the CDK2 immune complex regardless of the acteoside treatment. Since the increased binding of CKIs to CDK complexes subsequently reduces the kinase activity of CDK-cyclin complex, we next investigated the effect of acteoside on in vitro kinase activities, which were directly measured by forming an immune complex with histone H1 (for CDK2) or with GST–Rb fusion protein (for CDK4 or CDK6). As shown in Figure 6B, HL-60 treated with acteoside at 30 μM for 96 h strongly reduced the histone H1-associated kinase activities of CDK2 and Rb-associated kinase activities of CDK4 and CDK6 in response to acteoside treatment. We further found that decreased CDK2 activity was absolutely caused by the reduction of CDK2 protein levels instead of decreased kinase activity of CDK complexes. Collectively, these results suggest that p21CIP1/WAF1 and p27KIP1 proteins might play a key role in G1 phase arrest though their increased binding to CDK4 and CDK6 in the acteoside-treated HL-60 cells, which leads to the down-regulation of the kinase activities of CDK4 and CDK6 and hence to cell cycle arrest.

Acteoside induces differentiation via cell cycle arrest

Considering that pRb is one of the most relevant targets of CDKs (34), we evaluated the degree of phosphorylation of this tumor suppressor protein.
protein in western blot, by using whole extracts from the control or acteoside-treated cells. In HL-60 control cells, pRb was almost completely hyperphosphorylated, whereas in 30 μM acteoside-treated cells, a progressive loss of phosphate groups was evident after 48 h, as indicated by a change in the mobility of pRb immunoreactive bands (Figure 7A). To examine whether acteoside treatment can affect the amount of complex formed by Rb with E2F1, we performed immunoprecipitation with anti-Rb antibody followed by western blot using anti-E2F1 antibody. Rb/E2F1 complex was markedly increased after acteoside treatment compared with control (Figure 7B). Next, we examined the effects of acteoside on the protein levels of cell cycle regulators in HL-60 cells. Protein extracts were harvested from HL-60 cells exposed to 30 μM acteoside for the indicated times and subjected to western blot analysis using the specific antibodies for the cell cycle-related proteins. The experiments were repeated three times with similar results. All results are presented as n-fold of the expression levels respect to non-treated cells.

Effect of acteoside on differentiation of HL-60 cells

In order to determine whether growth inhibition via cell cycle arrest by acteoside is associated with terminal differentiation, we performed a NBT reduction test, and measured esterase activity, phagocytic activity and CD14 and CD66b surface antigen expressions of acteoside-treated HL-60 cells. Treatment of HL-60 cells with 30 μM acteoside resulted in a time-dependent increase in α-naphthyl acetate esterase activity, but the effect of acteoside on naphthyl AS-D chloroacetate esterase activity was relatively small (Figure 8B). Moreover, cells treated with acteoside also showed an apparent increase in phagocytic activity (Figure 8C). In addition, 30 μM acteoside significantly increased the expression of membrane antigen CD14, whereas it did not show any influence on the expression of CD66b (Figure 8D). These results indicate that acteoside induces differentiation of human promyelocytic leukemia cells to monocyte/macrophage lineage.

Acteoside increased TGF-β1 mRNA expression and phosphorylation of Smad2/3 in HL-60 cells

TGF-β1 belong to a family of growth factors that regulate essential functions such as proliferation, differentiation, cellular senescence and apoptosis. Therefore, the effect of acteoside on TGF-β1 expression was examined. Immunoassay analyses revealed that acteoside dose-dependently stimulated TGF-β1 mRNA level in human leukemia HL-60 (Figure 9A). To further confirm acteoside-mediated growth inhibition involving TGF-β1/Smad signaling, we performed western blot to explore critical molecular changes of this pathway. Acteoside produced a significant increase in phosphorylation of Smad2/3 in a time-dependent manner (Figure 9B). These data suggested that acteoside activates TGF-β1 production and phosphorylate Smad proteins in HL-60 cells.
and by repairing DNA damage caused by oxidative stress (38). Kinase activities were assayed using histone H1 (for CDK2) and Rb (for CDK4 and CDK6) as substrates.

The cells were incubated with or without 30 μM acteoside for 96 h. Whole cell lysates were immunoprecipitated with anti-CDK2, anti-CDK4 and anti-CDK6 antibodies and immunoprecipitates were collected. These immunoprecipitated proteins were separated in 12% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and probed with anti-21 or anti-p27 antibodies. The proteins were detected by enhanced chemiluminescence. (IP: immunoprecipitation, WT: western). (B) The kinase activities of CDKs after acteoside exposure. Total cell lysates from the control cells and the cells that were treated with acteoside at a dose of 30 μM for 96 h were immunoprecipitated with anti-CDK2, anti-CDK4 or anti-CDK6 antibodies. Kinase activities were assayed using histone H1 (for CDK2) and Rb (for CDK4 and CDK6) as substrates.

Discussion

The phenylpropanoid glycosides, natural polyphenol constituents of plants, are widely found in many dicotyledon families. Several phenylpropanoid glycosides have been shown to have a wide range of biological activities, including antioxidant (22,23) and antitumor effects (27). The results of the present study using seven phenylpropanoid glycosides isolated from the stems of C. trichotomum and F. sieboldiana var. angustata demonstrate that phenylpropanoids possess a wide range of activities, and that these depend on the natures of the substituents attached to the glucose backbone. In the present study, acteoside and isoacteoside, which have two catechol moieties and one rhamnose group, were found to be potently cytotoxic to various cancer cells (Table I), suggesting that caffeoyl substituents on the glucose ring of the phenylpropanoid structure are crucial for cytotoxicity. Moreover, the cytotoxicity of leucosceptoside A was approximately a half that of the acteoside derivatives, thus implying that the number of catechol moieties is also an important cytotoxic feature. Consistent with this trend, martynoside and isomartynoside, which have no catechol moiety, showed no cytotoxic effect at <150 μM. On the other hand, calceolarioside A and B were slightly less cytotoxic than leucosceptoside A, although these possess two catechol moieties, which indicate that the rhamnose group is also an important anticancer feature. Moreover, the positions of the caffeoyl substituents on the glucose ring has a secondary effect on cytotoxicity, as acteoside and calceolarioside A were more cytotoxic to various cancer cells than isoacteoside and calceolarioside B, respectively.

Acteoside has been reported to induce differentiation and apoptosis by inhibiting telomerase activity in human gastric carcinoma cells (35) by exhibiting anti-proliferative activity toward some tumor cells (36), by inhibiting microsomal lipid peroxidation as an effective Reactive oxygen species (ROS) scavenger (e.g. for \( \text{O}_2^- \) and \( \text{OH}^- \)) (37) and by repairing DNA damage caused by oxidative stress (38). The present study demonstrates that acteoside potently causes HL-60 cell cycle arrest, and that this leads to the inhibition of the cell proliferation and the induction of differentiation. Moreover, we found that acteoside inhibits HL-60 proliferation in a dose- and time-dependent manner, by the trypan blue exclusion method (Figure 2B). This observation prompted us to investigate the effect of acteoside on cell cycle regulation and characteristics of HL-60 differentiation. Flow cytometric data of acteoside-treated HL-60 cells revealed that the cell cycle was blocked by acteoside at the G1 to S phase transition (Figure 3).

The results obtained in the present study provide convincing evidence that acteoside exerts its effects on cell cycle progression mainly via an up-regulation of p21\(^{\text{CIP1/WAF1}}\) (39) and p27\(^{\text{KIP1}}\) proteins and mRNA expressions in HL-60 cells (Figure 5). When cell cycle phase distributions are compared with alternations in cell cycle regulatory molecules, it was apparent that strong CKI up-regulation is one of the major causes of acteoside-induced G1 arrest and cell growth inhibition. The mammalian cell cycle is regulated by complex machinery, in which CDKs, CKIs and cyclins play essential roles (39). CKIs are tumor suppressor proteins that down-regulate cell cycle progression by binding to active CDK–cyclin complexes, thereby inhibiting their kinase activities (40,41). The important CKIs include p21\(^{\text{CIP1/WAF1}}\), a universal inhibitor of CDKs whose expression is mainly regulated by the p53 tumor suppressor protein (39), and p27\(^{\text{KIP1}}\), that is also up-regulated in response to anti-proliferative signals (40). Additionally, our data suggest that CKI up-regulation by acteoside involves a p53-independent pathway because HL-60 cells lack functional p53.
Among the CDKs that regulate the cell cycle, CDK2, CDK4 and CDK6 are known to be activated in association with D-type cyclins or cyclin E during G1 progression and G1–S transition (6). This study reveals that CDK2 and CDK6 and that cyclin D1, cyclin D2, cyclin D3 and cyclin E expressions are reduced by acteoside in HL-60 cells but that of CDK4 is not. In addition, proteins were found to accumulate in association with G1 arrest, and were largely complexed with both CDK4 and CDK6, respectively. The decreased CDK2, CDK6, cyclin D1, cyclin D2, cyclin D3 and cyclin E levels and the increased forms of p21 CIP1/WAF1-CDK4, p21 CIP1/WAF1-CDK6, p27KIP1-CDK4 and p27KIP1-CDK6 complexes support the notion that acteoside markedly attenuates CDK4- and CDK6-associated kinase activities in HL-60 cells. Although we increased the amounts of CDK2 antibodies, the complexes between CDK2 and p21CIP1/WAF1 and p27KIP1 were not affected, indicating that the reduction of CDK2 activities was solely dependent on the reduction of the expression level of CDK2 protein instead of binding with p21CIP1/WAF1 and p27KIP1 proteins. Furthermore, reduced CDK4 and CDK6 kinase activities were found to be associated with the underphosphorylation of the Rb protein, which is known to sequester the transcription factor, E2F, and thereby to prevent cell cycle progression. In addition, electrophoretic mobility shift assay experiments confirmed that acteoside reduced E2F1-DNA-binding capacity in a time-dependent manner. Overall, G1 blockage in HL-60 cells appears to be mediated by the down-regulations of CDK4- and CDK6-associated kinase activity in association with the induction of CKIs, like p21CIP1/WAF1 and p27KIP1.

These anti-proliferative effects were also related to the terminal differentiation. Terminal differentiation in the diverse cell types, which occurs either spontaneously or as a consequence of a treatment with the specific inducing agents, correlates with an irreversible loss of the proliferative potential (42). In this regard, the differentiation-inducing effect of acteoside was further examined in the present study. Acteoside-induced differentiation of HL-60 cells toward the monocyte/macrophage lineage, as determined using differentiation markers such as NBT-reducing ability (Figure 8A), the appearance of esterase activity, increased phagocytic activity and a marked increase in the expression of cell surface antigen CD14, which has been reported to be an antigenic monocyte marker. Thus, our results indicate that acteoside is a novel and potent inducer of HL-60 human leukemia cell to monocyte/macrophage differentiation.

TGF-β is a pluripotent cytokine that controls multiple cellular responses including the induction of cell growth inhibition, differentiation, cellular senescence, wound healing and apoptosis (43–45). These cellular responses are thought to define the role of TGF-β as a tumor suppressor. It inhibits the cell cycle through a partial block in the cell transition from G1 to S phase by down-regulating components of the cell cycle and up-regulating cell cycle inhibitors (46). In addition, HL-60 cells express Smad2 and Smad3, which are important direct downstream targets of TGF-β type I receptor and also express Smad4, which forms heteromeric complexes with Smad2 and Smad3 to enter the nucleus as transcription regulators (47).
Experiments were repeated three times with similar results. All results were observed by western blot analysis using specific antibodies. Significances between treated groups were determined using the Student’s t-test.

In summary, acteoside was found to inhibit the cell proliferation of HL-60 cells not only by arresting the G1 phase cell cycle through the down-regulation of the CDK4- and CDK6-associated kinase activities in association with the induction of CKIs such as p21cip1/waf1 and p27kip1 but also by inducing differentiation via increased TGF-β1 signaling. Finally, these results suggest that acteoside may be useful as one of the investigational drugs for treating leukemia patients.

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References


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