Panaxadiol selectively inhibits cyclin A-associated Cdk2 activity by elevating p21\textsuperscript{WAF1/CIP1} protein levels in mammalian cells

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We show that panaxadiol (PD), a ginseng saponin with a dammarane skeleton, selectively interferes with the cell cycle in human cancer cell lines. PD inhibited DNA synthesis in a dose-dependent manner with IC\textsubscript{50} values ranging from 0.8 to 1.2 \textmu M in SK-HEP-1 cells and HeLa cells. PD-treated cells were arrested at G\textsubscript{1}/S phase, which coincided from 0.8 to 1.2 \textmu M with decreases in Cyclin A±Cdk2 activity, but not in Cyclin E±Cdk2 and Cdc2 activities. The intracellular levels of p21\textsuperscript{WAF1/CIP1} were significantly and selectively elevated in a dose- and time-dependent manner in PD-treated HeLa cells. Similarly, levels of the p21\textsuperscript{WAF1/CIP1} protein that is associated with the Cyclin A±Cdk2 complex increased, and these increases correlated well with the down-regulation of Cyclin A±Cdk2 activity. Thus, PD selectively elevates p21\textsuperscript{WAF1/CIP1} levels and thereby arrests the cell cycle at G\textsubscript{1}/S phase by down-regulating Cyclin A±Cdk2 activity.

Materials and methods

Materials
PD was prepared from the acid-hydrolysate of butanol-soluble fraction of ginseng. Dulbecco’s modified Eagle’s medium and calf serum were obtained from Life Technologies (Palo Alto, CA). General chemicals were purchased from Sigma (St Louis, MO). \textsuperscript{[\gamma-\textsuperscript{32}P]}ATP was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Cell culture
HeLa cells and SK-HEP-1 cells were obtained from Seoul National University Cancer Center and maintained as a monolayer culture in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% (by volume) heat-inactivated calf serum, 100 U/ml penicillin and 100 \mu g/ml streptomycin.

\textsuperscript{[\textsuperscript{3}H]}Thymidine incorporation assay
HeLa and SK-HEP-1 cells were plated at a density of 2.5 \times 10\textsuperscript{4} cells onto 24 well plates in 1 ml 5\% CS-DMEM. Twenty-four hours after seeding, cells were incubated with 0.5\% CS-DMEM containing varying concentration of PD and 0.5 \mu Ci \textsuperscript{[\textsuperscript{3}H]}thymidine for 24 h. At the end of the incubation, unincorporated \textsuperscript{[\textsuperscript{3}H]}thymidine was removed by washing cells with phosphate buffered saline (PBS). Cells were then fixed with trichloroacetic acid, digested with 0.2 N NaOH/0.5\% SDS, and incorporated radioactivity was quantified by a liquid scintillation counter (Wallac, Sweden).

Flow cytometry analysis
HeLa cells were plated at density of 1 \times 10\textsuperscript{5} cells/60 mm dish and cultured in 5\% calf-serum containing DMEM, and treated with or without 5 \mu M PD containing DMEM for increased times. At the end of incubation, cells were trypsinized and fixed with 75\% ethanol and stored at 4°C until analysis. Cells were suspended in DAPI solution (Partec, Germany) and cell cycle analysis was performed using a flow cytometer (Partec, Germany). The percentage of cells in each phase of the cell cycle was analyzed by FloMax\textsuperscript{\textregistered} software.

Abbreviations: CDK, cyclin-dependent kinase; Cyclin A±Cdk2, Cyclin A-dependent kinase 2; CKIs, Cyclin-dependent kinase inhibitors; DAPI, 4,6-diamidino-2-phenylindole; PD, panaxadiol.
Immunoblot analysis

Cells were washed with ice-cold PBS and solubilized in IP lysis buffer containing 20 mM Tris pH 7.5, 0.5% Triton X-100, 2 mM MgCl₂, 1 mM DTT, 1 mM EGTA, 50 mM β-glycerol phosphate, 25 mM NaF, 1 mM Na₃VO₄, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml antipain and 1 μM phenylmethyl sulfonyl fluoride (PMSF). After incubation on ice for 1 h, the insoluble materials were removed by centrifugation at 12 000 × g for 15 min. An aliquot (30 μg of protein) from each sample was resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and electrotransferred onto a PVDF membrane (Gelman, Ann Arbor, MI). The membrane was blocked with 5% non-fat milk and probed with mouse monoclonal antibodies to p21^WAF1/CIP1 or p27^KIP1 (Upstate Biotechnology, Lake Placid, NY), Cyclin A or Cyclin E (Santa Cruz, Santa Cruz, CA), or polyclonal rabbit antibodies (Santa Cruz) to Cdk2, Cdc2, Cyclin A and Cyclin E, respectively. Each immune-complex was then incubated for 15 min at 30°C in 50 μl kinase assay buffer supplemented with 5 μg histone H1 (Gibco BRL, Carlsbad, CA), 10 μCi ['γ-32P]ATP (10 μM), 5 μM PKI and 20 mM EGTA, and the proteins were resolved on a 12% SDS–PAGE. Cyclin-associated kinase activities were then determined by autoradiography.

Immunoprecipitation and histone H1 kinase assay

An aliquot (500 μg of protein) from each cell extract was pre-cleared with protein A–agarose beads (Upstate Biotechnology), and the supernatant was incubated with polyclonal rabbit antibodies to Cyclin A for 4 h. Immunocomplexes associated with Cyclin A were collected after incubating for 2 h with protein A–agarose beads. The immune-complexes were then resolved by SDS–PAGE and analyzed for their components by immunoblotting using corresponding mouse monoclonal antibodies. Immune-complexes of Cdk2, Cdc2, Cyclin A and Cyclin E were obtained from aliquots (200 μg of protein) of each cell extract by immunoprecipitating with specific antibodies against Cdk2, Cdc2, Cyclin A and Cyclin E, respectively. Each immune-complex was washed three times with IP lysis buffer and twice with kinase assay buffer containing 50 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM DTT, 1 mM EGTA, 50 mM β-glycerol phosphate, 25 mM NaF, 0.1 mM Na₃VO₄, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml antipain and 1 mM PMSF. The immune-complexes were then incubated for 15 min at 30°C in 50 μl kinase assay buffer supplemented with 5 μg histone H1 (Gibco BRL, Carlsbad, CA), 10 μCi ['γ-32P]ATP (10 μM), 5 μM PKI and 20 mM EGTA, and the proteins were resolved on a 12% SDS–PAGE. Cyclin-associated kinase activities were then determined by autoradiography.

Results

Panaxadiol suppresses the proliferation of human cancer cell lines by selectively arresting the cell cycle at G1/S phase

We first assessed the suppressive effect of PD on the proliferation of HeLa cells and SK-HEP-1 cells by a[^3H]thymidine incorporation assay. After treatment with PD (1–10 μM) for 24 h,[^3H]thymidine uptake was markedly suppressed in both cell lines with a half-maximal inhibition value of ~1.2 and 0.8 μM in HeLa cells and SK-HEP-1 cells, respectively (Figure 1). To examine whether the decrease in[^3H]thymidine incorporation in PD-treated cells is a consequence of the cell cycle being arrested at a specific phase, we analyzed the cell cycle of HeLa cells after treatment with PD by flow cytometry. The results indicated that asynchronous HeLa cells were arrested in the G1/S phase in response to PD treatment. Percentage of cells in the G1/S phase increased in a time-dependent fashion by >95% of the total cells 16 h after treatment with PD, while those of cells in G2/M phase inversely decreased to <5% of the total cell populations during the same time periods (Figure 2). In contrast, in asynchronous control cells, percentages of cells in G1/S and G2/M phases were ~80 and 20%, respectively, after 16 h incubation. These results clearly indicated that PD induces the cell cycle arrest in G1/S phase of HeLa cells.

Panaxadiol down-regulates the activity of Cyclin A-dependent Cdk2

As PD arrests the cell cycle in G1/S phase, we investigated whether this arrest results from the selective inhibition of the Cyclin–Cdk2 activity that is responsible for cell cycle progression. An immune-complex kinase assay showed that Cyclin–Cdk2 activity is selectively down-regulated in a dose-dependent manner while Cyclin–Cdc2 activity remains unaltered even at a dose (10 μM) of PD (Figure 3A) that is high enough to completely arrest cell growth (Figure 1). We next examined whether either Cyclin A- or Cyclin E-dependent Cdk2 activity is selectively suppressed in PD-treated HeLa cells. An immune-complex assay showed that Cyclin A-dependent kinase activity, but not Cyclin E-dependent kinase activity, is significantly down-regulated in PD-treated HeLa cells (Figure 3B). These findings suggest that the selective inhibition of Cyclin A–Cdk2 activity is responsible for the cell cycle arrest at G1/S phase in PD-treated HeLa cells. To rule out the possibility that PD directly inhibits Cyclin A–Cdk2 activity, we performed an in vitro kinase assay with the Cyclin A–Cdk2 kinase immune-complex. The results showed that Cyclin A–Cdk2 kinase activity is not detectably altered by incubation with increasing concentrations (up to 20 μM) of PD, while the positive control, roscovitine (1 μM), a known inhibitor of Cdk2 and Cdc2 kinases (8), effectively inhibits Cyclin A–Cdk2 activity (Figure 3C). Thus, the inhibitory effect of PD on Cyclin A–Cdk2 activity appears to be mediated through an indirect action, such as by the modulation of upstream signals.

Down-regulation of Cyclin A–Cdk2 activity is attributed to elevated p21[^WAF1/CIP1] levels in PD-treated cells

To elucidate the mechanism by which Cyclin A–Cdk2 activity is down-regulated in PD-treated HeLa cells, we examined whether the levels of the different protein components of the Cyclin A–Cdk2 complex are altered following PD treatment (Figure 4). The immunoblots indicated that the levels of Cyclin A, Cdk2, Cyclin E and Cdc2 remained unaltered in cells treated with increasing concentrations of PD (Figure 4). Interestingly, however, p21[^WAF1/CIP1] levels were markedly elevated in a PD dose-dependent manner, and they were 8–9 fold higher in cells treated with 10 μM PD compared with vehicle-treated cells. In contrast, p27^KIP1 levels were unchanged under the same experimental conditions. Additionally, p21[^WAF1/CIP1] levels were detectably elevated 8 h after treatment with 5 μM PD, a concentration that is sufficient to arrest the cell cycle at G1/S phase as determined by flow...
cytometry. Furthermore, p21\textsuperscript{WAF1/CIP1} levels gradually increased in a time-dependent fashion in PD-treated cells (Figure 5A), while the levels of the other components remained unchanged. These results strongly suggest that the down-regulation of Cyclin A–Cdk2 activity can be primarily attributed to elevated p21\textsuperscript{WAF1/CIP1} levels following PD treatment. To provide direct evidence that increased p21\textsuperscript{WAF1/CIP1} levels are responsible for the down-regulation of Cyclin A–Cdk2 activity, we examined whether levels of the p21\textsuperscript{WAF1/CIP1} protein that is directly associated with the Cyclin A–Cdk2 complex are also increased following PD treatment. Immunoblotting analysis of the Cyclin A-immune-complex indicated that levels of p21\textsuperscript{WAF1/CIP1} protein associated with the Cyclin A–Cdk2 complex are significantly elevated in a PD dose-dependant manner (Figure 5B). As expected, the levels of the p27\textsuperscript{KIP1} and Cdk2 proteins that are associated with the immune-complex were not changed. Thus, the selective down-regulation of Cyclin A–Cdk2 activity in PD-treated cells can be mainly attributed to elevated p21\textsuperscript{WAF1/CIP1} protein levels. Collectively, the data suggest that PD arrests the cell cycle at G1/S phase by selectively down-regulating Cyclin A–Cdk2 activity by a mechanism that elevates p21\textsuperscript{WAF1/CIP1} protein levels.

Discussion
In the present study, we have described the mechanism of action by which PD, a diol-type of ginseng saponin with a dammarane skeleton, arrests growth of HeLa cells at G1/S phase. As determined by a [\textsuperscript{3}H]thymidine incorporation assay, the IC\textsubscript{50} values of 1.2 and 0.8 \textmu M for the growth inhibition of HeLa cells and SK-HEP-1 cells, respectively, suggest that PD exhibits a similar potent anti-proliferative activity against different cancer cell types. Interestingly, PD induces cell cycle arrest of cells at G1/S phase, as indicated by the results of flow cytometric analysis (Figure 2). Our results show that in PD-treated cells, 95.8% of cells are arrested at G1/S phase after 16 h of PD treatment and only 4.2% of cells are in the cell cycle stage of G2/M phase. In contrast, asynchronous control cells normally proceed to cell cycle progression during these time periods. These results suggest that PD inhibits cell proliferation by specifically blocking the cell cycle at G1/S phase. It is well documented that progression through the G1/S transition and S phase is governed by the Cyclin–Cdk2 kinases (3). Cdk2 activity is positively controlled by the levels of its regulatory subunits, Cyclin A and Cyclin E, and negatively regulated by levels of the endogenous Cyclin-dependent
kinase inhibitor proteins p21\textsuperscript{WAF1/CIP1} and p27\textsuperscript{KIP1}. To examine whether PD induces cell cycle arrest at G\textsubscript{1}/S phase by modulating Cdk2 activity, we determined the Cdk2 and Cdc2 activity in HeLa cells following PD treatment. Interestingly, Cyclin A-Cdk2 activity, but not Cyclin E-Cdk2 or Cdc2 activity, was selectively down-regulated in PD-treated HeLa cells (Figure 3A and B). This observation agrees well with the result from the flow cytometric analysis that showed that HeLa cell growth is arrested at G\textsubscript{1}/S phase in response to PD treatment, because Cyclin A-Cdk2 kinase activity, but not the Cyclin E-Cdk2 or Cdc2 kinase activity, is responsible for progression through S phase (1,2,8).

\textit{In vitro} immune-complex kinase assays showed that Cyclin A-Cdk2 activity is not detectably inhibited by incubation with increasing PD doses (up to 20 \textmu M), while roscovitine, a specific Cdk2 kinase inhibitor, effectively inhibits the kinase activity at 1 \textmu M (Figure 3C). Therefore, we ruled out the possibility that PD directly inhibits Cyclin A-Cdk2 activity. These results indicate that the down-regulation of Cyclin A-Cdk2 activity in PD-treated cells can be attributed to indirect mechanisms. To elucidate these mechanisms, we first determined whether the levels of the components that constitute the Cdk2 complexes are altered. The results showed that levels of Cyclins A and E, Cdk2 and Cdc2 are not altered following treatment with increasing doses of PD. Interestingly, p21\textsuperscript{WAF1/CIP1} levels, but not p27\textsuperscript{KIP1} levels, are selectively elevated in a PD concentration-dependent fashion (Figure 4).

![Fig. 3](image3.png) Specific inhibition of Cyclin A-associated Cdk2 activity by PD. HeLa cells were incubated with varying concentrations of PD for 24 h, and cell lysates were prepared. Histone H1 kinase activity associated with the immune-complex precipitated by (A) anti-Cdk2, anti-Cdc2, (B) anti-Cyclin A and anti-Cyclin E antibodies was determined as described in Materials and methods. (C) Cell lysates from exponentially growing HeLa cells were immunoprecipitated by anti-Cyclin A antibody, and the histone kinase reaction assay was performed in the presence of PD and roscovitine.

![Fig. 4](image4.png) Effect of PD on the levels of several cell cycle regulatory proteins. HeLa cells were incubated with varying concentrations of PD for 24 h. Cell lysates were prepared and immunoblot analysis was performed as described in Materials and methods. The levels of p21\textsuperscript{WAF1/CIP1} were increased in PD-treated cells.

![Fig. 5](image5.png) PD selectively induces p21\textsuperscript{WAF1/CIP1} in HeLa cells. (A) Time-dependent effect of PD on the p21\textsuperscript{WAF1/CIP1} level in cells. Cells were incubated with 5 \textmu M PD and harvested at the indicated times. Immunoblot analysis was performed against the indicated proteins as described in Materials and methods. (B) p21\textsuperscript{WAF1/CIP1} associated with Cyclin A-Cdk2 is increased in PD-treated cells. Cell lysates were prepared as described in Figure 4, and were immunoprecipitated with a polyclonal antibody to human Cyclin A. Immune-complexes were resolved by SDS-PAGE and analyzed by immunoblotting using specific monoclonal antibodies to Cyclin A, Cdk2, p27\textsuperscript{KIP1} and p21\textsuperscript{WAF1/CIP1}.
p21\textsuperscript{WAF1/CIP1} levels are elevated in PD-treated cells. Furthermore, levels of the p21\textsuperscript{WAF1/CIP1} protein that is associated with the Cyclin A–Cdk2 complex are markedly elevated in a PD dose-dependent manner, while those of other components in the immune-complex including Cyclin A, Cdk2 and p27\textsuperscript{KIP1} are not altered. These results clearly support the idea that the PD-induced down-regulation of Cyclin A–Cdk2 activity can be primarily attributed to elevated p21\textsuperscript{WAF1/CIP1} levels. To support this idea, we tested if PD-induced cell cycle arrest and Cyclin A–Cdk2 activity inhibition might be recovered after withdrawal of PD from the culture medium. The results suggest that the down-regulated Cdk2 activity in cells treated with 5 \textmu M PD is effectively recovered in an incubation time-dependent fashion almost to levels of the untreated-control cells (Figure 6A and B). Importantly, protein levels of Cdk2-associated p21\textsuperscript{WAF1/CIP1} that have been induced in response to 5 \textmu M PD treatment decreased to the control levels after the PD-withdrawal (Figure 6C). These results support the notion that PD-induced cell cycle arrest is attributed to the down-regulated Cdk2 activity by inducing p21\textsuperscript{WAF1/CIP1} levels and this arresting effect is recovered by withdrawal of PD from the culture medium.

Our earlier reports have shown that ginsenosides-Rs3 (G-Rs3) and -Rs4 (G-Rs4), which are different types of ginseng saponins, can induce cell cycle arrest in human hepatoma SK-Hep1 cells, and that this is associated with the selective elevation of p21\textsuperscript{WAF1/CIP1}. Interestingly, PD is structurally different from G-Rs3 and G-Rs4: the side chain of PD at C-20 is cyclized, whereas those of G-Rs3 and G-Rs4 are open and straight, and the –OH at C-3 of PD is free, while those of G-Rs3 and G-Rs4 are attached to acetylated Glc-Glc (Figure 7). However, these three ginsenosides are structurally similar in that they share a common dammarane skeletal structure with –OH groups attached at C-3 and C-12. It is interesting to note that panaxatriol has the same dammarane skeleton as PD, but neither exhibits cell cycle-arresting activity nor down-regulation of Cyclin-associated kinase activity (data not shown here). PD structurally differs from panaxatriol at C-6.–OH. It is speculated that the –OH groups attached to C-3 and C-12 are functionally important, but the additional –OH group at C-6 in panaxatriol may exhibit an opposite effect. These structural features may explain why the diol-type of ginsenosides such as G-Rs3, G-Rs4 and PD all elevate p21\textsuperscript{WAF1/CIP1} levels and thereby down-regulate the activities of Cyclin-dependent kinases.

**Fig. 6.** Cdk2 activity is effectively recovered after PD-withdrawal from the culture medium. Cells were plated and cultured in DMEM containing 5% calf-serum and treated for 24 h with 5 \textmu M of PD in DMEM. PD-containing medium was then replaced with 5% CS-containing DMEM. Cells were thereafter harvested at 12 and 24 h. (A) Restoration of Cdk2 activity and levels of Cdk2-associated p21\textsuperscript{WAF1/CIP1} protein were determined by an immuno-complex kinase assay and immunoblot assay, respectively. (B) Densitometric representation of the relative Cdk2 activity to control value. (C) Protein levels of Cdk2-associated p21\textsuperscript{WAF1/CIP1} were densitometrically analyzed and presented as the relative protein levels to the control value. Lanes: 1, control; 2, vehicle control; 3, 24 h treatment with 5 \textmu M PD; 4, control for 12 h recovery; 5, 12 h recovery of 5 \textmu M PD treatment; 6, control for 24 h recovery; 7, 24 h recovery of 5 \textmu M PD treatment.
In conclusion, we suggest that PD, a pseudoaglycone of diol-type ginsenosides, displays an anti-proliferative activity by arresting the cell cycle at G1/S phase. This effect is potentially achieved through the down-regulation of Cyclin A±Cdk2 kinase activity, which is tightly associated with elevated p21\textsuperscript{WAF1/CIP1} levels in PD-treated cells. We therefore propose that PD can be used to arrest the cell cycle at G1/S phase through the selective elevation of p21\textsuperscript{WAF1/CIP1} levels in various types of cancer cells.

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