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Pyrrolidine Dithiocarbamate Inhibition of Luteolin-Induced Apoptosis through Up-regulated Phosphorylation of Akt and Caspase-9 in Human Leukemia HL-60 Cells

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Previously, we observed that luteolin effectively inhibited cell growth and induced apoptosis in HL-60 cells. In that study, we also explored the modulatory effects and molecular mechanisms of pyrrolidine dithiocarbamate (PDTC) on the cytotoxicity of luteolin to HL-60 cells. In this study, we found that PDTC was able to inhibit luteolin-induced cell apoptosis in a dose-dependent manner. When HL-60 cells were treated with PDTC for 0.5 h before 60 μM luteolin treatment, the DNA ladder disappeared. Moreover, flow cytometry showed that PDTC had dose dependently decreased the percentage of apoptotic HL-60 cells and had not interfered with luteolin's ability to change the mitochondrial membrane potential or its ability to trigger the release of cytochrome *c* to cytosol. Detection by Western blotting, however, did show that PDTC had interfered with luteolin's ability to cleave poly(ADP-ribose)-polymerase and DNA fragmentation of factor-45. Three hours after the PDTC-pretreated HL-60 cells were treated with 60 μM luteolin, the product cleaved from Akt started to appear. Therefore, not only was PDTC able to stop the apoptosis of HL-60 cells treated with luteolin, it was also found to increase phosphorylation of Akt and caspase-9. These results suggest that in the luteolin-induced apoptotic pathway, phosphorylation of procaspase-9 by survival signals might play an important role in the ultimate fate of HL-60 cells.

KEYWORDS: PDTC (pyrrolidine dithiocarbamate); luteolin; apoptosis; caspase-9; Akt

INTRODUCTION

Flavonoids are widespread in fruits, vegetables, seeds, and medicinal herbs. All of them might be one type of polyphenolic flavonoids, which have the diphenylpropane (C6C3C6) skeleton, including monomeric flavanols, flavones, flavanols, and flavanones, and all have been intensively studied for their role in human health, including cancer prevention. Epidemiological studies have shown that the consumption of vegetables, fruits, and tea is associated with delaying or preventing carcinogenesis. Luteolin (shown in **Figure 1**) is a flavone that can be found in large amounts in thyme but is present also in many other plants, including beets, cabbage, cauliflower, and Brussels sprouts (*1*). It has been shown to be a potential inhibitor of tumor cell proliferation and estrogen-induced mammary carcinogenesis and, therefore, may have potential as a dietary anticarcinogenic agent (*2*−*6*). In a previous study, luteolin was able to decrease the mitochondrial membrane potential, trigger the release of cytochrome *c* to cytosol, and subsequently induce the processing of procaspase-9 and procaspase-3, which led to the cleavage of poly(ADP-ribose)polymerase (PARP) and DNA fragmentation of factor-45 (DFF-45). In that same study, luteolin induced the cleavage of proapoptotic Bcl-2 proteins and the cleavage of the antiapoptotic Bcl-2 proteins (*7*). Recently, reactive oxygen species (ROS) have been found to play an important role in the induction of apoptosis (*8*, *9*). In this study, we first studied the effect of different antioxidants, including allopurinol (ALL),

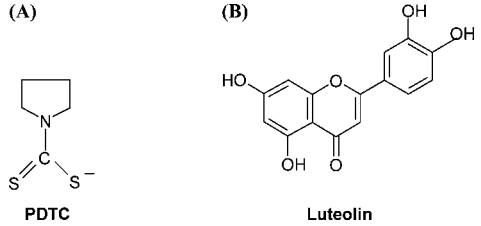

Figure 1. Chemical structures of (**A**) PDTC and (**B**) luteolin.

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catalase (CAT), diphenylene iodonium (DPI), *N*-acetylcysteine (NAC), pyrrolidine dithiocarbamate (PDTC), and superoxide dismutase (SOD), on luteolin-induced apoptosis. Only two were found to inhibit luteolin-induced apoptosis, SOD at high doses only and PDTC.

PDTC (shown in **Figure 1**) has been found to both inhibit and induce apoptosis, depending on cell type. PDTC, an antioxidant and radical scavenger, has been shown to be an inhibitor of agonist-induced apoptosis in human leukemia HL-60 cells and thymocytes (*10*). Other studies have reported that the effects of PDTC-induced apoptosis depend on the cell types and the presence of Cu and Zn (*11*), although this difference may be not because it is an antioxidant but because it is a chelator. PDTC-induced apoptosis occurs mainly through its inhibiting effects on viral macromolecule synthesis but not through its antioxidant effects (*12*). Apoptosis is defined as a type of cell death involving the concerted action of a number of intracellular signaling pathways, including members of the caspases family of cytsteine proteases, stored in most cells as zymogens or procaspases (*13*). Akt has been implicated to be an apoptosis suppressor in signal transduction pathways (*14*). Akt could inhibit caspase-9 activity by directly phosphorylating caspase-9 and inhibiting its protease activity and, therefore, suppressing downstream caspases and apoptosis (*15*, *16*).

In the present study, we used flow cytometry and Western blotting to investigate the effects of PDTC on the luteolin-induced apoptosis. We found that PDTC dose dependently inhibited the luteolin-induced apoptosis in human leukemia HL-60 cells by modulating Akt, which phosphorylated and reduced the activity of caspase-9. These findings are important because knowledge regarding phosphorlylation of capsase-9 and signal transduction is crucial in designing preventive and curative therapies using flavonoids and antioxidants such as PDTC for cancer in humans.

MATERIALS AND METHODS

Materials. Luteolin was obtained from Versuchsstation Schweizerischer Brauereien (Zürich, Switzerland) and dissolved in dimethyl sulfoxide (DMSO) prior to use. PDTC was obtained from Sigma Chemical Co. (St. Louis, MO). The antibodies to DFF-45 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-caspase-3 and -9, cytochrome *c*, anti-Akt, anti-phospho-Akt, and PARP antibodies were from PharMingen (San Diego, CA); anti-phospho-caspase-9 monoclonal antibody (Thr 125) was obtained from Cell Signaling Technology (New England Biolabs, Ipwich, MA). The horseradish peroxidase-conjugated anti-mouse and rabbit IgG propidium iodide (PI) that we used were obtained from Sigma.

Cell Culture. Human leukemia HL-60 cell lines were obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI-1640, supplemented with 15% fetal calf serum (Gibco BRL, Grand Island, NY), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 2 mM L-glutamine (Life Technologies, Inc., Grand Island, NY) and kept at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Cell Viability Assay. Cells were placed into 96 well plates at a density of 1×10^5 cells/100 μL/well. After overnight growth, cells were pretreated with various antioxidants and luteolin (60 μM) for 12 h; final concentrations of DMSO were less than 0.1%. Cell viability was analyzed using a Luminescent ATP Detection Assay Kit (Packard BioScience B.V.). Briefly, 50 μL of cell lysate was used to assay luminescent ATP. The plate was first kept for 1 min in the dark. Luminescence was measured on a Microplate Scintillation Luminescence Counter (Molecular Devices, MaxII, CA) in single photon counting mode for 0.1 min/well.

Apoptotic Ratio Analysis. The cell apoptotic ratio was analyzed by flow cytometry. Cells were harvested, washed twice with phosphate-buffered saline (PBS), and fixed in 70% ethanol for at least 2 h at −20 °C. Those that were fixed were washed with PBS, incubated with 1 mL of PBS containing 0.5 μg/mL RNase A and 0.5% Triton X-100 for 30 min at 37 °C, and then stained with 50 μg/mL PI. The stained cells were analyzed by a FACScan laser flow cytometer (Becton Dickinson, San Jose, CA) connected to ModFit LT cell cycle analysis software (Verity Software, Topsham, ME).

DNA Extraction and Electrophoresis Analysis. HL-60 cells (2×10^5 cells/mL) were harvested, washed with PBS, and then lysed with digestion buffer containing 0.5% sarkosyl, 0.5 mg/mL proteinase K, 50 mM Tris (hydroxymethyl) aminomethane (pH 8.0), and 10 mM EDTA at 56 °C for 3 h. They were treated with RNase A (0.5 μg/mL) for another 2 h at 56 °C. The DNA was extracted by phenol/chloroform/isoamyl alcohol (25/24/1) before loading and analyzed by 2% agarose gel electrophoresis. The agarose gels were run at 50 V for 120 min in TBE buffer (Tris-borate/EDTA electrophoresis buffer). About 20 μg of DNA was loaded in each well and observed and photographed under UV light.

Analysis of Mitochondrial Membrane Potential. The change of mitochondrial transmembrane potential ($\Delta\Psi_m$) was monitored by flow cytometry. Briefly, HL-60 cells were harvested after a 1 h treatment with either 60 μM luteolin or 50 μM PDTC. The pretreated HL-60 cells were first pretreated with PDTC for 0.5 h, treated with 60 μM luteolin, and harvested 1 h later. The mitochondrial transmembrane potential of all samples, including controls, those treated with luteolin alone and PDTC alone, and those pretreated with PDTC followed by luteolin, were analyzed by flow cytometry using 40 nM 3,3′-dihexyloxacarbocyanine (Molecular Probe, Eugene, Oregon). The samples were analyzed for fluorescence using a FACScan flow cytometer (Becton Dickinson). Histograms were analyzed using Cell Quest software.

Cytochrome *c* Release. HL-60 cells were treated with 60 μM luteolin, 50 μM PDTC, or pretreated with 50 μM PDTC followed by 60 μM luteolin treatment. Cells were harvested at different times, washed twice in ice cold PBS, and prepared by resuspending in buffer A [250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiolthione, 17 μg/mL phenylmethylsulfonyl fluoride, 8 μg/mL aprotinin, and 2 μg/mL leupeptin (pH 7.4)] and incubated on ice for 30 min. Cells were passed through a syringe needle 20 times. Unlysed cells and nuclei were pelleted by centrifugation at 750*g* for 10 min. The supernatant was then spun at 10000*g* for 15 min. The pellet was then resuspended in buffer A and taken to represent the mitochondrial fraction. The remaining supernatant was centrifuged at 100000*g* for 30 min. The resulting supernatant from this final centrifugation was taken to represent the cytosolic fraction. The release of cytochrome *c* release was determined by Western blot.

Western Blotting. HL-60 cells were first treated with 60 μM luteolin, 50 μM PDTC, or pretreated with 50 μM PDTC for 0.5 h followed by 60 μM treatment for 12 h. They were then harvested and homogenized in a lysis buffer (50 mM Tris-HCl, pH 8.0; 5 mM EDTA; 150 mM NaCl; 0.5% NP-40; 0.5 mM phenylmethylsulfonyl fluoride; and 0.5 mM dithiothreitol) for 30 min on ice. The cell debris were centrifuged at 10000*g* at 4 °C for 30 min. The proteins in the supernatant were measured by bicinchoninic acid assay. Briely, 50 μg of total cellular proteins was resolved by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (10% for PARP and DFF-45; 12% for caspase-3, caspase-9, and cytochrome *c*), transferred onto poly(vinylidene difluoride) membranes (Amersham, Arlington, IL), and then probed with primary antibody followed by the addition of horseradish peroxidase-labeled secondary antibody. The expression of protein was detected by measuring the chemiluminescence of the blotting agent (ECL, Amersham Corp.), and then, the membranes were exposed to Kodak X-Omat film.

Statistical Analysis. Data are presented as means ± standard error (SE) for the indicated number of independently performed experiments. Statistical analysis was done by one-way Student's *t*-test.

RESULTS

Effect of Antioxidant on Luteolin-Treated HL-60 Cells. A previous study showed that luteolin was a potent inducer of

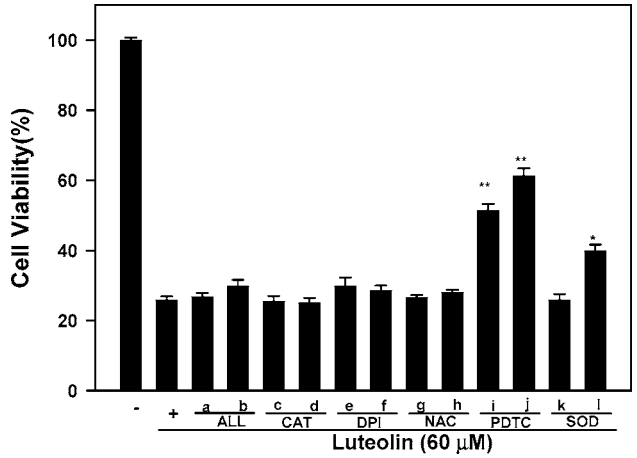

Figure 2. Effects of antioxidant on luteolin-treated HL-60 cells. HL-60 cells were treated with different concentrations of ALL, CAT, DPI, NAC, PDTC, and SOD for 0.5 h followed by luteolin (60 μM) treatment for another 12 h. The cell viability was determined by a Luminescent ATP detection assay kit. Each value is presented as the mean ± SE of three independent experiments. ALL (a, 50 μM; b, 100 μM), CAT (c, 200 U/mL; d, 400 U/mL), DPI (e, 20 μM; f, 40 μM), NAC (g, 2.5 mM; h, 5 mM), PDTC (i, 20 μM; j, 40 μM), and SOD (e, 100 μg/mL; f, 200 μg/mL); * < 0.05 and ** < 0.01.

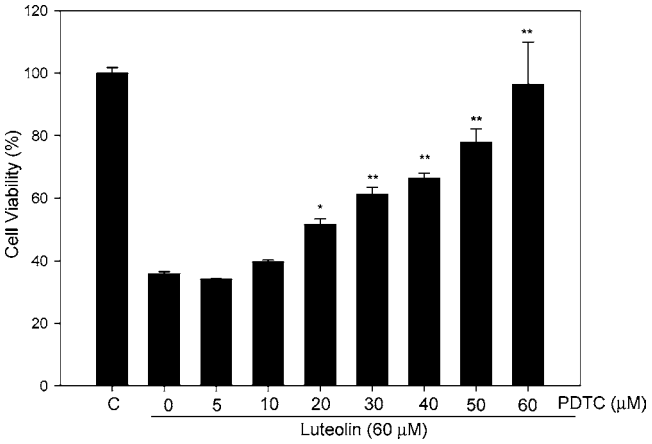

Figure 3. Effects of PDTC on luteolin-treated HL-60 cells. HL-60 cells were pretreated with various concentrations of PDTC as indicated in the figure for 0.5 h before incubating with 60 mM luteolin for 12 h. The cell viability was determined by a Luminescent ATP detection assay kit. Each value is presented as the mean ± SE of three independent experiments; ** < 0.01.

apoptosis in HL-60 cells (*7*). Recently, ROS has been found to play an important role in the induction of apoptosis. Therefore, using the antioxidants ALL, CAT, DPI, NAC, PDTC, and SOD, we investigated whether ROS production was essential to luteolin-inhibited cell survival. Pretreatment with PDTC and high doses of SOD, but not ALL, CAT, DPI, and NAC, protected HL-60 cells from luteolin-induced cytotoxicity (**Figure 2**); PDTC's effect was dose-dependent (**Figure 3**).

PDTC Inhibition of Luteolin-Induced DNA Fragmentation in Leukemia Cell Lines. We measured DNA fragmentation in cells treated with luteolin alone and cells treated first with different concentrations of PDTC and later with luteolin and found that PDTC inhibited luteolin-induced DNA fragmentation in a dose-dependent manner (**Figure 4**).

PDTC Induction of Dose-Dependent Decreases in Luteolin-Induced Leukemia Cell Apoptotic Ratio. Using flow cytometry, we tested the effects of different concentrations of

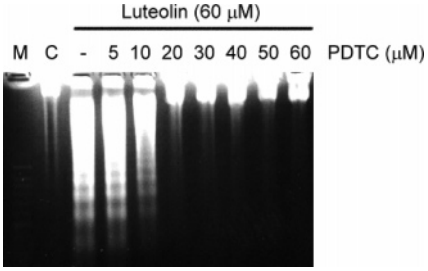

Figure 4. Effects of PDTC on luteolin-induced DNA fragmentation. HL-60 cells were pretreated with various concentrations of PDTC as indicated for 0.5 h before incubating with 60 μM luteolin for 12 h. Internucleosomal DNA fragmentations were analyzed by agarose electrophoresis. M, 100 base pairs DNA ladder size maker; C, control. This experiment was repeated three times with similar results.

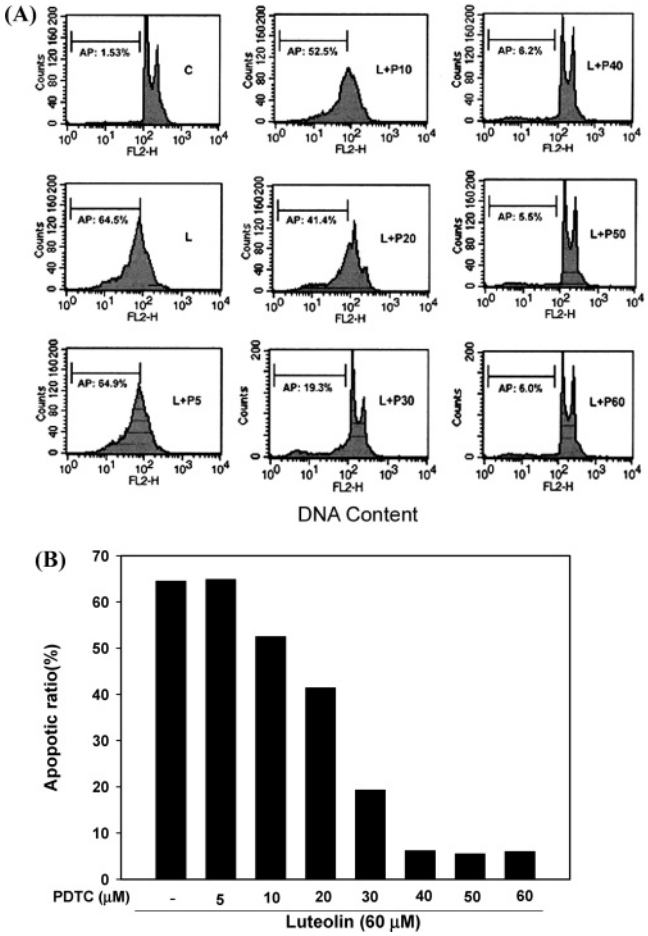

Figure 5. Effects of PDTC on luteolin-induced apoptotic ratios. HL-60 cells were pretreated with various concentrations of PDTC as indicated for 0.5 h before incubating with 60 μM luteolin for 12 h. The ratios of sub-G1 cells were analyzed by flow cytometry. Sub-G1 represents apoptotic cells with a lower DNA content. Each value is presented as the mean ± SE of three independent experiments.

PDTC pretreatment on apoptotic ratios in human promyelocytic leukemia HL-60 cells (**Figure 5**). The percentage of apoptotic HL-60 cells had decreased sharply from 41.4% in HL-60 pretreated with 20 μM PDTC to 19.3% in those pretreated with 30 μM PDTC. The greater the dosage of PDTC is, the more reduced the apoptotic ratio is. This finding shows that PDTC can potently suppress luteolin-induced apoptosis in HL-60 cells.

Effects of PDTC on the Luteolin-Induced Cleavage of PARP and DFF-45. Western blotting was used to study the

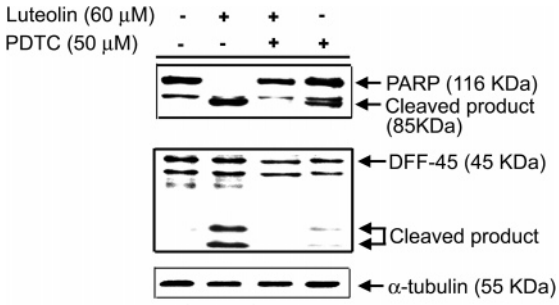

Figure 6. PDTC inhibited the cleavage of poly(ADP-ribose) polymerase and DFF-45 in luteolin-treated HL-60 cells. Western blotting analysis of PARP and DFF-45 in HL-60 cells treated with DMSO, luteolin, luteolin and PDTC, or PDTC only at 12 h, respectively. HL-60 cells were pretreated with PDTC for 0.5 h before incubating with 60 μM luteolin for 12 h. At the end of the incubation time, the total protein was extracted for PARP, DFF-45, and α-tubulin analysis using PARP, DFF-45, and α-tubulin-specific antibodies as described in the Materials and Methods. This experiment was repeated three times with similar results.

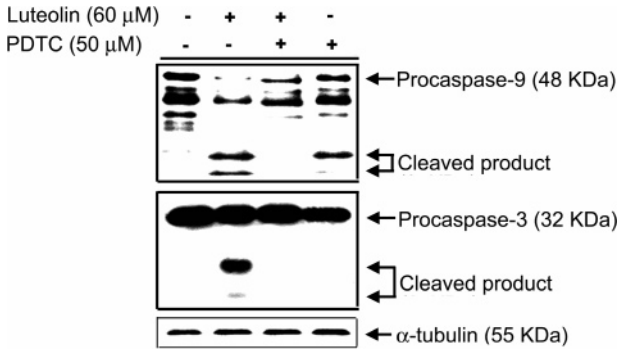

Figure 7. PDTC inhibited the cleavage of caspase-3 and -9 in luteolin-treated HL-60 cells. HL-60 cells were pretreated with PDTC for 0.5 h before incubating with 60 μM luteolin for 12 h. The processing of procaspase-3 and -9 was detected by Western blotting analysis using specific antibody against caspase-3 and -9. This experiment was repeated three times with similar results.

effects of PDTC on the luteolin-induced cleavage of PARP and DFF-45. The proteolytic cleavage of PARP and DFF-45 was in both luteolin and PDTC samples. However, in cells treated with both PDTC and luteolin, there was no accumulation of an 85 kDa fragment or a decrease of a 116 kDa protein (**Figure 6**).

Blocking of Luteolin-Induced Cleavage of Caspase-3 and -9 by Pretreatment with PDTC. The cleavage of PARP and DFF-45 is caused by activation of caspase-3, which has been reported to play a central role in many types of apoptosis (*17, 18*). We used Western blotting to study the effect of PDTC on the cleavage of procaspase-3 and procaspase-9 (**Figure 7**). Cleavage of procaspase-3 and procaspase-9 was induced by luteolin alone and slightly induced by PDTC alone after 12 h of treatment each. However, in cells treated with both, no cleavage of either was found after 12 h.

Disruption of Mitochondrial Membrane Potential and Release of Cytochrome *c*. Disruption of mitochondria has been reported to cause the release of cytochrome *c* and result in the cleavage and activation of caspase-9 (*19*−*21*). A decrease in mitochondrial membrane potential is thought to cause the disruption of the outer mitochondrial membrane and contribute to the release of cytochrome *c* (*22*). The release of cytochrome *c* results in the activation of caspase-9 and subsequently leads to apoptosis. We used flow cytometry and Western blotting to

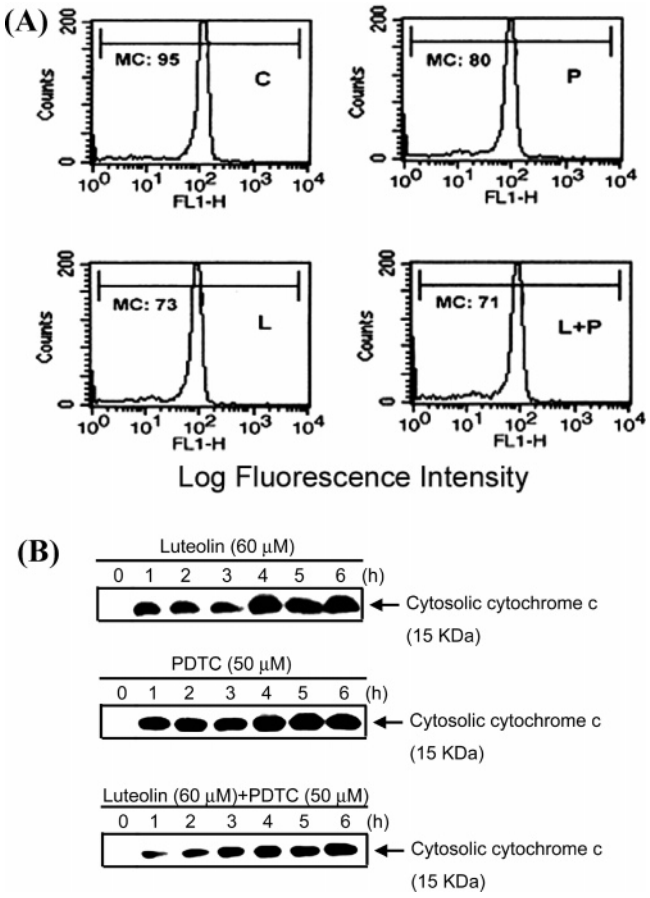

Figure 8. Decrease of mitochondria membrane potential and release of cytochrome *c* into cytosol. (**A**) HL-60 cells were treated with 60 μM luteolin and 50 μM PDTC for 1 h and pretreated with 50 μM PDTC for 0.5 h followed by 60 μM luteolin treatment for 1 h. The relative mitochondrial membrane potential ($\Delta\Psi_m$) was measured by fluorescent emission. The numbers represent the mean of the relative fluorescent intensity. C, control cells; L, luteolin treatment; P, PDTC treatment; and L + P, pretreated with PDTC followed by luteolin treatment. (**B**) HL-60 cells were treated with 60 μM luteolin and 50 μM PDTC and pretreated with 50 μM PDTC for 0.5 h followed by 60 μM luteolin treatment at the time periods indicated. Subcellular fractions were prepared as described in the Materials and Methods, and cytosolic cytochrome *c* was detected by Western blotting. This experiment was repeated three times with similar results.

find out whether the reduction of mitochondrial membrane potential could cause the release of cytochrome *c* into the cytosol. The mitochondrial membrane potential was reduced after 1 h in all three (**Figure 8**). We detected cytochrome *c* in the cytosol at 1, 2, 3, 4, 5, and 6 h after treatment. These observations demonstrated that the release of luteolin-triggered cytochrome *c* from mitochondria was not blocked by PDTC in HL-60 cells.

PDTC Up-Regulation of Phosphorylation of Akt and Caspase-9 in Luteolin-Treated HL-60 Cells. One previous study suggested that although phosphorylation and inactivation of caspase-9 might, Akt promotes cell survival (*23*). Cardone et al. also indicated that Akt could prevent activation of caspase-9 in the mitochondria pathway by phosphorylation and, therefore, suppress downstream caspases and apoptosis (*24*). Bagli et al. demonstrated that luteolin could inhibit VEGF-induced activation of Akt, a survival signal of the PI3K/Akt pathway (*25*). In this study, we examined the expression of Akt at different time points after 60 μM luteolin treatment. As shown in **Figure 9A**, there was no visible change of Akt expression,

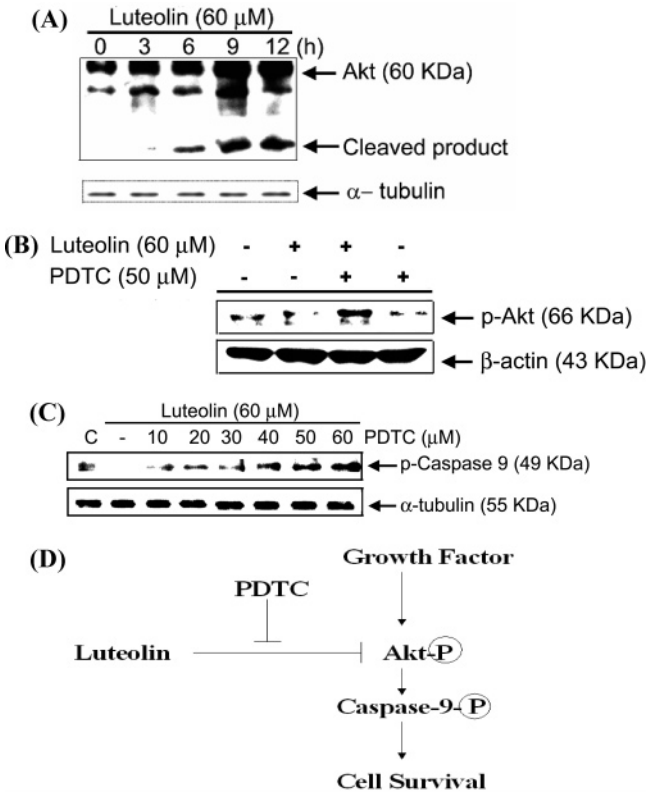

Figure 9. (**A**) Luteolin induced the cleavage and inactivation of Akt. HL-60 cells were treated with 60 μM luteolin for the indicated time. The expression of Akt was detected by Western blotting analysis using a specific antibody against Akt. (**B**) The effect of PDTC on luteolin-induced apoptotic cells through phosphorylation and activation of Akt. HL-60 cells were treated with luteolin, PDTC, and 60 μM luteolin followed by 50 μM PDTC treatment. The expression of Akt was detected by Western blotting analysis using specific antibodies against Akt. (**C**) PDTC recovered the phosphorylation of caspase-9 in luteolin-induced apoptotic HL-60 cells. HL-60 cells were treated with 0, 10, 20, 30, 40, and 50 μM PDTC followed by 60 μM luteolin for 3 h of treatment. The phosphorylated caspase-9 was detected by Western blotting analysis using a specific antibody against phosphocaspase-9. This experiment was repeated three times with similar results. (**D**) Schematic representation of action mechanism by which PDTC suppressed the luteolin-induced apoptosis in HL-60 cells. Luteolin inhibited Akt phosphorylation and, therefore, blocked the phosphorylation of caspase-9. PDTC might have resulted in cell survival by recovering the phosphorylation of Akt and caspase-9 that was inhibited by luteolin.

but the cleavage product of Akt began to appear after 3 h and then steadily increased in a time-dependent manner. We used Western blotting to determine whether phosphorylation of Akt had occurred in these samples. We only found phosphorylation of Akt to be increased in HL-60 cells pretreated with both PDTC and luteolin, not in those treated with either chemical separately (**Figure 9B**). To determine whether and to what extent PDTC might cause phosphorylation and inactivation of caspase-9 in luteolin-treated HL-60 cells, we treated the cells for 0.5 h with 10, 20, 30, 40, or 50 μM PDTC for 0.5 h and then treated them all with 60 μM luteolin for 3 h. PDTC not only increased the phosphorylation of caspase-9, but it increased it dose-dependently (**Figure 9C**).

DISCUSSION

PDTC is a low molecular weight thiol compound that has many functions in biological systems. It has been described as both an antiapoptosis-inducing agent (*26*, *27*) and apoptosis-inhibiting agent (*10*, *28*). In our previous study, we observed that luteolin could trigger cytochrome *c* released to cytosol, induce the processing of procaspase-9 and -3, and subsequently lead to the cleavage of PARP and DFF-45 (*7*). Therefore, in this study, we aimed to examine the effect of PDTC on luteolin-inducing apoptosis in HL-60 cells. We found that PDTC was able to inhibit luteolin-induced apoptosis in these cells (**Figures 2** and **3**), a finding that we confirmed by a DNA fragmentation test (**Figure 4**). The percentage of apoptotic cells analyzed by flow cytometry also indicated that PDTC dose dependently decreased the luteolin-induced apoptotic ratio (**Figure 5**), suggesting that it may act at some point along the luteolin-induced apoptotic pathway to inhibit the apoptotic effect in HL-60 cells.

We tried to identify at which point in the apoptotic cascade PDTC did this. Downstream, we found no cleavage of PARP or DFF-45 products in the cells pretreated cells (**Figure 6**), nor did we find any cleavage upstream in caspase-3 and -9 products (**Figure 7**). Therefore, we assumed the decrease of mitochondrial membrane potential ($\Delta\Psi_m$) and release of cytochrome *c* to cytosol did not happen in the PDTC-pretreated cells (**Figure 8**). Nevertheless, PDTC did inhibit the luteolin-induced cleavage of caspase-9 and caspase-3, suggesting that PDTC might have acted upstream from caspase-9.

Akt, which is thought to suppress apoptosis in signal transduction pathways, is inactivated by luteolin (*14*). In our HL-60 cells treated by luteolin alone, the cleavage product of Akt began to appear at 3 h and continued to increase over time, indicating that luteolin might have inactivated Akt by cleavage. Bagli et al. demonstrated that luteolin could inhibit VEGF-induced activation of Akt, a survival signal of the PI3K/Akt pathway (*25*). Akt has been found to be able to inhibit caspase-9 activity by directly phosphorylating caspase-9 and inhibiting protease activity and, therefore, suppressing downstream caspases and apoptosis (*15*, *24*). Caspases could be directly regulated by protein phosphorylation (*23*, *29*). Alteration in phosphatase activity can be induced by PDTC, and phosphorylation and inactivation of caspase-9 might be used by Akt to promote cell survival and, therefore, suppress downstream caspases and apoptosis (*16*, *23*, *29*). Because PDTC is able to inhibit luteolin-induced apoptosis on HL-60 cells through the upregulation of phosphorylation and activation of Akt, it may be able to cause the phosphorylation of caspase-9 downstream. Because Akt appeared to be cleaved slightly at 3 h when HL-60 cells were treated with luteolin, we chose 3 h to be the time point for testing the effect of PDTC on phosphorylation of caspase-9. HL-60 cells were treated with 0, 10, 20, 30, 40, and 50 μM PDTC followed by 60 μM luteolin for 3 h of treatment. We found that PDTC dose dependently increased phosphorylation of luteolin-inhibited caspase-9 (**Figure 9**).

Opportunities for future research abound. First, PDTC only failed to cause Akt phosphorylation and alter the phosphorylation status of caspase-9 when it was used alone to treat the HL-60 cells, suggesting the possibility that other proapoptotic proteins, such as Smac/DIABLO, HtrA2, and Apaf-1, may also be released in luteolin-induced apoptosis. Apaf-1 and procaspase-9 are known to be required for cytochrome *c*-induced proteolytic processing of caspase-3 (*30*). Which ones, in particular, could be investigated. Further studies could also investigate the chemical interactions between PDTC and luteolin and other signal transducers. Although we did not study it directly, more studies need to be done on the effects of PDTC

and flavonoids, for we have noticed that they produce an antagonistic effect when used in combination. The precise mechanism through which PDTC and luteolin regulate Akt pathway remains unknown. Also, because PDTC is a known inhibitor of the action of NKκB, it may act as an inhibitor of luteolin-induced apoptosis and further investigation might be needed to investigate this possibility.

In conclusion, we have demonstrated that PDTC inhibits luteolin-induced apoptosis, which it might do by causing the phosphorylation of caspase-9 in human leukemia HL-60 cells. Therefore, phosphorylation of caspase-9 might play an important role in determining the ultimate fate of HL-60 cells in the luteolin-induced apoptotic pathway. To the best of our knowledge, this study represents the first to provide evidence that in HL-60 cells pretreated with PDCT, Akt signal pathway participates in the up-regulation of the phosphorylation of caspase-9, consequently leading to the inhibition of luteolin-induced apoptosis. These findings are important because knowledge regarding phosphorylation of capsase-9 and signal transduction is crucial in designing preventive and curative therapies using flavonoids and antioxidants such as PDTC for cancer in humans.

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