Piceatannol-Induced Apoptosis Is Reversed by N-Acetyl-l-cysteine through Restoration of XIAP Expression

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Piceatannol, a naturally occurring stilbene derivative mainly found in grapes, possesses apoptotic activity in various cancer cell lines, in addition to potent antioxidant activity. In the current study, we showed that piceatannol exhibits potent cytotoxic effects in all tested leukemia cell lines (THP-1, HL-60, U937, and K562). These effects were accompanied by induction of DNA damage, an increase in the proportion of cells in the sub-G1 phase of the cell cycle, and inhibition of reactive oxygen species (ROS) generation. However, N-acetyl-l-cysteine (NAC), a strong ROS scavenger, significantly inhibited piceatannol-induced apoptosis, suggesting that piceatannol-induced apoptosis does not occur via inhibition of ROS generation. Piceatannol also resulted in a significant increase in mitochondrial depolarization, along with a decline in Bcl-2 expression, which was not restored by NAC. Conversely, ectopic Bcl-2 overexpression moderately inhibited piceatannol-induced apoptosis. Furthermore, piceatannol strongly inhibited X-linked inhibitor of apoptosis protein (XIAP) expression, which was restored by NAC. A transient knockdown of XIAP significantly increased piceatannol-induced apoptosis in the presence of NAC, suggesting that XIAP downregulation increases piceatannol-induced apoptosis, and that NAC could reverse this effect by increasing XIAP expression. Taken together, these results suggest that piceatannol induces apoptosis in human leukemia cell lines by downregulating XIAP expression, regardless of antioxidant activity.

Key words piceatannol; N-acetyl-l-cysteine; apoptosis; B-cell lymphoma 2; X-linked inhibitor of apoptosis protein

Leukemias are associated with an abnormal proliferation of white blood cells and are generally classified into one of two broad classes, lymphocytic and myelogenous leukemias.1) U.S. statistics for the year 2013 indicate that there were 48610 new diagnoses of leukemia, while the estimated number of deaths from leukemia was 23720.2) However, over the past decade, the annual death rate has been declining as a result of improvements in early diagnosis based on the analysis of morphological and immunophenotypic characteristics of neoplastic cells.3,4) Despite major therapeutic advances witnessed in the past 10–20 years, some forms of leukemias remain resistant to the available chemotherapeutic modalities. However, the identification of novel therapeutic targets could lead to the development of new antileukemic medicines. In this regards, chemotherapeutic agents that target key proteins in the death receptor, mitochondrial, and convergent pathways of caspase activation hold promise as novel treatments against leukemia.

Initially isolated from Euphorbia lagascae, piceatannol (3,5,3′,4′-tetrahydroxystilbene) is a hydroxylated analog of resveratrol that contains an additional phenolic group at position 3.5) Piceatannol has more potent antioxidant, anticancer, and antiproliferative activities than resveratrol.6) The antioxidant properties of piceatannol could be exploited for the prevention and treatment of multiple diseases owing its effect on the activation of nuclear factor erythroid-derived 2-like 2 (Nrf2), which upregulates antioxidant and phase II detoxification enzymes.7) In addition, the presence of ortho-dihydoxy structures in piceatannol contributes to its antioxidant activity, which enhances survival of cancer cells against hydroxyl radical-mediated cell death.8,9) Recently, Fu et al.10) reported that piceatannol significantly prevents β-amyloid-induced neuronal cell death by activating the phosphatidylinositol 3-kinase (PI3K)/Akt pathway which transmits cell survival signals. This result suggests that antioxidant activity of piceatannol prevents cells against reactive oxygen species (ROS)-mediated cell damage and death, ultimately leading to cell survival. In addition to these survival effects through antioxidant activity, piceatannol is also a promising anticancer therapeutic agent. Recently, Du et al.11) have shown that the antineoplastic effect of piceatannol could be mediated by upregulation of miR-181a. Schmeel et al.12) have shown that piceatannol inhibits the Wnt/β-catenin pathway, leading to selective toxicity to multiple myeloma cells. These results suggest that aside from ROS targets, other molecules and pathways appear to be involved in piceatannol-mediated apoptosis.

In the current study, we showed that piceatannol effectively increases apoptosis of leukemia cells by upregulating mitochondrial depolarization, and downregulating Bcl-2 and X-linked inhibitor of apoptosis protein (XIAP) expression, regardless of the canonical ROS-related apoptosis. We also

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found that N-acetyl-L-cystein (NAC) inhibits piceatannol-induced apoptosis by activating XIAP expression, which suggest that the antioxidant activity of piceatannol does not contribute to its pro-apoptotic properties, since both piceatannol and NAC possess potent ROS-scavenging activities.

MATERIALS AND METHODS

Reagents and Antibodies Piceatannol, propidium iodide (PI), NAC, H$_2$O$_2$, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Piceatannol was dissolved in dimethyl sulfoxide. Caspase-3 activity assay kit was obtained from R&D Systems (Minneapolis, MN, U.S.A.). A caspase-3 inhibitor, z-DEVD-fmk, a caspase-9 inhibitor, z-LEHD-fmk, a caspase-8 inhibitor, z-IETD-fmk, and a specific mitochondrial dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolylcarbocyanine iodide (JC-1) were obtained from Calbiochem (San Diego, CA, U.S.A.). RPMI 1640 medium was purchased from Invitrogen Corp. (Carlsbad, CA, U.S.A.) and fetal bovine serum (FBS) was purchased from GIBCO-BRL (Gaithersburg, MD, U.S.A.). Antibodies against Bcl-2, caspase-3, and XIAP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibody against β-actin was obtained from Sigma. Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin were purchased from GE Healthcare, U.S.A.

Cell Culture and Viability Assay Human leukemia THP-1, HL-60, U937, and K562 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and cultured in RPMI1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco BRL) at 37°C and 5% CO$_2$. Bcl-2-overexpressing U937 cells were a generous gift from Dr. T. K. Kwon (Department of Immunology, Keimyung University School of Medicine, Taegu, Korea). Cell viability was determined using an MTT assay, which is based on the conversion of MTT to formazan by mitochondrial enzyme.

DNA Fragmentation Assay Cells were treated with various concentrations of piceatannol and lysed in lysis buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% Triton X-100 for 30 min. The lysates were vortexed and cleared by centrifugation at 10000×g for 20 min. The fragmented DNA in the supernatant was extracted using an equal volume of neutral phenol–chloroform–isoamylalcohol (25:24:1, v/v/v) and analyzed electrophoretically on 1.0% agarose gel containing ethidium bromide.

Flow Cytometric Analysis Cells were harvested and washed with phosphate buffered saline (PBS), fixed in ice-cold 70% ethanol, and stored at 4°C. The cells were suspended in a cold PBS containing 100 µg/mL ribonuclease (RNase) A, 50 µg/mL PI, 0.1% (w/v) sodium citrate, and 0.1% (w/v) NP-40, and incubated on ice for 30 min in the dark. Flow cytometric analysis was carried out using flow cytometry (FACS Caliber, Becton Dickinson, San Jose, CA, U.S.A.) and Cell Quest software was used to determine the relative DNA content based on the presence of a red fluorescence.

Detection of ROS Level ROS production was assessed using fluorogenic probe 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA, Calbiochem). In brief, human leukemia U937 cells were incubated with 25 µM piceatannol and/or 10 µM NAC in the presence of 500 µM H$_2$O$_2$ for 1 h. Then, 30 µM H$_2$DCFDA was added. After a 30-min incubation, stained cells were washed and detected by fluorescence measurement of H$_2$DCFDA (λEX/EM=485 nm/535 nm).

Protein Extraction and Western Blot Analysis Cells were treated with piceatannol for 24 h and harvested with ice-cold PBS. For the isolation of total cellular protein, the cells were gently lysed for 20 min in ice-cold lysis buffer [20 mM sucrose, 1 mM EDTA, 20 mM Tris–Cl (pH 7.2), 1 mM diithiothreitol (DTT), 10 mM KCl, 1.5 mM MgCl$_2$, 5 µg/mL pepstatin A, 10 µg/mL leupeptin, and 2 µg/mL aprotinin]. The protein concentrations were measured using a Bio-Rad protein assay (Bio-Rad Lab., Hercules, CA, U.S.A.) according to the manufacturer’s instructions. Equal amount of protein was subjected to electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, U.S.A.). The blots were probed with specific antibodies for 1 h, incubated with the diluted enzyme-linked secondary antibody and visualized by enhanced chemiluminescence according to the recommended procedure (Amersham).

Assay of Caspase-3 Activity Caspase-3 activity was assayed using a colorimetric assay kit according to the manufacturer’s protocol (R&D Systems). Briefly, cells were lysed in a lysis buffer for 30 min in an ice bath. The lysates were centrifuged at 12000×g for 10 min, and 100 µg protein was incubated with 50 µg reaction buffer and 5 µg colorimetric tetrapeptides, DEVD-p-nitroaniline (pNA) for caspase-3 at 37°C for 2 h. The optical density of the reaction mixture was quantified spectrophotometrically at a wavelength of 405 nm.

Mitochondrial Membrane Potential Assay Mitochondrial membrane potential was measured by flow cytometry with the lipophilic cationic probe JC-1. JC-1 is a ratio metric, dual-emission fluorescent dye that is internalized and concentrated by respiring mitochondria and can reflect changes in its potential. The cells were treated with piceatannol for 24 h and harvested with ice-cold PBS. For the transfection, 450 µM JC-1 for 20 min at 37°C. The cells were subsequently washed once with cold PBS, re-suspended in a total volume of 500 µL and analyzed using flow cytometry.

Transient Knockdown of XIAP U937 cells were seeded on a 24-well plate at a density of 1×10$^5$ cells/mL and transfected with XIAP-targeted silencing RNA (siRNA, Santa Cruz Biotechnology) for 48 h. For the transfection, 450 µL growth medium was added to 20 nm siRNA duplex with the transfection reagent G-Fectin (Genolution Pharmaceuticals Inc., Seoul, Republic of Korea).

Statistical Analysis All data are presented as means±standard error (S.E.). Significant differences among the groups were determined using the unpaired Student’s t-test and one-way ANOVA. p<0.05 was accepted as being statistically significant. All the results shown in each of the figures in this article are representative of at least three independent experiments.

RESULTS

Piceatannol Increases Apoptosis Independently of Its Antioxidant Activity The effect of piceatannol on cell growth was evaluated by treating a variety of leukemia cell
lines (THP-1, HL-60, U937, and K562) with increasing concentrations of piceatannol (PIC) for 24 h and measuring cell viability using an MTT assay. The concentrations used were selected on the basis of preliminary cytotoxicity assessments. As shown in Fig. 1A, treatment with piceatannol significantly decreased the viability of all leukemia cell lines tested in a dose-dependent manner. To evaluate piceatannol-induced cell death in more detail, we analyzed the levels of fragmented DNA and cell cycle distribution of the treated leukemia cell lines. Treatment with piceatannol for 24 h induced a ladder pattern of internucleosomal fragmentation, a hallmark of apoptosis, in all leukemia cell lines (Fig. 1B). In addition, treatment with piceatannol resulted in an accumulation of cells in the sub-G1 phase of the cell cycle, suggesting that cells underwent apoptosis after exposure to piceatannol. Surprisingly, NAC, a potent antioxidant, completely suppressed piceatannol-induced apoptosis (Fig. 1C). To determine whether piceatannol-induced apoptosis is related to its antioxidant or oxidant activity, we measured ROS generation in the presence of H2O2. As expected, piceatannol itself significantly decreased ROS generation induced by H2O2, comparable to the effects of NAC treatment. A combined treatment with piceatannol and NAC also inhibited ROS levels to the same extent as treatment with either piceatannol or NAC alone (Fig. 1D). These results indicate that piceatannol potently induces apoptosis in leukemia cells, not increasing ROS production.

**Piceatannol Causes Apoptosis of Leukemia Cells by Activating the Caspase Cascade**

To investigate whether caspase-3 is related to piceatannol-induced apoptosis, we analyzed caspase-3 expression and activity in U937 cells. The results revealed that treatment with piceatannol upregulates the levels of active caspase-3 (cleavage form); however, NAC completely blocks piceatannol-induced caspase-3 cleavage (Fig. 2A). The role of caspase-3 in piceatannol-induced apoptosis was further investigated by pretreating the cells with z-DEVD-fmk, a specific caspase-3 inhibitor, and NAC for 1 h, followed by treatment with piceatannol. As shown in Fig. 2B, pretreatment with z-DEVD-fmk and NAC significantly attenuated piceatannol-induced caspase-3 activation. Additionally, the piceatannol-induced accumulation of cells in the sub-G1 phase was significantly suppressed by z-DEVD-fmk, z-IETD-fmk, and z-LEHD-fmk, indicating that piceatannol-induced apoptosis is mediated through the activation of caspases (Fig. 2C). NAC also downregulated the sub-G1 population induced by piceatannol. These results suggest that piceatannol induces apoptosis in leukemia cells through the caspase-dependent
A Decrease in Bcl-2 Partially Enhances Piceatannol-Mediated Apoptosis Concomitant with Mitochondrial Depolarization

To further investigate piceatannol-induced apoptosis, we focused on the role of Bcl-2. As expected, piceatannol inhibited Bcl-2 expression, whereas NAC itself did not induce any changes in Bcl-2 expression (Fig. 3A). Unexpectedly, piceatannol-induced downregulation of Bcl-2 was not reversed by NAC, suggesting that NAC prevents piceatannol-treated cell death through a mechanism that does not involve Bcl-2 expression. In addition, ectopic expression of Bcl-2 in U937 cells allowed partial recovery from piceatannol-induced cell death (Fig. 3B). We next evaluated mitochondrial depolarization in U937 cells upon piceatannol and NAC treatment. We observed that piceatannol-induced mitochondrial depolarization was not restored by NAC treatment (Fig. 3C). These results indicate that downregulation of Bcl-2 partially neutralizes piceatannol-induced apoptosis. Additionally, our results show that piceatannol induces mitochondrial depolarization.

NAC Reverses Piceatannol-Induced Apoptosis through Recovery of XIAP Expression

Since simultaneous inhibition of XIAP and Bcl-2 renders cells sensitive to apoptosis-inducing agents, we investigated whether piceatannol downregulates XIAP expression, which would point to a novel mechanism for piceatannol-induced apoptosis. As expected, piceatannol significantly downregulated XIAP expression in THP-1 and U937 cells, much less prominent in HL60 and K562 cells (Fig. 4A); however, XIAP expression unaltered by NAC (Fig. 4B). To test whether NAC was able to reverse piceatannol-induced XIAP downregulation, the cells were simultaneously treated with NAC and piceatannol. NAC significantly restored XIAP expression downregulated by piceatannol in all leukemia cell lines (Fig. 4C). We next determined whether silencing of XIAP blocks piceatannol-induced apoptosis and NAC-mediated cell survival. U937 cells were transfected with control and specific siRNA targeting XIAP for 48 h, and then exposed to piceatannol and/or NAC for 24 h. The results indicate that XIAP expression was significantly reduced in cells transfected with XIAP siRNA compared to cells transfected with control siRNA (Fig. 4D). In addition, transient knockdown of XIAP enhanced the piceatannol-induced increase in the proportion of cells in the sub-G1 phase. Remarkably, XIAP siRNA attenuated the NAC-induced cell survival in the presence of piceatannol. These results suggest that NAC reverses piceatannol-induced apoptosis by upregulating XIAP in leukemia cells, thereby leading to cell survival.

DISCUSSION

Piceatannol is a naturally occurring hydroxylated analog of resveratrol, mainly found in grapes, rhubarb, and sugar...
cane. In addition, it is believed to be somewhat chemically unstable, because of its natural cis–trans configuration that makes it prone to oxidation. 6,8) Piceatannol exhibits a potent antioxidant activity, which promotes cell survival in the face of several cellular stresses. 7,11,14) In addition to its antioxidant activity, piceatannol also exhibits antineoplastic activity in various cancer cells derived from the skin, prostate, bladder, and breast. 15) Interestingly, Morales and Haza 16) have confirmed that piceatannol induces apoptosis in HL-60 cells through a ROS-independent cell death pathway. Additionally, many researchers have found that ROS cause severe damages in cancer cells; thus, the use of bioactive compounds that can modulate oxidative stress in neoplastic cells appears to be a promising strategy for cancer treatment. 17) Further studies are needed to confirm the potential of piceatannol as an anticancer medicine.

ROS are key molecules in the apoptotic process, since they can induce apoptosis upon excessive accumulation inside cells. 19) When cells are incapable of counteracting ROS accumulation, mitochondrial dysfunction may occur, which perturbs the conversion of molecular oxygen to water. In damaged mitochondria, an imbalance between oxidants and antioxidants can alter biochemical reactions, which ultimately results in elevated ROS and defective antioxidant defenses, causing cell death. ROS can damage or modify DNA, but can also cause oxidation and peroxidation of lipid and proteins inside the cells. 19,20) ROS also downmodulate the levels of non-enzymatic antioxidants such as glutathione (GSH), which subsequently leads to the activation of complex signaling pathways, including some that are driven by the intrinsic apoptotic processes. 21) NAC, an antioxidant sulfhydryl substance that is well-known as an antidote to acetaminophen overdose, is a precursor in the formation of GSH that ultimately prevents damages to key cellular components. 22,23) Since the side effects of systemic NAC administration are generally mild, it can be used clinically for its ability to support antioxidant activity during toxic assault, stress, infection, and inflammatory conditions. 24) In the current study, we found that piceatannol induces apoptosis by activating caspase-3 which is a main executioner of apoptosis; therefore, caspase-8 and caspase-9 inhibitors significantly inhibit piceatannol-induced apoptosis, which suggests that piceatannol induces apoptosis via the activation of extrinsic and intrinsic pathways. In addition, piceatannol activated apoptosis without ROS accumulation; however, combined treatment with NAC and piceatannol completely restored cell survival, which suggests that piceatannol-

Fig. 3. Piceatannol Induces Apoptosis by Downregulating Bcl-2 Expression

(A) U937 cells were treated with 10 µM NAC and/or 25 µM piceatannol (PIC) for 24h, and cell lysates were prepared for Western blotting to detect the Bcl-2 protein. (B) Bcl-2-expressing U937 cells were seeded at a density of 1×10⁵ cells/mL and treated with 25 µM PIC and sub-G₁ populations were detected by flow cytometry as an index of apoptosis. Western blot analysis was performed to confirm ectopic Bcl-2 expression in U937 cells (upper panel). (C) Mitochondrial depolarization was determined by fluorescence measurement of JC-1. All data are presented as the mean±S.E. Significant differences between the groups were determined using the unpaired Student’s t-test and one-way ANOVA [*, p<0.05 vs. untreated control and PIC-treated U937/Vec (B); *, p<0.05 vs. untreated group and NAC-treated group (C)]. UT, untreated; PIC, piceatannol; NAC, N-acetyl-L-cysteine.
induced apoptosis occurs through a ROS-independent cell death pathway. NAC also attenuated piceatannol-induced caspase activity, which likely contributes to cell survival. Surprisingly, piceatannol and NAC, both potent antioxidants, lead to different cellular responses. Our results also revealed that piceatannol downregulates Bcl-2 expression, and that this effect is accompanied by mitochondrial depolarization. Interestingly, NAC did not restore the piceatannol-induced Bcl-2 decline and mitochondrial depolarization, suggesting that piceatannol increases apoptosis by inhibiting mitochondrial-mediated Bcl-2 expression, which are not targets of NAC. In addition, XIAP represents a group of structurally interrelated proteins that block apoptosis in the heart of the apoptotic machinery by cooperating with Bcl-2. XIAP is constitutively expressed in a variety of malignant tumors including prostate cancer, hepatoma, as well as acute and chronic leukemia. In the current study, we observed that piceatannol significantly downregulates expression of XIAP; however, NAC restores downregulated XIAP expression induced by piceatannol, and transient knockdown of XIAP inhibits NAC-mediated cell survival, suggesting that NAC reverses the effect of piceatannol-induced cell death by activating XIAP expression.

CONCLUSION

In summary, the current study reveals that piceatannol is a potential therapeutic candidate for treatment of human leukemia by targeting the downregulation of Bcl-2 and XIAP, and that these effects are independent of piceatannol’s antioxidant activity. However, further studies are needed to elucidate the pleiotropic mechanisms of action of piceatannol and NAC. These effective antioxidants might lead to the development of novel effective chemotherapeutic agents for the treatment of leukemias.

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**Conflict of Interest** The authors declare no conflict of interest.

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