Abstract. Background: Chemotherapy agents, particularly those that can induce apoptosis, are the major intervening strategy in the treatment of leukemia. In this study, we investigated the effects of baicalin (a compound obtained from Scutellaria baicalensis Georgi and S. rivularis Benth Labiateae) on the viability, induction of apoptosis and associated mechanism in human leukemia HL-60 cells. 

Materials and Methods: The cell viability and apoptosis was examined by flow cytometric analysis. The results showed that baicalin induced cytotoxicity in a dose- and time-dependent manner through the activation of caspase-3, as shown by treatment of HL-60 cells with an inhibitor of caspase-3 (z-VAD-fmk). Baicalin increased the levels of ROS, Ca²⁺ and decreased mitochondrial membrane potential in HL-60 cells. Western blot demonstrated that baicalin promoted the levels of Gadd153, Bax, cytochrome c and caspase-3 and -12, but decreased the levels of Grp78 and Bcl-2 in HL-60 cells. Conclusion: Baicalin was found to induce apoptosis in HL-60 cells through multiple pathways.

Baicalin is one of the active compounds of Scutellaria baicalensis Georgi and S. rivularis Benth (Labiateae) that has been widely employed in traditional Chinese herbal medicine for centuries. It is a glucuronic compound of baicalein, and both baicalin and baicalein have been demonstrated to be able to induce cell death, apoptosis or necrosis (1-3). Baicalin has been shown to be effective in human T-cell leukemia virus-1 (4), in the treatment of HIV-infected patients (3) and on bladder (5) and prostate cancer cells (6). Many studies have demonstrated that baicalin inhibits cell proliferation (3), modulates cell cycle (5), induces cell apoptosis (6), and up-regulates the expression of p53, bax and p21 proteins (4, 7). Based on the reports from earlier investigators, it was indicated that baicalin has a cytotoxic effect on leukemia cells (3, 4). Recently, it was reported that baicalin-induced apoptosis is mediated by the Bcl-2-dependent, but not p53-dependent pathway in human leukemia cell line (8). However, the role reactive oxygen species (ROS) play in baicalin-induced apoptosis in human leukemia HL-60 cells is not clear. Therefore, in the present study, the association of ROS production with baicalin-induced apoptosis in HL-60 cells was investigated.

Materials and Methods

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Human promyelocytic leukemia cell line (HL-60). The HL-60 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC) and was cultured in RPMI-1640 media containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), and maintained at 37 °C in a humidified cell culture incubator containing 5% CO2.

Cell viability of HL-60 cells treated with baicalin determined by flow cytometry. The HL-60 cells were plated in 24-well plates at a density of 2x10⁵ cells/well and grown for 24 h. The concentrations of baicalin (0, 25, 50, 100 and 150 µM) were added to the cells while only adding DMSO (solvent) for the control regimen and grown at 37°C, 5% CO2 and 95% air for 6, 12, 24 and 48 h treatment. The flow cytometric assay was used, as described previously (9, 10), for determining cell viability.

Flow cytometry analysis of DNA content for apoptosis from HL-60 cells treated with baicalin. Approximately 2x10⁵ HL-60 cells/well in 24-well plate with 100 µM baicalin were incubated for 6, 12, 24, 48 and 72 h. The cell apoptotic ratio was analyzed by flow cytometry. Cells were harvested, washed twice with phosphate-buffered saline (PBS), and fixed gently (drop by drop) in 70% ethanol for at least 2 h at -20°C. Fixed cells 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 in the dark, and then stained with 50 µg/mL PI. The stained cells were analyzed using a FACScan laser flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an argon ion laser at 488 nm wavelength connected to ModFit LT cell cycle analysis software (Verity Software, Topsham, ME, USA) (9, 10).

Caspase-3 activity of HL-60 cells treated with baicalin. Approximately 2x10⁵ HL-60 cells/well in 24-well plate with concentrations of baicalin (0, 25, 50, 100, and 150 µM) were incubated for 24 h. Cells were harvested by centrifugation. A 50 µL of 10 µM substrate solution (PhiPhiLux is a unique class of substrates for caspase-3) was then added to the cell pellet (1x10⁵ cells per sample). Cells were incubated at 37°C for 60 min then washed once by adding 1 mL of ice cold PBS and re-suspended in 1 mL fresh PBS. A substrate of PhiPhiLux (OncoImmunin, Inc., Gaithersburg, MD, USA) which can penetrate into the cell nucleus, is converted to the fluorescent form when it is cleaved by the protease activity of caspase-3. Cells were analyzed with a flow-cytometry (Becton-Dickinson, San Jose, CA, USA) equipped with an argonion laser at 488 nm wave-length. The caspase-3 activity was determined and analyzed, as described previously (9-11).

Detection of ROS in HL-60 cells after treatment with baicalin by flow cytometry. Approximately 2x10⁵ HL-60 cells/well in 24-well plate were incubated with baicalin (0, 25, 50, 100 and 150 µM) for 2 h. The cells were harvested and washed twice, re-suspended in 500 ml of 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) (10 µM) and incubated at 37°C for 30 min and analyzed by flow cytometry, as described previously (9,10).

Effects of baicalin on the production of Ca²⁺ in HL-60 cells. Approximately 2x10⁵ HL-60 cells/well in 24-well plate were incubated for 24 h with baicalin (0, 25, 50, 100 and 150 µM). The cells were harvested and washed twice, once for apoptosis analysis and the other for re-suspension in Indo 1/AM (1-[2-Amino-5-(6-carboxyindol-2-yl)phenoxyl]-2-(2'-amino-5'-methylphenoxy)ethane-N,N',N'-tetra acetic Acid Pentaacetoxymethyl Ester) (3 µg/ml) and incubated at 37°C for 30 min and analyzed by flow cytometry, as described previously (9, 10).

Effects of baicalin on the mitochondrial membrane potential (ΔΨm) in HL-60 cells. Approximately 2x10⁵ HL-60 cells/well in 24-well plate were incubated for 24 h with baicalin (0, 25, 50, 100 and 150 µM) to detect the changes in ΔΨm. The cells were harvested and washed twice, re-suspended in 500 ml of 3,3'-Dihexyloxcarbocyanine (DiOC₆) (4 mol/L) and incubated at 37°C for 30 min; then analyzed by flow cytometry, as described previously (9, 10).

Western blotting for examining the effect of baicalin on p53, bax, Bcl-2, Gpr78, Gadd153, cytochrome c, caspase-3 and -12 of HL-60 cells. The total protein was collected from HL-60 cells after treatment with baicalin (0, 25, 50, 100 and 150 µM) for 48 h and the p53, bax,
Bcl-2, Grp78, Gadd153, cytochrome c, caspase-3 and -12 were measured using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as described elsewhere (12-14).

Statistical analysis. Student’s t-test and Tukey’s test were used to analyze the statistical differences between the baicalin-treated and control groups.

Results

Effects of baicalin on cell viability of HL-60 cells. The results from flow cytometric analysis indicated that the percentage of viable cells was significantly different between baicalin-treated and control groups. Increasing the dose of baicalin and/or time of incubation led to a decrease in the percentage of viable cells. The effects of baicalin were dose- and time-dependent (Figure 1A and B).

Induction of apoptosis by baicalin on HL-60 cells. We investigated the occurrences of apoptosis from HL-60 cells by treatment with baicalin. As shown in Figure 2, 100 µM baicalin induced apoptosis in a time-dependent manner.

Inhibition of baicalin-induced caspase-3 activity and apoptosis by the caspase inhibitor z-VAD-fmk on HL-60 cells. We examined whether or not caspase-3 activation is involved in the apoptosis of cells which were triggered by baicalin. We examined capase-3 activity using flow cytometric analysis and the result indicated that baicalin induced caspase-3 activity and these effects are dose-dependent (Table I). The HL-60 cells were pretreated with the cell permeable broad-spectrum caspase inhibitor (z-VAD-fmk), 3 hours prior to the treatment with baicalin. After treatment with z-VAD-fmk and baicalin, inhibition of baicalin-mediated caspase-3 activation in HL-60 cells was accompanied by the marked attenuation of baicalin-induced apoptotic cell death (Figure 3A and B).

Detection of ROS in HL-60 cells after treatment with various concentrations of baicalin by flow cytometry. The ROS level is significantly different between baicalin-treated groups and the controls. Baicalin increased ROS levels is examined at 30 min after cells were exposed to baicalin and these effects are dose-dependent (Table I).
Figure 4. continued

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Effects of baicalin on the levels of $Ca^{2+}$ from HL-60 cells. The $Ca^{2+}$ level is significantly different between the baicalin-treated groups and the controls. Increasing the dose of baicalin led to an increase in the level of $Ca^{2+}$ in HL-60 cells (Table I). The effects of baicalin on the levels of $Ca^{2+}$ production are dose-dependent.
Effects of baicalin on the mitochondrial membrane potential ($\Delta \Psi_m$) of HL-60 cells. The $\Delta \Psi_m$ level is significantly different between baicalin-treated groups and the controls. Increasing the dose of baicalin led to a decrease in the $\Delta \Psi_m$ in HL-60 cells (Table I).

Western blotting for Grp78, Gadd153, Bax, Bcl-2, cytochrome c, caspase-3 and -12 of HL-60 cells. The results (Figure 4; Panel A: Grp78; Panel B: Gadd153; Panel C: Bax; Panel D: Bcl-2; Panel E: cytochrome c; Panel F: caspase-3 and Panel G: caspase-12) indicate that the levels of Gadd153, Bax, cytochrome c, caspase-3 and-12 increased; Grp78 and Bcl-2 decreased. This may have led to the apoptosis in these examined cells. Figure 5 shows the Western blot of cytochrome c from total cells, mitochondria and cytosol and indicates that baicalin promoted cytochrome c release from the mitochondria.

Discussion

Although many studies have demonstrated that baicalin induces cytotoxicity and apoptosis in human cancer cells, there is still no available information about the involvement of Gadd153 in baicalin-induced apoptosis. In growing cells, Gadd153 is ubiquitous expressed at very low levels, but is highly expressed in response to numerous cellular stresses (15). It is known that agents that strongly affect endoplasmic reticulum (ER) function are identified as strong inducers of Gadd153, such as thapsigargin which depletes ER calcium stores, tunicamycin which blocks protein glycosylation, and dithiothreitol which disrupts disulfide bond formation (16), suggesting that induction of Gadd153 is highly responsive to ER stress. Therefore, the association of Gadd153 levels and ROS in HL-60 cells after exposure to baicalin was investigated.

The present in vitro studies demonstrated that baicalin exhibited a concentration-dependent and growth inhibitory effect on HL-60 leukemia cells. The results showed that HL-60 cells were affected by baicalin-induced apoptosis through the activation of both caspase-3 and caspase-12 pathways. At least 3 apoptosis pathways have been determined (17-19): (i) apoptosis mediated by ligation of death ligands to their receptors (‘extrinsic’); (ii) apoptosis mediated by mitochondria-involved signalling (‘intrinsic’); (iii) the endoplasmic reticulum (ER)-induced apoptotic cell death. Specific markers of ER stress, such as caspase-12 and Gadd153 were further investigated. The results showed that baicalin induces ROS production causing the increase in Gadd153 levels which may promote two pathways; one leading to the activation of caspase-12 followed by apoptosis and the other leading to the decrease of MMP levels followed by cytochrome c release, promoting caspase-3 activation leading to apoptosis. Caspase-12 is located on the cytoplasmic side of ER and is proteolytically activated following ER stress and m-calpain activation (20). It is known that ER stress is associated with agent-induced apoptosis in cancer cells (21, 22), and ER dysfunction can activate the unfolded protein response via the activation of two divergent processes: suppression of the initiation process in global protein synthesis and expression of glucose-regulated protein 78 (Bip/Grp78) and the C/EBP homologous transcription factor CHOP/Gadd153 (21). Moreover, caspase-12 plays a central role in the initiation of ER stress-induced cell death in the mouse system, and is no need for the mitochondrial pathway of death to take place (23). Our results also showed that baicalin increased the levels of Ca$^{2+}$ in examined HL-60 cells. It was reported that ER might act as a third subcellular compartment implicated in apoptotic execution. The ER is the predominant site for protein synthesis and folding, and for cellular calcium storage (24); accumulation of misfolded...
Figure 6. Proposed baicalin mechanism of action for apoptosis in HL-60 cells. Baicalin increased the levels of ROS which may have caused ER stress (Gadd153 increased expression) followed by the increase of Bax, caspase-3 and -12 expressions before leading to apoptosis in HL-60 cells.
proteins and changes in Ca^{2+} homeostasis in ER result in ER stress and lead to apoptotic cell death (23).

It was reported that pure baicalin and/or extract from Sho-saiko-to, a traditional Japanese medicinal herbal preparation for treating various chronic liver diseases, exert anti-tumor effects on human hepatoma and pancreatic cancer cell lines and rat glioma cells (25, 26). Baicalin has a more potent effect than baicalein (which does not carry a cancer cell lines and rat glioma cells (25, 26). Baicalin has anti-tumor effects on human hepatoma and pancreatic preparation for treating various chronic liver diseases, exerting on ER stress and lead to apoptotic cell death (23).

In this study, it was also shown that baicalin promoted the levels of Bax, but inhibited the levels of Bcl-2, in HL-60 cells; these factors might lead to the decrease of the levels of ΔΨ_{m} followed by apoptosis. Mitochondria are one of the most susceptible organelles to apoptotic stimulus and have, next to their respiratory function, a crucial role in apoptotic signalling. Bcl-2 and several anti-apoptotic relatives (such as Bcl-xL and Mcl-1) have the function to associate with and maintain the integrity of the mitochondrial outer membranes and prevent apoptosis (30).

Although the induction of apoptosis in human leukemia cells by baicalin was reported, the exact mechanism and the role of ROS and Ca^{2+} and Gadd153 were still unknown. However, various evidence from other studies suggest that induction of apoptosis by baicalin might be mediated through different pathways. Other investigators showed that baicalein could inhibit the activity of topoisomerase II in the human hepatocellular carcinoma cell lines, and the induced apoptosis was not conducted via the regulation of the Fas antigen (31). An interesting point is that both baicalin and baicalein have been shown to be selective inhibitors of lipoxgenase and cyclooxygenase in platelets and leukocytes (32, 33). Although it was reported that baicalin can induce the activity of quinone reductase in a murine hepatoma cell line (33), it remains to be further investigated through which pathways apoptosis is promoted in the different leukemia cancer cells by baicalin.

Conclusion

Our study has shown that baicalin is cytotoxic to the human leukemia HL-60 cell line through the ROS-mediated induction of apoptosis. In other words, baicalin can induce apoptosis through two caspases pathways: one is the mitochondrial-dependent pathway (caspase-3 dependent) and the other is the mitochondrial-independent pathway (caspase-12 dependent) as shown in Figure 6. Therefore, baicalin could be a promising chemopreventive agent for leukemia.

Acknowledgements

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References


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