

## RESEARCH COMMUNICATION

# Baicalin Induces Apoptosis in Leukemia HL-60/ADR Cells via Possible Down-regulation of the PI3K/Akt Signaling Pathway

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### Abstract

**Background:** The effect and possible mechanism of traditional Chinese medicine, baicalin, on the PI3K/Akt signaling pathway in drug-resistant human myeloid leukemia HL-60/ADR cells have been investigated in this current study. **Methods:** HL-60/ADR cells were treated by 20, 40, 80  $\mu\text{mol/L}$  baicalin followed by cell cycle analysis at 24h. The mRNA expression level of the apoptosis related gene, Bcl-2 and bad, were measured by RT-PCR on cells treated with 80  $\mu\text{mol/L}$  baicalin at 12, 24 and 48hr. Western blot was performed to detect the changes in the expression of the proteins related to HL-60/ADR cell apoptosis and the signaling pathway before and after baicalin treatment, including Bcl-2, PARP, Bad, Caspase 3, Akt, p-Akt, NF- $\kappa\text{B}$ , p-NF- $\kappa\text{B}$ , mTOR and p-mTOR. **Results:** Sub-G1 peak of HL-60/ADR cells appeared 24 h after 20  $\mu\text{mol/L}$  baicalin treatment, and the ratio increased as baicalin concentration increased. Cell cycle analysis showed 44.9% G0/G1 phase cells 24 h after baicalin treatment compared to 39.6% in the control group. Cells treated with 80  $\mu\text{mol/L}$  baicalin displayed a trend in decreasing of Bcl-2 mRNA expression over time. Expression level of the Bcl-2 and PARP proteins decreased significantly while that of the PARP, Caspase-3, and Bad proteins gradually increased. No significant difference in Akt expression was observed between treated and the control groups. However, the expression levels of p-Akt, NF- $\kappa\text{B}$ , p-NF- $\kappa\text{B}$ , mTOR and p-mTOR decreased significantly in a time-dependent manner. **Conclusions:** We conclude that baicalin may induce HL-60/ADR cell apoptosis through the PI3K/AKT signaling pathway.

**Keywords:** Baicalin - HL-60/ADR cells - AKT signaling pathway - apoptosis

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### Introduction

The PI3K/Akt signaling pathway is a well-established and important intracellular signal transduction pathway. Studies have shown that it plays a key role in the inhibition of apoptosis and the promotion of cell proliferation by affecting the activation state of a variety of downstream effector molecules. It is closely related to the occurrence and development of a variety of human tumors such as hematopoietic system tumors (Jucker et al., 2002; Xu et al., 2003; Levine et al., 2005; Oda et al., 2005). Recent studies have shown that some traditional Chinese medicine or Chinese medicine extracts can affect tumor cell apoptosis through the PI3K/Akt signaling pathway. Many Chinese researchers have used traditional Chinese medicine such as osthole, emodin, meisoindigo, curcumin, and epigallocatechin gallate ester to study induced tumor cell apoptosis (Zheng et al., 2007; Cui et al., 2008; Liu et al., 2008; Wang et al., 2011; Xu et al., 2011), and have obtained encouraging results. Leukemia is a malignant tumor of the hematopoietic system. Leukemia cell resistance to chemotherapeutic drugs is now believed

to be an important cause of chemotherapy failure. It has thus become popular in recent years to study induced leukemia cell apoptosis through the intervention of the PI3K/Akt signaling pathway to overcome drug resistance. Baicalin (chemical structural formula: C<sub>21</sub>H<sub>18</sub>O<sub>11</sub>) is an active ingredient extracted from *Scutellaria* (Labiatae). Many studies have shown that baicalin can play an anti-tumor role through various means (Hoffman and Liebermann, 2008; Wierstra and Alves, 2008; Zhang et al., 2009). For example, the promotional effect of prostaglandin synthetase on tumor cells can be blocked by reducing cyclooxygenase-2 expression to inhibit prostaglandin E<sub>2</sub> production. And tumor cell apoptosis can be induced by suppressing cell proliferation, blockage of cell cycle progression, reducing Akt phosphorylation and inhibiting telomerase activity. Extracellular matrix metalloproteinases can also be inhibited to against tumor angiogenesis. Our previous studies have showed that baicalin has the same growth inhibition effect on the drug-sensitive human myeloid leukemia HL-60 and drug-resistant HL-60/ADR cells (Chen et al., 2008; Zheng et al., 2009). In this current study, we have further investigated

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the possible mechanism of the PI3K/Akt signaling pathway in baicalin-treated HL-60/ADR cells, which would serve to provide a theoretical basis to overcome drug resistance of leukemia cells.

## Materials and Methods

### Agents

Baicalin (MW: 446.35, HPLC-determined purity: 98.12%) was purchased from Nanjing Qingze Medical Technology Co., Ltd, dissolved in dimethyl sulfoxide (DMSO, Sigma Co.) to a stock concentration of 50,000  $\mu\text{mol/L}$ , and stored at  $-20\text{ }^{\circ}\text{C}$  in the dark.

### Cell culture

The HL-60/ADR cells were doxorubicin-induced acute myeloid leukemia cells produced by Tianjin Institute of Hematology, Chinese Academy of Medical Sciences, and maintained by Fujian Institute of Hematology in RPMI 1640 medium (Gibco Co.) with 0.4  $\mu\text{g/L}$  doxorubicin (ADR) and 10% fetal bovine serum (Tianjin Haoyang Biological Manufacture Co., Ltd.) in a  $37\text{ }^{\circ}\text{C}$ , 5%  $\text{CO}_2$  humidified incubator. The medium was changed to ADR-free RPMI1640 medium two weeks before experiments. Logarithmic growth phase cells were used in the experiments.

### Cell cycle analysis

Cells were collected 24 h after baicalin treatment at different concentrations (20, 40, and 80  $\mu\text{mol/L}$ ) with untreated control cells, and used for cell cycle analysis based on the instructions of the Cell Cycle Test Plus DNA Reagent Kit (Beckton Dickinson). The cells were resuspended in 1 ml buffer solution for three times, and the cell concentration was adjusted to  $1.0 \times 10^6$  /ml. Two hundred and fifty  $\mu\text{l}$  Solution A was added and mixed at room temperature for 10 min before the addition of 200  $\mu\text{l}$  Solution B. The solution was incubated at room temperature for 10 min before 200  $\mu\text{l}$  Solution C was added and mixed. The samples were incubated on ice for another 10 min followed by flow cytometry (BECKMAN COULTER FC500) detection.

### RT-PCR detection of the mRNA expression of the *Bcl-2* and *bad* genes

Cells were collected 12, 24, and 48 hours after 80  $\mu\text{mol/L}$  baicalin treatment with untreated control cells. After cell washing in PBS, the Trizol extraction kit (Gibco BRL) was used to extract total RNA. The mRNA expression of the *Bcl-2* and *bad* genes was detected by RT-PCR with  $\beta$ -actin as an internal reference.  $\beta$ -actin upstream primer: 5'-GGCATGGGTCAGAAGGATT CC-3'; downstream primer: 5'-ATGTCACGCACGATTTCCCGC-3'; the size of the PCR-amplified product: 500 bp. *Bcl-2* upstream primer: 5'-CGACGACTTCTCCCGCCGCTACCGC-3'; downstream primer: 5'-CCGCATGCTGGGGCCGTACAGTTCC-3'; the size of the PCR-amplified product: 318 bp. *Bad* upstream primer: 5'-GAGGAAGATGAAGGGATGGA-3'; downstream primer: 5'-TCAACGCTGTACGTCAGCTC-3'; the size of the synthesized product: 400 bp. The upstream primer of

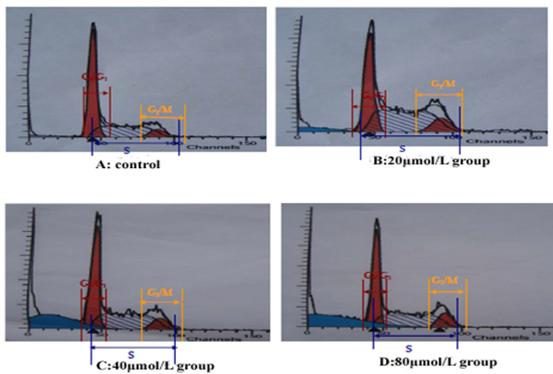
the negative control: 5'-AGTTCACCTTGATGCCGTTC-3'; downstream primer: 5'-TGCTTCAGCCGCTACCC-3'; the size of the synthesized product: 286 bp. The total volume of the PCR reaction was 25  $\mu\text{L}$ , including 1.5  $\mu\text{L}$  cDNA, 2.5  $\mu\text{L}$  10  $\times$  Mg-free buffer, 1.5  $\mu\text{L}$  25 mM  $\text{MgCl}_2$ , 0.5  $\mu\text{L}$  10 mM dNTP, 0.5  $\mu\text{L}$  25 pmol/ $\mu\text{L}$  primers, and 1.5 U TaqDNA polymerase (Sino-American Biotechnology Co., Ltd.). The PCR-reaction was performed as follows using a Model 2400 thermocycler (PE): 1 cycle of 5 min pre-denaturation at  $99\text{ }^{\circ}\text{C}$ , then 35 cycles of 30 s at  $94\text{ }^{\circ}\text{C}$ , 30 s annealing at  $60\text{ }^{\circ}\text{C}$ , 30 s extension at  $72\text{ }^{\circ}\text{C}$ , and followed by 1 cycle of 5 min extension at  $72\text{ }^{\circ}\text{C}$ . The PCR ratios of the target genes to  $\beta$ -actin were used for semi-quantitative analysis. The experiments were repeated three times.

### Western blot detection of the expression of the proteins including *Bcl-2*, *PARP*, *Bad*, *Caspase-3*, *Akt*, *p-Akt*, *NF- $\kappa$ B*, *p-NF- $\kappa$ B*, *mTOR*, and *p-mTOR*

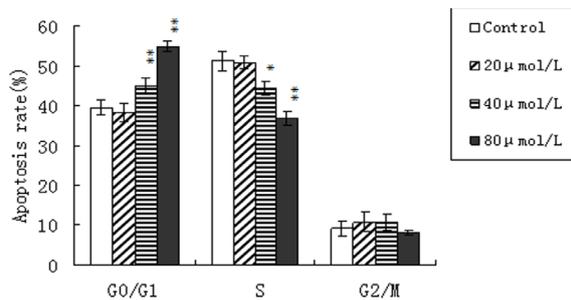
The sample cells were collected 12, 24 and 48 hours after 80  $\mu\text{mol/L}$  baicalin treatment with untreated control cells. After PBS washing, the cells were lysed at a ratio of  $10^6$  cells in 100  $\mu\text{l}$  lysis buffer with 1  $\mu\text{l}$  protease inhibitors (Pierce Co.), followed by 30 min incubation at  $4\text{ }^{\circ}\text{C}$  and 6 min centrifugation at 12,000 r / min. The supernatant was then aspirated. The CBB-G250 staining assay was performed to determine protein content. After 5 min denaturation at  $99\text{ }^{\circ}\text{C}$ , the same amount of protein samples was added to each lane. After discontinuous 10% SDS-polyacrylamide gel electrophoresis, the samples were transferred to cellulose acetate membrane. Based on the instructions of different monoclonal antibody kits, western-blot detection was performed briefly as follows: 1 hour blocking of the cellulose acetate membrane, then the addition of the diluted primary antibody, and incubation overnight at  $4\text{ }^{\circ}\text{C}$ . After membrane washing in the wash solution, 1:3000 diluted horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG was added followed by 1 hour incubation at room temperature. After membrane washing in the wash solution again, the substrate was added for development.  $\beta$ -actin was used in the enhanced chemiluminescence (ECL) experiments as an internal reference to ensure consistency in the amount of sample proteins.  $\beta$ -actin and caspase-3 mAb (NeoMarkers Co.), *Bcl-2* and *NF- $\kappa$ B* (eBioscience Corp.), *PARP* (Santa Cruz Co.), *Bad* mAb (Cell signal Transduction Co.), *Akt*, *p-Akt*, *p-NF- $\kappa$ B*, *m-TOR*, and *p-mTOR* (Cell Signaling Technology Corp.), protease inhibitors (Pierce Co.), Protein Detector LumiGLO Western Blot Kit containing horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (KPL Co.) were used in the research. The primary antibody dilution was 1:100, except *Bcl-2*, *NF- $\kappa$ B*, and phosphor-*NF- $\kappa$ B* were 1:200 dilutions. The secondary antibody Dilutions were all performed at 1:2000 dilutions. The experiments were repeated three times.

### Statistical analysis

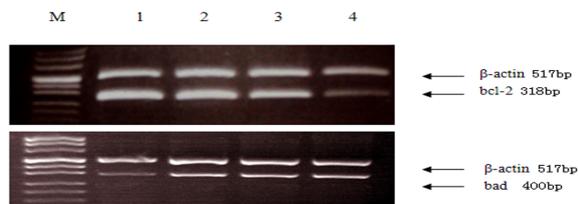
The obtained data were analyzed by t-test using the SPSS11.5 statistical package and represented as  $X \pm \text{SD}$ .



**Figure 1.** Cell cycle analysis of HL-60/ADR cells after 24h of baicalin treatment. A: control, B: 20 μmol/L group, C: 40 μmol/L group, D: 80 μmol/L group



**Figure 2.** Cell Cycle Analysis of HL-60/ADR Cells after 24h of Baicalin Treatment



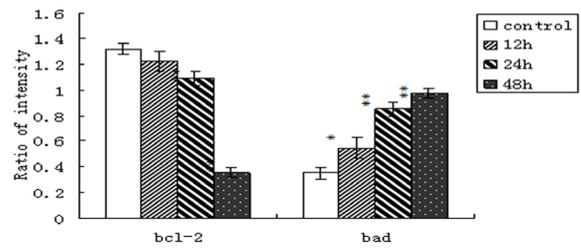
**Figure 3.** Effects of Baicalin at 80 μmol/L on the mRNA Expression of Apoptotic Related Genes of HL-60/ADR Cells for Different Time by RT-PCR. M: PUC8 marker Lane 1:control; Lane 2:12h group; Lane 3:24h group; Lane 4:48h group

## Results

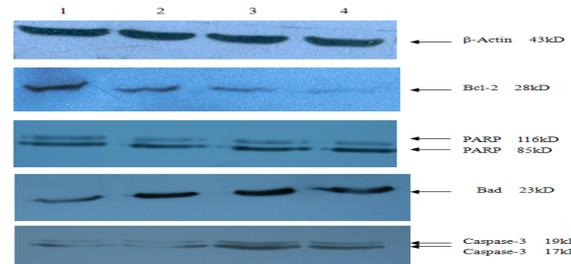
### DNA ploidy analysis

A 5.80% sub-G1 peak appeared 24 h after 20 μmol/L baicalin treatment, and the ratio increased as baicalin concentration increased (25.46% for 40 μmol/L and 28.98% for 80 μmol/L). Cell cycle analysis showed 39.57% G0/G1 phase cells in the control group and 44.94% in the sample group 24 h after baicalin treatment. The difference between the groups was statistically significant ( $P < 0.05$ ). Yet the percentage of G2 and S phase cells decreased as shown in Figure 1 (A-D) and Figure 2. RT-PCR detection of the mRNA expression of the Bcl-2 and bad genes

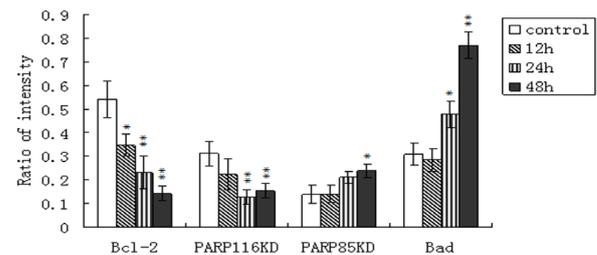
The amplified band intensity of Bcl-2 and bad gene mRNA in the electrophoresis images was scanned using β-actin as an internal reference. No reduction of the Bcl-2 gene expression in HL-60/ADR cells was observed after 80 μmol/L baicalin treatment for 12 hr; However, a trend in decreasing of Bcl-2 expression was shown with treatment time and dramatically decreased at 48 hr. Yet the mRNA expression of the bad gene gradually increased



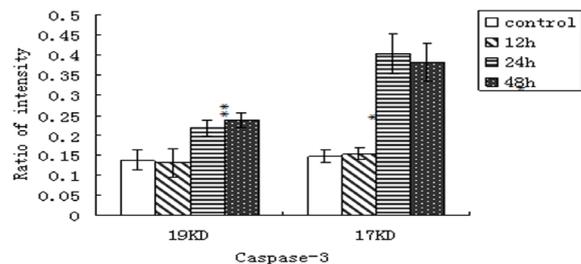
**Figure 4.** The Ratio of Intensity of Baicalin at 80 μmol/L on the bcl-2 and bad mRNA Expression of HL-60/ADR Cells for Different Time by RT-PCR



**Figure 5.** Effects of Baicalin at 80 μmol/L on the Expression of Apoptotic Related Proteins of HL-60/ADR Cells by Western Blot Method. Lane 1:control; Lane 2:12h group; Lane 3:24h group; Lane 4: 48h group



**Figure 6.** The Ratio of Intensity of Baicalin at 80 μmol/L on the Expression of Apoptotic Related Proteins of HL-60/ADR Cells by Western Blot Method

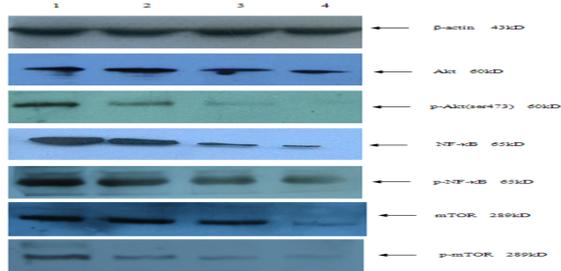


**Figure 7.** The Ratio of Intensity of Baicalin at 80 μmol/L on the Expression of Caspase-3 proteins of HL-60/ADR Cells by Western Blot Method

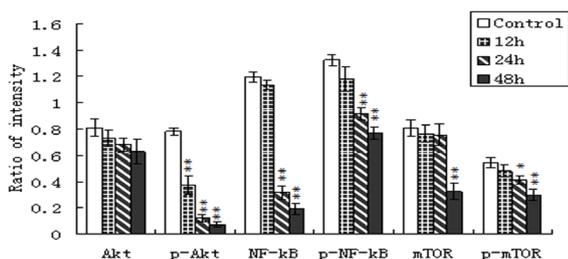
with treatment time. The difference from the control group was statistically significant (Figure 3-4).

### The effect of baicalin on the expression of apoptosis-related proteins

The same concentration (80 μmol/L) of baicalin was added to the HL-60/ADR cells at three different times, and the proteins were extracted accordingly. Western blot analysis was performed with consistent results, i.e., 12, 24, and 48 h after 80 μmol/L baicalin treatment, the expression level of the Bcl-2 and PARP (116 kD) proteins decreased significantly while that of the PARP (85 kD),



**Figure 8. Effects of Baicalin at 80 μmol/L on the Protein Expression of PI3K/Akt Signal Transduction Pathway of HL-60/ADR Cells by Western Blot Method.** Lane 1:control; Lane 2:12h group; Lane 3: 24h group; Lane 4: 48h group



**Figure 9. The Ratio of Intensity on the Protein Expression of PI3K/Akt Signal Transduction Pathway of HL-60/ADR Cells**

Caspase-3 (17 kD, 19 kD), and Bad proteins gradually increased (Figure 5-7).

*The effect of baicalin on protein expression in the PI3K/Akt signaling pathway*

The effect of baicalin on the PI3K/Akt signaling pathway in HL-60/ADR cells was time-dependent. After 80 μmol/L baicalin treatment, the expression level of Akt in HL-60/ADR cells showed insignificant difference from the control. Yet the expression level of p-Akt, NF-κB, p-NF-κB, mTOR, and p-mTOR decreased significantly in a time-dependent manner (Figure 8-9). This shows that baicalin can inhibit the activity of the PI3K/Akt signaling pathway in HL-60/ADR cells.

**Discussion**

Baicalin is an active ingredient extracted from *Scutellaria*. Many studies have shown that baicalin has an inhibitory effect on the growth of a variety of solid tumors (Miocinovic et al., 2005; Wang et al., 2008; Du et al., 2010). There are few reports, however, on the effect of baicalin on human myeloid leukemia cell lines. Our preliminary studies have showed that baicalin could effectively inhibit HL-60 cell proliferation and induce apoptosis (Zheng et al., 2009).

HL-60/ADR is a doxorubicin-resistant human myeloid leukemia cell line. In order to explore whether baicalin has the same inhibitory effect on HL-60/ADR cell growth and induce apoptosis, the MTT assay was previously performed in vitro and showed a significant inhibitory effect of baicalin. The effect of induced apoptosis was confirmed by Annexin V FITC/PI double staining flow cytometry, and the IC50 was approximately 28.3 μmol/L

48 h after dosing without significant difference from that in the baicalin-treated non-resistant HL-60 cells (Chen et al., 2008). This suggested the same inhibitory effect on both the drug-resistant and non-resistant cell lines. Apoptosis is genetically controlled autonomous cell death. Its initiation and implementation is a complex process regulated by multiple genes and signaling pathways. In-depth investigation has been performed in this current study using DNA ploidy analysis and apoptosis-related protein and signaling pathway detection.

DNA ploidy analysis performed 24 h after dosing showed that baicalin could induce a typical dose-dependent sub-peak (apoptotic peak) in HL-60/ADR cells. As shown in cell cycle analysis, the number of the G0/G1 phase cells increased significantly while that of the S phase cells decreased significantly as the drug concentration increased, suggesting the cell cycle-delaying effect of baicalin to arrest the HL-60/ADR cells in G0/G1 phase and prevent the cells from entering S phase.

The Bcl-2 gene family is an important member in the regulation of cell apoptosis, which is divided into two categories based on the function, namely the anti-apoptotic genes such as Bcl-2 and Bcl-xl and the pro-apoptotic genes including bax, bak, Bcl-x (s), bik, bid, bad, etc. The study by Shieh et al. (2006) showed that baicalin can induce apoptosis in human leukemia cell lines by down-regulating the expression of Bcl-2. Our results showed that the mRNA and protein expression levels of the Bcl-2 gene decreased in the HL-60/ADR cells after baicalin treatment while those of the pro-apoptotic gene bad gradually increased. This indicates the involvement of the Bcl-2 gene family in the regulation of baicalin-induced HL-60/ADR cell apoptosis.

At least three apoptosis pathways have been found so far, namely the mitochondrial pathway, death receptor pathway and endoplasmic reticulum pathway. All the pathways led to cell apoptosis by ultimately triggering caspase. The caspase family are cysteine-containing aspartate-specific proteases which play a key role in apoptosis. The protease families result in cell apoptosis by activating cascade reactions, in which caspase-3 is common (Worf and Suicidal, 1999). Caspase-3 is usually present in a state of non-activated zymogen (procaspase-3). Under a variety of apoptotic stimuli, procaspase-3 is hydrolyzed to active caspase-3 and cleaved with other caspase substrates such as PARP [poly (ADP-ribose) polymer-ase] which is related to DNA repair and genetic integrity. In the initiation of cell apoptosis, PARP (116 kD) is cleaved by caspase-3 at Asp216-Gly217 into two 31 kD and 85 kD fragments. The two DNA-binding zinc finger structures in PARP are thus separated from the C-terminal catalytic domain and lose their normal function. As a result, the activity of the PARP-negatively regulated Ca<sup>2+</sup>/Mg<sup>2+</sup>-dependent endonuclease increased to cleave the DNA between nucleosomes and induce apoptosis (Ivana Scovassi and Diederich, 2004). Our results showed that the expression of PARP (116 kD) in HL-60/ADR cells gradually decreased while that of the 85 kD PARP fragment and the 17 kD and 19 kD caspase-3 fragments increased significantly with baicalin treatment time, suggesting the involvement of the Caspase family in the apoptosis of the baicalin-induced HL-60/ADR cells.

The PI3K/Akt signaling pathway is a recently discovered important intracellular signal transduction pathway. Studies have shown that it plays a key role in the inhibition of cell apoptosis and the promotion of cell proliferation by affecting the activation state of a variety of downstream effector molecules. It is closely related to the occurrence and development of many human tumors including hematological malignancies (Xu et al., 2003; Friedrichs et al., 2011; Sun et al., 2011). Akt is a potent kinase for Bad as a member of the Bcl-2 family. Bad can normally bind with Bcl-2 or Bcl-x and form a complex with apoptotic activity. Activated Akt can phosphorylate Bad at the site of Ser136, thus blocking the formation of the Bad-Bcl-2/Bcl-xL dimer, disabling the pro-apoptotic function of Bad, and effectively blocking Bad-induced apoptosis. Our results showed that the expression of Akt in HL-60/ADR cells was not significantly affected by baicalin treatment while that of Akt and p-Akt gradually decreased in a time and dose-dependent manner. Combined with the effect on the expression of apoptosis-related proteins (Bcl-2, Bad and PARP), we conclude that baicalin may lift the suppression of the pro-apoptotic protein Bad and induce cell apoptosis by inhibiting intracellular Akt activity and down-regulate its expression.

NF- $\kappa$ B is a dimer transcription factor located in the nucleus with its main anti-apoptotic function in tumor cells. Its activity depends on the phosphorylation of the I $\kappa$ B kinase (IKK) complex. Vandermoere et al. showed in their studies (2005) that Akt can directly or indirectly regulate I $\kappa$ B kinase (IKK) activity and lead to NF- $\kappa$ B nuclear translocation and activation and the transcription of NF- $\kappa$ B-dependent survival genes. Akt phosphorylates NF- $\kappa$ B and activates its transcriptional function, which enhances the expression of the cell survival member Bcl-xL in the Bcl-2 family and promotes cell survival. The study by Harikumar et al. (2009) also showed that the plant extract, xanthohumol, can be used to increase the apoptosis of leukemia cells and myeloma cells as xanthohumol inhibits IKK phosphorylation and degradation, thereby inhibiting NF- $\kappa$ B nuclear translocation, its binding with DNA, and its transcriptional activity. The activity of the anti-apoptotic genes such as survivin, Bcl-xL, cyclinD1, and c-myc is also down-regulated to inhibit the production of anti-apoptotic products, thereby promoting leukemia cell apoptosis. Our results have shown that the expression of NF- $\kappa$ B and p-NF- $\kappa$ B in HL-60/ADR cells gradually decreased with time after baicalin treatment, suggesting that baicalin can indirectly inhibit NF- $\kappa$ B phosphorylation by inhibiting Akt activity, and down-regulate the anti-apoptotic effect of NF- $\kappa$ B.

The mammalian target of rapamycin, mTOR, is another important target molecule in Akt downstream signaling. It can be triggered by Akt phosphorylation and ultimately affect cell proliferation. As such, it is an important target in the treatment of hematopoietic system tumors (Kawauchi et al., 2009). Marinov et al. (2009) found in their studies an increase of mTOR activity in small cell lung cancer cell lines and in vivo. The rapamycin derivative, RAD001, can be used to inhibit mTOR activity and increase etoposide cytotoxicity in small cell lung cancer cell lines. High Akt and Bcl-2 expression cells

were found more sensitive to RAD001. Our results of this current study showed decreased expression of mTOR and p-mTOR in HL-60/ADR cells in a time-dependent manner after baicalin treatment, suggesting a close relationship between the affected activity of the AKT/mTOR signaling pathway and baicalin-induced apoptosis.

In conclusion, our experimental results showed that baicalin can inhibit the proliferation and promote the apoptosis of the HL-60/ADR cells with a possible mechanism involving the down-regulation of the activity and expression of Akt, p-Akt, NF- $\kappa$ B, p-NF- $\kappa$ B, mTOR, and p-mTOR, which further down-regulates downstream apoptosis-related proteins such as Bcl-2, PARP, Bad and Caspase-3.

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