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Baicalein Induces Apoptosis in SCC-4 Human Tongue Cancer Cells *via* a Ca^{2+}-dependent Mitochondrial Pathway

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Abstract. *Background: The effects of baicalein on SCC-4 human tongue cancer cells were examined to better understand its effect on apoptosis and associated possible signal pathways in vitro. Materials and Methods: Apoptosis induction, reactive oxygen species (ROS), cytoplasmic Ca^{2+}, mitochondrial membrane potential (MMP) and caspase-3 activity were analyzed using the flow cytometric assay. Apoptosis-associated proteins, such as p53, BAX, BCL-2, cytochrome c, caspase-3 and -9, EndoG and AIF were determined by Western blotting. Results: Our results showed that baicalein promoted the levels of p53, BAX, cytochrome c, capase-3 and -9 and reduced the level of BCL-2, which were associated with the induction of apoptotic cell death of SCC-4 cells. A release of cytochrome c from mitochondria into cytosol was demonstrated and an activation of caspase-3, which led to the occurrence of apoptosis in SCC-4 cells treated with baicalein as determined by Western blot. In order to understand the role of Ca^{2+} in the induction of apoptosis, cells were pre-treated with BAPTA (intracellular calcium chelator) and baicalein. It was shown that the MMP was restored, and the level of cytoplasmic Ca^{2+} suppressed, the proportion of cells undergoing apoptosis was also markedly diminished. Our data suggest that cellular Ca^{2+} modulates baicalein-induced cell death via a Ca^{2+}-dependent mitochondrial death pathway in SCC-4 cells.*

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Key Words: Baicalein, reactive oxygen species, cytochrome *c*, cytoplasmic Ca^{2+}, caspase-3, mitochondrial death pathway, apoptosis, SCC-4 cells.

Many studies have demonstrated that flavonoids from natural plants exhibit a variety of biological activities, such as the anti-inflammatory, antioxidant, antitumor and antiviral actions (1). Baicalein (5,6,7-trihydroxyflavone) is a flavonid derived from the root of *Scutellaria baicalensis* Georgi, a medicinal plant traditionally used in Chinese herbal medicine (2). It was reported that baicalein ameliorated all the considered inflammatory symptoms in dextran sulfate sodium-induced colitis in mice *in vivo* experiments (3). Many experiments also showed that baicalein is a free-radical scavenger, an antioxidant (4-7) and exhibits a cytoprotective effects (8-11). Baicalein was also protective against benzo[α]pyrene and aflatoxin B1-induced genotoxicity (12). Baicalein has been reported to be an antiinflammatory agent (13) and an inhibitor of prostaglandin E2 (14). Recently, it was reported that baicalein inhibited hydrogen peroxide-induced apoptosis *via* ROS-dependent heme oxygenase 1 gene expression (15).

It is well-known that the best strategy for killing cancer cells is to induce cancer cell apoptosis. Antitumor agents can trigger apoptosis and then lead to rapid elimination of tumor cells (16, 17). Interference with the apoptotic process is considered a crucial part of cancer prevention and therapy (18). Another important factor is the interactions among BCL-2 family proteins (pro-apoptotic and anti-apoptotic proteins) which are also involved in cell death or survival (19, 20). Although many reports have demonstrated that baicalein induced apoptosis in human cancer cells including breast cancer (21), hepatoblastoma (22), prostate cancer (23) and gastric cancer cells (24), there is no available information to address the mechanism of baicalein-induced apoptosis in SCC-4 human tongue cancer cells. The aim of the present study was to investigate the effects and the role of Ca^{2+} and caspase-3 in the induction of apoptosis by baicalein in SCC-4 human tongue cancer cells.

Materials and Methods

Chemicals and reagents. Baicalein, Tris-HCl, triton X-100 and propidium iodide (PI) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), potassium phosphates and dimethyl sulfoxide (DMSO) were purchased from Merck Co. (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). Caspase-3 activity assay kits were bought from Boehringer Mannheim (Mannhein, Germany).

SCC-4 human tongue cancer cell line. The SCC-4 cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). SCC-4 cells have been cultured for several generations and have been checked for viability as described previously. The cells were placed into 75 cm^3 tissue culture flasks and grown at 37˚C under a humidified 5% CO_2 and 95% air at one atmosphere in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin (10 ng/ml penicillin and 10 ng/ml streptomycin) and 1% glutamine (25).

Baicalein effect on caspase-3 activity. The SCC-4 cells were plated in 6-well plates at a density of $5x10^5$ cells/well and grown for 24 hours. Then cells were grown in 25 and 75 μM baicalein, with DMSO (solvent) alone for the control regimen, at 37˚C in a humidified 5% CO_2 for 24 hours. The caspase-3 activity was analyzed by flow cytometry (Becton Dickinson FACS Calibur) as described elsewhere (26-28).

Baicalein effect on reactive oxygen species (ROS). The SCC-4 cells were treated with or without 0, 25 or 75 μM of baicalein (or pre-treated with 10 μM caspase inhibitor Ac-DEVD-CHO for 3 hours) for 24 hours to detect the changes of ROS. The cells were harvested and washed twice, re-suspended in 500 μl of 2,7-dichlorodihydrofluorescein diacetate (10 μM) (DCFH-DA, Sigma) and incubated at 37˚C for 30 min, before being analyzed by flow cytometry (26-28).

Baicalein effect on Ca^{2+} concentrations. The SCC-4 cells were treated with or without 0, 25 or 75 μM of baicalein for 24 hours then to detect the changes in Ca^{2+} concentrations, and then they were harvested and washed twice for re-suspension in Indo 1/AM (3 μg/ml) (Calbiochem, La Jolla, CA, USA), incubated at 37˚C for 30 min, and then analyzed by flow cytometry (26-28).

Baicalein effect on mitochondrial membrane potential (MMP). Cells were treated with 75 μM baicalein for 24 hours. The cells were harvested and washed twice, re-suspended in 500 μl of $DiOC_6$ (4 mol/L), incubated at 37˚C for 30 min, and then analyzed by flow cytometry (26-28).

Effect of baicalein on cytoplasmic Ca^{2+}, MMP, and proportion of apoptosis in human tongue cancer SCC-4 cells pretreated with BAPTA. The level of Ca^{2+}, MMP and apoptosis of the SCC-4 cells was determined by flow cytometry (Becton Dickinson FACS Calibur) using the Indo 1/AM (Calbiochem). Cells were pre-treated with or without 10 μM BAPTA-AM (intracellular calcium chelator) for 3 hours then with 25 and 75 μM baicalein for 24 hours. The cells were harvested and washed twice, for apoptosis analysis and for re-suspension in Indo 1/AM (3 μg/ml), incubated at 37˚C for 30 min, and then analyzed by flow cytometry (26-28) for Ca^{2+} concentration, MMP and apoptosis.

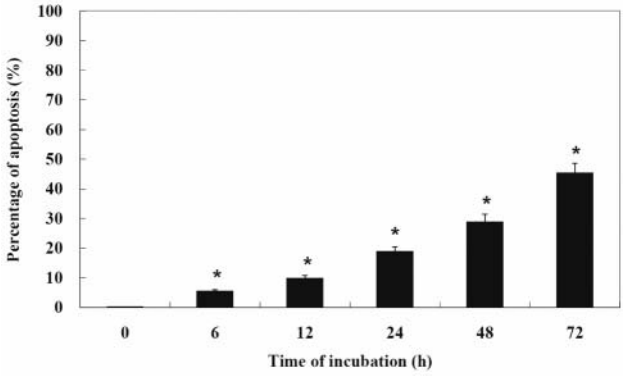

Figure 1. *Baicalein induced apoptosis in SCC-4 human tongue cancer cells. PI staining analysis for the effects of baicalein on human tongue cancer SCC-4 cell's apoptosis. The SCC-4 cells were incubated with 75 μM baicalein for 6, 12, 24, 48 and 72 h and apoptosis was determined by flow cytometric analysis as described in Materials and Methods. Data represents mean±S.D. of three experiments (*$p<0.05$).*

Effect of baicalein on the expressions of p53, BAX, BCL-2, cytochrome c, caspase-3 and -9, EndoG and AIF. The total proteins were collected from SCC-4 cells treated with or without 0, 25 or 75 μM of baicalein for 48 hours. Subsequently, p53, BAX, BCL-2, cytochrome *c*, caspase-3 and -9, EndoG and AIF were measured by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. The levels of cytochrome *c* in the mitochondria and in the cytosol were also determined. The SCC-4 cells after treatment with or without 75 μM baicalein were harvested and disrupted. They were then centrifuged to obtain cytosolic and mitochondria fractions and underwent further examination in regards to the level of cytochrome *c* by Western blot as described elsewhere (28, 29).

Statistical analysis. Statistical calculations of the data were performed using an unpaired Student's *t*-test and Tukey's test. Statistical significance was set at $p<0.05$.

Results

Induction of apoptosis by baicalein. After SCC-4 cells were treated with 75 μM baicalein for 0, 6, 12, 24, 48 and 72 hours, apoptosis was detected by PI staining method and then analyzed by flow cytometry. As shown in Figure 1, baicalein induced apoptosis in a time-dependent manner.

Effect of baicalein on caspase-3 activity. Caspase-3 activity increased in baicalein-treated SCC-4 cells and increasing baicalein dose led to increasing caspase-3 activity, as shown in Table I.

Effect of baicalein on ROS. The increase of the levels of ROS was detected in 24 hours after cells being treated with

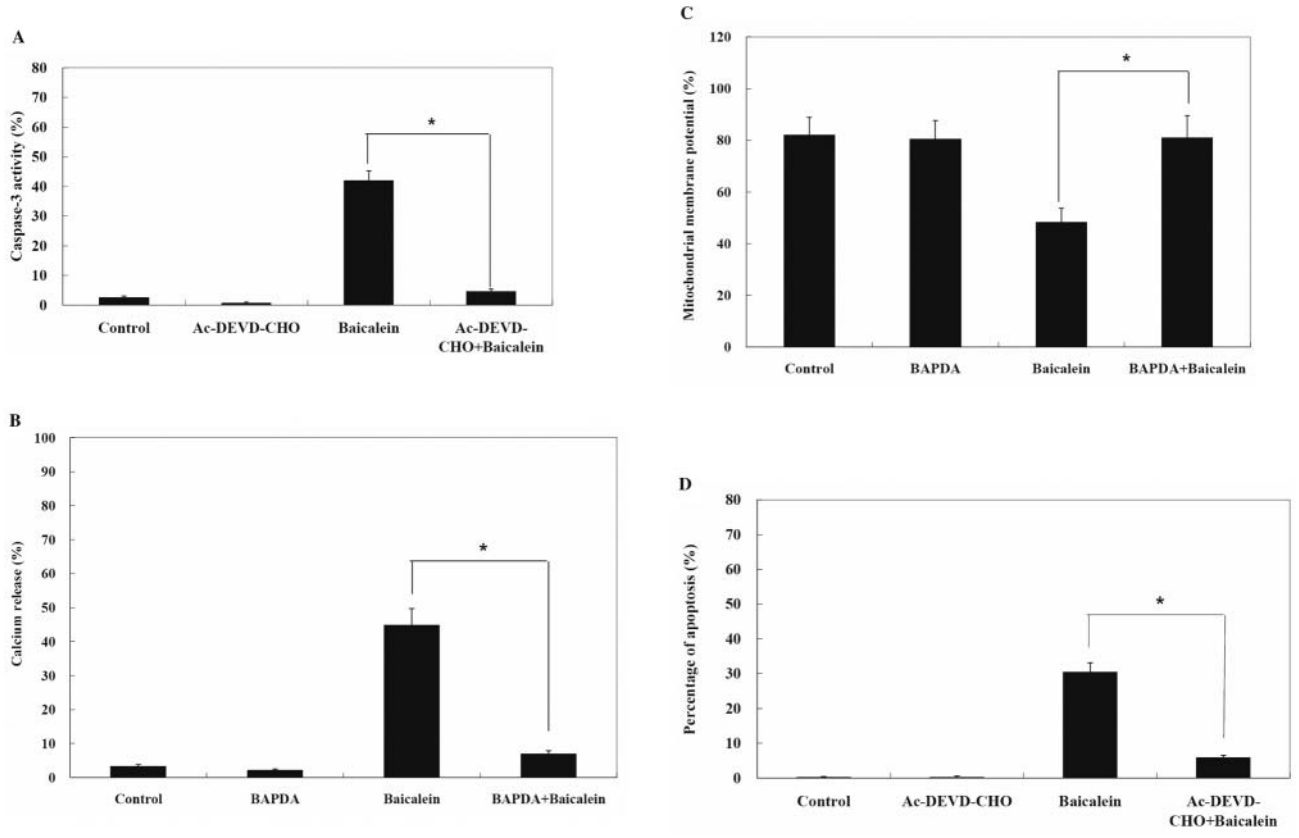

Figure 2. *Caspase-3 inhibitor inhibited baicalein induced apoptosis in human tongue cancer SCC-4 cells. The SCC-4 cells were pre-treated with BAPTA for 3 h then treated with 75 μM baicalein before cells were harvested for caspase-3 activity, Ca^{2+} concentration, MMP levels and apoptosis determinations as described in Materials and Methods. Data represents mean±S.D. of three experiments. *$p<0.05$. (Panel A: caspase-3 activity; panel B: Ca^{2+} concentration; panel C: MMP levels; and panel D: apoptosis).*

baicalein and increasing dose led to an increase in ROS production (Table I).

Effect of baicalein on the levels of cytoplasmic Ca^{2+} from human tongue cancer SCC-4 cells. The increase of the levels of cytoplasmic Ca^{2+} was detected 24 hours after treatment with baicalein, dose-dependently (Table I).

Effect of baicalein on the MMP levels. Mitochondria membrane potential (MMP) declined in SCC-4 cells treated with 25 and 75 μM baicalein (Table I).

Effect of Ac-DEVD-CHO on baicalein promoted caspase-3 activity in human tongue cancer SCC-4 cells. SCC-4 cells were pretreated with or without Ac-DEVD-CHO (inhibitor of caspase-3) followed by treatment with 75 μM baicalein for 24 h. Caspase-3 activity was increased in baicalein-treated cells of SCC-4, but decreased in caspase-3 inhibitor (Ac-DEVD-CHO) pretreatment in baicalein-treated SCC-4 cells, as shown in Figure 2A.

Table I. *Flow cytometric analysis of intracellular ROS and Ca^{2+} levels, MMP and caspase-3 activity in SCC-4 cells treated with baicalein.*

Baicalein (μM)	% of control			
	ROS	Ca^{2+}	MMP	Caspase-3
0	1.2±0.6	4.8±1.2	96.0±6.8	2.9±0.8
25	19.6±2.9[b]	32.4±2.8[b]	78.4±7.2[b]	26.6±3.4[b]
75	54.8±6.8[a]	61.2±5.2[a]	54.2±4.8[a]	64.2±7.4[a]

The SCC-4 cells ($5x10^5$ cells/ml) were treated with various concentrations of baicalein. The zero concentration was defined as control. The stained cells were determined by flow cytometry as described in the Materials and Methods section. Values are means±SD (n=3). Dates not sharing the same letter are significantly different by Tukey's test (*$p<0.05$).

Effects of BAPTA on baicalein-induced changes in levels of cytoplasmic Ca^{2+}, MMP and apoptosis of SCC-4 human tongue cancer cells. The changes of the levels of cytoplasmic Ca^{2+},

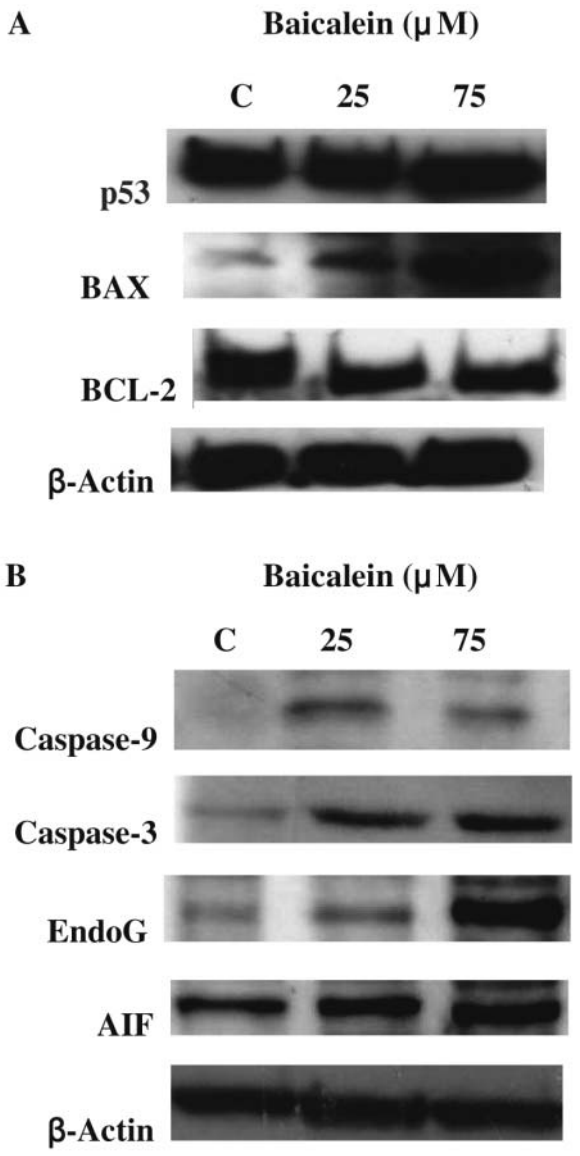

Figure 3. *Western blotting showing that baicalein affects apoptosis-associated proteins in SCC-4 human tongue cancer cells. Representative Western blot showing changes on the levels of p53, BAX, BCL-2, cytochrome c, caspase-9, caspase-3, EndoG and AIF in human tongue cancer SCC-4 cells after treated with baicalein. The SCC-4 cells ($5x10^6$/ml) were treated with 0, 25 and 75 μM baicalein for 48 h, then total protein was prepared and determined as described in Materials and Methods, then followed by evaluation of the protein levels (Panel A: p53, BAX and BCL-2; panel B: caspase-9, caspase-3, EndoG and AIF). Expressions were estimated by Western blotting as described in Materials and Methods.*

MMP and apoptosis in baicalein-treated SCC-4 cells were greatly affected by pretreatment with BAPTA. The decline of MMP induced by baicalein was reversed by BAPTA. After cells were pretreated with BAPTA, the increase in cytoplasmic Ca^{2+} was suppressed and the proportion of apoptosis was also markedly diminished (Figure 2B, C and D).

Effects of baicalein on the levels of p53, BAX, CLl-2, cytochrome c, caspase-3 and -9, EndoG and AIF from SCC-4 human tongue cancer cells. The increase in the expressions of p53, BAX, cytochrome *c*, caspase-3 and -9, EndoG and AIF, and the decrease in the expression of BCL-2 in baicalein-treated SCC-4 cells may contribute to the occurrence of apoptosis (Figure 3). The results also demonstrated that the increase of cytochrome *c* was derived from its release from the mitochondria to the cytosol (Figure 4).

Discussion

Although many experiments have shown that baicalein induced apoptosis in human cancer cell lines there is no available information regarding the effects of baicalein on SCC-4 human tongue cancer cells. In this study, we demonstrated that baicalein induced apoptosis in SCC-4 cells *via* a Ca^{2+}-associated mitochondrial and caspase-3-dependent pathway. Our results from Western blotting demonstrated that baicalein promoted production of p53, BAX, cytochrome *c*, EndoG and AIF and reduced the levels of BCL-2 which led to the disruption of mitochondrial membrane potential (MMP) and the release of cytochrome *c* from the mitochondria to the cytosol. This finding also points out that baicalein may be useful in clinical trials in the future for tongue cancer patients.

Our data indicated that baicalein-induced apoptosis also involved a decrease of the MMP, which is in agreement with the reports showing that apoptosis is accompanied by a loss of MMP (30, 31) and exposure of phosphatidylserine at the surface of the cell (32-35). It is well-known that mitochondrial alterations constitute a critical event of the apoptotic cascade (36). Our results also showed that baicalein promoted cytochrome *c* release (Figure 4) which is in agreement with other reports which indicated that the reduction of MMP is an early reversible step of apoptosis, followed by cytochrome *c* release in many cell types based on the $DiOC_6$ uptake as shown by flow cytometric analysis (37). Therefore, baicalein induced apoptosis in SCC-4 cells through a mitochondria-dependent pathway.

Many investigators have demonstrated that baicalein induced apoptosis in human cancer cells and the apoptogenic action of baicalein was associated with caspase activation, mitochondrial dysfunction and modulation of BCL-2 family proteins (21-24). However, information regarding the role of Ca^{2+} in the induction of apoptosis caused by baicalein is scarce. Based on our results, baicalein elevated cytoplasmic Ca^{2+} which is involved in the induction of apoptosis (based on pretreatment with BAPTA (Ca^{2+} chelator) in baicalein-treated SCC-4 cells), MMP was restored, the level of cytoplasmic Ca^{2+} was reduced and the proportion of

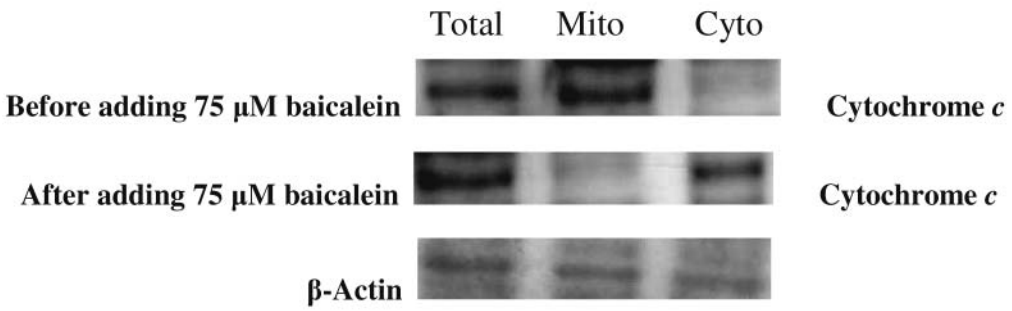

Figure 4. *Representative Western blot showing changes on the levels of cytochrome c in SCC-4 human tongue cancer cells after being treated with baicalein. The SCC-4 cells (5x10^6/ml) were treated with 75 μM baicalein for 48 h before mitochromia and/or cytosolic fraction and total protein were prepared and determined. The evaluation of the protein levels (cytochrome c from total, mitochondria and cytosol fraction) were estimated using Western blotting as described in Materials and Methods.*

apoptosis was also markedly diminished. This conclusion is confirmed by other reports demonstrating that under normal conditions, the Ca^{2+} influx increases and cells die (38). Furthermore, other investigators also showed that Co^{2+} blocks Ca^{2+} influx, resulting in the protection of the cells despite high ROS levels (21). Our results also showed baicalein induced ROS production (Table I). It is well-known that in normal amounts ROS is involved in maintaining human physiological functions, however overproduction of ROS can be detrimental and has been shown to participate in the etiology of several human diseases such as cancer, inflammation and diabetes.

It is also reported that flavonols may interact directly with a Ca^{2+} channel which leads to prevention of its opening that is responsible for the final demise of the cell (39). Whether or not they may prevent the signaling mechanism between high ROS levels and the opening of the Ca^{2+} channel needs further investigation. We summarize the possible signal pathway (Figure 5) demonstrating that baicalein induced apoptosis through a mitochondrial and caspase-3-dependent pathway and that AIF and EndoG are involved in these events.

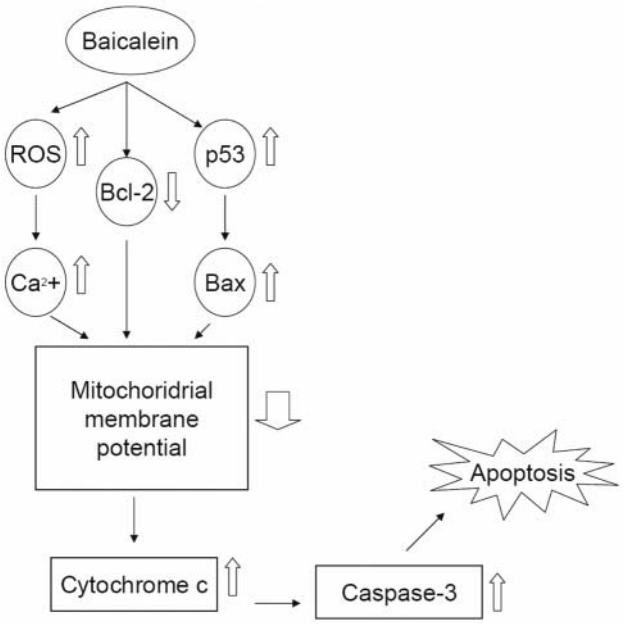

Figure 5. *Proposed model of baicalein mechanism of action for apoptosis in SCC-4 human tongue cancer cells. Baicalein increased the production of BAX, cytochrome C, ROS and Ca^{2+} and decreased MMP levels led to cytochrome c release and caspase-3 activation before causing apoptosis in SCC-3 cells.*

Acknowledgements

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