

Baicalein Induces Apoptosis in SCC-4 Human Tongue Cancer Cells via a Ca²⁺-dependent Mitochondrial Pathway

YUH-TZY LIN¹, JAI-SING YANG², HUI-JU LIN^{3,4}, TZU-WEI TAN², NOU-YING TANG⁵, JO-HUA CHAING⁸, YUNG-HSIEN CHANG⁶, HSU-FENG LU⁷ and JING-GUNG CHUNG^{8,9}

¹Department of Nursing and Management, Jen-Teh Junior College of Medicine, Taiwan;
Departments of ²Pharmacology and ⁸Biological Science and Technology, ⁵School of Chinese Medicine,
⁶Graduate Institute of Integration Chinese and Western Medicine, China Medical University, Taichung, Taiwan;
³Department of Ophthalmology, China Medical University Hospital, Taichung, Taiwan;
⁴Department of Life Science, Tunghai University, Taichung, Taiwan;
⁷Department of Clinical Pathology, Cheng Hsin Rehabilitation Medical Center, Taipei, Taiwan;
⁹Department of Biotechnology, Asia University, Wufeng, Taichung County 41354, Taiwan, R.O.C.

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 Ca²⁺, 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 Ca²⁺ 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 Ca²⁺ suppressed, the proportion of cells undergoing apoptosis was also markedly diminished. Our data suggest that cellular Ca²⁺ modulates baicalein-induced cell death via a Ca²⁺-dependent mitochondrial death pathway in 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 Ca²⁺ and caspase-3 in the induction of apoptosis by baicalein in SCC-4 human tongue cancer cells.

Correspondence to: Jing-Gung Chung, Ph.D., Department of Microbiology, School of Medicine, China Medical University, No 91, Hsueh-Shih Road, Taichung 404, Taiwan, R.O.C. Tel: +88 642205 3366, Fax: +88 642205 3764, e-mail: jgchung@mail.cmu.edu.tw

Key Words: Baicalein, reactive oxygen species, cytochrome c, cytoplasmic Ca²⁺, caspase-3, mitochondrial death pathway, apoptosis, SCC-4 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 (Mannheim, 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 cm³ tissue culture flasks and grown at 37°C under a humidified 5% CO₂ 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 5x10⁵ 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% CO₂ 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 Ca²⁺ 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 Ca²⁺ 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 DiOC₆ (4 mol/L), incubated at 37°C for 30 min, and then analyzed by flow cytometry (26-28).

Effect of baicalein on cytoplasmic Ca²⁺, MMP, and proportion of apoptosis in human tongue cancer SCC-4 cells pretreated with BAPTA. The level of Ca²⁺, 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

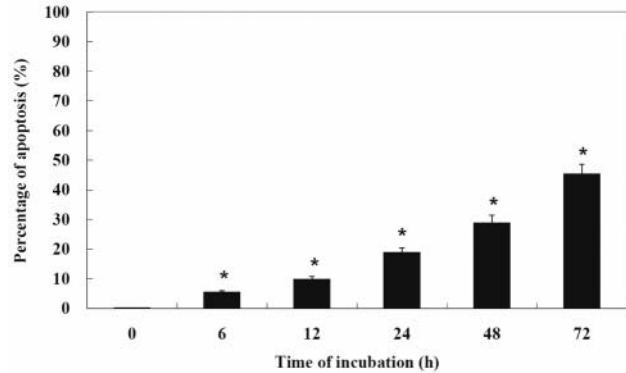


Figure 1. Baicalein induced apoptosis in SCC-4 human tongue cancer cells. PI staining analysis for the effects of baicalein on human tongue cancer SCC-4 cell's apoptosis. The SCC-4 cells were incubated with 75 µM baicalein for 6, 12, 24, 48 and 72 h and apoptosis was determined by flow cytometric analysis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

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 Ca²⁺ concentration, MMP and apoptosis.

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

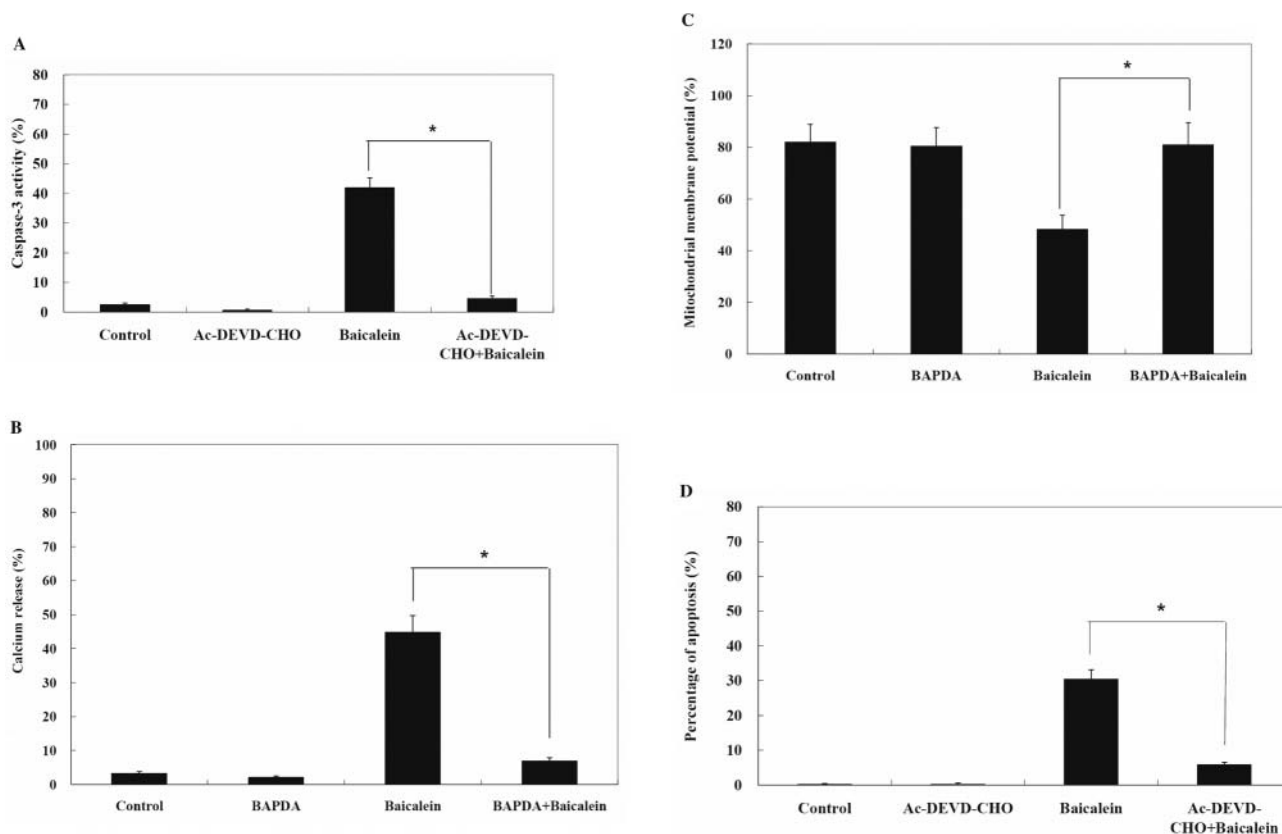


Figure 2. Caspase-3 inhibitor inhibited baicalein induced apoptosis in human tongue cancer SCC-4 cells. The SCC-4 cells were pre-treated with BAPTA for 3 h then treated with 75 μ M baicalein before cells were harvested for caspase-3 activity, Ca^{2+} concentration, MMP levels and apoptosis determinations as described in Materials and Methods. Data represents mean \pm 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 μ M 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 μ M 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 (μ M)	% of control			
	ROS	Ca^{2+}	MMP	Caspase-3
0	1.2 \pm 0.6	4.8 \pm 1.2	96.0 \pm 6.8	2.9 \pm 0.8
25	19.6 \pm 2.9 ^b	32.4 \pm 2.8 ^b	78.4 \pm 7.2 ^b	26.6 \pm 3.4 ^b
75	54.8 \pm 6.8 ^a	61.2 \pm 5.2 ^a	54.2 \pm 4.8 ^a	64.2 \pm 7.4 ^a

The SCC-4 cells (5×10^5 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 \pm 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+} ,

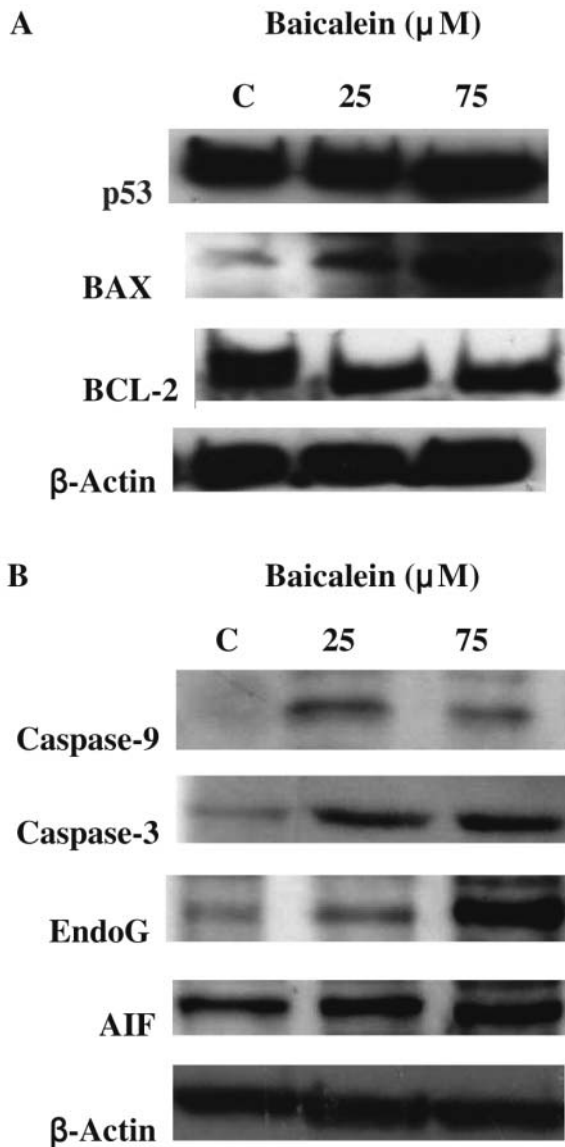


Figure 3. Western blotting showing that baicalein affects apoptosis-associated proteins in SCC-4 human tongue cancer cells. Representative Western blot showing changes on the levels of p53, BAX, BCL-2, cytochrome *c*, caspase-9, caspase-3, EndoG and AIF in human tongue cancer SCC-4 cells after treated with baicalein. The SCC-4 cells ($5 \times 10^6/ml$) were treated with 0, 25 and 75 μ M 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, CLI-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 DiOC₆ 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

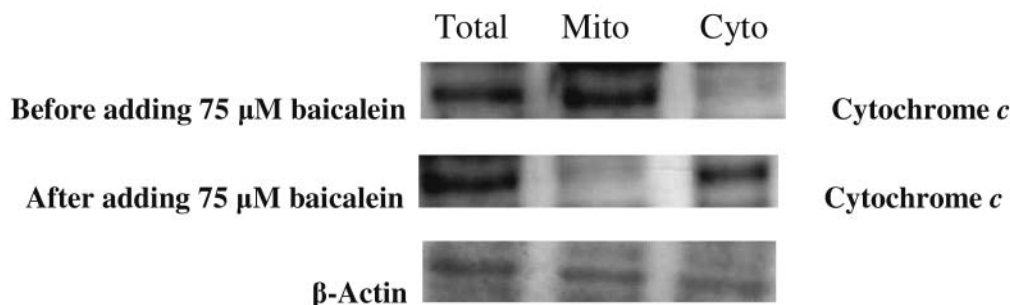


Figure 4. Representative Western blot showing changes on the levels of cytochrome c in SCC-4 human tongue cancer cells after being treated with baicalein. The SCC-4 cells ($5 \times 10^6/ml$) were treated with $75 \mu M$ baicalein for 48 h before mitochondria and/or cytosolic fraction and total protein 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.

Acknowledgements

This work was supported by grant CMU95-127 from China Medical University, Taichung, Taiwan, R.O.C.

References

- 1 Middleton E Jr: Some biological properties of plant flavonoids. *Ann Allergy* 61: 53-57, 1988.
- 2 Kim YO, Leem K, Park J, Lee P, Ahn DK, Lee BC, Park HK, Suk K, Kim SY and Kim H: Cytoprotective effect of *Scutellaria baicalensis* in CA1 hippocampal neurons of rats after global cerebral ischemia. *J Ethnopharmacol* 77: 183-188, 2001.
- 3 Hong T, Jin GB, Cho S and Cyong JC: Evaluation of the anti-inflammatory effect of baicalein on dextran sulfate sodium-induced colitis in mice. *Planta Med* 68: 268-271, 2002.

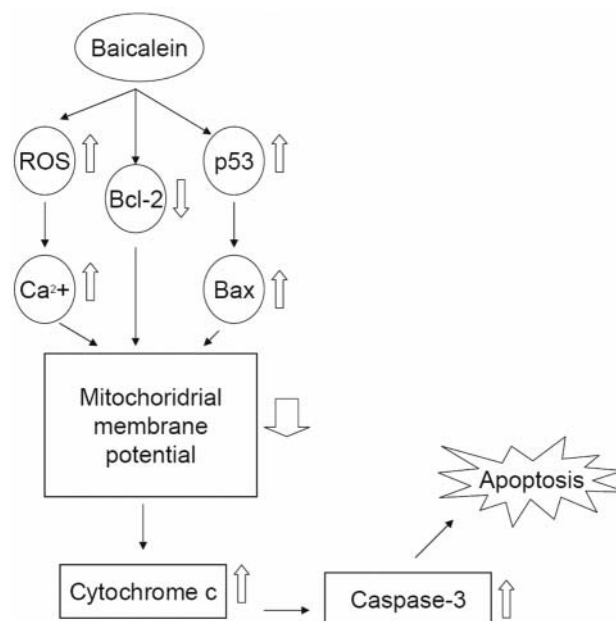


Figure 5. Proposed model of baicalein mechanism of action for apoptosis in SCC-4 human tongue cancer cells. Baicalein increased the production of BAX, cytochrome C, ROS and Ca^{2+} and decreased MMP levels led to cytochrome c release and caspase-3 activation before causing apoptosis in SCC-3 cells.

- 4 Hamada H, Hiramatsu M, Edamatsu R and Mori A: Free radical scavenging action of baicalein. *Arch Biochem Biophys* 306: 261-266, 1993.
- 5 Gao Z, Huang K, Yang X and Xu H: Free radical scavenging and antioxidant activities of flavonoids extracted from the radix of *Scutellaria baicalensis* Georgi. *Biochim Biophys Acta* 147: 2643-2650, 1999.
- 6 Shieh DE, Liu LT and Lin CC: Antioxidant and free radical scavenging effects of baicalein, baicalin and wogonin. *Anticancer Res* 20: 2861-2865, 2000.
- 7 Chen ZY, Su YL, Bi YR, Tsang SY and Huang Y: Effect of baicalein and acetone extract of *Scutellaria baicalensis* on canola oil oxidation. *J Am Oil Chem Soc* 77: 73-78, 2000.

- 8 Gao Z, Huang K and Xu H: Protective effects of flavonoids in the roots of *Scutellaria baicalensis* Georgi against hydrogen peroxide-induced oxidative stress in HS-SY5Y cells. *Pharmacol Res* 43: 173-178, 2001.
- 9 Ishige K, Schubert D and Sagara Y: Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. *Free Radic Biol Med* 30: 433-446, 2001.
- 10 Lebeau A, Esclaire F, Rostene W and Pelaprat D: Baicalein protects cortical neurons from beta-amyloid (25-35) induced toxicity. *Neuroreport* 12: 2199-2202, 2001.
11. Choi J, Conrad CC, Malakowsky CA, Talent JM, Yuan CS and Gracy RW: Flavones from *Scutellaria baicalensis* Georgi attenuate apoptosis and protein oxidation in neuronal cell lines. *Biochim Biophys Acta* 1571: 201-210, 2002.
- 12 Ueng YF, Shyu CC, Liu TY, Oda Y, Lin YL, Liao JF and Chen CF: Protective effects of baicalein and wogonin against benzo[a]pyrene- and aflatoxin B(1)-induced genotoxicities. *Biochem Pharmacol* 62: 1653-1660, 2001.
- 13 Lin CC and Shieh DE: The anti-inflammatory activity of *Scutellaria rivularis* extracts and its active components, baicalin, baicalein and wogonin. *Am J Chin Med* 241: 31-36, 1996.
- 14 Nakahata N, Kutsuwa M, Kyo R, Kubo M, Hayashi K and Ohizumi Y: Analysis of inhibitory effects of scutellariae radix and baicalein on prostaglandin E2 production in rat C6 glioma cells. *Am J Chin Med* 263: 311-323, 1999.
- 15 Lin HY, Shen SC, Lin CW, Yang LY and Chen YC: Baicalein inhibition of hydrogen peroxide-induced apoptosis *via* ROS-dependent heme oxygenase 1 gene expression. *Biochim Biophys Acta*, 2007
- 16 Li Y, Maher P and Schubert D: Requirement for cGMP in nerve cell death caused by glutathione depletion. *J Cell Biol* 139: 1317-1324, 1997.
- 17 Woynarowska BA and Woynarowska JM: Preferential targeting of apoptosis in tumor versus normal cells. *Biochim Biophys Acta* 1587: 309-317, 2002.
- 18 Lowe SW and Lin AW: Apoptosis in cancer. *Carcinogenesis* 21: 485-495, 2000.
- 19 Gross A, McDonnell JM and Korsmeyer SJ: Bcl-2 family members and the mitochondria in apoptosis. *Genes Dev* 13: 1899-1911, 1999.
- 20 Reed JC: Bcl-2 family proteins. *Oncogene* 17: 3225-3236, 1998.
- 21 Tong W, Ding X and Adrian T: The mechanisms of lipoxygenase inhibitor-induced apoptosis in human breast cancer cells. *Biochim Biophys Res* 296: 942-948, 2002.
- 22 Chang WH, Chen CH, Gau RJ, Lin CC, Tsai CL, Tsai K and Lu FJ: Effect of baicalein on apoptosis of the human Hep G2 cell line was induced by mitochondrial dysfunction. *Planta Med* 68: 302-306, 2002.
- 23 Pidgeon GP, Kandouz M, Meram A and Honn KV: Mechanisms controlling cell cycle arrest and induction of apoptosis after 12-lipoxygenase inhibition in prostate cancer cells. *Cancer Res* 62: 2721-2727, 2002.
- 24 Wong BC, Wang WP, Cho CH, Fan XM, Lin MC, Kung HF and Lam SK: 12-Lipoxygenase inhibition induced apoptosis in human gastric cancer cells. *Carcinogenesis* 22: 1349-1354, 2001.
25. Lu HF, Hsueh SC, Ho YT, Kao MC, Yang JS, Chiu TH, Huang SY, Lin CC and Chung JG: ROS mediates baicalin-induced apoptosis in human promyelocytic leukemia HL-60 cells through the expression of the Gadd153 and mitochondrial-dependent pathway. *Anticancer Res* 27(1A): 117-125, 2007.
- 26 Lin HL, Yang JS, Yang JH, Fan SS, Chang WC, Li YC and Chung JG: The Role of Ca(2+) on the baicalin-induced Apoptosis in Mouse-Rat Hybrid Retina Ganglion Cells (N18). *Neurochem Res* 31: 383-393, 2006.
- 27 Yang JH, Hsia TC, Kuo HM, Chao PD, Chou CC, Wei YH and Chung JG: Inhibition of lung cancer cell growth by quercetin glucuronides *via* G2/M arrest and induction of apoptosis. *Drug Metab Dispos* 34: 296-304, 2005.
- 28 Lin TP, Huang AC, Wei HC, Lin JG and Chung JG.: Ethyl 2-[N-p-chlorobenzyl-2'-methyl] anilino-4-oxo-4,5-dihydrofuran-3-carboxylate (JOT01007) induces apoptosis in human cervical cancer Ca Ski cells. *In Vivo* 21: 397-406, 2007.
29. Kim HR, Kim EJ, Yang SH, Jeong ET, Park C, Lee JH, Youn MJ, So HS and Park R: Trichostatin A induces apoptosis in lung cancer cells *via* simultaneous activation of the death receptor-mediated and mitochondrial pathway. *Exp Mol Medicine* 36: 616-624, 2006.
- 30 Lin SY, Jang JH and Surh YJ: Induction of cyclooxygenase-2 and peroxisome proliferators-activated receptor-gamma during nitric oxide-induced apoptotic PC12 cell death. *Ann NY Acad Sci* 1010: 648-658, 2003.
- 31 Kluza J, Clark AM and Bailly C: Apoptosis induced by the alkaloid sampangine in HL-60 leukemia cells: correlation between the effects on the cell cycle progression and changes of mitochondrial potential. *Ann NY Acad Sci* 1010: 331-334, 2003.
- 32 Mathur A, Hong Y, Kemp BK, Barrientos AA and Erusalimsky JD: Evaluation of fluorescent dyes for the detection of mitochondrial membrane potential changes in cultured cardiomyocytes. *Cardiovasc Res* 46: 24-27, 2000.
- 33 Isenberg JS and Klaunig JE: Role of the mitochondrial membrane permeability transition (MPT) in rotenone-induced apoptosis in liver cells. *Toxicol Sci* 53: 340-351, 2000.
- 34 Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST and van Oers MH: Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* 84: 1415-1420, 1994.
- 35 Zang G, Gurtu V, Kain SR and Yan G: Early detection of apoptosis using a fluorescent conjugate of annexin V. *Biotechniques* 23: 525-531, 1997.
- 36 Hajra KM and Liu JR: Apoptosome dysfunction in human cancer. *Apoptosis* 9: 691-704, 2004.
- 37 Ozgen U, Savasan S, Buck S and Ravindranath Y: Comparison of DiOC₆ uptake and annexin V labeling for quantification of apoptosis in leukemia cells and non-malignant T lymphocytes from children. *Cytometry* 42: 74-78, 2000.
- 38 Esposti MD, Hatzinisiriou I, McLennan H and Ralph S: Bcl-2 and mitochondrial oxygen radicals. New approaches with reactive oxygen species-sensitive probes. *J Biol Chem* 274: 29831-29837, 1999.
- 39 Li Y, Maher P and Schubert DA: Role for 12-lipoxygenase in nerve cell death caused by glutathione depletion. *Neuron* 19: 453-463, 1997.

Received June 21, 2007

Revised October 4, 2007

Accepted October 16, 2007