Abstract. Baicalein is a purified flavonoid extracted from the roots of Scutellaria baicalensis or Scutellaria radix. Although previous studies have suggested that Baicalein possesses an in vitro anti-hepatocellular carcinoma activity, its in vivo effects and mechanisms of action are still not completely understood. In this study, Baicalein at concentrations of 40-120 µM exhibited significant cytotoxicity to three hepatocellular carcinoma (HCC) cell lines but marginal cytotoxicity to a normal liver cell line in vitro. Compared to a standard chemotherapy drug, 5-fluorouracil (5-FU), Baicalein had greater effect on HCC cells but less toxicity on normal liver cells. Treatment with Baicalein dramatically reduced mitochondrial transmembrane potential, and activated caspase-9 and caspase-3. Blockade of Baicalein-induced apoptosis with a pan-caspase inhibitor partially attenuated Baicalein-induced growth inhibition in HCC. Baicalein treatment significantly inhibited tumor growth of HCC xenografts in mice. Induction of apoptosis was demonstrated in Baicalein-treated xenograft tumors by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Furthermore, Baicalein treatment dramatically decreased the levels of phosphorylation of MEK1, ERK1/2 and Bad in vitro and in vivo. Overexpression of human MEK1 partially blocked Baicalein-induced growth inhibition. Consequently, these findings suggest that Baicalein preferentially inhibits HCC tumor growth through inhibition of MEK-ERK signaling and by inducing intrinsic apoptosis.

Introduction

Hepatocellular carcinoma (HCC) is one of the common cancers in Asia and Africa. The incidence of HCC is increasing in Europe and the United States (1). Although HCC can be cured at the early stage by surgical resection, most patients can not be diagnosed at the early stage since tumors are asymptomatic (2). Current treatment options for HCC patients at the late stage include chemotherapy, chemoembolization, ablation, and proton beam therapy. These treatment options remain disappointed in clinic. HCC patients will relapse and rapidly progress to the advanced stages with vascular invasion and multiple metastases, which lead to a low 5-year survival rate of less than 7% (3). HCC patients who have surgically resectable localized tumors show a better prognosis. However, even these patients have a dismal 5-year survival rate of 15 to 39% (4). Clearly, there is an urgent need to search for new therapies for this lethal disease.

We have reported that chrysanthemum indicum extract (CIE), a Chinese herbal extraction, exerts a significantly inhibitory effect on HCC cells (MHCC97H) in previous studies (5,6). One particular point to stress is that CIE appears to have no cytotoxic effect on normal liver cells, highlighting an advantage of the herbal treatment. Herbal medicine flavonoids have recently received increasing attention because of the beneficial effects of anti-tumor and...
as chemopreventive agents (2-5). Baicalein (5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one) is a purified flavonoid with defined chemical structure (Fig. 1) and is extracted from the roots of *Scutellaria baicalensis* or *Scutellaria radix*. Although the anti-tumor activity of Baicalein in HCC has been reported *in vitro* (7,8), little is known about the underlying mechanisms of action on HCC, as well as the anti-tumor effect *in vivo*.

Previously genetic and expression profiling analyses of human HCC have led to the identification of key oncogenes and tumor-suppressor genes in liver carcinogenesis (9). They are mostly associated with the mitogen-activated protein kinase (MAPK) pathway (9). Constitutively activated extracellular signal-regulated kinases (ERK) have been shown to increase proliferation of human HCC cells (10). So far there is no report that investigates the effects of Baicalein on ERK in HCC. In this study, we have investigated the effects of Baicalein on HCC cells *in vitro* and *in vivo*, especially the effects of Baicalein on ERK in HCC. We have demonstrated that inhibition of MAPK/ERK signaling and induction of apoptosis by Baicalein treatment are critical mechanisms by which Baicalein inhibits HCC growth.

**Materials and methods**

**Reagents.** Baicalein was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS), penicillin, and streptomycin were ordered from Gibco-BRL (Rockville, MD, USA). The apoptosis detection kit was from Nanjing KeyGen Biotech Co. Ltd. (Nanjing, China). MTT (3-(4,5-dimethyl-2-thiazole)-2,5-diphenyltetrazolium bromide) was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Caspase inhibitor z-VAD-fmk and anti-cytochrome c were purchased from Beyotime (Haimen, China), anti-MEK1 and anti-Phospho-MEK1 (Thr386) anti-Phospho (Thr386) MEK1 (p-MEK1) were from Millipore Co. (Billerica, MA, USA). Anti-ERK1/2, anti-Phospho (Thr202/Tyr204) ERK1/2 (p-ERK1/2), anti-Phospho-MEK1/2 (p-MEK1/2), anti-caspase-3, anti-Bad and anti-Phospho-Bad(Ser112) antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA).

**Animals.** Male BALB/c nude mice (4-week-old) were purchased from the Beijing Experimental Animal Center and maintained in the Laboratory Animal Center of Xi’an Jiaotong University, in accordance with the University Institutional Animal Care and Use Committee. HepG2 cells (2x10⁶) suspended in 200 µl of DMEM were injected subcutaneously into the right inguinal area of the 6-week-old male nude mice. All animals developed palpable tumors. Mice were divided into two groups (n=6 per group): group I, treatment with vehicle DMSO as the control group; group II, treatment with 20 mg/kg/day Baicalein via oral administration. Treatments were started one week after the injection of HepG2 HCC cells. Resulting tumors were measured using a vernier caliper every two days following the tumor cell injections, and tumor volumes were calculated using the formula: volume = (length x width³)/2 and expressed as mean size ± standard error.

**Cell culture.** Human HCC cell lines (HepG2, BEL-7402, SMMC-7721) and human normal liver cell line (HL-7702) were purchased from Shanghai Institute of Cell Biology (Shanghai, China). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mmol/l glutamine. All cells were incubated at 37°C with 5% CO₂.

**Construction of expression plasmids and transfection.** The full-length pcDNA3.1 (Invitrogen, Paisley, UK) MEK1 vector was made by cloning of the full-length PCR product of MEK1 with KOD® DNA polymerase (Toyobo, Osaka, Japan). All the plasmid sequences were confirmed by DNA sequencing. For transient transfection experiments, cells were plated 24 h before transfection in a 6-well plate at a density of 2x10⁵. Lipofectamine 2000 (Invitrogen) was used to perform transfection with 4.0 µg pcDNA3.1(+)MEK1 vector or 4.0 µg pcDNA3.1(+) empty vector (as a negative control) according to the manufacturer's protocol.

**Assessment of cell viability and apoptosis.** Cell viability was determined by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously reported (11). In brief, after treatment of cells with or without the indicated agent and/or serum for 48 h, the cells were washed twice with PBS and incubated with 0.5 mg/ml MTT (Sigma) for 4 h. The reagent was absorbed by living cells and eventually formed an insoluble blue formazan product. After the incubation period, cells were washed with PBS, solubilized with dimethyl sulfoxide (DMSO), and quantified using a microplate reader at the absorbance of 550 nm. The inhibition rate was determined using SPSS software (version 17.0, SPSS Inc, Chicago, IL, USA).

**Apoptotic and/or necrotic cells were evaluated by Annexin V binding and propidium iodide (PI) uptake using an Annexin V-FITC/PI kit as previously described (12). Briefly, tumor cells were plated at a density of 1x10⁵ cells per well into 6-well plates for 24 h. The cells were treated with various concentrations of Baicalein (0, 40, 80 and 120 µM) and incubated at 37°C for 24 and 48 h. The cells were washed with cold PBS and resuspended in Annexin V binding buffer. The cells were stained with Annexin V-FITC for 15 min, washed, and then stained with PI. The samples were analyzed by flow cytometer with CellQuest software.

**Detection of mitochondrial membrane potential (MMP) [µm].** Loss of MMP [µm] was assessed by flow cytometry, using a fluorescent indicator Rh123, as previously described (13,14).
Briefly, cells were treated with Baicalein at different concentrations (0, 20, 40 and 60 μM) for 24 h. Then, Rh123 working solution was added to the culture at a final concentration of 2 μg/ml and then incubated in the dark at 37°C for 30 min. Cells were then washed with PBS, and fluorescence of Rh123 was detected immediately using a FACSCalibur, at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Caspase-3 and caspase-9 activity assay. Cell lysates were prepared by incubating 2x10^6 cells/ml in extraction buffer (25 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, and 150 mM NaCl, 1% Triton X-100, 25 μg/ml leupeptin, and 25 μg/ml aprotinin) for 30 min on ice. Lysates were centrifuged at 12,000 x g for 15 min. Cellular extracts (30 μg) were then incubated in a 96-well microtitre plate with 20 ng Ac-DEVD-pNA (caspase-3 activity) or Ac-LEHD-pNA (caspase-9 activity) (Beyotime) for 2 h at 37°C. Caspase activity was measured by cleavage of the Ac-DEVD-pNA or Ac-LEHD-pNA substrate to pNA, the absorbance of which was measured at 405 nm. Relative caspase activity was calculated as a ratio of emission of treated cells to untreated cells.

Western blot analysis. Western blot analysis was executed as previously described (15). Whole-cell extracts were prepared from Baicalein-treated or control-treated cells cultured in 6-well plates. After incubation, cells were harvested and resuspended in lysis buffer, washed with ice-cold PBS and lysed in extraction buffer (40 mmol/l Tris-HCl, pH 7.5, 150 mmol/l KCl, 1 mmol/l EDTA, 1% Triton X-100, 100 mmol/l NaVO₄, 1 mmol/l PMSF) supplemented with the protease inhibitor cocktail. The protein (50 μg) was separated on 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS) at 37°C, and then incubated with rabbit anti-MEK1 antibody (1:1,000), rabbit anti-p-MEK1 antibody (1:1,000), mouse anti-ERK1/2 antibody (1:1,000), rabbit anti-p-ERK1/2 antibody (1:1,000), rabbit anti-Bad antibody (1:1,000), rabbit anti-p-Bad antibody (1:1,000) or mouse anti-β-actin antibody (1:500) in TBS containing 5% non-fat milk for 12 h at 4°C. Horseradish peroxidase-linked anti-mouse IgG (1:5,000) or horseradish peroxidase-linked anti-rabbit IgG (1:5,000) was used as a secondary antibody (in TBS containing 5% non-fat milk for 3 h at room temperature), and antigen-antibody complexes were detected using an enhanced chemiluminescence kit (Amersham, ECL Plus, Freiburg, Germany). Densitometry values for western blot analysis and antibody array experiments were estimated by the ImageQuant TL software (GE Healthcare, Buckinghamshire, UK) and expressed as arbitrary units (a.u.). Multiple film exposures were used to verify the linearity of the samples analyzed and to avoid saturation of the film.

Immunohistochemical procedures. The expressions and intracellular localizations of MAPK/ERK in HCC and mice xenograft were examined immunohistochemically. Antigen retrieval was performed by microwave oven for 15 min in TEG buffer (10 mM Tris, 0.5 mM ethylene glycol tetraacetic acid, pH 9.0). Incubation with primary antibody for 60 min at room temperature was followed by detection of the primary antibody using the Advance™ HRP system (Dako). The chromogen 3,3’-diaminobenzidine was applied and all the staining was performed using the Autostainer Plus Link Instrument (Dako). After washing, the slides were counterstained with Meyer’s hematoxylin for 30 sec. The following antibodies were used: p-MEK1/2 (dilution factor 1:100), pERK1/2 (dilution factor 1:100), PCNA (dilution factor 1:100). All antibodies mentioned above were from Cell Signaling Technology.

Terminal dUTP nick end labeling (TUNEL) analysis. Xenograft tumors were resected and fixed in formalin for 24 h, and imbedded in paraffin and 5-micron of sections were prepared. TUNEL assay was performed using an apoptag peroxidase in situ apoptosis detection kit (Chemicon International, Temecula, CA, USA). Briefly, the sections were digested using proteinase K and the endogenous peroxidase activity was blocked using 3% hydrogen peroxide in PBS. The sections were then placed in equilibration buffer and incubated with working strength of TdT enzyme in a humidifying chamber at 37°C for 1 h. The reaction was terminated with a stop/wash buffer provided with the kit. The apoptotic nuclei were stained by direct immunoperoxidase detection of digoxigenin-labeled DNA in test sections.

Statistical analysis. Data are presented as the mean ± standard errors from at least three independent experiments and analyzed using Student’s t-test. p<0.05 was considered statistically significant. All statistical tests and corresponding p-values were two sided.

Results

Baicalein preferentially inhibits HCC cells and spares normal liver cells. In order to investigate whether or not Baicalein has any differential cytotoxicity to HCC and normal liver cells as CIE does (5,6). We examined the cytotoxic activity of Baicalein in three HCC lines (HepG₂, BEL-7402 and SMMC-7721) and one normal liver cell line (HL-7702). The anti-tumor effects of Baicalein were examined by an MTT assay after treatment with 20 to 120 μM of Baicalein for 48 or 72 h. As shown in Fig. 2A, the viability of HepG₂, BEL-7402 and SMMC-7721 cells was significantly reduced by Baicalein treatment in a time- and dose-dependent manner, whereas the normal liver cell line (HL-7702) was hardly affected. To further examine the cytotoxicity of Baicalein in HepG₂, HCC cells and normal liver HL-7702 cells, 5-FU was used as a treatment benchmark in comparison with Baicalein. While 5-FU had a 50% inhibiting concentration (IC₅₀) of 1.05 mM in HepG₂ cells, Baicalein had an IC₅₀ value of 68.32 μM (Fig. 2B). As shown in Fig. 2B, the inhibitory effect of Baicalein on HL-7702 normal liver cells at its IC₅₀ concentration was significantly lower than that of 5-FU at its IC₅₀ concentration. Baicalein had an inhibition rate of 5.43±1.00%, whereas 5-FU inhibited more than 35% on normal liver cells at its IC₅₀ concentration in normal liver cells (Fig. 2B).

Baicalein reduces mitochondrial transmembrane potential and induces intrinsic apoptosis. To explore the mechanisms by which Baicalein inhibits HCC growth, HepG₂ HCC cells were first examined by phase contrast microscopy for any apoptotic characteristics after incubation with Baicalein at different concentrations.
concentrations (0, 40, 80, 120 µM) for 24 h. As shown in Fig. 2C, the control-treated cells showed a typical polygonal and intact appearance, whereas the Baicalein-treated cells displayed cellular shrinkage (40, 80 µM), rounding (120 µM), poor adherence (120 µM) and round floating shapes (120 µM). To determine the effect of Baicalein on apoptosis in detail, HepG2 HCC cells were treated with different concentrations of Baicalein (40, 80 or 120 µM), for 24 h and then subjected to an Annexin V analysis on flow cytometry. As shown in Fig. 3A, Baicalein induced marked apoptosis in HepG2 cells in a concentration-dependent manner. After short treatment for 24 h, the numbers of early apoptotic cells accompanied some late apoptotic cells were significantly increased in the Baicalein-treated HepG2 cells when compared with control-treated HepG2 cells (Fig. 3A). After longer treatment for 48 h, the numbers of late apoptotic and necrotic cells were also dramatically increased along with early apoptotic cells in the Baicalein-treated HepG2 cells (Fig. 3A).

The decrease of mitochondrial transmembrane potential (MMPΔψm) has been reported as an early event of apoptosis (16) and can be detected by the decline of rhodamine 123 fluorescence. To determine whether or not Baicalein-induced apoptosis involves the MMPΔψm, we used a fluorescent indicator Rh123 to detect the MMPΔψm in HepG2 cells that were treated with 20-60 µM of Baicalein for 24 h. As shown in Fig. 3B, after exposure to different doses of Baicalein, the cells exhibited dose-dependent decline of Rh123 staining. At the doses of 40 and 60 µM, Baicalein-treated cells had significant lower values of rhodamine 123 fluorescence (3,258.11±355.90, 2,705.45±276.17) than the control (4,703.24±698.91, p<0.05), further indicating that Baicalein can induce apoptosis in liver cancer cells (Fig. 3B).

To further determine whether apoptosis induced by Baicalein was a mitochondrial-dependent pathway, we tested whether cytochrome c could be released from the mitochondria into the cytoplasm. As shown in Fig. 3C, levels of cytochrome c release from the mitochondria increased dose-dependently in the Baicalein-treated HepG2 cells at concentrations ranging from 40 to 120 µM. To further investigate whether apoptosis induced by Baicalein was a caspase-dependent pathway, we tested whether mitochondrial-related caspases were activated by Baicalein treatment. Our research showed that caspase-9 and caspase-3 activities were highly increased dose-dependently on exposure to Baicalein in HepG2 cells.
Furthermore, we treated HepG2 cells with Baicalein in the presence of 10 M pan-caspase inhibitor (z-VAD-fmk) or DMSO (as a control). MTT assay showed that z-VAD-fmk partially attenuated Baicalein-induced inhibition on HepG2 cells (Fig. 3D), suggesting that apoptosis induction is an important cause for Baicalein-induced growth inhibition in HCC.

Figure 3. Baicalein induces intrinsic apoptosis in HCC cells and inhibition of apoptosis partially blocks Baicalein-induced growth inhibition. HepG2 cells were treated with Baicalein at indicated concentrations. (A) Detection of Baicalein-induced apoptosis by Annexin V flow cytometry assay. Flow cytometry was performed after 24 and 48 h of treatment. Induction rate of apoptosis was calculated as percentage of Annexin V+/PI+ cells in HepG2. Early apoptotic cells, late apoptotic cells and necrotic cells were measured according to manufacturer's protocol. *p<0.05 significant difference vs control. This assay was repeated three times in duplicate. (B) Detection of Baicalein-induced apoptosis by mitochondrial membrane potential (MMP Δψm). MMP Δψm of HepG2 cells was determined by flow cytometry 24 h after Baicalein treatment. Results presented are representative of 3 independent experiments. M1, HCC cells contained Rho123 in cytoplasm; M2, HCC cells contained Rho123 in mitochondria; *p<0.05 vs control group. (C) Detection of Baicalein-induced apoptosis by cytochrome c release. Cytochrome c release into the cytoplasm was detected by western blot analysis. (D) Detection of Baicalein-induced apoptosis by caspase 3/9 activity. Caspase 3/9 activities were detected by commercially available ELISA kits as described in Materials and methods. (E) Role of Baicalein-induced apoptosis on growth inhibition of HepG2 cells treated with Baicalein in the presence of 10 µM pan-caspase inhibitor z-VAD-fmk or DMSO as a control. *p<0.05 vs control.
Baicalein inhibits MEK/ERK signaling in vitro. Western blot analysis has been utilized to evaluate the effect of Baicalein on phosphorylation levels of MEK and ERK in HepG2 cells. As shown in Fig. 4A and 4C, Baicalein inhibited MEK1 and ERK1/2 phosphorylation at a concentration-dependent manner in HepG2 cells. The phosphorylation level of Bad (Ser 112), which is an anti-apoptosis protein activated by the MEK/ERK pathway in tumor cells (17), was also measured 24 h after Baicalein treatment. Baicalein reduced levels of phosphorylated Bad of Ser 112 in a dose-dependent manner (Fig. 4A and 4C).

Roles of MEK-ERK signaling in Baicalein activity. To determine whether this Baicalein-induced growth inhibition depends on the MEK-ERK pathway, HepG2 cells were transfected with a plasmid pcDNA3.1(+)MEK1 expressing human MEK1. Ectopic expression of MEK1 led to an enhanced activity of MEK-ERK pathway indicated by increased phosphorylation of MEK1 and ERK1/2 (18) (Fig. 4B). Importantly, HepG2 cells with ectopic expression of MEK1 (higher MEK-ERK activity) became relatively resistant to Baicalein-induced growth inhibition (Fig. 4). Overexpression of MEK1 partially attenuated Baicalein-induced inhibition of ERK1/2 phosphorylation (Fig. 4C). Overexpression of MEK1, in part, blocked Baicalein-induced growth inhibition in vitro (Fig. 4D). These data suggest that inhibition of MEK-ERK is one of critical mechanism by which Baicalein inhibits HCC cells.

Baicalein suppresses HCC xenograft growth, inhibits MEK-ERK phosphorylation, and induces apoptosis in vivo. In the animal study, the control group received diluent vehicle treatment only, whereas the treatment group received Baicalein 20 mg/kg/day. This in vivo dosage was selected by our pilot experiments that showed significant tumor inhibition but without significant side effects. The mice were treated with Baicalein daily for 21 days. As shown in Fig. 5A, Baicalein-treated mice exhibited a statistically significant tumor volume reduction (p<0.01) compared with the control group. The average tumor volume of control and treatment group were 3.25±0.56 cm³ and 1.02±0.40 cm³, respectively. After treatment for 21 days, the mice were sacrificed, xenograft tumors were resected and the tumor weight of xenograft were measured. As shown in Fig. 5B and 5C, tumor sizes and weights in Baicalein-treated mice were dramatically smaller than those in control-treated
mice. The control-treated mice had a median tumor weight of 2.12 g, whereas the \textit{Baicalein}-treated mice had a median tumor weights of 0.41 g (Fig. 5B).

In order to confirm the \textit{in vitro} observation of \textit{Baicalein}-induced apoptosis (Fig. 3), \textit{Baicalein}-induced apoptosis in xenograft tumors was evaluated with the terminal deoxy- nucleotidyl transferase dUTP nick end labeling (TUNEL) assay. As shown in Fig. 6A, \textit{Baicalein}-treated tumors had greater TUNEL-positive cells than control-treated tumors. In agreement with the \textit{in vitro} observations, p-MEK1/2 and p-ERK1/2 expression were markedly inhibited in \textit{Baicalein}-treated tumors as illustrated by immunohistochemical analysis (Fig. 6A) and western blot analysis (Fig. 6B and 6C). MEK-ERK signaling associated Bad phosphorylation (Serine 112) was also decreased by \textit{Baicalein} treatment (Fig. 6B and 6C). Above data confirm the \textit{in vitro} results and show that \textit{Baicalein} treatment can significantly suppress HCC tumor growth and MEK-ERK signaling, and can induce apoptosis \textit{in vivo}.

**Discussion**

\textit{Baicalein} alone, or in combination with other herbs, has recently been shown to have cytostatic effect on several cancer cell lines \textit{in vitro} (7,19) and also \textit{in vivo} (20,21). \textit{Baicalein} has shown the advantage of inhibiting the growth of cancer cells while leaving normal cells relatively unaffected in several studies (22,23). In this report, we confirmed that \textit{Baicalein} had anti-cancer effect against HCC cells \textit{in vitro}. We have further demonstrated that \textit{Baicalein} had much lower cytotoxicity to normal liver cells in comparison with 5-FU. 5-FU can be beneficially used for hepatic arterial infusion chemotherapy (24) or intra-peritoneal administration (25) as treatment for HCC. However, toxicity issue limits its clinical application. Our data showed that \textit{Baicalein} had greater effect on HCC cells but less toxicity on normal liver cells than 5-FU. Thus, \textit{Baicalein} is potentially more acceptable than 5-FU in clinic and deserves further clinical trials. To our knowledge, this is the first study to evaluate the potential of \textit{Baicalein} \textit{in vivo} treatment of HCC xenografts. Significant reduction of tumor mass was observed after a 3-week treatment. The \textit{in vivo} effect of \textit{Baicalein} on HCC tumors strongly support \textit{Baicalein} as a potential new chemodrug for anti-HCC treatment.

Whether \textit{Baicalein} inhibits HCC cells via apoptosis induction is still controversial. A high proportion of necrotic HCC cells after \textit{Baicalein} treatment was observed by Matsuzaki \textit{et al} (8). \textit{Baicalein} was also reported to induce caspase-related apoptosis in cancer cells (26). In the present study, we confirmed a pro-apoptotic effect of \textit{Baicalein} on HCC cells by using several methods. Although it was widely reported that mostly chemotherapy reagent induced mitochondrial
signaling apoptosis (27), the mechanisms and pathways involved in Baicalein-induced apoptosis on HCC cells are still unclear. Our data illustrated a decrease of MMP ∆ψm and released of cytochrome c from mitochondria, and the following activation of caspase-9 and caspase-3, suggesting a mitochondrial signaling-related apoptosis was induced by Baicalein in HCC cells. We have further demonstrated that induction of apoptosis is important for Baicalein effect. z-VAD-fmk is a pan-caspases inhibitor which could nullify caspases activity (28). Our results confirmed that z-VAD-fmk did blocked Baicalein effects, suggesting that caspase-dependent apoptotic pathways were involved in Baicalein-induced inhibition on HCC cells.

The exact molecular mechanism by which Baicalein inhibits cell growth is still not known. There are few studies suggesting that MEK-ERK pathway could be the downstream signaling in response to Baicalein (29-31). Our study has demonstrated that inhibition of MEK-ERK pathway is critical for Baicalein action in HCC. The experiments of MEK overexpression showed that without inhibition of MEK-ERK pathway, Baicalein-induced growth inhibition was significantly attenuated. In fact, extracellular signal-regulated kinase (ERK) cascade plays critical roles in the development of HCC (32). ERK is a serine/threonine kinase that can be activated by hepatocyte growth factor (HGF) (33) and its receptor the c-Met proto-oncogene (34). ERK is activated in HepG2 cells after treatment with HGF and constitutive expression of Ha-Ras (35,36). ERK inhibitor is suggested as a potential anti-HCC agent (37-39). Sorafenib is the first targeted therapy drug that has demonstrated an improved overall survival benefit in patients with advanced HCC (40-42). Sorafenib can inhibit tumor cell proliferation in vitro by targeting the Raf/MEK/ERK signaling pathway at the level of Raf kinase (43) and by targeting angiogenesis (44). p-ERK could be a useful biomarker predictive of sensitivity to sorafenib (45), suggesting the critical role of the MAPK/ERK signaling in HCC. Again, our study supports the notion that down-regulation of the MAPK/ERK activity is beneficial in HCC treatment.

Bad is a pro-apoptotic protein and its function is modulated by phosphorylation at two sites, Ser-112 and Ser-136 (46,47). the MAPK-activated pp90-ribosomal S6 kinase family can catalyze the phosphorylation of Bad (48). Underphosphorylated Bad interacts with anti-apoptotic Bcl-2 members and anchors on the mitochondria to induce apoptosis whereas phosphorylated Bad is sequestered in the cytoplasm by 14-3-3 proteins that attenuate Bad induced apoptosis (50). Our results indicate that Baicalein downregulates the phosphorylation level of Bad, suggesting that Bad is one of the downstream targets of Baicalein-induced inhibition of ERK. Baicalein-induced apoptosis in hepatocellular cells could be through Bad-related regulation, which needs to be further determined.
Taken together, this study found that 

**Baicalein** is an effective anti-HCC agent with low cytotoxicity to normal liver cells. This study provides evidence to show that inhibition of MAPK-ERK signaling and induction of intrinsic apoptosis are the critical mechanisms by which **Baicalein** inhibits HCC growth.

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**References**


