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Baicalein inhibits the migration and invasive properties of human hepatoma cells

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ABSTRACT

Flavonoids have been demonstrated to exert health benefits in humans. We investigated whether the flavonoid baicalein would inhibit the adhesion, migration, invasion, and growth of human hepatoma cell lines, and we also investigated its mechanism of action. The separate effects of baicalein and baicalin on the viability of HA22T/VGH and SK-Hep1 cells were investigated for 24 h. To evaluate their invasive properties, cells were incubated on matrigel-coated transwell membranes in the presence or absence of baicalein. We examined the effect of baicalein on the adhesion of cells, on the activation of matrix metalloproteinases (MMPs), protein kinase C (PKC), and p38 mitogen-activated protein kinase (MAPK), and on tumor growth in vivo. We observed that baicalein suppresses hepatoma cell growth by 55%, baicalein-treated cells showed lower levels of migration than untreated cells, and cell invasion was significantly reduced to 28%. Incubation of hepatoma cells with baicalein also significantly inhibited cell adhesion to matrigel, collagen I, and gelatin-coated substrate. Baicalein also decreased the gelatinolytic activities of the matrix metalloproteinases MMP-2, MMP-9, and uPA, decreased p50 and p65 nuclear translocation, and decreased phosphorylated I-kappa-B (IKB)-β. In addition, baicalein reduced the phosphorylation levels of PKCα and p38 proteins, which regulate invasion in poorly differentiated hepatoma cells. Finally, when SK-Hep1 cells were grown as xenografts in nude mice, intraperitoneal (i.p.) injection of baicalein induced a significant dose-dependent decrease in tumor growth. These results demonstrate the anticancer properties of baicalein, which include the inhibition of adhesion, invasion, migration, and proliferation of human hepatoma cells in vivo.

Introduction

Metastasis, the major cause of death among cancer patients (Mehlen and Puisieux, 2005), involves a distinct sequence of events that are not yet fully elucidated. These events include changes in cell–extracellular matrix (ECM) interactions, disruption of intercellular adhesions, separation of single cells from solid tumor tissue, degradation of the ECM, and transit of tumor cells into this structure (Weigelt et al., 2005).

Abbreviations: MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; PKC, protein kinase C; (IKB)-β, phosphorylated I-kappa-B.

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A fundamental role in tumor metastasis has been attributed to matrix metalloproteinases (MMPs), which display altered expression in different human cancers (López-Otín and Hunter, 2010). Generally, elevated expression of MMPs in primary tumors and metastases is associated with cancer progression, poor prognosis, and hence shorter survival times. Proteinases involved in the degradation of the ECM (MMP-2, MMP-9, uPA, uPAR, PAI) are regarded as biomarkers of malignant hepatocellular carcinoma (HCC), and are thus related to the prognosis and therapeutic outcomes of HCC (Tretiakova et al., 2009).

HCC is the fifth most frequent neoplasm worldwide and the third most common cause of tumor-related death (Parkin et al., 2002). Although surgery remains the only truly effective therapeutic approach to the treatment of HCC, most patients with HCC are ineligible for surgical intervention. Protein kinase C α (PKCα) is associated with cell migration and invasion and is highly expressed in the poorly differentiated human HCC cell lines, HA22T/VGH and SK-Hep1 (Hsieh et al., 2007). Thus, it has been speculated that p38 mitogen-activated protein kinase (MAPK) may directly affect the invasiveness of SK-Hep1 and HA22T/VGH cells mediated via activation of PKCα, which contributes to the development of HCC (Hsieh et al., 2006). There is, therefore, an urgent need to gain further insight into the molecular mechanisms underlying the biology of HCC and, thereby, identify potential new molecular targets for developing novel treatments that are more effective and less toxic than existing therapies and surgical approaches.

Epidemiological studies now indicate that an increased intake of dietary flavonoids is associated with a decreased risk of inflammation, hypertension, cardiovascular disease, and bacterial and viral infections (Arts and Hollman, 2005; Knekt et al., 2002). Flavonoids are secondary products of plants and are thus ingested daily from fruits, vegetables and other foods. Studies of dietary flavonoids have revealed a broad spectrum of biological activities for these molecules, including the inhibition of cell proliferation in cell culture, induction of apoptosis, alterations in the activity of certain intracellular enzymes, and antioxidant properties (Havsteen, 2002). Most importantly, flavonoids show almost no toxic effects on normal peripheral blood and myeloid cells, or on normal hepatocytes (Chen et al., 2009; Hwang et al., 2006). Baicalein, a bioactive flavonoid extracted from the roots of *Scutellaria baicalensis* or *Scutellaria radix*, has been shown to exert antitumor activity (Li-Weber, 2009). In addition, this compound causes cell cycle arrest and suppresses the proliferation of cancer cells. Moreover, baicalein induces apoptosis in a variety of human cancer cell lines (Chen et al., 2000; Po et al., 2002; Kuo et al., 2009). However, the precise mechanisms underlying the anti-metastatic abilities of baicalein remain unclear.

In our current study, we characterized two flavonoids that are structurally similar, baicalein and baicalin, in that they contain a 7-hydroxyl or 7-glycoside substitution in their A-ring. We hypothesized that baicalein would function as a chemopreventive agent in a comparable manner to baicalin. We evaluated the in vivo antitumor potential of this agent by investigating whether baicalein would attenuate the invasiveness of HCC cells via the inhibition of cell–ECM interactions, the suppression of MMPs, uPA and NF-κB translocation.

Materials and methods

Chemical reagents and antibodies. Baicalein, baicalin, gelatin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Anti-phospho-p44/42 MAPK or anti-p44/42 MAPK, anti-phospho-PKCα, anti-phospho-p38 and anti-phospho-IκBβ (Thr19/Ser23) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-MMP2, MMP-9, NF-κB-p50, p65/RelA, Histone H1 and IκBβ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. The HA22T/VGH (BCRC No. 60168) cell line was purchased from the Bioresources Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan). SK-Hep1 cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured with Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS), penicillin G, and streptomycin (Sigma Chemicals, St. Louis, MO, USA) in a humidified atmosphere containing 5% CO_2 at 37 °C. All experiments were performed using plastic tissue culture flasks, dishes, or microplates (Nunc, Naperville, Denmark).

Cell growth and proliferation assay. Cell viability was determined using the MTT quantitative colorimetric assay as previously described (Kuo et al., 2006a). Cells were seeded and incubated with the various agents. Thereafter, the medium was changed and cells were incubated with MTT (0.5 mg/mL) for 4 h. The viable cell number is directly proportional to the production of formazan, which is measured spectrophotometrically ($\lambda = 563$ nm) after solubilization with isopropanol. Cell growth was determined by counting the cells at the indicated time points with a Coulter counter, combined with a trypan blue (0.2%) exclusion assay.

Apoptosis assays. Annexin V-FITC (Biosource International, USA) was used to quantify the percentage of cells undergoing apoptosis (Kuo et al., 2006a). Flow cytometric analysis was performed with a FACSCaliber using CellQuest software. Data were analyzed with CellQuest and WinMDI software.

Cell cycle distribution analysis. Flow cytometric analysis of the baicalein-treated cells was performed using a FACScan (Becton Dickinson Immunocytometry Systems, UK) (Kuo et al., 2006b). The DNA content of the stained nuclei was analyzed by flow cytometry. The distribution of DNA content was expressed as G1, S, and G2/M phases.

Boyden chamber assay. A Boyden chamber, comprising an upper and a lower compartment, was used to analyze tumor cell migration. Human hepatoma cells were allowed to grow as discrete colonies and were treated with baicalein or baicalin as previously described (Lin et al., 2008). Migration assays were carried out in a 48-well chemotaxis chamber (Neuro Probe, Inc.). The number of cells that migrated to the lower side of the membrane was then determined.

Matrigel invasion assay. SK-Hep1 cells and human HA22T cells were incubated with DMEM in 10% fetal calf serum and then collected by trypsinization. Cells (1×10^5/mL) in serum-free medium were added to the inner cup of the 48-well transwell chamber that had been coated with 50 μL of matrigel (1:10 dilution in serum-free medium). After 24 h, cells that had migrated through the matrigel and filter membrane composed of 8-μm pores were fixed, stained, and counted under a light microscope (Lin et al., 2008).

Scratch assays. Scratch assays were performed as previously described (Lin et al., 2008). After the attached cells had reached confluence, a 4-mm scratch was made through the cell monolayer. The cells were then washed twice with phosphate-buffered saline (PBS; pH 7) and incubated with culture medium in the absence (control) or presence of baicalein or baicalin at the appropriate concentrations. Photographs of treated cells moving within the scratch were taken and analyzed these digital frames.

Cell–matrix adhesion assay. Cells were pretreated with various concentrations of the test compounds for 24 h, and single-cell suspensions were then plated and cultured for 30 min on 24-well dishes that had been pre-coated with matrigel, type I collagen, and gelatin. Non-specific binding to pre-coated dishes was blocked by preincubating with 2% BSA in PBS for 2 h at room temperature. After a 30 min incubation at 37 °C, non-adherent cells were removed by

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washing with PBS and adherent cells were fixed by ethanol. After staining with 0.1% crystal violet for 10 min, fixed cells were lysed with 0.2% Triton X-100 and their absorbance was measured at 550 nm (Yoon et al., 2001).

Preparation of total cell extracts and immunoblotting analysis. Cellular lysates were prepared by suspending 1×10^6 cells in 200 μL of lysis buffer. The protein content in the supernatant was quantified using the BCA protein quantitation assay and immunoblotted on Immobilon-P membranes (Millipore, Bedford, MA, USA) using the indicated secondary antibodies. Signals were detected using an enhanced chemiluminescence western blot kit as described previously (Kuo et al., 2011).

Preparation of nuclear extracts. Nuclear extracts were prepared as described previously with some modifications (Kuo et al., 2006a). Briefly, the cell pellet was then resuspended in cytosolic buffer on ice for 15 min and, after centrifugation at 2000 *g* for 10 min, the supernatants were collected as cytoplasmic extracts. The nuclear pellet was resuspended in 50 μL of extraction buffer and incubated at 4 °C with occasional vortexing for 20 min. The mixture was finally centrifuged at 14,000 rpm for 5 min. The supernatant was collected, and its protein concentration was then measured.

Zymography. MMP-2 and MMP-9 enzymatic activities were assayed and prepared as described previously with some modifications (Lin et al., 2008). The area of the photo images in the gel was determined by measuring the numbers of pixels using ImageGauge 3.46 software (Fujifilm, Inc.).

In vivo treatments with baicalein. BALB/c-nu mice were purchased from the National Laboratory Animal Center Taiwan. BALB/c-nu female nude mice at 4–6 weeks old (18–20 g), were maintained under specific pathogen-free (SPF) conditions and were supplied with sterilized food and water. Trypsinized SK-Hep1 cells (1×10^6 cells/0.2 mL) were injected subcutaneously into the flanks of female athymic BALB/c-nu mice (4–6 weeks old). After tumor inoculation, mice were divided randomly into four groups of eight mice each. The control animals were treated daily with 0.1 mL DMSO (0.25%; i.p.), and the test animals were treated with baicalein (5, 10, 20 mg/kg/day; i.p.) for five days. Tumor volumes were monitored on the fourth day, and then measured at four-day intervals using calipers; calculation was based on the following formula: length × width^2 × π/6 (Kuo et al., 2006b). To monitor drug toxicity levels, the body weights of the mice were measured every week. After 32 days, the mice were sacrificed, and the tumors were removed and weighed. In addition, a pathologist examined the organs of each mouse, including their liver, lungs, and kidneys.

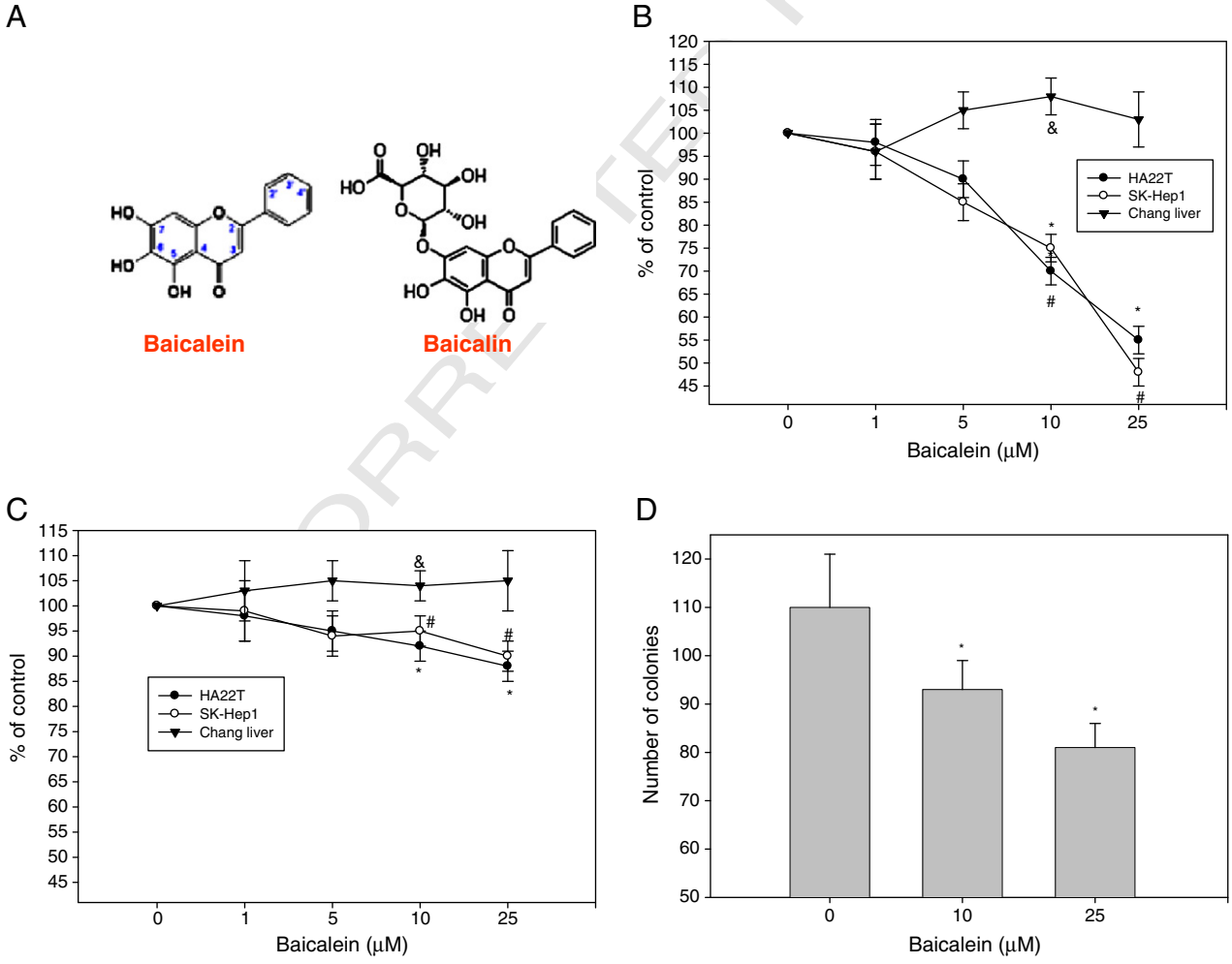

Fig. 1. Cellular viability of HA22T, SK-Hep1, and Chang liver cells treated with the flavonoids baicalein and baicalin. (A) The structures of baicalein and baicalin. (B,C) HA22T, SK-Hep1, and Chang liver cells were treated with baicalein or baicalin, and after 24 h their viability was measured using an MTT assay. Data are reported as mean ± SD of three independent experiments, each performed in triplicate. *$P<0.05$ indicates significant difference compared to an untreated group of HA22T. #$P<0.05$, compared with a control group of SK-Hep1. &$P<0.05$, compared with a control group of Chang liver cells. (D) Cell clonogenic assays to assess the effect of baicalein treated in SK-Hep1 cells for 10 days. SK-Hep1 was plated out at 300 cells per dish in 100-mm dishes (10 mL medium/dish). The cells were then fixed and stained with crystal violet, and the number of survivors (i.e., colonies containing >50 cells) scored.*$P<0.05$ indicates significant difference compared to an untreated group of SK-Hep1.

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Statistical analyses. Data were reported as the mean ± standard deviation (SD) of three independent experiments and were analyzed by one-way analysis of variance (ANOVA). The data were analyzed using the SAS software statistical package "SigmaPlot," version 9.0 (SAS Institute Inc., Cary, NC, USA).

Results

Effects of baicalein and baicalin on the proliferation of hepatoma cells

Because of their structural resemblance (Fig. 1A), we hypothesized that baicalein would have chemopreventive properties comparable to that of baicalin. We used the poorly differentiated HCC cell lines HA22T/VGH and SK-Hep1, which are PKCα-positive, to test the potency of these two flavonoids as cell growth inhibitors, and we employed a cell invasiveness model to examine their antitumor properties (Hsieh et al., 2007, 2006). After seeding the cells in 24-well plates and incubating with 1, 5, 10, and 25 μM baicalein or baicalin for 24 h, cell viability was determined using an MTT assay of logarithmically growing hepatoma cells (Figs. 1B and C). Cell viability increased within 24 h of treatment with 1.0 μM baicalein and baicalin. However, a further increase in the concentration of baicalein decreased cell viability in media containing 10% FBS. Following the addition of 10 and 25 μM baicalein alone, the viability of HA22T/VGH and SK-Hep1 cells was reduced to 75% and 48%, respectively ($P<0.01$). Baicalin did not show significant cytotoxic effects in Chang liver cells, which are widely used as a model for normal human hepatocytes in studies of cell signaling and apoptosis (Fernandez-Martinez et al., 2006). To confirm the results of the MTT assay, we performed a trypan blue dye exclusion assay and obtained similar findings (data not shown). Survival ability of SK-Hep1 cells has been evaluated by the clonogenic assay at 72 h after baicalein exposure. The result showed addition of

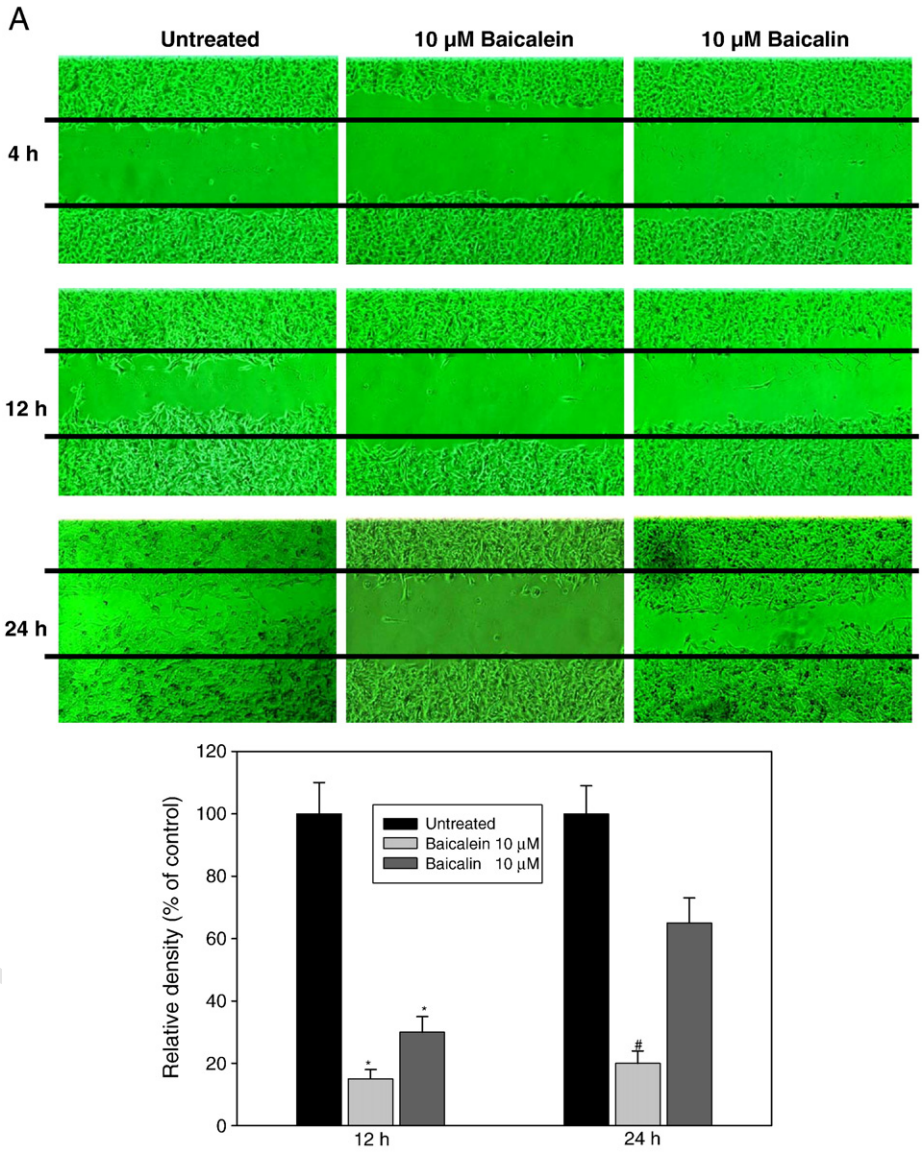

Fig. 2. Effects of baicalein and baicalin treatment on both cell migration and growth of SK-Hep1 cells as measured by the scratch-wound assay. (A) Cells were incubated with indicated concentrations of baicalein or baicalin for the indicated time points, and were measured by the scratch assay as described in Materials and methods. The percentage of surface area filled by the SK-Hep1 cells was subsequently quantified by densitometric analysis and compared to that of control (100%) as shown just below the gel data. Data are presented as mean ± SD of three independent experiments. *$P<0.05$, compared with control group for 12 h. #$P<0.05$, compared with control group for 24 h. (B) Quantitation of the percentage of cell death was determined by annexin V/PI-FITC. One of three independent experiments is shown. (C) After treatment with baicalein and mitomycin C for 24 h, the cells were fixed and stained with propidium iodide, and the DNA content was analyzed by flow cytometry (FACS). The percentage of cells in each phase (G1, S, and G2/M) of the cell cycle was calculated and expressed. Data are presented as mean ± SD of three independent experiments.

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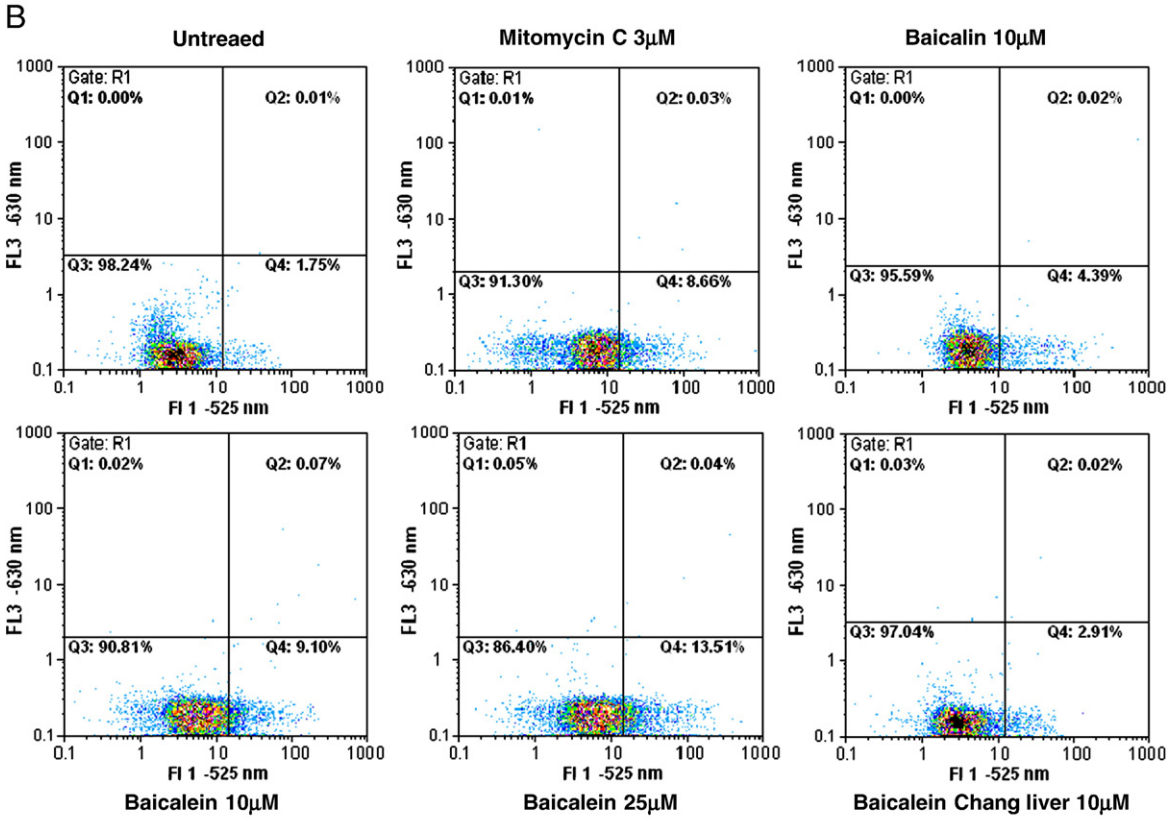
B



C

	G1	S	G2/M
Untreated	55±3	31±2	14±3
Baicalin 10 μM	60±3	25±2	15±3
Baicalein 10 μM	65±3	21±3	14±5
Mitomycin C	4±3	92±3	4±2

Fig. 2 (*continued*).

10 and 25 μM baicalein, a degree of long term survivors of SK-Hep1 cells was reduced to 80% and 70%, respectively ($P<0.01$), which is consistent with our previously MTT assay (Fig. 1D).

Baicalein-mediated reduction in hepatoma cell proliferation, migration and invasion

A scratch-wound assay was performed to determine the effects of baicalein and baicalin on SK-Hep1 cell growth. The extension of the cell population was quantified by estimating the percentage of recolonization of the wound surface after 24 h. A continuous rapid movement was observed for all cells. The resultant movement of the hepatoma cell migration front was clearly evident at 24 h, where a highly confluent (90%–100%) monolayer region gradually migrated into the cell-free "scratch" region. In the presence of baicalein, this movement was significantly reduced after 12 and 24 h of incubation, although it was not completely inhibited at 24 h (Fig. 2A). Baicalein and baicalin induced significant cell death as determined by annexin V-FITC/PI dye. The extent of apoptosis was quantified as a percentage of annexin V-positive cells. Twenty-four hours after the addition of 10 or 25 μM of baicalein, the extent of apoptosis was 9% or 14%, respectively; however, 25 μM baicalein-induced apoptosis was abolished in Chang liver cells (Fig. 2B) compared to untreated control. The number of cells in each cell cycle phase was determined by flow cytometry. As shown in Fig. 2C, after treatment with baicalein, the percentage of cells in the G0/G1 phase increased in a dose-dependent manner; those in the G1 phase increased to 65%, while those in the S and G2/M phase decreased to 21% and 14%, respectively. The effect of mitomycin was used to examine growth inhibition as a standard control in SK-Hep1 cells. The migration and invasiveness of HA22T/VGH and SK-Hep1 cells were also significantly reduced by treatment with baicalein (Figs. 3A and B). However, baicalin did not have a significant effect on HCC cells migration and invasion (data not shown).

Effects of baicalein and baicalin on the adhesion of hepatoma cells to ECM proteins and cells

Cell–matrix interactions play a unique role in cancer cell invasion as they affect protease expression, cell movement, and cell survival (Giancotti and Ruoslahti, 1999; Larsen et al., 2006). Hence, we examined whether baicalein and baicalin had any impact on these interactions. When SK-Hep1 cells were preincubated for 6 h with these compounds, baicalein markedly reduced the number of cells attached to matrigel, type I collagen, and gelatin alone in a dose-

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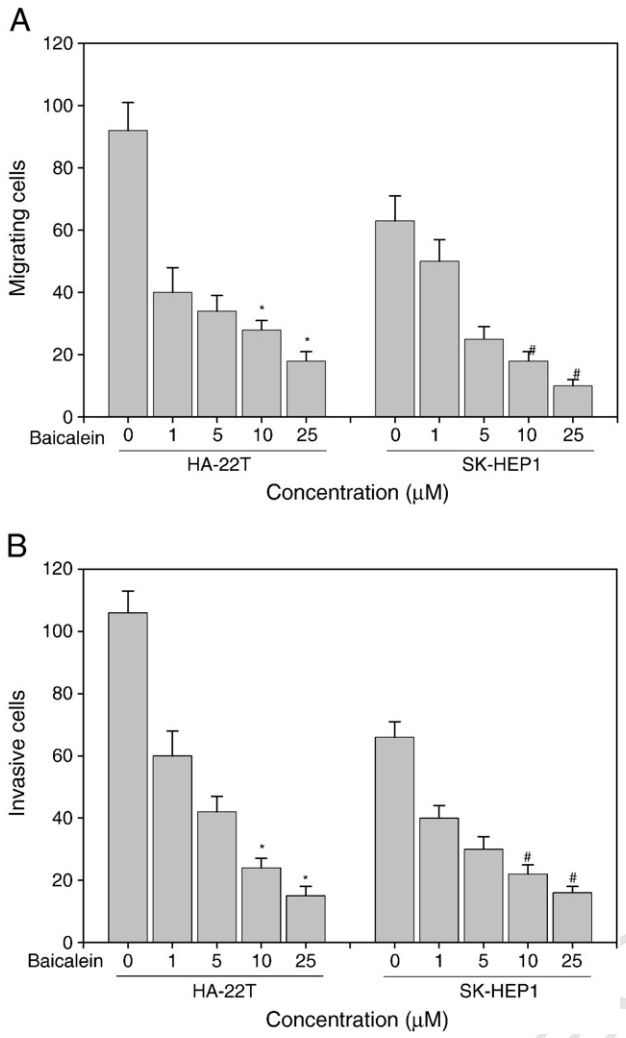

Fig. 3. Effects of baicalein on in vitro migration and invasion of HA22T and SK-Hep1 cells. (A) For the migration assay, HA22T and SK-Hep1 cells were assayed. Cells preincubated with various concentrations of baicalein were plated onto the upper wells of the chamber. FBS (10%) was added to the lower wells for 24 h to induce cell migration. After 24 h, cells on the bottom side of the filter were fixed, stained, and counted. Migration was assessed by counting migrated cells in five microscopic fields per well at 200× magnification. Cell migration minus spontaneous migration in PBS was designated as control. (B) For the invasion assay, the lower and upper parts of transwells were coated with matrigel to which cells and various concentrations of baicalein were added. HA22T and SK-Hep1 cells preincubated with drug were plated onto the upper wells of the chamber. Data represent the mean ± SD of at least three independent experiments, each performed in triplicate. $^{*}P<0.05$ indicates significant differences compared to the untreated group of HA22T. $^{\#}P<0.05$, compared with control group of SK-Hep1.

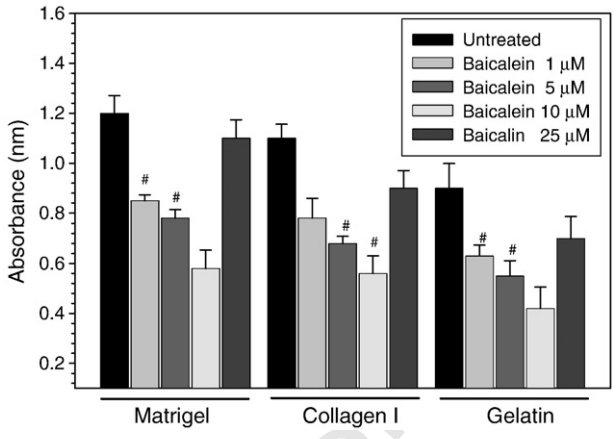

Fig. 4. Effect of baicalein on cell–matrix adhesion. For the cell–matrix attachment assay, SK-Hep1 cells, which were preincubated with baicalein 1–10 μM or baicalin 25 μM for 6 h, were seeded onto the matrigel, type I collagen, or gelatin-coated wells. After 30 min, unattached and attached cells were collected and counted. Data are reported as mean ± SD of three independent experiments, each performed in triplicate. $^{\#}P<0.05$, compared with control group of SK-Hep1.

dependent manner (Fig. 4). In contrast, baicalin did not significantly affect cell–ECM protein interactions.

Effects of baicalein on MMP and uPA activities

When tumor cells become detached from neighboring cells by releasing their intercellular junctions during metastasis, the extracellular matrix is proteolytically degraded to allow migration and invasion of these cells (Clark et al., 2008). Hepatoma cells constitutively secrete high levels of such proteinases, specifically MMP-2, MMP-9, and uPA (Sakamoto et al., 2000; Zhou et al., 2000). To clarify whether baicalein has an inhibitory effect on the activities of the MMPs and uPA, we employed gelatin and fibrin zymography, respectively, and MMP and uPA bands were confirmed by size markers. Incubation of SK-Hep1 cells with baicalein for 24 h markedly reduced the MMP-2 and uPA activities in a concentration-dependent manner but only slightly decreased MMP-9 activity (Fig. 5A). Several transcriptional factors are known to regulate the expression of the MMPs, most notably NF-κB, which regulates MMP-9, MMP-2, and uPA (Yan and Boyd, 2007). Using an ELISA MMP-2/MMP-9 activity assay we observed that baicalein decreased MMP-2 and MMP-9 activity by 0.7 ± 0.2 and 0.5 ± 0.1-fold compared to control (n = 3, $^{*}P<0.05$), indicating inactivation of the MMP-2 and MMP-9 invasive signaling pathway. In addition, the p38 inhibitor SB203580 also decreased MMP-2 and MMP-9 activity by 0.4 ± 0.1 and 0.3 ± 0.1-fold compared to control (n = 3, $^{*}P<0.05$) (Fig. 5B).

Inhibitory effects of baicalein on the expression of NF-κB and the MMPs, in addition to the phosphorylated proteins PKCα, p38, and ERK1/2 MAPK

NF-κB is a key signaling molecule that mediates the expression of many genes. It has been reported that, under unstimulated conditions, HCC cell lines with varying degrees of poor differentiation (including SK-Hep1 cells) are involved in the activation of NF-κB (Liu et al., 2000). To further determine the molecular mechanisms underlying the baicalein-mediated suppression of NF-κB transactivation, we measured the protein levels of transcription factors regulated by baicalein. We observed that treatment with baicalein significantly decreased the nuclear translocation of NF-κB/P50, and total lysate protein p65/RelA, and the phosphorylation of IKB-β (Fig. 6A). To investigate whether NF-κB bind the promoter region in SK-Hep1, we performed quantitative analysis for NF-κB p50 binding activity in vitro by using TF ELISA kits from Panomics (Huang et al., 2011). The results from those experiments showed that treatment of SK-Hep1 cells with baicalein resulted in decreased binding activities. Treatment of SK-Hep1 cells with baicalein also resulted in decreased levels of MMP-9 and MMP-2 (Fig. 6B). To then determine the role of p38 MAPK in PKCα-mediated hepatoma malignant invasion, we evaluated the effects of baicalein and baicalin on the phosphorylation of PKCα, p38, and ERK1/2 MAPK. The activities of PKCα and p38 MAPK were significantly decreased by baicalein (Fig. 6C), whereas baicalin had no effect.

In vivo inhibition of SK-Hep1 tumor growth by baicalein

To evaluate the in vivo effects of baicalein on tumor growth, SK-Hep1 cells were xenografted into nude mice as described previously (Kuo et al., 2006a). The results of our previous toxicity assessment

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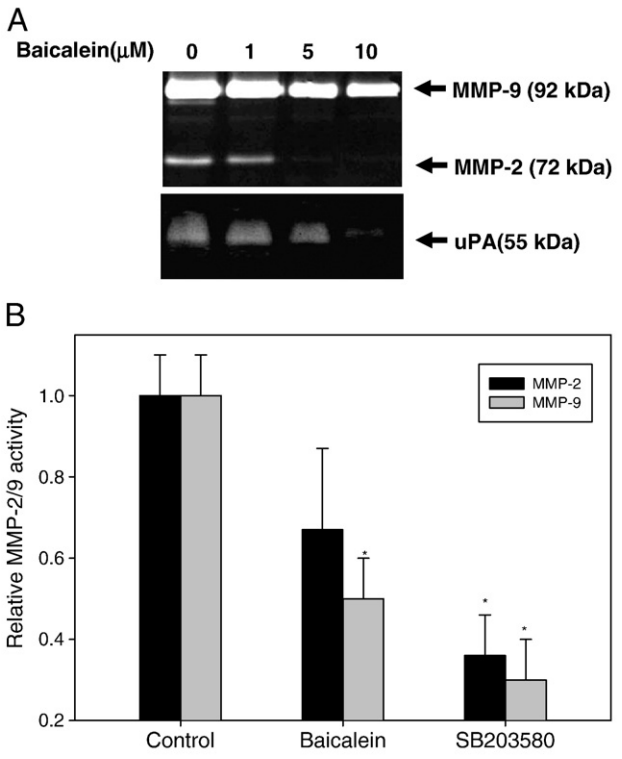


Fig. 5. Effects of baicalein on NF-κB-dependent transcriptional activity and on activities of MMP-9, MMP-2, and uPA. (A) To measure activities of MMP-9, MMP-2, and uPA, SK-Hep1 cells were treated with various concentrations of baicalein 1–10 μM. After treatment for 24 h, conditioned media were collected, and gelatin zymography was performed. Protein levels were quantified by densitometric analysis, with that of control being set at 100%. (B) SK-Hep1 cells grown to confluence were treated with 10 μM of baicalein and 10 μM of SB203580 for 24 h. Cell culture supernatant activity was measured as described under "Amersham Matrix Metalloproteinase-9 (MMP-9) Biotrak Activity Assay System." MMP-2 and MMP-9 activities were normalized and expressed relative to control (C = control; * versus control, n = 3, $P<0.05$).

experiments suggested that the in vivo dosage of baicalein should not exceed 20 mg/kg (data not shown); therefore, concentrations of 5, 10, and 20 mg/kg were used in these experiments. The time course of SK-Hep1 xenograft growth with and without baicalein treatment is shown in Fig. 7A. By evaluating the xenograft tumor volume, a significant inhibition of tumor growth by baicalein was observed in both a dose- and time-dependent manner. In the baicalein-treated (5, 10, 20 mg/kg/day) mice at 32 days after cell implantation, the SK-Hep1 xenograft volumes were inhibited to 40%, 15%, and 8%, respectively, of those in the control group. At the end of the experiment, the xenograft tumors were removed and weighed. Baicalein was found to significantly decrease the solid tumor mass when compared to the control group (Figs. 7B and C). No signs of toxicity were observed in any of the transplanted nude mice as assessed by body weight and microscopic examination of individual organs (data not shown). In addition, another marker of HCC differentiation, PKCα, was examined by immunohistochemistry. The results showed that baicalein treatment reduced the number of PKCα-positive cells (Fig. 7D). These results indicate the antitumor action of baicalein on SK-Hep1 cells in vivo.

Discussion

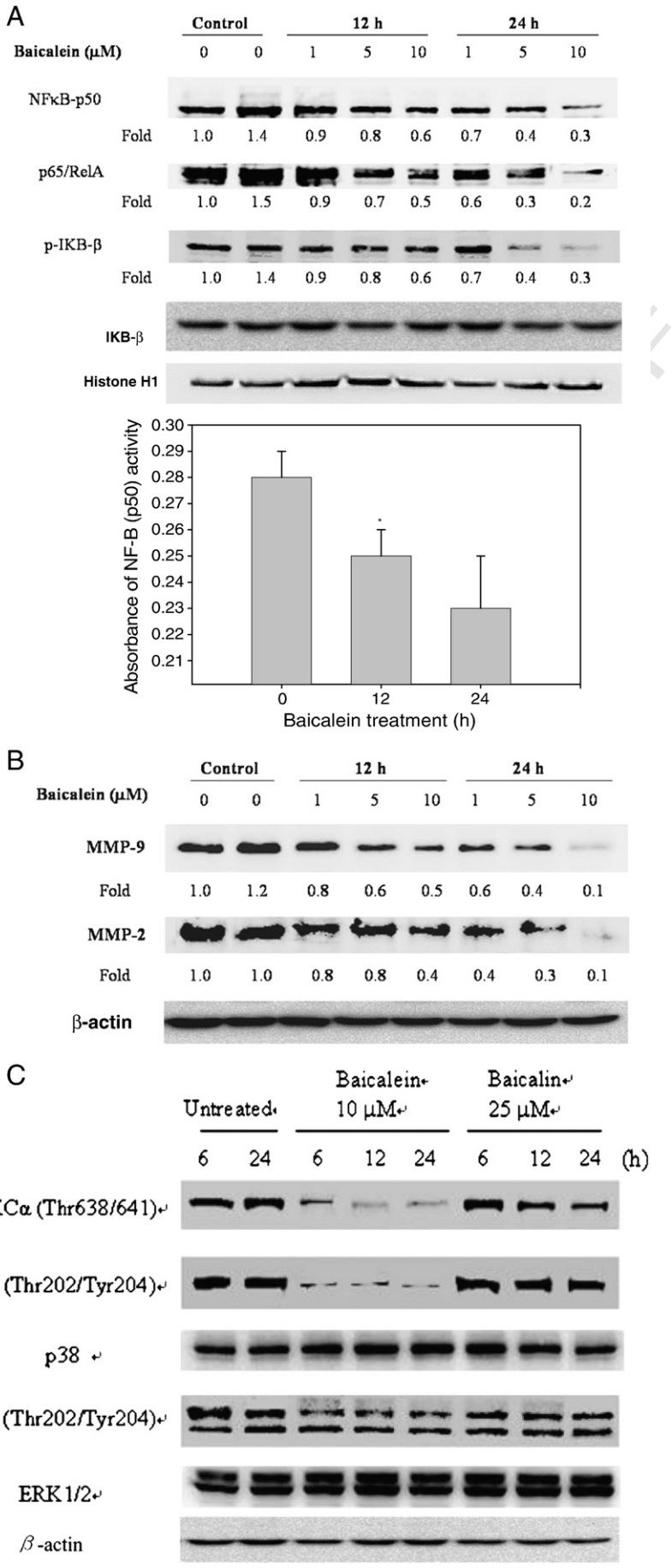
We show here that baicalein suppresses HCC metastasis at doses below its toxic range and also significantly inhibits the invasive properties of SK-Hep1 HCC cells. Non-toxic levels of baicalein also markedly reduced cell-collagen attachment, whereas the structurally related flavonoid baicalin had no effect on adhesion. Many earlier reports have shown the importance of cancer cell–matrix interactions (Chakraborti et al., 2003) for promoting cell migration, proliferation, and ECM degradation (Humphries et al., 2004). In addition, it has been shown that prevention of tumor cell adhesion and migration is related to the inhibition of tumor cell invasion of the basement membrane, and that agents that inhibit cell attachment in vitro decrease the invasiveness and/or metastatic potential of tumor cells in vivo (Lin et al., 2007; Roskelley et al., 2001; Pan et al., 2009). Hence, cellular interactions with the ECM, which promote adhesion and migration, are thought to be required for primary tumor invasion, migration, and metastasis (Huynh et al., 2009). As it has been shown previously that baicalein inhibits the growth of various human cancer cell types (Li-Weber, 2009), we demonstrate in our present analyses that 6 h of pretreatment with 10 μM baicalein significantly decreased the attachment of SK-Hep1 cells to type I collagen. In contrast, the related flavonoid baicalin showed no inhibitory effects on attachment.

The effects of relatively low concentrations of baicalein on the movement of hepatoma cells were also striking. SK-Hep1 cells treated with baicalein showed significant alterations in terms of migration and invasion. In previous studies, cell movements through tissue have been shown to play a primary role in cancer progression (Larsen et al., 2006). This process requires a series of distinct but concerted biological events in which the matrix-degrading proteinases plays an essential role. These events include tumor cell attachment to the ECM components, and the degradation of the matrix by tumor cell-associated proteases (Clark et al., 2008). As our present study demonstrates that baicalein down-regulates MMP-9 and MMP-2, we propose that these inhibitory effects are a plausible explanation for the suppressive effects of baicalein on invasion.

Baicalein exerts its actions by inhibiting NF-κB, suppressing the nuclear translocation of NF-κB-p50 and p65/RelA, and decreasing the phosphorylation of IKB-β in SK-Hep1 cells. In this respect, baicalein shows similar properties to those of genistein (Wan et al., 2008), ursolic acid (Cha et al., 1996), and 1α,25-dihydroxyvitamin D_3 (Bao et al., 2006). However, baicalein has properties distinct from these compounds in all other cases. In addition, baicalein not only directly affects NF-κB transcription factors but also affects signaling molecules such as PKCα and p38 MAPK. It is thus possible that baicalein may be a novel, effective, anti-metastatic agent for the treatment of HCC by inducing the downregulation of PKCα and p38 MAPK as well as inhibiting the expression of MMP-9 and MMP-2, thereby blocking cell migration and invasion pathways.

The key roles that p38 plays in the production of pro-inflammatory cytokines and in the signal relays from cytokine receptors have led to the evaluation of a large number of p38 MAPK inhibitors in clinical trials (Wagner and Nebreda, 2009). In any case, chronic inflammation is a potent cancer promoter and cytokines are important survival factors for cancer cells (Mantovani et al., 2008), suggesting that p38 inhibition might be beneficial for the treatment of inflammation-associated cancers, such as colon cancer and possibly HCC. Furthermore, the NF-κB signaling pathway is another potential target for the development of hepatoprotective agents, including cases of human HCC (Karin, 2006). Accumulating evidence has shown that baicalein and baicalin are the principal active components of *Scutellaria* and are potent inducers of apoptosis in cancer cells. The anticancer constituents of *Scutellaria* are not limited to baicalein and baicalin, however, and include a number of minor flavonoids, such as wogonin, chrysin, luteolin, and apigenin, which have also been reported to possess potent antitumor activities (Li-Weber, 2009). In addition, very few studies have reported on the mechanisms by which baicalein, with a 7-hydroxyl substitution on its A-ring, inhibits the invasion of human HCC cells (Huang et al., in press). Q3 The question of which targets will be clinically relevant to allow the eventual treatment of HCC is also an important one.

Our current study also reveals that treatments with baicalein compounds can prevent the incidence of HCC and further reduce its



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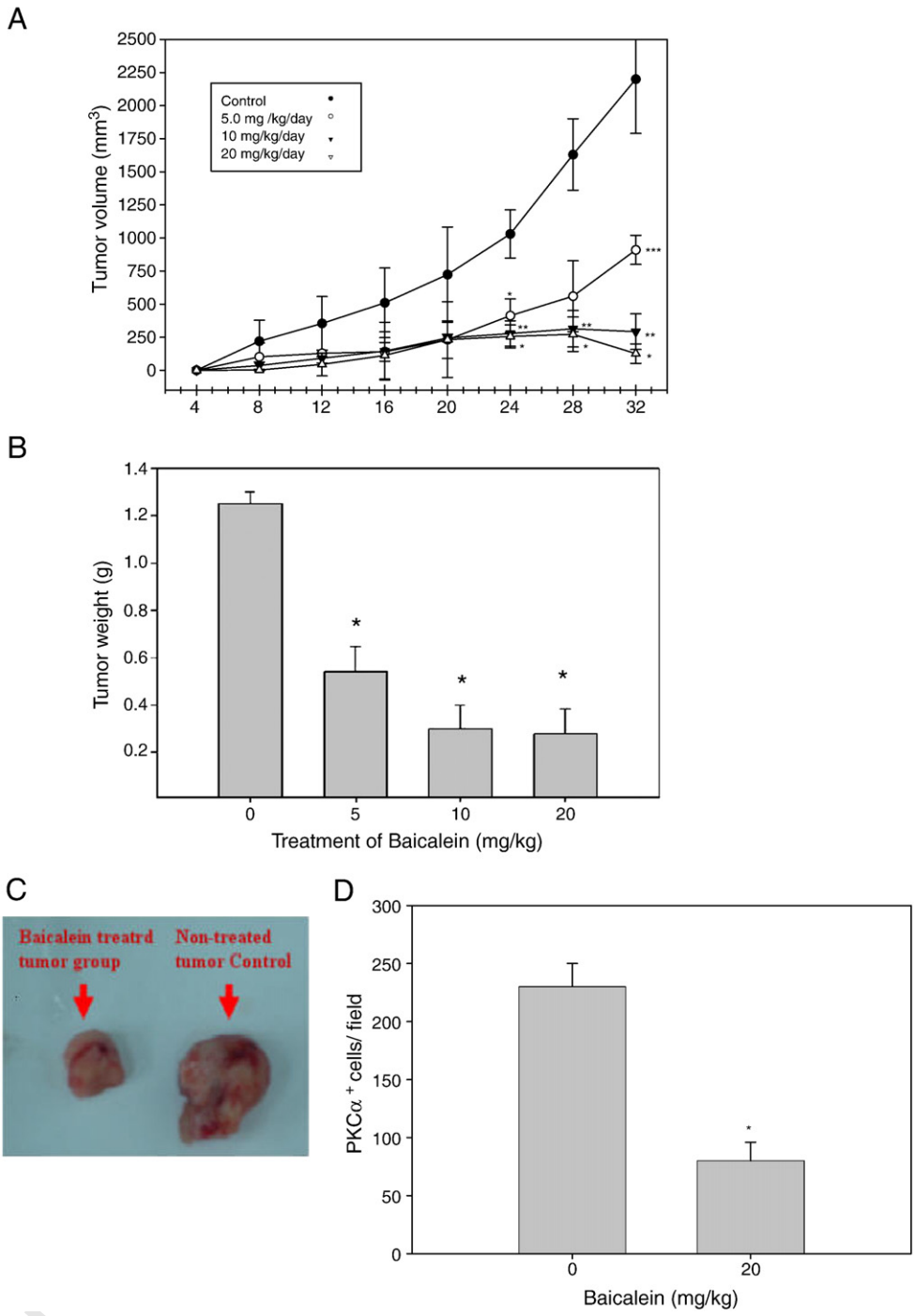

Fig. 7. In vivo growth inhibition of implanted SK-Hep1 cells by baicalein. SK-Hep1 cells were implanted subcutaneously into nude mice. Mice were treated without or with baicalein by i.p. injection (5, 10, 20 mg/kg/day). (A) Tumor volume was monitored every four days. (B) Tumors were excised and weighed on Day 34. (C) On Day 34 mice were sacrificed by local dislocation and the primary tumors were separated from the surrounding muscles and dermis. (D) A section of tumor was stained with anti-PKCα as described in the text. The positive cells were counted from 10 fields (400× magnification) of each tumor sample. The results from statistical analysis are the means of PKCα-positive cells and were calculated per microscope field from six animals per group. $^{*}P<0.05$, compared with control group.

Fig. 6. The expression levels of NF-κB-related proteins and MMP as well as phosphorylation and protein levels of PKCα, p38, and ERK1/2 after treatment with baicalein. (A) Inhibitory effect of baicalein on the phosphorylation of IκBβ as well as on p50 and p65 nuclear translocation. Cells were pretreated with baicalein for 12 h and 24 h. Nuclear and whole cell lysate proteins were prepared and analyzed by western blot, with Histone H1 and β-actin serving as loading control. NF-κB p50 activation was determined by a TF ELISA assay. All bar graphs represent absorbance, mean ± SEM. $^{*}P<0.05$ versus untreated control.(B) SK-Hep1 cells were treated with the indicated concentration of baicalein for 12 h and 24 h. Total cell lysates treated with baicalein for the indicated time were analyzed by SDS-PAGE and subsequently immunoblotted with antisera against MMP-9 and MMP-2, with β-actin serving as an internal control. (C) SK-Hep1 cells were incubated for 6–24 h in the absence or presence of baicalein. Total cell lysates were prepared and subjected to western blot analysis. Protein levels of phosphorylated PKCα, p38, and ERK1/2 as well as non-phosphorylated p38, ERK1/2 and β-actin were detected with the indicated antibodies. Protein levels were quantified by densitometric analysis with the control being set at 100%.

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tumorigenic properties, most notably metastasis. This study strongly indicates that the specific structural features of baicalein play a significant role in its properties. Our results suggest that the hydroxyl substitutions in the A-ring (C7) of this flavonoid are necessary for its anti-metastatic properties against HCC. In contrast, the glycoside substitutions of the OH groups on the A-ring of baicalin perhaps a high dose would have a significant effect similar to those obtained for baicalein. Recently it has been reported that both the C5 and C7 hydroxyl groups of the A-ring play a significant role in the bioactivities of the flavonoids (Lotito and Frei, 2006). Substitutions of these C5 or C7 hydroxyl groups confer apoptotic properties on flavonoids by modulating their capacity to induce intracellular reactive oxygen species (ROS) production, coupled with the activation of the ERK signaling pathway (Kim et al., 2008). Based on our current knowledge of flavonoids (Echeverry et al., 2010), we compared the anti-metastatic properties of hydroxyl substitutions in the A-ring (C7) of baicalein to further examine the structure–activity relationships associated with this molecule with regard to the differential modulation of key signaling pathways. Consistently, when SK-Hep1 cells were grown as xenografts in nude mice, treatment with baicalein induced a significant dose-dependent decrease in tumor growth. However, further studies of the anti-metastatic properties of baicalein in vivo are still required to validate these findings.

In conclusion, we show that baicalein inhibits several essential steps in the onset of metastasis. These include cell–matrix interactions and the activities of invasion-associated proteases and their inhibitors. In contrast, the related molecule baicalin had no marked effect on HCC cell invasion, cell matrix interactions, or the expression of proteases, but did affect sustainable the phosphorylation levels of PKCα and p38 proteins. Finally, our in vivo analyses demonstrated that baicalein decreases the growth of SK-Hep1 cell tumor xenografts in nude mice by inhibiting cell proliferation. Taken together, these results suggest that the suppression of invasion and metastasis in HCC by baicalein is strictly dependent on the hydroxyl substitution of its A-ring (C7).

Uncited reference

Li et al., 2010

Conflict of interest

The authors declare that there are no conflicts of interest.

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