Colon cancer chemopreventive effects of baicalein, an active enteric microbiome metabolite from baicalin

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Abstract. Baicalin is a major constituent of *Scutellaria baicalensis*, which is a commonly used herbal medicine in many Asian countries. After oral ingestion, intestinal microbiota metabolism may change parent compound's structure and its biological activities. However, whether baicalin can be metabolized by enteric microbiota and the related anticancer activity is not clear. In this study, using human enteric microbiome incubation and HPLC analysis, we observed that baicalin can be quickly converted to baicalein. We compared the antiproliferative effects of baicalin and baicalein using a panel of human cancer cell lines, including three human colorectal cancer (CRC) cell lines. *In vitro* antiproliferative effects on CRC cells were verified using an *in vivo* xenograft nude mouse model. Baicalin showed limited antiproliferative effects on some of these cancer cell lines. Baicalein, however, showed significant antiproliferative effects in all the tested cancer cell lines, especially on HCT-116 human colorectal cancer cells. *In vivo* antitumor results supported our *in vitro* data. We demonstrated that baicalein exerts potent S phase cell cycle arrest and pro-apoptotic effects in HCT-116 cells. Baicalein induced the activation of caspase 3 and 9. The *in silico* modeling suggested that baicalein forms hydrogen bonds with residues Ser251 and Asp253 at the active site of caspase 3, while interactions with residues Leu227 and Asp228 in caspase 9 through its hydroxyl groups. Data from this study suggested that baicalein is a potent anticancer metabolite derived from *S. baicalensis*. Enteric microbiota play a key role in the colon cancer chemoprevention of *S. baicalensis*.

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Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide (1,2). In the United States, it is expected that there will be 132,700 newly diagnosed CRC cases and 49,700 CRC-related deaths in 2015 (2), indicating the inadequacies of currently available measures (1,3). Herbal medication is an approach that has recently gained more attention for colorectal cancer (CRC) management (4,5). It is known that botanicals have been a significant resource to several of the currently used efficacious chemotherapeutic agents (6,7). The identification of non-toxic natural compounds from herbal medicines remains an essential step in advancing CRC therapeutics (8,9).

The root of *Scutellaria baicalensis* is a widely used herbal medicine in the traditional medical systems of China and Japan for a variety of inflammation related ailments (10-13). The major constituents of this botanical are a group of flavonoid glycosides, including baicalin, and wogonoside, of which baicalin is the major constituent in the herb (14,15).

S. baicalensis is most often orally administered. After oral ingestion, the constituents in the herb inevitably come into contact with intestinal microbiota. Many of these constituents could be transformed by the intestinal bacteria before being absorbed (16). As reported before, for natural glycosides such as ginsenosides, the most common metabolic pathway is the deglycosylation reaction induced by intestinal bacteria via the stepwise cleavage of the sugar moieties (17-19). After deglycosylation, compared to their parent compounds, the intestinal microbiome metabolites may have more potent biological activity (20-22).

Anticancer activities of *S. baicalensis* and its constituents were reported, but previous studies focused more on its natural sourced flavonoid glycosides (23,24). We recently observed that the major constituent of *S. baicalensis*, baicalin, can be

converted to baicalein by glycoside hydrolases or hydrolyzing during the herb's processing or storage (20,25), but whether the enteric microbiome will metabolize baicalin is still unclear. In addition, although attempts have been made to evaluate the anticancer activities of the two compounds (26,27), a chemopreventive effect comparison between baicalin and baicalein on CRC has not been performed.

In this study, using the human microbiome, we determined biotransformation from baicalin to baicalein. We compared the antiproliferative effects of baicalin and baicalein using a panel of human cancer cell lines, including three human CRC cell lines. The *in vitro* antiproliferative effects on CRC cells were verified using an *in vivo* xenograft nude mouse model. Then, we selected HCT-116 colon cancer cells, which are most sensitive to baicalein treatment, for further mechanistic observations, including cell cycle arrest and apoptosis induction. Due to the fact that caspases are highly conserved in multicellular organisms and function as central regulators of apoptosis, levels of caspase expression were subsequently determined. Finally, the possible binding modes of baicalein at the catalytic domains of caspase 3 and 9 were simulated using the receptor-ligand docking analysis.

Materials and methods

Chemicals and materials. All cell culture plasticware were obtained from Falcon Labware (Franklin Lakes, NJ, USA) and Techno Plastic Products (Trasadingen, Switzerland). Trypsin, McCoy's 5A, Leibovitz's L-15, RPMI-1640 and DMEM media, and phosphate-buffered saline were obtained from Mediatech, Inc. (Herndon, VA, USA). Penicillin and streptomycin were obtained from Sigma-Aldrich (St. Louis, MO, USA). The MTS assay kit, CellTiter 96 Aqueous Solution Cell Proliferation Assay, was obtained from Promega (Madison, WI, USA). The Annexin V-FITC apoptosis detection kit was obtained from BD Biosciences (Rockville, MD, USA). PI/RNase staining buffer was obtained from BD Biosciences Pharmingen (San Diego, CA, USA). Caspase 3 and 9 ELISA kits were obtained from BioVison (Mountain View, CA, USA). Baicalin and wogonoside were obtained from Indofine Chemical Co. Inc. (Hillsborough Township, NJ, USA). Baicalein and wogonin were obtained from Sigma-Aldrich.

Plant materials and extraction. The roots of *Scutellaria baicalensis* were obtained from Chengde (Hebei, China). The voucher samples were deposited at the Tang Center for Herbal Medicine Research at The University of Chicago. Dried *S. baicalensis* roots were ground to powder, and the powdered roots were extracted with 70% ethanol for 2 h. The extraction method was boiling under reflux. The filtrate was collected and the extraction procedure was repeated one more time on the residue. The combined filtrate was condensed under vacuum and lyophilized to yield dried *S. baicalensis* extract (SbE).

Biotransformation of SbE by human fecal microflora. Fecal samples were obtained from five adult volunteers, who were non-smokers and had not consumed antibiotics for ≥3 months before the study. The samples were collected by the donors in plastic cups, and were processed within 30 min of passage. All five fecal samples were mixed and an aliquot of 5 g of the mixed feces was homogenized with 20 ml of phosphate buffer (pH 7.0) to obtain a fecal slurry. The slurry was filtered through muslin to remove particulate material. One microliter of the fecal slurry was mixed with 4 ml anaerobic medium containing 2.5 mg of SbE. They were anaerobically incubated at 37˚C for 0, 2 or 8 h. Then, 1 ml of reaction mixture was extracted three times with 400 μl n-butanol/each time. The combined n-butanol solution was dried under nitrogen steam spray in a water bath (60˚C). Then the residue was dissolved in methanol. The methanol solution was centrifuged at 17,000 x g for 10 min before HPLC analysis.

High performance liquid chromatography (HPLC) analysis. The HPLC system was a Waters 2960 instrument (Milford, MA, USA) with a quaternary pump, an automatic injector, a photodiode array detector (Model 996), and Waters Empower software for peak identification and integration. The separations were carried out on a Phenomenex Prodigy ODS(2) column (150x2.0 mm, 5 μm). A binary gradient solvent system of acetonitrile (eluent A) - 0.03% (v/v) phosphoric acid in water (eluent B) was used as follows: 13% A and 87% B (0 min), 28% A and 72% B (17 min), 35% A and 65% B (27 min), 90% A and 10% B (30-31 min), 13% A and 87% B (34-39 min). The flow-rate of 0.8 ml/min was used and absorbance was detected at 280 nm. All tested solutions were filtered through Millex 0.2-μm nylon membrane syringe filters before use. The contents of the constituents were calculated using standard curves of flavonoids.

Cell lines and cultures. The human colorectal cancer cell lines HCT-116 (McCoy's 5A), SW-480 (Leibovitz's L-15), HT-29 (McCoy's 5A), NSCLC non-small cell lung cancer cells (DMEM), and human breast cancer cell lines MCF-7 (RPMI-1640), MDA-MB-231 (RPMI-1640) were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were grown in the indicated medium supplemented with 10% FBS and 50 IU penicillin/streptomycin in a humidified atmosphere with 5% CO_2 at 37˚C.

Cell proliferation analysis. Baicalin and baicalein were dissolved in DMSO and were stored at -20˚C before use. Cells were seeded in 96-well plates ($1x10^4$ cells/well). After 24 h, indicated concentrations of drugs were added to the wells. The final concentration of DMSO was 1%. Controls were exposed to culture medium containing 1% DMSO without drugs. All experiments were performed in triplicate and repeated 3 times. Following the indicated incubation period, cell proliferation was evaluated using an MTS assay according to the manufacturer's instructions. Briefly, the medium was replaced with 100 μl of fresh medium and 20 μl of MTS reagent (CellTiter 96 Aqueous Solution) in each well, and the plate was returned to the incubator for 1-2 h. A 60-μl aliquot of medium from each well was transferred to an ELISA 96-well plate and its absorbance at 490 nm was recorded. Since 1% DMSO did not influence the proliferation of the two cell lines, results were expressed as percent of control (DMSO vehicle set at 100%).

Cell cycle analysis. HCT-116 cells were seeded in 24-well tissue culture plates. On the second day, the medium was changed and cells were treated with test compounds. Cells

were incubated for 48 h before they were harvested. These cells were fixed gently with 80% ethanol in a freezer for 2 h and were then treated with 0.25% Triton X-100 for 5 min on an ice bath. Cells were resuspended in 300 μl of PBS containing 40 μg/ml propidium iodide (PI) and 0.1 mg/ml RNase (28). Then the cells were incubated in the dark for 20 min at room temperature, and cell cycle analysis was performed using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA) and FlowJo 7.1.0 software (Tree Star, Ashland, OR, USA). For each measurement, ≥10,000 cells were counted.

Apoptotic analysis. The apoptosis assay was performed by flow cytometry following a previously described procedure (29). Briefly, HCT-116 cells were seeded in 24-well tissue culture plates. After culturing for 1 day, the medium was changed and test compounds were added. After treatment for 48 h, cells floating in the medium were collected. The adherent cells were detached with trypsin. Then, culture medium containing 10% FBS (and floating cells) was added to inactivate trypsin. After gentle pipetting, the cells were centrifuged for 5 min at 1,500 g. The supernatant was removed and cells were stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) according to the manufacturer's instructions. Untreated cells served as controls. The cells were analyzed immediately after staining using a flow cytometer. For each measurement, ≥20,000 cells were counted.

Caspase 3 and 9 analyses. HCT-116 cells were seeded in 6-well tissue culture plates. After 24 h, the medium was changed and baicalein was added. After treatment for 24 h, cell lysates were collected. Expression levels of caspase 3 and 9 were determined by the colorimetric method according to the manufacturer's instructions. Briefly, cell lysates were diluted with 50 μl of 2X reaction buffer (containing 10 mM DTT) to a protein concentration of 0.5 mg/ml in an ELISA 96-well plate. Then, 5 μl of colorimetric tetrapeptide substrate (DEVD-pNA for caspase 3 and LEHDpNA for caspase 9) and cell lysate were added, and plate was incubated at 37˚C for 24 h. Then, the absorbance was recorded at 405 nm. The change in caspase activity was calculated as absorbance of baicalein treated cells/absorbance of untreated controls.

Receptor docking analysis. The possible binding modes of baicalein at the catalytic domains of human caspase 3 and caspase 9 were predicted using the docking program Surflex-Dock (Tripos, St. Louis, MO, USA). The structure of baicalein was generated (through Ligand model in Sybyl), and protein crystal structures were obtained (PDB code 3H0E for caspase 3 and 2AR9 for caspase 9). To prepare for docking analysis, the protein structures were prepared by adding hydrogen atoms and missing sidechain atoms and removing water molecules. Intermolecular interaction between baicalein and caspases were analyzed, and the key pharmacophore in the ligand was identified (30,31).

Statistical analysis. Data are presented as mean ± standard error (SE) (n=3). A one-way ANOVA was employed to determine the statistical significance of the results. When necessary, a Student's t-test was used to compare the two groups. The level of statistical significance was set at $P<0.05$.

Results

Baicalin metabolism by human enteric microbiome to produce baicalein. HPLC analysis was used to monitor SbE flavonoid changes during the biotransformation of human enteric microbiome. As shown in Fig. 1, four flavonoids were detected in SbE, i.e., baicalin, wogonoside, baicalein and wogonin. Baicalin is the major constituent in SbE. To determine whether fecal compounds influence SbE flavonoid analysis, we assayed the vehicle fecal sample. A major peak at retention time (Rt) of 9.061 min was observed in the chromatogram of the fecal sample. For the SbE, the closest flavonoid peak to this fecal peak is baicalin, with an Rt of 11.366 min. This fecal peak was separated from the baicalin peak at baseline. Thus, compounds from fecal sample did not influence SbE flavonoid determination.

When SbE was cultured with human enteric microbiome for 2 h, compared to un-transformed SbE, the baicalin peak was significantly reduced. Data showed that 75.3% of baicalin in SbE was converted to baicalein after being cultured for 2 h. Furthermore, after being cultured for 8 h, baicalin was not detected, while baicalein was the major constituent in the reaction mixture, indicating that all baicalin in SbE was converted to baicalein. Similar results were also observed in wogonoside. Our data showed that the gut microbiome can quickly transform baicalin to baicalein.

Antiproliferative effects of baicalin and baicalein. In this study, six human cancer cell lines from three of the most common human cancers were used, including colorectal cancer (HCT-116, SW-480 and HT-29), non-small cell lung cancer (NSCLC), and breast cancer (MCF-7 and MDA-MB-231).

As shown in Fig. 2A-C, while 48-h treatment with baicalin inhibited cancer cell growth in relatively high concentrations, baicalein caused much stronger growth suppression in all three colorectal cancer cell lines. At 60 μM, only baicalin showed 7.6±1.7% of an antiproliferative effect on HCT-116 cells ($P<0.05$ vs. control), while no significant effects were observed on the other two cancer cell lines. In the same concentration (60 μM), baicalein inhibited cancer cell growth by 73.7±3.4% in HCT-116, 40.3±2.9% in SW-480, and 22.3±1.8% in HT-29 cells, respectively (all $P<0.01$ vs. control). Among the three cell lines, baicalein showed the most potent antiproliferative effects in HCT-116 cells with an IC_{50} value of 40.1 μM.

Baicalein also showed significant antiproliferative effects on the other three cancer cell lines, but NSCLC cells were not sensitive to baicalein treatment. Compared to baicalein, baicalin showed weaker antiproliferative effects on NSCLC and MDA-MB-231 cells (Fig. 2D and F). MCF-7 cells were the only exception. Although baicalein showed significant effects on MCF-7 cells at concentrations even as low as 20 μM, when treatment concentration was increased to 100 μM, baicalin still did not inhibit cancer cell growth (Fig. 2E).

Our data suggested that baicalin showed less antiproliferative effects in the tested concentrations. Its enteric microbiome metabolite, baicalein, showed very significant antiproliferative effects in different human cancer cell lines, especially HCT-116 cells (Fig. 2).

Antitumor effects of baicalin and baicalein in xenograft tumor model. To confirm the *in vitro* antiproliferative effect

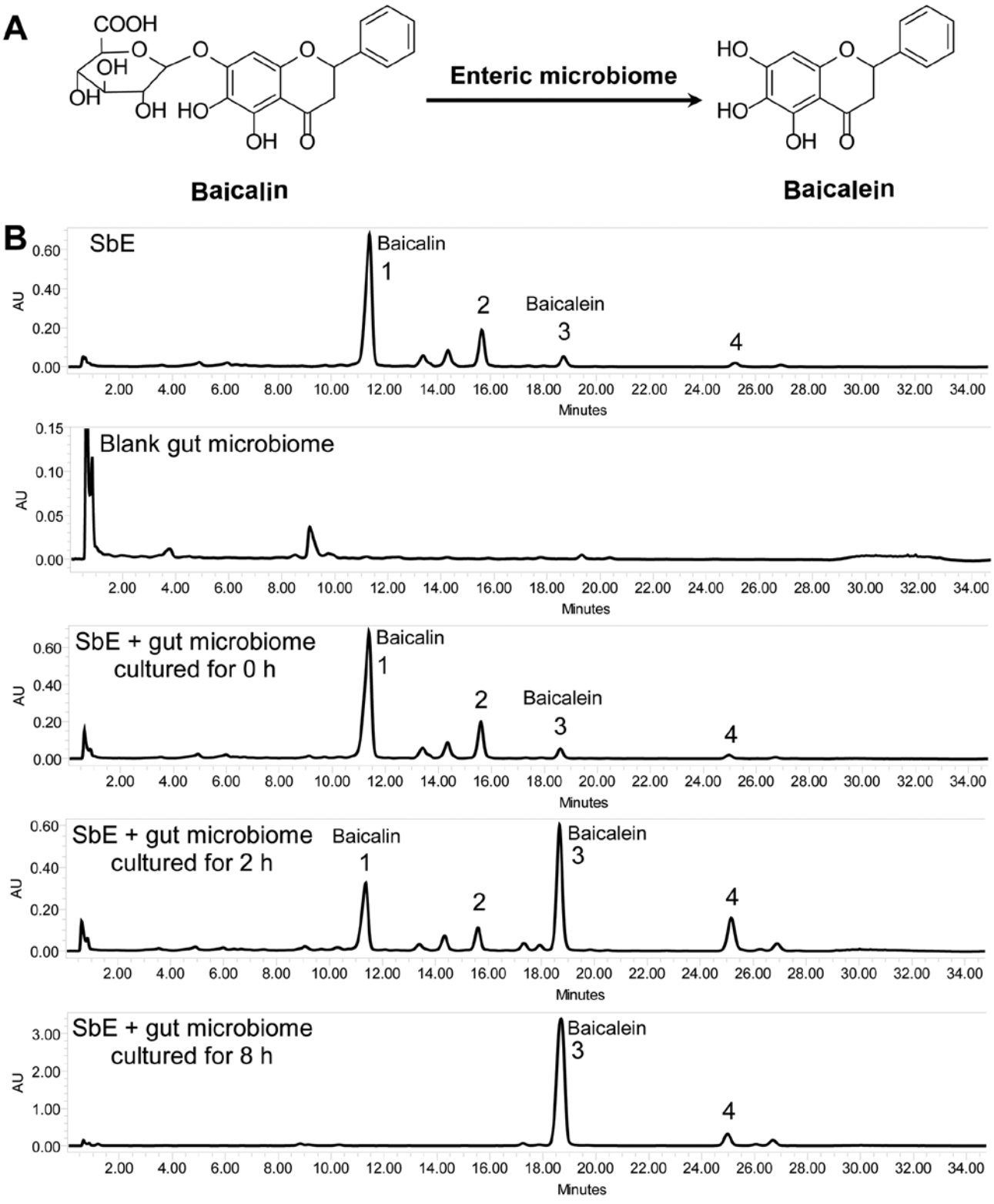

Figure 1. Biotransformation of *S. baicalensis* extract (SbE) by the human enteric microbiome. (A) Chemical structures of baicalin and baicalein. (B) HPLC analysis of *S. baicalensis* flavonoids transformed by enteric microbiome. Chromatograms of SbE, gut microbiome, SbE + gut microbiome, without culture (0 h), or cultured at 37˚C for 2 and 8 h are shown. Peak number: 1, baicalin; 2, wogonoside; 3, baicalein; 4, wogonin.

of baicalein on HCT-116 colorectal cancer cells, the *in vivo* antitumor activities of baicalin and baicalein were evaluated. Firefly luciferase-tagged HCT-116 cells were inoculated into the flanks of athymic nude mice. Beginning on day 1, animals were also administered with baicalin or baicalein at 30 mg/kg or vehicle intraperitoneally every other day. Tumor growth was measured by xenogeny bioluminescence imaging on a weekly basis. Representative xenogen imaging results at weeks 0-4 are shown in Fig. 3A. Tumor size at indicated time-points as assessed by imaging signal intensities is summarized

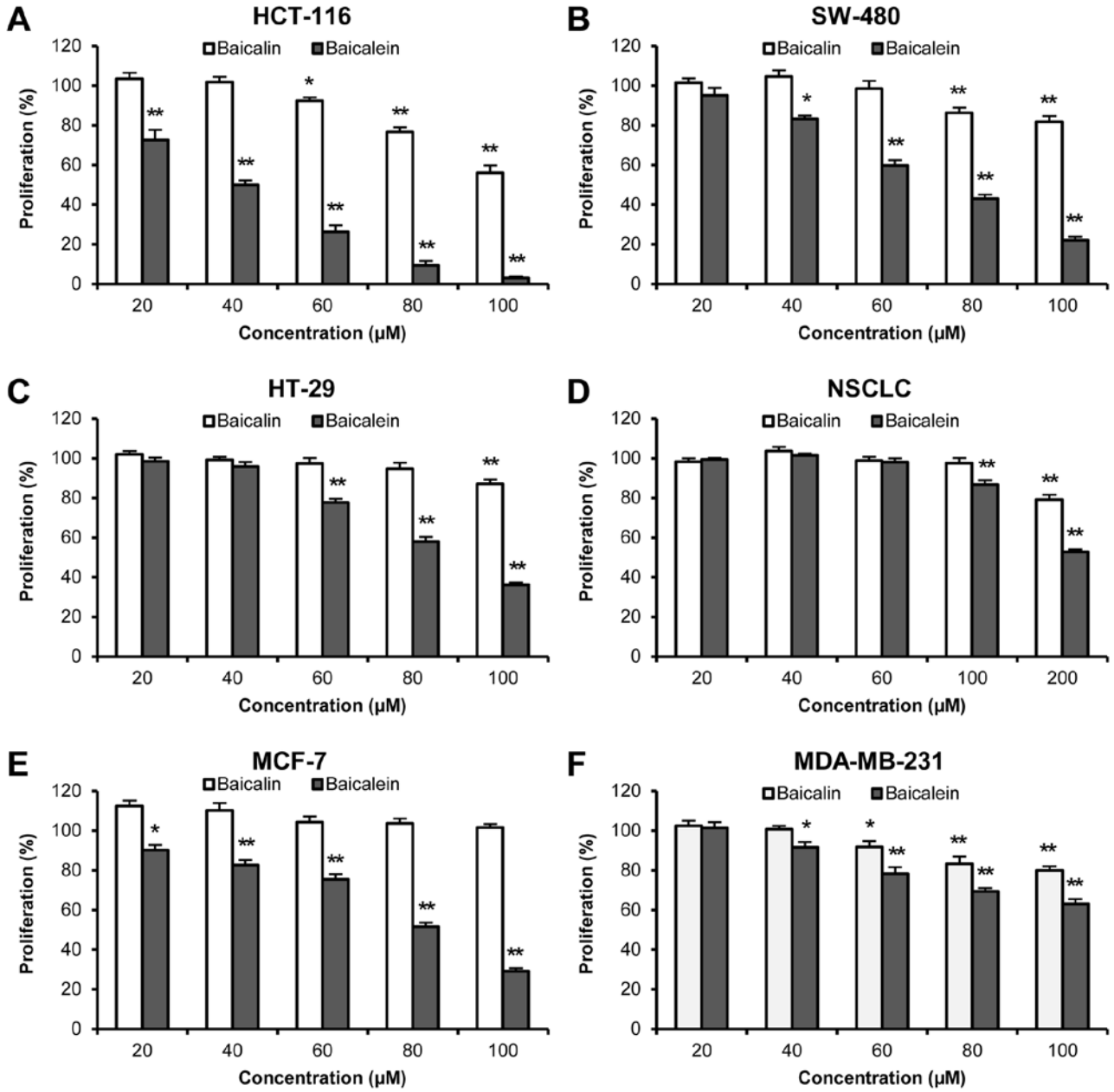

Figure 2. Effects of baicalin and baicalein on the proliferation of different human cancer cell lines assayed by the MTS method. Cell lines used include colorectal cancer (SW-480, HCT-116, HT-29), non-small cell lung cancer cells (NSCLC) and breast cancer (MCF-7, MDA-MB-231). Cells were treated with 10-200 μM of tested compounds for 48 h. *P<0.05; and **P<0.01 vs. control (100%).

in Fig. 3B. The data showed that at weeks 2 and 3, baicalin suppressed tumor growth, but there is no significant differences compared to control. At week 4, baicalin significantly inhibited tumor growth (P<0.05). For the baicalein, at week 2, baicalein exhibited significantly decreased xenogeny imaging signal intensities compared with those of the control (P<0.05). Weeks 3 and 4 exhibited more significant antitumor effects than week 2 (both P<0.01). Our data suggested the metabolite baicalein showed more significant antitumor effects than those of its parent compound baicalin. The enteric microbiome metabolism plays an important role in enhancing the anticancer activity of SbE.

Effects of baicalin and baicalein on cell cycle. Since HCT-116 colorectal cancer cells were sensitive to baicalein treatment both *in vitro* and *in vivo*, we selected this cell line for further mechanistic evaluations. As shown in Fig. 4, compared to the control, the effects of baicalein on the cell cycle profile were observed at concentrations as low as 20 μM. Treatment of HCT-116 cells with 20, 40 and 60 μM baicalein for 48 h decreased G1 phase to 25.0, 19.0 and 26.7%, respectively, compared to 41.8% in vehicle treated cells, while increasing S phase to 49.7, 60.7 and 73.6%, respectively, compared to 43.3% in vehicle treated cells (all P<0.01). Thus, baicalein significantly decreased the number of cancer cells in G1 phase and increased the number of cancer cells in S phase. On the other hand, baicalin treatment did not influence the cell cycle at 20 and 40 μM. Treatment with 60 μM of baicalin for 48 h changed cell proportions by increasing G1 phase and decreasing G2/M phase (P<0.05 vs control). These results

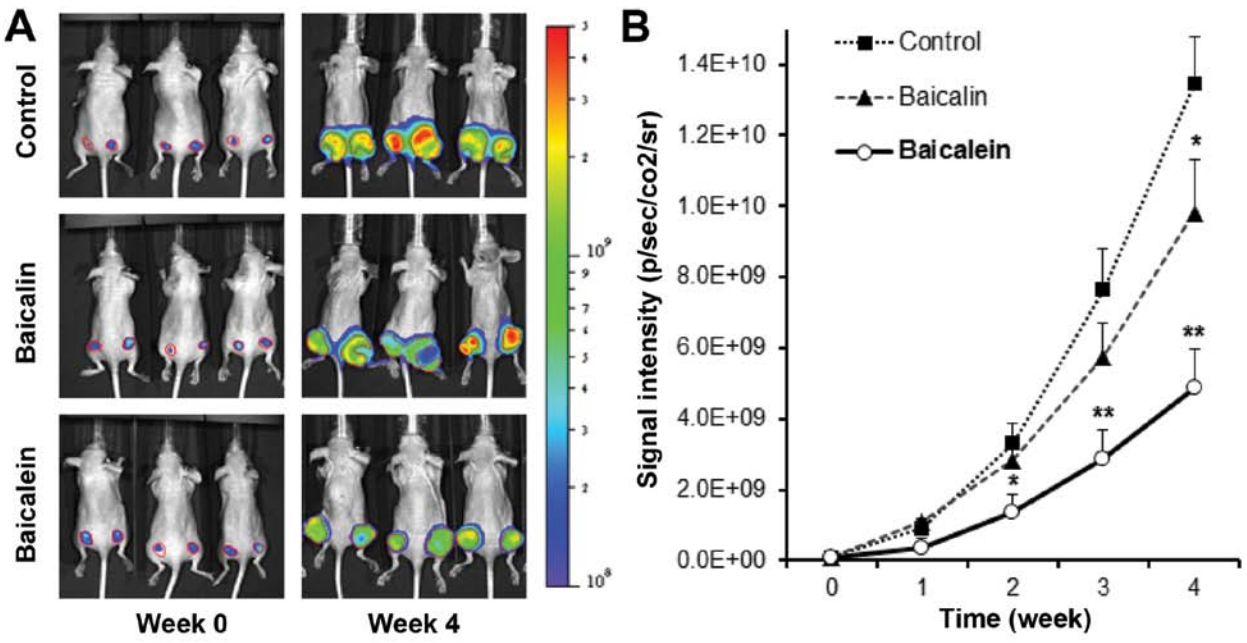

Figure 3. *In vivo* antitumor activity of baicalein in a xenograft mouse model. Firefly luciferase-tagged HCT-116 cells were injected into both flanks of athymic mice subcutaneously (n=10/group), and the tumor sizes after treatment with solvent control or 30 mg/kg/day of baicalein were measured on a weekly basis by xenogen bioluminescence imaging. (A) Representative xenogen imaging results are shown. (B) Quantitative analysis of xenogen bioluminescence imaging. Average tumor sizes at the indicated time-points are represented with imaging signal intensities (in photons/second/cm^2/steradian) as mean ± standard error. *P<0.05, **P<0.01 vs. control.

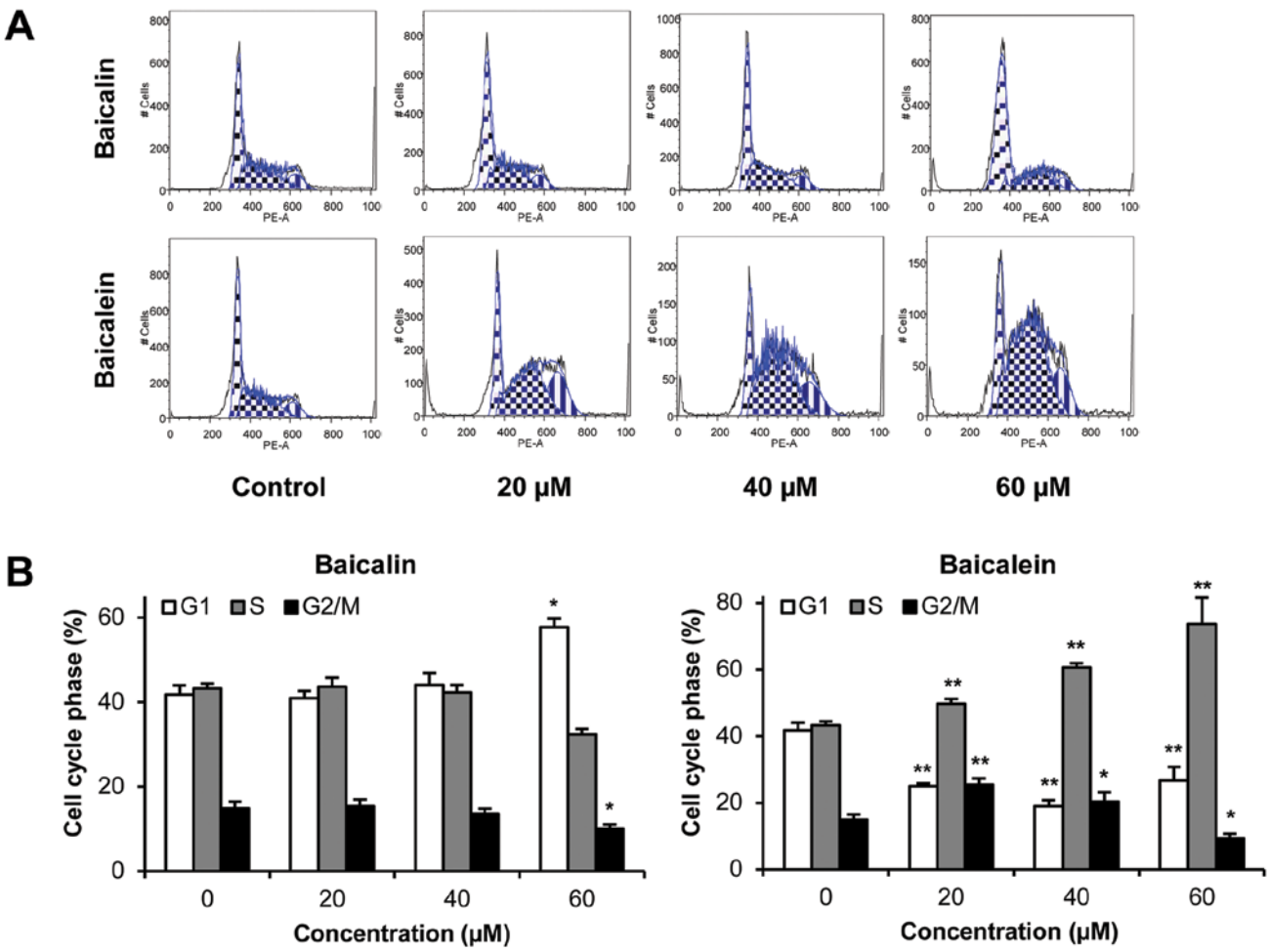

Figure 4. Cell cycle analysis of HCT-116 cells using flow cytometry. After the HCT-116 cells were treated with 20-60 μM of baicalin or baicalein for 48 h, the cells were stained with propidium iodide (PI). (A) Typical cell cycle profiles. (B) Data are presented as the means ± SE of triplicate experiments. *P<0.05, and **P<0.01, vs. control.

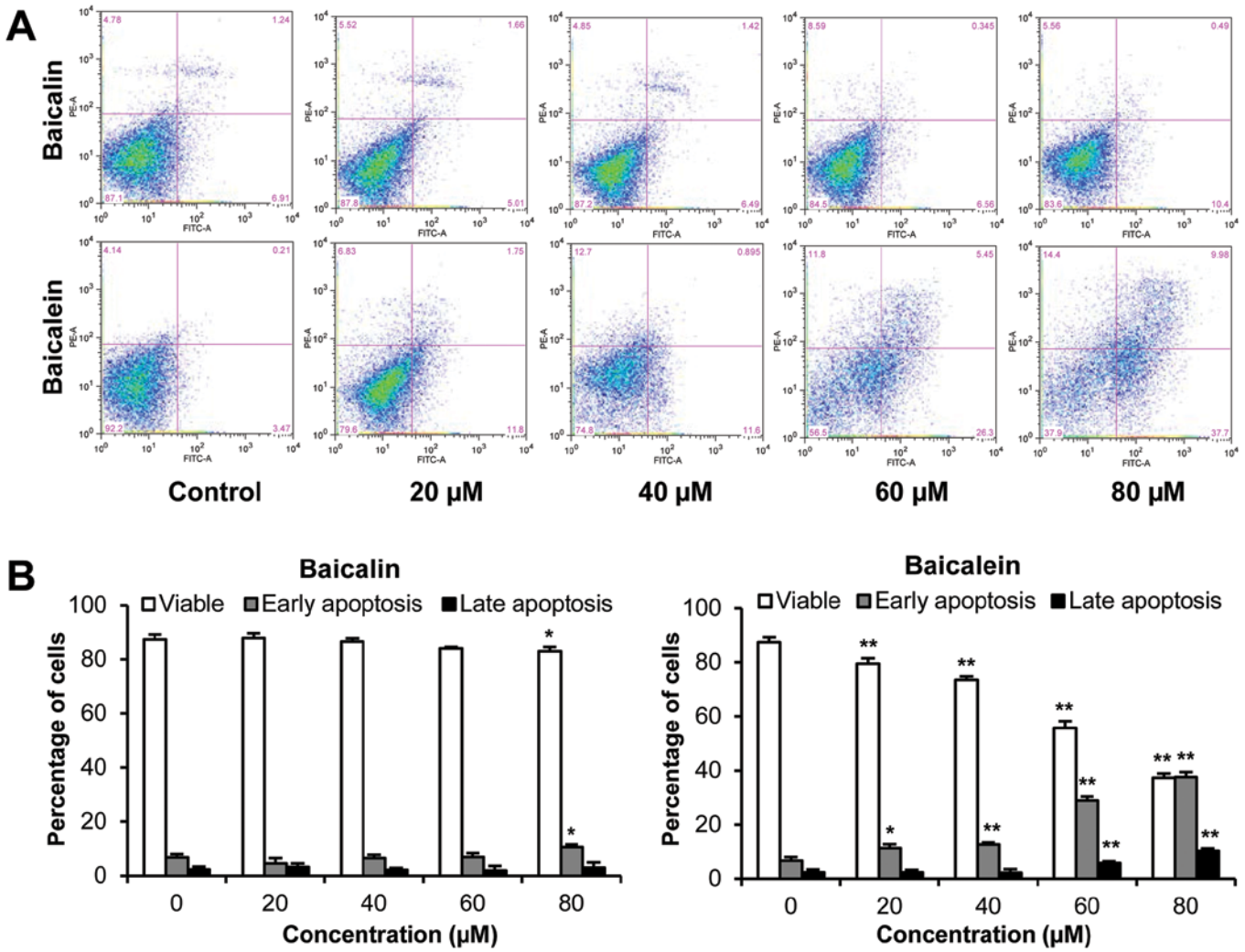

Figure 5. Apoptosis assay using flow cytometry after staining with Annexin V-FITC/propidium iodide (PI). HCT-116 cells were treated with 20-80 μM of baicalin or baicalein for 48 h. (A) Representative scatter plots of PI (y-axis) vs. Annexin V (x-axis). (B) Percentage of viable, early apoptotic and late apoptotic cells. Data are presented as the means ± SE of triplicate experiments. *P<0.05, and **P<0.01 vs. control.

suggested that the metabolite baicalein, not parent compound baicalin, significantly induced S phase cell cycle arrest in HCT-116 cells.

Effects of baicalin and baicalein on apoptosis. The apoptotic effects of baicalin and baicalein were evaluated by flow cytometry after staining with Annexin V and PI. Annexin V can be detected in both early and late stages of apoptosis, whereas PI stained cells only in late apoptosis or necrosis. Early apoptotic cells were positive for Annexin V and negative for PI (lower right quadrant); late apoptotic cells stained for both Annexin V and PI (upper right quadrant). As shown in Fig. 5, following treatment with 20, 40, 60 and 80 μM of baicalein for 48 h, compared to the control (6.7%), the percentage of early apoptotic SW-480 cells was increased to 11.2, 12.5, 28.8 and 37.5%, respectively (P<0.05, P<0.01, P<0.01 and P<0.01). However, baicalin did not induce apoptosis at the concentrations of 20-60 μM. Only 80 μM of baicalin showed an antiproliferative effect, which induced 10.5% of early apoptotic cells (P<0.05). These data demonstrate that baicalein significantly induces cell apoptosis.

Effects baicalein on activities of caspase 3 and 9. Caspase 3 and caspase 9 are two key proteins of the caspase family of proteases, which are highly conserved in multicellular organisms and function as central regulators of apoptosis. They have been identified as playing a key role in the progression of apoptosis (32). To characterize the potential mechanism of baicalein's anticancer activity, we assayed the activities of two caspases since baicalein increased cancer cell apoptosis. As shown in Fig. 6A, treatment of HCT-116 cells with 40 μM baicalein for 24 h upregulated caspase 3 and 9 activities significantly. These activities were further enhanced with 60 μM of baicalein, increasing caspase 3 and 9 activities to 103.7±18.5 and 95.4±16.4% above those of vehicle treated cells, respectively (both P<0.01). Our results suggested that baicalein significantly induced the expression of caspase 3 and 9.

Molecular modeling of caspase 3 and 9 and the binding mode of baicalein. To further explore the potential effects of baicalein on caspase 3 and 9, we performed docking analysis to characterize the physical interactions of baicalein with these caspases. We examined baicalein docking for human

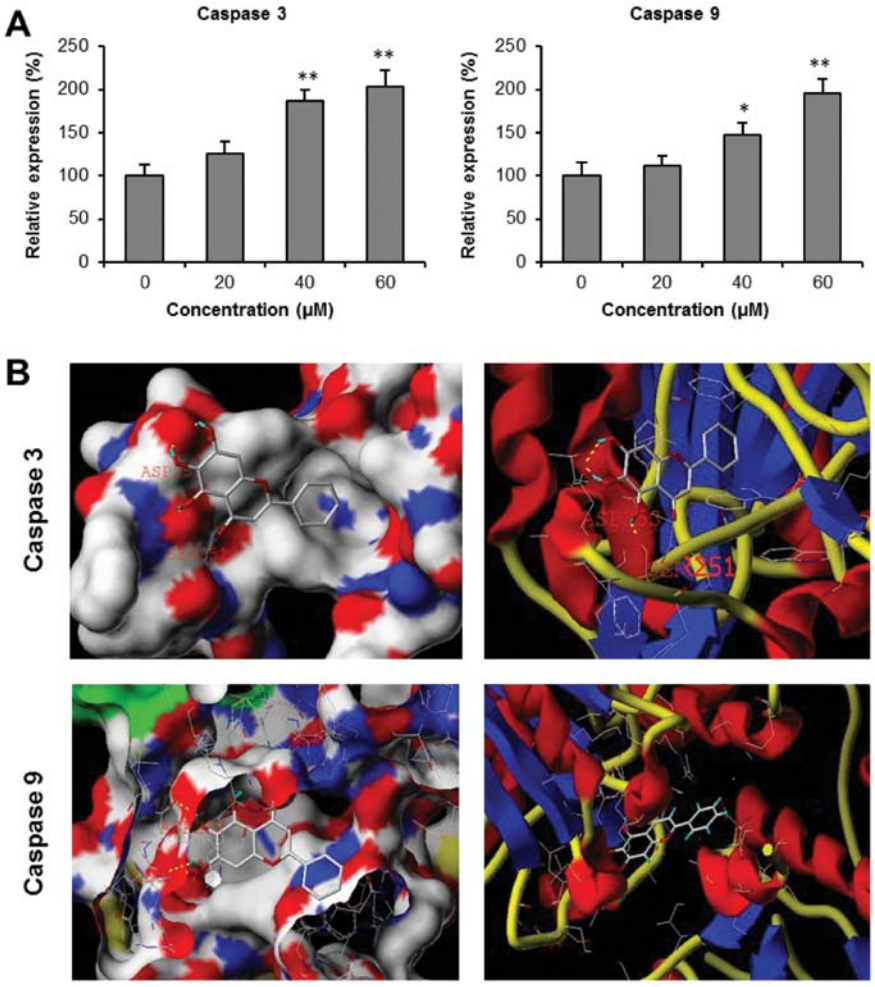

Figure 6. Effects of baicalein on caspase 3 and 9 activities in HCT-116 cells and docking analysis. (A) After treatment with 20-60 μM of baicalein for 24 h, cell lysates were prepared and enzymatic activities were measured by colorimetric assay for caspase 3 and 9. Results are normalized to each control in percentage and expressed as the means ± SE of triplicate experiments. *P<0.05, and **P<0.01 vs. control. (B) Three-dimensional docking model of baicalein at the binding site of human caspase 3 and caspase 9 proteins. The possible binding modes of baicalein at the catalytic domains of caspases were predicted using the docking program Surflex-Dock. The surface views are shown in the left panel, and stick-ribbon models on the right.

caspase 3 (PDB code: 3H0E) and human caspase 9 (PDB code: 2AR9). The Surflex-Dock program was used to predict the binding sites of baicalein to caspase 3 and 9. The energetically most favorable positions for baicalein interaction with these caspases are shown in Fig. 6B. The *in silico* modeling suggested that baicalein forms hydrogen bonds with residues Ser251 and Asp253 at the active site of caspase 3, while baicalein forms hydrogen bonds with residues Leu227 and Asp228 in caspase 9 through its hydroxyl groups. In addition, baicalein is predicted to show significant binding affinity for caspase 3 (CScore 3.76) and caspase 9 (CScore 3.18), suggesting that baicalein may directly interact with these caspases.

Discussion

Colorectal cancer is the second leading cause of cancer related death in the United States, and the second most prevalent cancer worldwide (1,2). The clinical CRC management involves diverse conventional modalities, including surgery, radiation, and chemotherapy (2,33). The complex characteristics of human cancer also require some complementary approaches, including herbal medication, to improve the therapeutic efficacy of conventional therapies (34,35). In the past 30 years, nearly 80% of approved anticancer drugs were derived from natural compounds (6). Herbal medicine has contributed significantly to CRC therapies and many of the novel compounds with significant anticancer properties are likely to be found in plant sources.

S. baicalensis is one of the most commonly used herbs in traditional medicine for the treatment of various inflammatory diseases in Asia. The representative constituents of *S. baicalensis* are a group of flavonoids that include glycosides (baicalin, wogonoside) and their aglycon metabolites (baicalein and wogonin), while baicalin occupies the major content of the total flavonoids (14,15). In recent years, the anticancer activities of *S. baicalensis* extract and its constituents were reported (36,37). However, most studies focused on its glycosides, such as baicalin, which possess only limited anticancer activities.

S. baicalensis is most often orally administered. In natural products research, many previous studies employed primarily reductionist approaches in screening compounds for bioactivity, and often only parent compounds were investigated. In fact, in the gut, parent flavonoid glycosides in *S. baicalensis* could be metabolized by the gut microbiome. However, whether

baicalin can be metabolized by the enteric microbiome and its microbial metabolite leads to modified anti-colorectal cancer activities are largely unclear.

In this study, we investigated the biotransformation of baicalin by the human enteric microbiome. In our pilot study, we selected 2, 8 and 24-h incubation periods, based on our previous ginseng research (25), and observed 8 h was enough to allow for the full metabolism of the parent compounds. In this study, we observed that after 2 h of incubation time, ¾ of baicalin was converted to baicalein. After 8-h incubation, all baicalin had been metabolized to baicalein. Similar results were also observed on another pair of compounds: >50% of wogonoside was converted to wogonin at 2 h. After 8-h incubation, all wogonoside had been converted to wogonin. Our data suggested that the human gut microbiome can effectively metabolize baicalin to baicalein. Due to the possibility that water soaking may structurally modify baicalin, as a control, we tested if water only (without microbiome) can convert baicalin to baicalein. Our results showed that after 8 h of culture, water soaking (without enzymes) did not induce such transformation. The experimental condition in this water control was different from those of our previously published report, in which botanical enzymes significantly converted baicalin to baicalein (15). Thus, our results suggested that the enteric microbiome played a critical role in metabolizing baicalin to baicalein.

To compare the antiproliferative effects between baicalin and baicalein, in addition to three human colorectal cancer cell lines (HCT-116, SW-480 and HT-29), we also used three other cancer cell lines from two common solid tumors, small cell lung cancer (NSCLC) and breast cancer (MCF-7 and MDA-MB-231). We observed that, compared to baicalin, baicalein showed much stronger antiproliferative effects on all the cancer cell lines. Basically, baicalin was not effective on NSCLC and MCF-7 cells. At 100 μM, baicalin did not inhibit cancer cell growth in these two cell lines. Although baicalin showed antiproliferative effects in other cancer cell lines, however, the active concentration was >60 μM. On the other hand, baicalein showed very significant antiproliferative effects on all the tested cancer cell lines.

For the three human colorectal cancer cell lines, HT-29 was relatively resistant to baicalein treatment, with an IC_{50} of 87.3 μM. Baicalein showed the most potent effects on HCT-116 cells, with an IC_{50} of 40.1 μM. Thus, we select this cell line to further validate its effects and explore anticancer metabolisms of action.

A subcutaneous HCT-116 tumor model using xenograft nude mice was established to confirm the *in vitro* antiproliferative effect of baicalein on colorectal cancer cells. Our data indicated that the daily administration of 30 mg/kg of baicalin inhibited the HCT-116 tumor growth at week 4. Compare to limited effects of baicalin, baicalein showed much stronger antitumor effects. After mice received 30 mg/kg of baicalein, HCT-116 tumor growth was significantly inhibited in weeks 2-4 (Fig. 3). The *in vivo* antitumor evaluation supported the *in vitro* antiproliferative effects that the enteric microbiome metabolite baicalein is an active anti-colorectal cancer compound.

Because the inhibition of cell cycle progression and induction of apoptosis are important mechanisms mediating the effects of many anticancer agents, in this study, we compared the activities of baicalin and baicalein on the cell cycle and apoptosis. Cell cycle effects of baicalin were observed only at high concentrations. At 60 μM, baicalin increased cell proportions in G1 phase and decreased G2/M phase. On the other hand, at as low as 20 μM, baicalein showed potent effects on the cell cycle. Compared to the control, baicalein significantly dose-dependently induced HCT-116 cell cycle arrest in S phase. We previously isolated the glycoside fraction (contains baicalin and wogonoside) and aglycon fraction (contains baicalein and wogonin) from SbE (36). Since the aglycon fraction showed potent antiproliferative effects, we assayed cell cycle effects of aglycon fractions on HCT-116 cells. Aglycon fraction induced cell cycle arrest in both S and G2/M phases (38). Data from this study supported our previous observations, that baicalein contributed to the S phase arrest that was also observed by the aglycon fraction treatment.

Effects of baicalin and baicalein on HCT-116 cell apoptosis were evaluated. Baicalein markedly induced colon cancer cell apoptosis in concentrations of 20-80 μM, while baicalin appeared to have positive effect at high concentration (80 μM). Because caspase 3 and 9 are situated at critical points in apoptotic pathways (39), to further explore the mechanism mediating baicalein-induced apoptosis, we assayed the activities of caspase 3 and 9. At concentrations of 40 and 60 μM, baicalein significantly upregulated the activities of these caspases. Our docking analysis further suggested interaction sites between baicalein and caspases. Baicalein induced apoptosis may be mediated by direct physical interactions with these targets. Our results suggest that the cancer cell growth inhibitory effect of baicalein was predominantly mediated by induction of apoptosis.

In conclusion, using the human enteric microbiome, the biotransformation and metabolic profile of *S. baicalensis* flavonoids was determined. Baicalin can be easily metabolized to baicalein. Using a panel of human cancer cell lines, we tested the antiproliferative effects of baicalin and baicalein. As a major parent compound from *S. baicalensis*, baicalin showed limited antiproliferative effects on some of these cell lines. Baicalein, however, showed significant antiproliferative effects in all the tested cancer cell lines, especially on HCT-116 human colorectal cancer cells. *In vivo* antitumor results supported our *in vitro* data. We demonstrated that baicalein exerts potent S phase cell cycle arrest and pro-apoptotic effects in HCT-116 cells. Baicalein upregulated the expression of caspase 3 and 9. Baicalein-induced apoptosis could be through direct physical interactions with these apoptotic regulators. Data from this study suggested that baicalein is a potent anticancer metabolite derived from *S. baicalensis*. Enteric microbiota play a key role in the colon cancer chemoprevention of *S. baicalensis*.

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