

# Inhibition of Cancer Cell Proliferation and Prostaglandin E<sub>2</sub> Synthesis by *Scutellaria Baicalensis*<sup>1</sup>

David Y. Zhang,<sup>2</sup> Josephine Wu, Fei Ye, Li Xue, Shiquan Jiang, Jizu Yi, Wandu Zhang, Huachen Wei, Max Sung, Wayne Wang, and Xiaoping Li

Department of Pathology [D. Y. Z., J. W., F. Y., L. X., S. J., J. Y., W. Z., W. W., X. L.], Department of Dermatology [H. W.], and Division of Medical Oncology, Department of Medicine [M. S.], Mount Sinai School of Medicine, New York University, New York, New York 10029

## ABSTRACT

*Scutellaria baicalensis* is a widely used Chinese herbal medicine that has been used historically in anti-inflammatory and anticancer therapy. The purpose of this study is to verify its anticancer activity on head and neck squamous cell carcinoma (HNSCC) *in vitro* and *in vivo* and to investigate its effect on cyclooxygenase-2 (COX-2), which converts arachidonic acid to prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and is highly expressed in HNSCC. Two human HNSCC cell lines (SCC-25 and KB) and a nontumorigenic cell line (HaCaT) were tested *in vitro* for growth inhibition, proliferation cell nuclear antigen expression, and COX-2 activity and expression after treatment with *Scutellaria baicalensis* extract. Its effects were compared with those of baicalein (a flavonoid isolated from *Scutellaria baicalensis*), indomethacin (a nonselective COX inhibitor), and celecoxib (a selective COX-2 inhibitor). Four nude mice with s.c. inoculation of KB cells were tested for its anticancer activity *in vivo* by oral administration of *Scutellaria baicalensis* at a dose of 1.5 mg/mouse (75 mg/kg), five times/week for 7 weeks. *Scutellaria baicalensis* and other agents demonstrated a strong growth inhibition in both tested human HNSCC cell lines. No growth inhibition of HaCaT cells was observed with *Scutellaria baicalensis*. The IC<sub>50</sub>s were 150 μg/ml for *Scutellaria baicalensis*, 25 μM for celecoxib, and 75 μM for baicalein and indomethacin. *Scutellaria baicalensis*, as well as celecoxib and indomethacin, but not baicalein, suppressed proliferation cell nuclear antigen expression and PGE<sub>2</sub> synthesis in both cell types. *Scutellaria baicalensis* inhibited COX-2 expression, whereas celecoxib inhibited COX-2 activity directly. A 66% reduction in tumor mass was observed in the nude mice. *Scutellaria baicalensis* selectively and effectively inhibits cancer cell growth *in vitro* and *in vivo* and can be an effective chemotherapeutic agent for HNSCC. Inhibition of PGE<sub>2</sub> synthesis via suppression of COX-2 expression may be responsible for its anticancer activity. Differences in biological effects of *Scutellaria baicalensis* compared with baicalein suggest the synergistic effects among components in *Scutellaria baicalensis*.

## INTRODUCTION

In the United States, there are 70,000 newly diagnosed patients with head and neck cancer each year (1). Squamous cell carcinoma is the predominant type. Despite improvements in treatment modalities during the past 40 years, the 46% 5-year survival rate has not dramatically changed (2). The development and search for more effective treatment modalities and/or adjuvant therapies to treat early and advanced stages of the disease has now become an important subject of research.

Early experiments revealed that PGs<sup>3</sup> are often highly produced in head and neck cancer patients (3). PGE<sub>2</sub> is the predominant type of PGs derived from the cell membrane arachidonic acid, the release of

which, in turn, is controlled by phospholipases (4). Klapan *et al.* (5) found elevated serum levels of PGE<sub>2</sub> in head and neck cancer patients, which decreased after tumor resection. Jung *et al.* (6) demonstrated that PGE<sub>2</sub> levels in tumor tissue were four times higher than in control tissue in seven patients undergoing surgery for HNSCC. Increased PGE<sub>2</sub> levels in tumor tissue are mainly because of the increase of the expression of COX, a key enzyme involved in the conversion of arachidonic acid to PGs (7). There are two isoforms of COX: COX-1 is constitutively expressed in all eukaryotic cells, whereas COX-2 is inducible and is increased under the certain conditions such as inflammation. Chan *et al.* (8) demonstrated that both COX-2 mRNA and protein are up-regulated in HNSCC. In their study, the COX-2 mRNA was increased ~150-fold, and COX-2 protein was increased ~50-fold in tumor tissue when compared with histologically normal appearing mucosa of HNSCC patients.

*Scutellaria baicalensis*, also known as Chinese skullcap or Huang Qin, is a widely used herb in traditional Chinese medicine with anticancer, antiviral, antibacterial, and anti-inflammatory properties (9–13). Historically, *Scutellaria baicalensis* has been used to treat respiratory tract infection, diarrhea, jaundice, hepatitis, and cancer. Recent investigations have shown that *Scutellaria baicalensis* alone, or in combination with other herbs, can inhibit cancer cell growth or induce apoptosis in breast, hepatocellular, pancreatic, prostatic, and urothelial carcinoma cell lines (14–18). Because *Scutellaria baicalensis* has a strong anti-inflammatory effect, we hypothesized that its anticancer activity may derive from inhibition of COX-2 pathway. The purpose of this study is to examine the anticancer activity of *Scutellaria baicalensis* on HNSCC both *in vitro* and in nude mice xenografted with HNSCC cells and to understand its molecular mechanisms with special emphasis on its effect on COX-2 pathway. The information generated from this study will help to support the development of human clinical trials in the future.

## MATERIALS AND METHODS

**Chemicals and Drugs.** The raw extract of *Scutellaria baicalensis* was prepared by boiling dried root of the plant in water, followed by a spray-drying process of the water extract. The powder form of the extract was supplied by Q-Herb Laboratory (New York, NY) and was dissolved in medium to 20 mg/ml, vortexed at room temperature for 1 min, and incubated at 37°C for 1 h while rotating before use. This solution was centrifuged at 5000 rpm for 10 min to remove any insoluble ingredients. The supernatant was passed through a 0.22-μm filter for sterilization and diluted with culture medium to final concentrations of 1.5–1500 μg/ml *Scutellaria baicalensis* extract. Fifty mM stock solutions of baicalein (Sigma, St. Louis, MO) and indomethacin (Sigma) and 10 mM stock solution of celecoxib [SC-58635; 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1]-benzene-sulfonamide; Searle Research and Development, St. Louis, MO] were prepared with DMSO (Sigma) and diluted with culture medium to final concentrations of 5–400 μM. Control cells received DMSO (0.25%) or culture medium only.

**Cell Lines and Tissue Culture.** Two human squamous cell carcinoma cell lines (KB and SCC-25) were purchased from American Type Culture Collection (Manassas, VA). The rationale of using two cell lines is that squamous cell carcinoma is a heterogeneous cancer and there may be a difference in their response to drugs. SCC-25 cell line was derived from tongue squamous cell

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<sup>2</sup> To whom requests for reprints should be addressed, at Molecular Pathology Laboratory Mount Sinai School of Medicine, Box 1122, One Gustave Levy Place, New York, NY 10021. Phone: (212) 659-8173; Fax: (212) 427-2082; E-mail: David.Zhang@mssm.edu.

<sup>3</sup> The abbreviations used are: PG, prostaglandin; HNSCC, head and neck squamous cell carcinoma; COX, cyclooxygenase; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCNA, proliferating cell nuclear antigen; EIA, enzyme immunoassay.

carcinoma and was grown in a 50:50 mixture of DMEM (Life Technologies, Inc., Rockville, MD) and F12 (Life Technologies, Inc.) containing 10% FBS (Life Technologies, Inc.) and 1% Antibiotic-Antimycotic (Life Technologies, Inc.). KB cell line was derived from an oral floor squamous cell carcinoma containing human papilloma virus 18 sequences and was cultured in DMEM containing 10% FBS and 1% Antibiotic-Antimycotic mixture. In addition, a nontumorigenic human oral squamous cell line, HaCaT, was used as a control for growth inhibition study (19, 20). HaCaT cell line was kindly obtained from Dr. Jonathon Garlick (State University of New York at Stony Brook, Stony Brook, NY) and cultured in MEM containing 10% FBS and 1% Antibiotic-Antimycotic mixture. Cell lines were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

**Cell Viability Assay.** The percentage of growth inhibition was determined by using a MTT (Sigma) assay to measure viable cells. A total of  $2.5 \times 10^3$  cells/well was seeded onto a 96-well plate for 24 h, treated with various concentrations of *Scutellaria baicalensis*, baicalein, indomethacin, and celecoxib, and incubated for an additional 3 days at 37°C. Subsequently, 10  $\mu$ l of MTT at a concentration of 5 mg/ml was added to each well, and cells were incubated for an additional 4–6 h. The supernatant was aspirated, and 100  $\mu$ l of DMSO were added to the wells to dissolve any precipitate present. The absorbance was then measured at a wavelength of 570 nm using an EL<sub>x</sub>800 reader (Bio-Tek Instruments, Inc., Winooski, VT).

**Cell Cycle Analysis.** A total of  $1.5 \times 10^5$  cells/well was plated onto 6-well plates and incubated for 24 h at 37°C. Various concentrations of *Scutellaria baicalensis*, baicalein, indomethacin, and celecoxib were added to the wells and incubated for an additional 3 days. Cells were then washed, pelleted, fixed with cold 70% ethanol for at least 30 min, and incubated with 100  $\mu$ g/ml RNase A and 50  $\mu$ g/ml propidium iodide in PBS at room temperature for 30 min. Samples were immediately analyzed by flow cytometry (Becton Dickinson, San Jose, CA). Cell cycle phase distribution was determined using Modfit software (Verity Software House, Topsham, ME).

**Immunohistochemical Analysis of PCNA Expression.** Harvested cells were applied to polylysine-coated slides by centrifugation at 800 rpm for 5 min in a Cytospin instrument (Sakura Finetek USA, Torrance, CA). Cells were air dried and fixed in 100% acetone for 10 min at 4°C. Slides were then incubated with 3% hydrogen peroxide to quench endogenous peroxidase activity for 5 min. After three washings with PBS, slides were incubated with serum blocking solution for 20 min at room temperature. The solution was blotted, and 2  $\mu$ g/ml primary mouse monoclonal anti-PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were added. Cells were then incubated at 4°C overnight. After three washings with PBS, the slides were incubated for 10 min with biotinylated goat antimouse antibodies at room temperature (Zymed Laboratory, San Francisco, CA). Slides were washed three more times with PBS and then incubated for 10 min with streptavidin-peroxidase conjugate at room temperature (Zymed Laboratory). Color was developed by the addition of diaminobenzoate chromogen (Zymed Laboratory), and slides were counterstained with hematoxylin. Cells with brown nuclear precipitations were regarded as positive. A minimum of 500 cells/slide was analyzed in a high-powered field using a light microscope, and the percentage of positive cells was determined.

**Western Blot Analysis.** SCC-25 cells were treated with *Scutellaria baicalensis*, and the proteins were extracted from the cells using a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP40, and 1 $\times$  protease inhibitors (Roche Applied Science, Indianapolis, IN). Fifteen  $\mu$ g of protein were fractionated by electrophoresis through a 10% SDS polyacrylamide gel, and the proteins then transferred onto a nitrocellulose membrane. The membrane was blocked for 1 h in a blocking buffer containing 5% dry milk, 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween 20 and then incubated with a polyclonal anti-COX-2 antibody (1:400 dilution; Cayman Chemical, Ann Arbor, MI) in the blocking buffer at 4°C overnight. The membrane was then incubated with an antirabbit antibody conjugated with horseradish peroxidase (Amersham, Arlington Height, IL), and the protein was detected using chemiluminescence method followed by autoradiography. The same membrane was then used to detect  $\beta$ -actin protein using a monoclonal anti- $\beta$ -actin antibody (1:10,000 dilution; Sigma) as described above.

**PGE<sub>2</sub> EIA.** A competitive PGE<sub>2</sub> EIA was used to quantify the level of PGE<sub>2</sub> released into culture media and was performed according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ). The supernatants of control and treated cells were collected, centrifuged at 2500

rpm for 2 min, and stored at -70°C until tested. Fifty  $\mu$ l aliquots of each sample were assayed in triplicate. Absorbance was measured at 450 nm on an EL<sub>x</sub>800 reader (Bio-Tek Instruments, Inc.). The minimal detectable concentration of PGE<sub>2</sub> by the assay is 50 pg/ml. The PGE<sub>2</sub> produced is expressed as pg/10<sup>6</sup> cells.

The effect of celecoxib and *Scutellaria baicalensis* on COX-2 activity was additionally examined using a cellular arachidonic acid conversion assay. The SCC-25 cells were incubated for 12 h, and the medium was aspirated and replaced with fresh medium containing *Scutellaria baicalensis* or celecoxib at IC<sub>50</sub> concentrations (150  $\mu$ g/ml for *Scutellaria baicalensis* or 25  $\mu$ M for celecoxib). The cells were additionally incubated for various periods of time, and the medium was aspirated. Fresh medium containing 100  $\mu$ M arachidonic acid (Cayman) was added, and the cells were incubated for additional 30 min. PGE<sub>2</sub> in the medium was measured as described above.

**Animals and Treatment Protocol.** Six-week-old female nude mice (NCR/NU) were purchased from Taconic (Cincinnati, OH) and received s.c. injections of KB cells ( $5 \times 10^6$ ) to the right flank. At 2 weeks, tumors reached 300 mm<sup>3</sup> in volume, and mice were randomized into two experimental groups ( $n = 4$ ). The treatment group was given 1.5 mg/mouse (75 mg/kg) *Scutellaria baicalensis* extract dissolved in water by oral gavage five times/week for duration of 7 weeks. Mice were weighed, and tumor volume assessed on a weekly basis. Tumor volume was measured by two perpendicular dimensions (long and short) using a caliper and calculated using the formula  $(a \times b^2) / 2$ , where  $a$  is the larger and  $b$  is the smaller dimension of the tumor.

## RESULTS

### Growth Inhibition of *Scutellaria Baicalensis* on HNSCC Cell

**Lines.** The percentage of growth inhibition of different agents on KB and SCC-25 cells was determined as the percentage of viable-treated cells in comparison with viable cells of untreated controls. *Scutellaria baicalensis* displayed a dose-dependent (Fig. 1A) and time-dependent (data not shown) inhibition on the growth of both cell lines. Both cells exhibited equal sensitivity to *Scutellaria baicalensis* and IC<sub>50</sub>s of both cell lines were determined to be 150  $\mu$ g/ml. The maximal inhibition of cell growth (>90%) was achieved at 1500  $\mu$ g/ml. On the other hand, *Scutellaria baicalensis* did not inhibit the growth of HaCaT cell, a nontumorigenic oral squamous cell line, even at a concentration of 750  $\mu$ g/ml (Fig. 1A), indicating that *Scutellaria baicalensis* selectively inhibited cancer cell growth. We additionally tested and compared the inhibitory effects of indomethacin (nonselective COX inhibitor), celecoxib (COX-2-specific inhibitor), and baicalein, one of the ingredients of *Scutellaria baicalensis*. The results show that both HNSCC cells are sensitive to these three agents (Fig. 1, B and C). However, celecoxib exhibited stronger growth inhibition (IC<sub>50</sub> = 25  $\mu$ M) than baicalein (IC<sub>50</sub> = 75  $\mu$ M) and indomethacin (IC<sub>50</sub> = 75  $\mu$ M). Time-dependent growth inhibition of both cell lines was observed with maximal growth inhibition between days 3 and 7 depending on the concentration of *Scutellaria baicalensis* (data not shown).

**Cell Cycle Arrest.** We additionally analyzed the effect of these agents on cell cycles (Table 1). Our results show that *Scutellaria baicalensis* caused a significant G<sub>0</sub>-G<sub>1</sub> phase arrest with concurrent decrease of S phase in both SCC-25 and KB cells, compared with the control cells (Fig. 2). However, baicalein caused a significant G<sub>2</sub>-M-phase arrest with concurrent decrease of G<sub>0</sub>-G<sub>1</sub> phase. These results suggest that different mechanisms are responsible for the anticancer activity of *Scutellaria baicalensis* and that ingredients other than baicalein in *Scutellaria baicalensis* acted at G<sub>0</sub>-G<sub>1</sub> phase. In contrast to baicalein, celecoxib caused a significant arrest at G<sub>0</sub>-G<sub>1</sub> phase, whereas indomethacin exerted mild arrest at G<sub>0</sub>-G<sub>1</sub> phase. In addition, *Scutellaria baicalensis* exerted no effect on the cell cycle distribution of HaCaT cells up to the concentration of 750  $\mu$ g/ml (data not shown).

**Inhibition of Cell Proliferation.** The inhibitory effect of *Scutellaria baicalensis* on both cells was additionally confirmed using the immunohistochemical marker, PCNA. PCNA/cyclin regulates the ini-

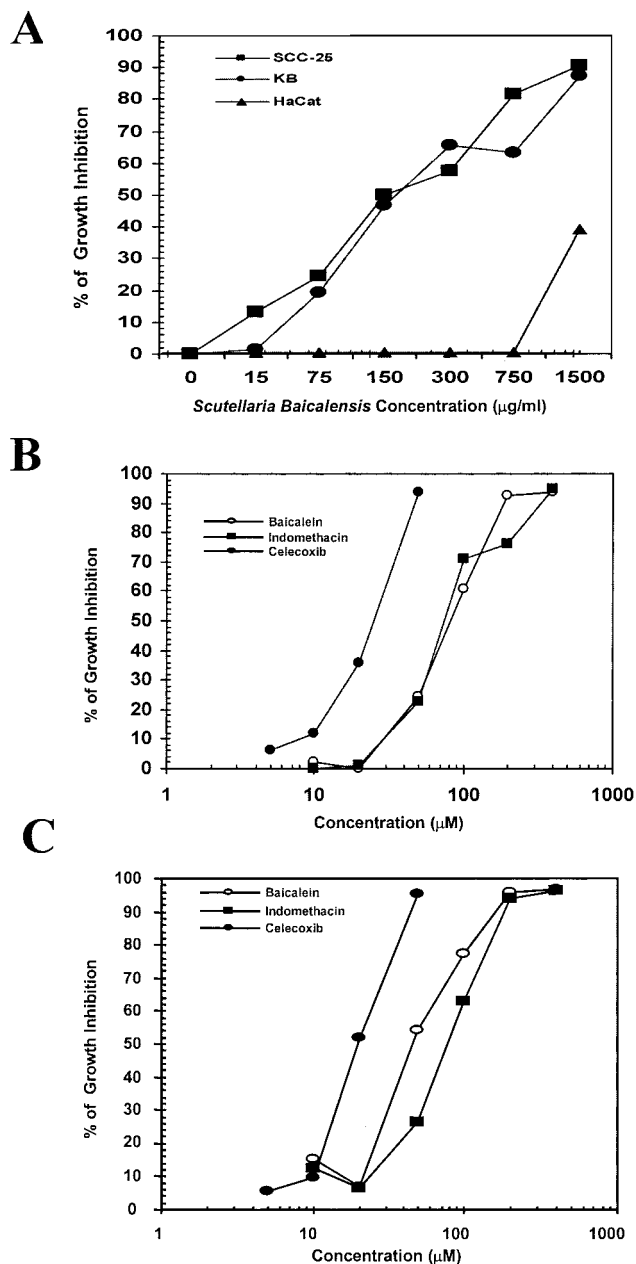


Fig. 1. Inhibition of HNSCC cell growth by *Scutellaria baicalensis*, baicalein, celecoxib, and indomethacin. Cells were seeded onto 96-well plate at  $2.5 \times 10^3$  cells/well and were treated with various agents at different concentrations, and percentage of growth inhibition was determined by MTT assay after 72-h treatment. A, dose-dependent growth inhibition of KB and SCC-25 cells was observed after treatment with *Scutellaria baicalensis* at concentrations ranging from 15 to 1500  $\mu\text{g/ml}$ . However, no significant inhibition to HaCaT cells was observed. Inhibition of HNSCC cell growth in SCC-25 (B) and KB cells (C) was observed after treatment with baicalein, indomethacin, and celecoxib at concentrations ranging from 5 to 400  $\mu\text{M}$ . Results are mean values  $\pm$  SD of independent experiments performed in triplicate (bars not evident because of minimal SD).

tiation of cell proliferation by mediating DNA polymerase and is elevated in the  $G_1$  and S phases of cell cycle (21). Our results show that there was a significant decrease of PCNA-positive cells after 72-h treatment with 150  $\mu\text{g/ml}$  *Scutellaria baicalensis*, indicating a suppression of cell proliferation (Fig. 3A). Both KB and SCC-25 cells displayed similar dose-dependent decreases in PCNA expression and had equal sensitivity to *Scutellaria baicalensis*. In Fig. 3B, KB and SCC-25 cells exhibited a dose-dependent reduction of PCNA expression after treatment with baicalein, indomethacin, and celecoxib.

However, COX-specific inhibitors, indomethacin and celecoxib, caused a more dramatic decrease of PCNA expression than baicalein in both HNSCC cell lines. These observations additionally confirmed the supportive role of  $\text{PGE}_2$  in cancer cell proliferation and indicated that different mechanisms of cell growth inhibition are involved. COX-specific inhibitors, indomethacin and celecoxib, reduce PCNA expression by inhibiting  $\text{PGE}_2$  production (see below), whereas baicalein decreases PCNA expression by a  $\text{PGE}_2$ -independent pathway. It is worthy to note that the change in PCNA expression correlated with the phase of the cell cycle that the agents acted upon. Although celecoxib suppressed cell proliferation at  $G_1$  phase with significant decrease of PCNA expression, baicalein-suppressed cell proliferation at  $G_2$ -M phase with less apparent effect on PCNA expression. These results indicate that PCNA expression is predominant at  $G_1$  and S phases.

**Inhibition of  $\text{PGE}_2$  Synthesis in HNSCC Cell Lines.** We observed high concentrations of  $\text{PGE}_2$  in both cell lines, which support the findings of previous investigations (22). However, SCC-25 cells produced a significantly higher level of  $\text{PGE}_2$  (300  $\text{pg}/10^6$  cells) than KB (80  $\text{pg}/10^6$  cells) after 12 h of incubation (Fig. 4A). This indicates heterogeneity in head and neck cancer cells. In the presence of *Scutellaria baicalensis*, a significant inhibition of  $\text{PGE}_2$  production in both cells was observed even at a concentration of 1.5  $\mu\text{g/ml}$ . At this concentration, no growth inhibition of cells was observed (Fig. 1A), indicating that  $\text{PGE}_2$  reduction precedes the inhibition of cell growth, thus confirming the role of  $\text{PGE}_2$  in stimulating cancer cell growth. Specific inhibition of  $\text{PGE}_2$  in both cell types was additionally confirmed by time course study. In the absence of *Scutellaria baicalensis*, there is a steady increase of  $\text{PGE}_2$  production, which reaches a plateau after 24 h (Fig. 4B). However,  $\text{PGE}_2$  levels in SCC-25 and KB cells remained at baseline levels in the presence of 150  $\mu\text{g/ml}$  *Scutellaria baicalensis*. At such concentration, no cell growth inhibition was observed at 12-h incubation (data not shown), additionally confirming the role of  $\text{PGE}_2$  in stimulating cancer cell growth. These results demonstrated that *Scutellaria baicalensis* inhibits the production of  $\text{PGE}_2$  in HNSCC, which, in turn, contributes to its growth inhibitory activity.

We additionally tested baicalein's effect on  $\text{PGE}_2$  synthesis in KB and SCC-25 cells, compared with those of indomethacin and celecoxib. Although baicalein displayed no effect on  $\text{PGE}_2$  synthesis, both indomethacin and celecoxib exhibited dose-dependent inhibition of  $\text{PGE}_2$  synthesis in both HNSCC cells at 12 h (Fig. 4C). These results confirmed that the increased level of  $\text{PGE}_2$  was attributable to the increase in COX-2 activity and/or COX-2 expression in both cells. Furthermore, no reduction of cell growth at even 1  $\mu\text{M}$  indomethacin or celecoxib was observed at 12-h incubation (data not show), additionally confirming that  $\text{PGE}_2$  reduction precedes the cell growth inhibition. Baicalein's ability to inhibit cell proliferation without affecting  $\text{PGE}_2$  synthesis indicates an alternative anticancer mechanism, independent of the COX pathway. It has been shown that

Table 1 Cell cycle distribution of SCC-25 and KB cells after treating with *Scutellaria baicalensis*, baicalein, celecoxib, and indomethacin

	SCC-25			KB		
	$G_0$ - $G_1$ (%)	S (%)	$G_2$ -M (%)	$G_0$ - $G_1$ (%)	S (%)	$G_2$ -M (%)
Control	60.43	24.37	15.2	59.85	17.8	22.35
<i>Scutellaria baicalensis</i>						
150 $\mu\text{g/ml}$	66.61	16.13	17.26	67.56	13.22	19.22
300 $\mu\text{g/ml}$	67.63	17.2	15.17	70.62	10.49	18.89
750 $\mu\text{g/ml}$	71.53	14.45	14.02	76.13	10.48	13.39
Baicalein (75 $\mu\text{M}$ )	48.11	25.79	26.1	39.89	27.58	32.53
Celecoxib (25 $\mu\text{M}$ )	73.03	14.93	12.04	67.77	14.95	17.28
Indomethacin (75 $\mu\text{M}$ )	61.98	23.28	14.74	64.8	14.53	20.67



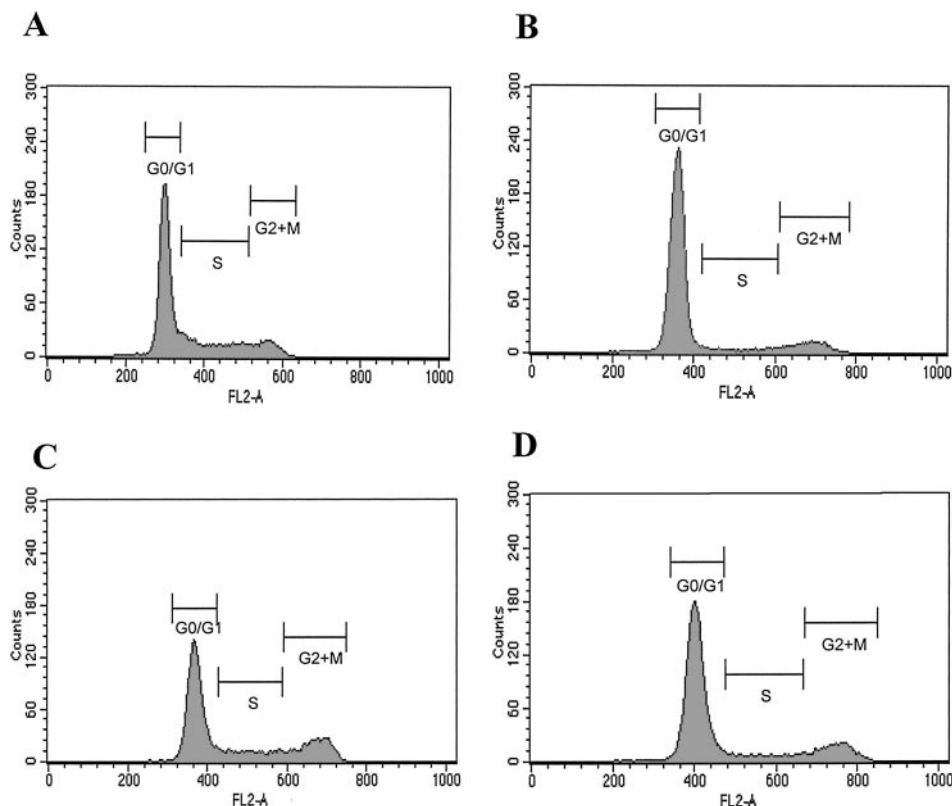


Fig. 2. Cell cycle analysis by flow cytometry: The SCC-25 (A and B) and KB (C and D) cells were treated with 750  $\mu\text{g/ml}$  *Scutellaria baicalensis* for 72 h (B and D), and cells were harvested and subjected to flow cytometry analysis. The histograms show that there was a significant increase of  $G_0$ - $G_1$  population with concomitant decrease of S population after treatment with *Scutellaria baicalensis* (B and D).

baicalein can inhibit leukotriene synthesis, a product in the lipoxygenase pathway of arachidonic acid metabolism, in cancer cells (23). Leukotriene  $B_4$  was tested by an EIA with no significant level of detection in both HNSCC cell lines (data not shown).

We also examined the effect of *Scutellaria baicalensis* and celecoxib on COX-2 activity using in cell arachidonic acid conversion assay. By providing exogenous arachidonic acid, which is the substrate of COX-2, the effect of arachidonic acid release from membrane (*i.e.*, protein kinase and phospholipase  $A_2$  activities) can be eliminated (24). Our results show that there is a significant decrease of  $\text{PGE}_2$  level in the presence of both *Scutellaria baicalensis* and celecoxib at  $\text{IC}_{50}$  doses. However, inhibition of COX-2 activity by celecoxib occurred earlier than by *Scutellaria baicalensis*: COX-2 activity decreased after 12 h for *Scutellaria baicalensis* and 2 h for celecoxib (Fig. 4D). The delayed inhibitory effects of *Scutellaria baicalensis* suggest that it inhibits  $\text{PGE}_2$  synthesis through a decrease of COX-2 expression, instead of direct inhibition of COX-2 activity.

**Inhibition of COX-2 Protein Expression.** We additionally investigated the effect of *Scutellaria baicalensis* on COX-2 protein expression in SCC-25 cells using Western blotting. We chose SCC-25 cells for this assay because they produce a higher level of  $\text{PGE}_2$ . The cells were treated with *Scutellaria baicalensis* at concentration of 300  $\mu\text{g/ml}$  for 4, 8, 12, 24, 48, and 72 h. Western blotting showed that a decrease of COX-2 expression did not occur until 48 h of incubation with *Scutellaria baicalensis* (Fig. 5A). These results may explain the delayed inhibition of  $\text{PGE}_2$  synthesis by *Scutellaria baicalensis* (Fig. 4D). There was a dose-dependent decrease of COX-2 expression on Western blot (Fig. 5B).

**Inhibition of Tumor Growth in HNSCC-xenografted Nude Mice.** To confirm the anticancer activity of *Scutellaria baicalensis*, an *in vivo* experiment was carried out. Nude mice were inoculated s.c. with KB cells and were treated with *Scutellaria baicalensis* by oral gavage at dose of 1.5 mg/mouse (75 mg/kg) daily. KB cells were used

because they grew well in nude mice, whereas SCC-25 grew poorly. Our results showed that there is a significant inhibition of the tumor growth in the treated mice as compared with the untreated mice (Fig. 6). Average tumor volume at 9 weeks was 693  $\text{mm}^3$  in the treated mice and 2224  $\text{mm}^3$  in the control mice. A tumor from one of the treated mice was nonpalpable after 7 weeks of treatment, indicating a complete remission of tumor after the drug administration. At a dose of 1.5 mg/mouse of *Scutellaria baicalensis*, no evidence of drug-related toxicity was observed in treated mice by comparing their body weight, organ histology, complete blood count, liver chemistry, or kidney function with those of controls (data not shown). Therefore, the herb was well tolerated by the animals.

## DISCUSSION

*Scutellaria baicalensis* has been shown to have a broad spectrum of biological activities, including anti-inflammatory and anticancer activity based on its long history in clinical applications. This study confirmed that *Scutellaria baicalensis* has strong dose-dependent anticancer activity, with the  $\text{IC}_{50}$  at 150  $\mu\text{g/ml}$  (Fig. 1A). Inhibition of cancer growth is because of its ability to cause  $G_0$ - $G_1$ -phase arrest. Inhibition of cell proliferation is additionally confirmed by its ability to reduce PCNA expression that correlates with decreased cell proliferation (25). We also investigated the effect of *Scutellaria baicalensis*, as well as COX-specific inhibitors on apoptosis using flow cytometry, TUNEL (terminal deoxynucleotidyl transferase-mediated nick end labeling) assay, and DNA ladder assay. No significant induction of apoptosis was observed at all concentrations of *Scutellaria baicalensis* (data not shown); however, an increased apoptosis was observed at higher concentration (100  $\mu\text{M}$ ) of celecoxib. These results indicate that inhibition of cancer cell growth by *Scutellaria baicalensis* is through the  $G_0$ - $G_1$ -phase arrest rather than apoptosis. The mode of action of *Scutellaria baicalensis* is similar to that of

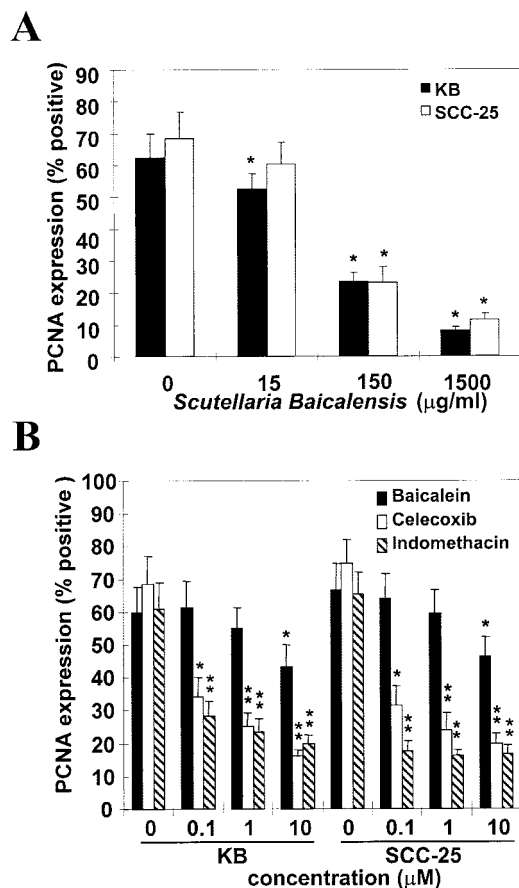


Fig. 3. Suppression of PCNA expression by *Scutellaria baicalensis*, baicalein, celecoxib, and indomethacin. KB and SCC-25 cells were treated with various agents for 72 h, and percentage of cells staining positive for PCNA were determined ( $n = 500$ ). A significant inhibition of PCNA expression in both cells was observed at 150 µg/ml for *Scutellaria baicalensis* (A) and 0.1 µM for celecoxib and indomethacin (B). However, no suppression of PCNA was observed until the concentration reached 10 µM for baicalein (B), indicating that baicalein was less effective on the suppression of PCNA expression. Results are mean values  $\pm$  SD (bars) of independent experiments performed in triplicate. Student  $t$  test was used to analyze the data. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ .

COX-2 inhibitors such as indomethacin and celecoxib, which also cause  $G_0$ - $G_1$  arrest, but different from baicalein, one of its components, which causes  $G_2$ -M-phase arrest (Table 1). It should be noted that *Scutellaria baicalensis* selectively inhibits cancer cell growth but has no effect on noncancerous HaCaT cells. This finding is consistent with a previous report that *Scutellaria baicalensis* selectively inhibits human tumor cell growth but is not toxic to human peripheral blood lymphocytes *ex vivo* (15).

The anticancer activity of *Scutellaria baicalensis* may result, at least in part, from the reduction of  $PGE_2$ . Our results confirmed previous reports of increased levels of  $PGE_2$  in HNSCC and the reduction of  $PGE_2$  in cancer cells resulting in an inhibition of cancer cell growth (6, 26). These observations are additionally supported by our results showing that, indomethacin and celecoxib, because of their specific inhibition of COX-reduced  $PGE_2$  production in cancer cells (Fig. 4C). Both agents also inhibit cancer cell growth *in vitro*. Our results also show that doses required to suppress  $PGE_2$  production as well as PCNA expression for *Scutellaria baicalensis* and other agents was 10-fold less than those for cell growth inhibition, indicating that decrease of  $PGE_2$  and PCNA precedes the growth inhibition. Alternatively, a COX-2-independent pathway may also play a role.

The molecular mechanism linking  $PGE_2$  level and cell proliferation remains unclear. It has been shown that  $PGE_2$  may regulate cancer cell proliferation in an autocrine and/or paracrine manner via the PG

receptors, especially  $EP_2$  (27). The stimulation of the receptors results in an increase of cyclic AMP level in cancer cells that triggers the signal transduction pathway leading to uncontrolled cell proliferation (28). One of the possible mechanisms is that inactivation of PG receptors by reducing the  $PGE_2$  synthesis by *Scutellaria baicalensis* may lead to reduced expression of cyclin D1 and cyclin E proteins, key factors that control the  $G_0$ - $G_1$  cell cycle (29). Our results show that both cell lines, although with different  $PGE_2$  levels, had equal sensitivity to *Scutellaria baicalensis* and other COX-specific inhibitors. Furthermore, *Scutellaria baicalensis* inhibited  $PGE_2$  synthesis, but baicalein did not. Some nonsteroidal anti-inflammatory drugs do not affect  $PGE_2$  production but exert anticancer activity. It is suggested that nonsteroidal anti-inflammatory drugs inhibit activation of nuclear factor- $\kappa B$ , a transcription factor that regulates the expression of genes protecting against cell death (30). Taken together, our results support the notion that there is a COX-2-independent pathway also responsible for anticancer activity of *Scutellaria baicalensis*.

Because COX-2 is the key enzyme in converting arachidonic acid to PGs, it is plausible that *Scutellaria baicalensis* may act on this enzyme by either reducing its activity or inhibiting its expression, leading to the decrease of  $PGE_2$ . Increased COX-2 protein expression has been found in various malignancies, including squamous cell carcinoma (8). We initially examined the effect of *Scutellaria baicalensis* on the COX-2 activity because a decreased  $PGE_2$  level was also seen in the presence of COX-2-specific inhibitor celecoxib (Fig. 4C). Our results show a significant decrease of COX-2 activity after 12 h of the treatment, whereas it was sharply decreased only 2 h after treatment with celecoxib (Fig. 4D). These results indicated that *Scutellaria baicalensis* may affect COX-2 expression rather than its activity. We then assayed the COX-2 mRNA expression using real-time reverse transcription-PCR, and insignificant decrease of mRNA expression was observed (data not shown). We additionally examined the COX-2 protein expression using Western blot analysis and found that there was a significant decrease of COX-2 protein in SCC-25 cells after *Scutellaria baicalensis* treatment. These results indicate that reduced COX-2 protein expression is mainly because of the increase of the protein turnover rate (*i.e.*, decreased stability). It is interesting to note that a decrease in  $PGE_2$  level does not parallel with COX-2 activity, *i.e.*,  $PGE_2$  synthesis is suppressed throughout the course of treatment (Fig. 4B), whereas COX-2 activity decreased at a later time (12 h; Fig. 4D), and COX-2 expression became suppressed at 48 h (Fig. 5), indicating that there are at least two independent mechanisms responsible for the decrease of  $PGE_2$ . We hypothesize that initial inhibition of  $PGE_2$  synthesis is through the inhibition of arachidonic acid release from cell membrane (*i.e.*, phospholipase  $A_2$ ), and the later inhibition is caused by reduction of COX-2 protein expression. These results suggest that *Scutellaria baicalensis* inhibits  $PGE_2$  production at multiple levels along the  $PGE_2$  synthesis pathway.

It is apparent that *Scutellaria baicalensis* exert its anticancer activities by multiple mechanisms. There are four major flavonoids, [baicalin (80%), wogonoside (16%), baicalein (2%), and wogonin (1%)], in addition to other compounds in trace amounts, found in *Scutellaria baicalensis* extract (31, 32). Our results show that the extract can inhibit  $PGE_2$  production, whereas its pure compound, baicalein, does not. However, both the extract and baicalein inhibits cancer cell growth *in vitro*. A recent study showed that wogonin significantly inhibit COX-2 activity in LPS-stimulated macrophages (24). These results indicate that there is a synergistic effect of various components in the herb, which act on different anticancer pathways such as COX-dependent and COX-independent pathways. These results strongly support the traditional use of raw herbs in decoction. Furthermore, because of the poor water solubility of pure flavonoids, *i.e.*,

Fig. 4. Inhibition of PGE<sub>2</sub> synthesis by *Scutellaria baicalensis*, baicalein, celecoxib, and indomethacin. The amount of PGE<sub>2</sub> present in the supernatant of the culture medium was measured by an enzyme immunoassay and expressed as picograms/10<sup>6</sup> cells. A, in the presence of *Scutellaria baicalensis* at concentrations ranging from 1.5 to 150 μg, both KB and SCC-25 cells demonstrated a dose-dependent suppression of PGE<sub>2</sub> synthesis after 12-h incubation. B, time-dependent inhibition of PGE<sub>2</sub> synthesis was also observed in KB and SCC-25 at concentration of 150 μg of *Scutellaria baicalensis*. Suppression of PGE<sub>2</sub> synthesis was observed through the course of treatment. C, both celecoxib and indomethacin inhibited PGE<sub>2</sub> synthesis even at a concentration of 0.1 μM. However, baicalein exerted no effect on PGE<sub>2</sub> synthesis. D, suppression of conversion of arachidonic acid to PGE<sub>2</sub> by *Scutellaria baicalensis* (150 μg/ml) and celecoxib (25 μM). A rapid inhibition (2 h) was observed in the presence of celecoxib, whereas a delayed inhibition (12 h) was observed for *Scutellaria baicalensis*. Results are mean values ± SD (bars) of independent experiments performed in triplicate (A and C). Student *t* test was used to analyze the data. \*, *P* < 0.05; \*\*, *P* < 0.001.

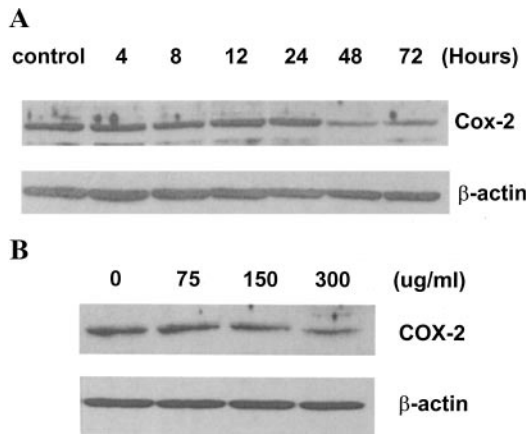
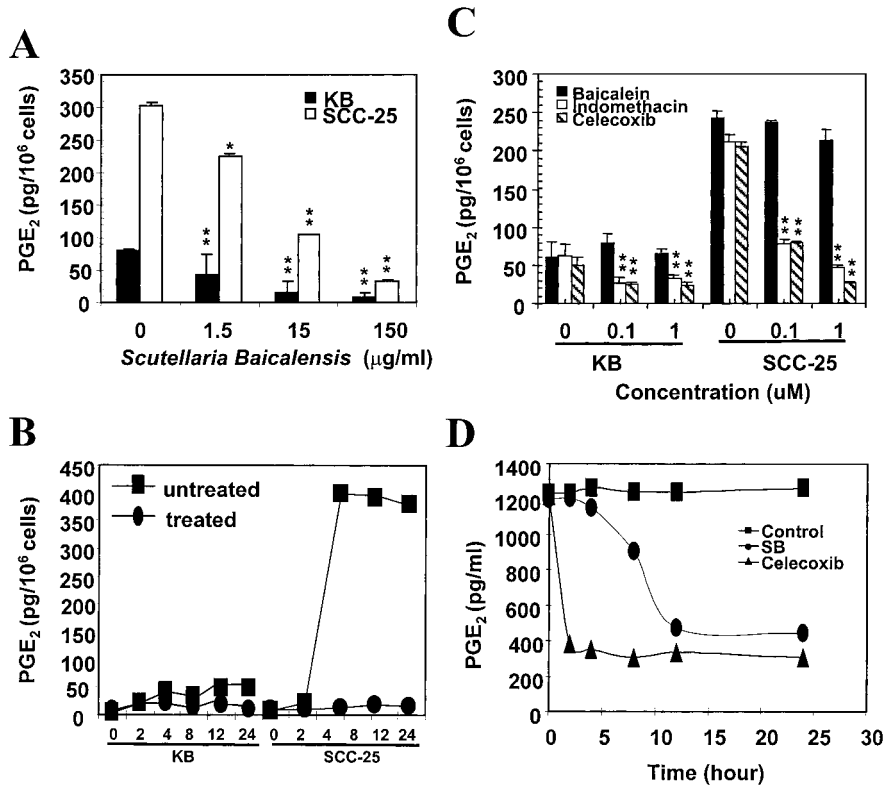


Fig. 5. Analysis of COX-2 protein expression by Western blot. A, SCC-25 cells were treated with *Scutellaria baicalensis* at 300 μg/ml for 4, 8, 12, 24, 48, and 72 h, and proteins were extracted from cells and separated through SDS-PAGE. A significant decrease of COX-2 protein expression was observed after 48-h treatment with *Scutellaria baicalensis* (top panel). β-Actin expression was used as an internal control (bottom panel). B, SCC-25 cells were treated with *Scutellaria baicalensis* at concentrations of 0, 75, 150, and 300 μg/ml for 72 h, and the amount of COX-2 proteins was determined as above. A dose-dependent decrease of COX-2 protein expression was observed after treatment with *Scutellaria baicalensis* (top panel). β-Actin expression was used as an internal control (bottom panel).

baicalein (data not shown), the use of herb extract is more attractive given its excellent water solubility and bioavailability.

Our *in vivo* study additionally confirmed the anticancer activity of *Scutellaria baicalensis*. Significant reduction of tumor mass was observed after a 4-week treatment (Fig. 6). These results indicate that the active ingredients of the herb extract can be readily absorbed in gastrointestinal tract and can reach an effective level in blood. Although the daily dose (75 mg/kg) used in this study is higher than the conventional human dose (45 mg/kg), it was well tolerated in this murine model. In a separate experiment, we administered *Scutellaria*

*baicalensis* at a daily dose of 750 mg/kg to nontumor-bearing nude mice for 9 weeks; no side effect or toxicity was observed (data not shown). In fact, no LD<sub>50</sub> of *Scutellaria baicalensis* in animal studies has been reported. Furthermore, the classic textbook of Chinese herbal

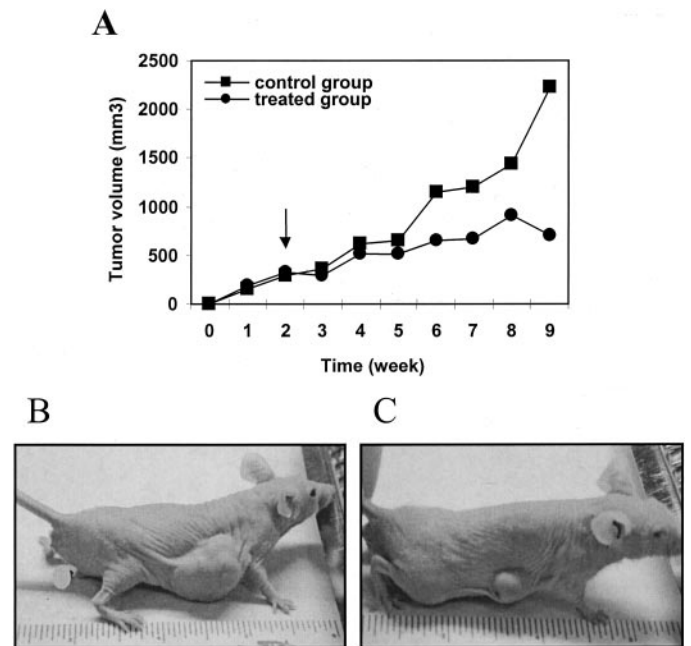


Fig. 6. Inhibition of tumor growth in nude mice xenografted with HNSCC by *Scutellaria baicalensis*. Two weeks after s.c. inoculation of KB cells, the mice were randomly divided into two groups, four mice in each group. In treatment group, *Scutellaria baicalensis* was given p.o. at a dose of 1.5 mg/mouse (75 mg/kg) five times/week for 7 weeks. A significant reduction of tumor volume was observed in the treatment group at 6 week (4 weeks after treatment; A). Representative photographs of an untreated mouse (B) and a control mouse (C). Arrow represents the starting week of *Scutellaria baicalensis* treatment.

medicine documenting several thousand years of clinical usage did not mention any serious toxicities.

In summary, we have demonstrated, for the first time, that *Scutellaria baicalensis* extract exhibits a strong anticancer activity *in vitro* and *in vivo*, especially on HNSCC that is usually resistant to chemotherapy. The anticancer activity of *Scutellaria baicalensis* can be attributed, in part, to its inhibitory effect on PGE<sub>2</sub> production via suppression of COX-2 expression and arachidonic acid release from cell membrane. Future studies will focus on the relationship between increased PGE<sub>2</sub> and cell cycle regulation and will determine its efficacy in clinical trials in patients with head and neck cancer.

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