The Antitumor Effects of *Angelica sinensis* on Malignant Brain Tumors *In vitro* and *In vivo*

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Abstract  
**Purpose:** In this study, we have examined the antitumor effects of chloroform extract of *Angelica sinensis* (AS-C), a traditional Chinese medicine, on glioblastoma multiforme (GBM) brain tumors *in vitro* and *in vivo*.

**Experimental Design:** *In vitro*, GBM cells were treated with AS-C, and the cell proliferation, changes in distributions of cell cycle, and apoptosis were determined. *In vivo*, human DBTRG-05MG and rat RG2 GBM tumor cells were injected s.c. or i.c. and were treated with AS-C. Effects on tumor growth were determined by tumor volume, magnetic resonance imaging, survival, and histology analysis.

**Results:** The AS-C displays potency in suppressing growth of malignant brain tumor cells without cytotoxicity to fibroblasts. Growth suppression of malignant brain tumor cells by AS-C results from cell cycle arrest and apoptosis. AS-C can up-regulate expression of cdk inhibitors, including p21, to decrease phosphorylation of Rb proteins resulting in cell arrest at the G0-G1 phase for DBTRG-05MG and RG2 cells. The apoptosis-associated proteins are dramatically increased and activated in DBTRG-05MG cells and RG2 cells by AS-C but RG2 cells without p53 protein expression. *In vitro* results showed AS-C triggered both p53-dependent and p53-independent pathways for apoptosis. In *in vivo* studies, AS-C not only can suppress growths of malignant brain tumors of rat and human origin but also shrink the volumes of *in situ* GBM, significantly prolonging survivals.

**Conclusions:** The *in vitro* and *in vivo* anticancer effects of AS-C indicate that it has sufficient potential to warrant further investigation and development as a new anti-brain tumor agent.

Brain tumors account for ~35,000 new cases of primary adult central nervous system tumors in the United States each year (1, 2). Although brain tumors comprise only ~1% of all cancers, they contribute to 2.5% of overall cancer mortality and are the third leading cause of cancer deaths in persons ages 15 to 34 years (3, 4). Glioblastoma multiforme (GBM), malignant gliomas, are highly vascularized and invasive neoplasms. Because the diffusely invasive properties of malignant gliomas make them nearly impossible to resect in toto, the standard treatment consists of surgery plus radiotherapy, eventually followed by chemotherapy (5–8). Despite this, the median survival of GBM patients is only ~50 weeks. This dismal prognosis warrants continued intensive investigation of new therapeutic options, with early introduction of promising agents into clinical trials.

Chemotherapy is usually reserved for recurrent tumors already treated with surgery and radiotherapy, or for tumors in which surgery was partial or not feasible and the effect of radiotherapy was limited (9). Various chemotherapy schemes are used; most of them consist of a combination of separate drugs usually administered at high doses. Toxicity, drug resistance, the relative impermeability of the blood-brain barrier, and a limited and transient benefit in patients are the main problems associated with standard chemotherapy regimens (10–14).

Several studies have shown that extracts from a number of herbal medicines or mixtures have anticancer potential *in vitro*, *in vivo*, or both (15–19). For example, alcohol extracts of *Ganoderma lucidum* can induce apoptosis in MCF-7 human breast cancer cells (16). An aqueous extract of *Paeoniae lactiflora* can inhibit growth of HepG2 and Hep3B hepatoma cells (18), whereas aqueous extracts of *Bu-Zhong-Yi-Qi Tang* (a mixture of 10 herbs) have also suppressed growth of hepatoma cells (17). The water-soluble ingredients of *Sho-Saiko-To* (a mixture of seven herbs) inhibit proliferation of KIM-1 human hepatoma cells and KMC-1 cholangiocarcinoma cells (19). Finally, *PE-SPE* (a mixture of eight herbs) was developed for clinical treatment of prostate cancer and has been shown to inhibit growth of colon cancer cells (20).

In traditional Chinese medicine, *dong quai* (*Angelica sinensis*, also called danggui or tang-kuei) is indicated for menstrual
disorders, including menopausal symptoms (21); it has also been widely used for conditions such as gastric mucosal damage, hepatic injury, impaired myocardial blood flow, and chronic glomerulonephritis (22–25). Furthermore, dong quai has been promoted in the United States for treatment of several gynecologic complaints (26). In these options, the different components in *A. sinensis* may involve in different pharmacologic activities.

However, few studies have been made of possible antitumor effects of *A. sinensis*. The antitumor effect of a chloroform extract of *A. sinensis* (AS-C) on malignant brain tumors was examined in this study. The results revealed that the AS-C extract had the ability to inhibit the growth and induce apoptosis of GBM tumor through p53-dependent and p53-independent pathways. Thus, *A. sinensis* may be a good source to provide the potent compound against human GBM tumor.

### Materials and Methods

#### Preparation of chloroform extracts and chemicals.

The roots of *A. sinensis* (Oliv.) were supplied by Chung-Yuan, Co. (Taipei, Taiwan; refs. 27, 28), with confirmation of identity by Professor Han-Ching Lin. A voucher herbarium specimen was deposited at the School of Pharmacy, National Defense Medical Center. A total of 12 kg dried and powdered rhizomes of *A. sinensis* were extracted thrice with chloroform (24 L per extraction) after three acetone extractions (24 L per extraction). The extracts were concentrated under reduced pressure to yield chloroform extract (31.67 g AS-C extraction from 100 g acetone extract).

Carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU); Sigma, St. Louis, MO] and Taxol (Sigma) were obtained from Bioresources Collection and Research Center (Hsin Chu, Taiwan). The J5 line of human hepatocellular carcinoma cells and BCM line of human breast cancer cells were kindly provided by Drs. M.J. Chou and C.S. Yang (Graduate Institute of Basic Medical Science, Chang Gung University; 28) and Dr. D.S. Yu (Department of Genitourinary, Tri-Service General Hospital; 29, 30), respectively. The DBTRG-05MG, RG2 line of rat GBM cells, SK-N-AS line of human neuroblastoma cells, SVEC mouse vascular endothelial cells, and Balb/3T3 mouse fibroblast cells were obtained from American Type Culture Collection (Rockville, MD). GST/VGH human GBM cells, GBM8401 human GBM cells, GBM8901 human GBM cells, N18 mouse neuroblastoma cells, and HL-60 human leukemia cells were obtained from Biosources Collection and Research Center (Hsin Chu, Taiwan). The J5 line of human hepatocellular carcinoma cells and BCM line of human breast cancer cells were kindly provided by Drs. M.J. Chou and C.S. Yang (Graduate Institute of Basic Medical Science, Chang Gung University; 28) and Dr. D.S. Yu (Department of Genitourinary, Tri-Service General Hospital; 29, 30), respectively. The DBTRG-05MG, GBM8401, GBM8901, BCM, HL-60, and J5 cells were maintained with 10% FBS in a humidified atmosphere containing 5% CO₂. The GBM tumor tissues (s.c. or i.c. GBM tumors with or without AS-C treatments), the tumors were harvested and fixed with 10% neutral formalin. Paraffin-embedded sections (7 μm per section) of tumors were stained by using In Situ Cell Death Detection kit. POD, western blot analysis.

**Cell lines and cell culture.** The DBTRG-05MG line of human GBM cells, RG2 line of rat GBM cells, SK-N-AS line of human neuroblastoma cells, SVEC mouse vascular endothelial cells, and Balb/3T3 mouse fibroblast cells were obtained from American Type Culture Collection (Rockville, MD). GST/VGH human GBM cells, GBM8401 human GBM cells, GBM8901 human GBM cells, N18 mouse neuroblastoma cells, and HL-60 human leukemia cells were obtained from Biosources Collection and Research Center (Hsin Chu, Taiwan). The J5 line of human hepatocellular carcinoma cells and BCM line of human breast cancer cells were kindly provided by Drs. M.J. Chou and C.S. Yang (Graduate Institute of Basic Medical Science, Chang Gung University; 28) and Dr. D.S. Yu (Department of Genitourinary, Tri-Service General Hospital; 29, 30), respectively. The DBTRG-05MG, GBM8401, GBM8901, BCM, HL-60, and J5 cells were maintained with RPMI 1640 containing 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO₂. The GBM tumor through p53-dependent and p53-independent pathways. Thus, *A. sinensis* may be a good source to provide the potent compound against human GBM tumor.

### Cell cycle analysis.

Brain tumor cell lines DBTRG-05MG and RG2 were cultured in growth medium with diluent for 48 hours; DMSO content was controlled at ≤0.02% and AS-C extract at 70 μg/mL (IC70). Cell cycle analysis was done by DNA staining with propidium iodide with flow cytometry. Briefly, 2 × 10⁶ adherent cells were detached by trypsinization and were resuspended in 0.8 mL 1× PBS. A total of 200 μL propidium iodide solution (50 μg/mL propidium iodide + 0.05 mg/mL RNase A; Sigma) was added, and cells were incubated at 4°C overnight. Cells were then incubated for at least 2 hours at room temperature before analysis began. A total of 2 × 10⁶ cells were analyzed for FL2 intensity using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA) and CellQuest analysis software (Becton Dickinson Immunocytometry Systems). The G0-G1 phase were gated in M1 (%G0-G1 phase = M1 × 2), G2-M phase were gated in M2 (%G2-M phase = M2 × 2), total cells were gated in M6 (% of phase = M6 − [(M1 × 2) + (M3 × 2)]), and sub-G1 phase were gated in M4.

**Terminal deoxynucleotidyl transferase–mediated nick end labeling assay.** Apoptotic cell death of drug-treated cells was assayed using In Situ Cell Death Detection kit, POD (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Briefly, cells were cultured on culture dishes and analyzed at the indicated time point (0, 6, 12, 24, 48, and 72 hours) after AS-C (70 μg/mL) treatment. In the AS-C–treated cell group, suspended cells were collected. For the control group, adherent and floating cells were collected. Cells were fixed with 3.7% formaldehyde at room temperature for 15 minutes on silane-coated glass slides (Muto Pure Chemicals, Tokyo Japan), washed once in 1× PBS, and incubated in cold permeabilization solution (0.1% Triton X-100 + 0.1% sodium citrate) after reducing the activity of endogenous peroxidase with 3% H₂O₂. Cells were washed with 1× PBS again and incubated with terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) reaction mixture for 60 minutes at 37°C. After washing with 1× PBS, counterstaining with propidium iodide for determination of cell count was done. For quantification of apoptosis, the resulting slides were viewed under a fluorescence microscope (Nikon, Kawasaki, Japan). For the histologic TUNEL staining of the GBM tumor tissues (s.c. or i.c. GBM tumors with or without AS-C treatments), the tumors were harvested and fixed with 10% neutral formalin. Paraffin-embedded sections (7 μm per section) of tumors were stained by using In Situ Cell Death Detection kit, POD. After dewaxing, rehydration, and proteinase digestion, the slides with tumor tissues were incubated with 100 μL TUNEL reaction mixture and covered with lid for 60 minutes at 37°C in a humidified atmosphere in the dark. Finally, slides were washed thrice with 1× PBS, mounted, and visualized under a fluorescence microscope.

**Western blot analysis.** DBTRG-05MG cells and RG2 cells were treated with AS-C (70 μg/mL) for different durations (0, 6, 12, 24, or 48 hours). Cell pellets were resuspended in lysis buffer (10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 0.5% CHAPS, 10% (v/v) glycerol, 5 mmol/L l-2-mercaptoethanol, and 0.1 mmol/L phenylmethylsulfonyl fluoride) and incubated on ice 30 minutes. Total cell lysate was collected after centrifugation at 13,000 rpm at 4°C for 20 minutes. The protein concentration of cell lysates was measured with BCA protein assay kit (Pierce, Rockford, IL) following the manufacturer’s instructions. Cell lysates (20 μg/lane) were run on 10% to 12% SDS-PAGE (Bio-Rad, Hercules, CA) gels and transferred to polyvinylidene difluoride membranes (Amersham Life Sciences, Piscataway, NJ) after separation. The membranes were blocked for 1 hour at room temperature with 5% skim milk and incubated with respective antibodies. Antibodies including anti-Fas, anti–Fas-L, anti– caspase 3, anti– caspase 8, anti– caspase 9, anti-Bax, anti-p16, anti-p21, and anti-p53 (1/100 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-phospho-p53 (Ser15; 1/2,000 dilution) and antisphospho-RR (Ser256 and Ser286; 1/2,000 dilution; Cell Signaling Technology, Beverly, MA) were used in this study. The immobilized primary antigen-antibody complex was detected with respective horseradish peroxidase–conjugated anti– mouse, anti–rabbit, or anti–goat IgG secondary antibodies (1/1,000 dilution; Santa Cruz Biotechnology) for 1 hour at room temperature.
and visualized with the ECL Plus chemiluminescence system (Amer-
sham, Arlington Heights, IL). We ensured that samples contained equal
amounts of protein by preparing SDS-PAGE gels in duplicate and
staining one of each set with Coomassie blue. The intensity of bands
was analyzed by GS-800 Calibrated Imaging Densitometer (Quantity
One 4.0.3 software; Bio-Rad).

Animal studies. The RG2 rat GBM cells and DBTRG-05MG human
GBM cells were used in animal experiments to monitor antitumor
activities of AS-C. Male F344 rats (230-260 g) and male Foxn1 nu/nu
mice (10-12 weeks) were obtained from the National Laboratory
Animal Center (Taipei, Taiwan). All procedures were done in
compliance with the standard operating procedures of the Laboratory
Animal Center of Tzu Chi University (Hualien, Taiwan).

Syngeneic F344 rats (six rats per group) were implanted s.c. on the
back with 1 × 10^6 RG2 cells; six animals in each group were treated by
s.c. injection with AS-C (500 mg/kg/d) and vehicle (50 mg/mL
propylene glycol and 10 mg/mL Tween 80 in distilled water; Standard
Chem. & Pharm., Tainan, Taiwan) far from the inoculated tumor sites
(>1.5 cm) on days 3, 6, and 9 after tumor cell implantation. In
addition, nude mice (six mice per group) were implanted s.c. with
5 × 10^6 DBTRG-05MG cells. The tumor-bearing animals were treated
with AS-C (i.p. or s.c. injection using 500 mg/kg/d in 50% of AS-
C SC500 group, respectively) or vehicle (s.c., control group) on day 5
after tumor cell implantation. Tumor size was measured by use of a
caliper and volume was calculated as L × H × W × 0.5236 (31).

Animals were sacrificed when tumor volume exceeded 25 cm^3 in rat
and 1000 mm^3 in mice and that day was used as the final survival day
for the rats and mice.

The cytotoxic effects of AS-C on in situ tumor were determined with
RG2 cells. Syngeneic animals (six animals per group) were implanted
intracerebrally (i.c.) into striatum with 5 × 10^6 RG2 cells and treated
with AS-C (500 mg/kg/d) or vehicle s.c. on days 4, 5, 6, 7, and 8 after
tumor cell implantation. Tumor volume was measured and calculated
by 3-T unit magnetic resonance imaging (General Electric, Milwaukee,
WI) with echo-planar imaging capability (Signa LX 3.8, General Electric;
ref. 32) in Buddhist Tzu Chi General Hospital (Hualien, Taiwan).

Briefly, rats were anesthetized with chloral hydrate (400 mg/mL, 1 mL/
100 g). Functional magnetic resonance imaging scanning was conducted
with a fast spin echo, echo-planar acquisition sequence in which the repetition
time was 6,000 milliseconds, the echo time was 102 milliseconds, the matrix image was 256 × 256 pixels, the field of
view was 5 × 5 cm, and the in-plane resolution was 80 µm. Twenty
slices (1.5 mm thick each) were obtained every 19.5 seconds for 6.5
minutes total time per rat.

As described above, DBTRG-05 MG cells (5 × 10^6) were implanted s.c.
on the backs of nude mice. Until the tumor volume reached 250 mm^3,
tumor-bearing mice were treated with single-dose AS-C (500 mg/kg) or
vehicle s.c., and mice were sacrificed to determine cytotoxicity in tumors
(microscopic slides prepared with H&E tissue staining) on day 10 after
treatment of AS-C. Tissue sections were observed and photographed
under a light microscope at magnifications of ×50 and ×400.

Immunohistochemical staining. Paraffin-embedded sections were
obtained from the tumors (s.c. or i.c. GBM tumors with or without AS-C
treatments) and were processed for immunohistochemical staining. A
goat polyclonal anti-Ki-67 antibody (1/100 dilution; Santa Cruz
Biotechnology) and a rabbit polyclonal anti-cleaved caspase 3 (Asp73)
antibody (1/1,000 dilution; Cell Signaling Technology) were used in
immunohistochemical studies for 4°C overnight incubation. Briefly,
the slides were treated with 3% hydrogen peroxide in 1× PBS for 10
minutes to block endogenous peroxidase activity after being dewaxed
and rehydrated. Next, the sections were washed thrice with TBS-T (1×
TBS containing 0.05% Tween 20) for 5 minutes each time and
nonspecific reactions were blocked by 10% fetal bovine serum in PBS
for 10 minutes at room temperature. Sections were incubated with the
anti-Ki-67 antibody and the immune complexes were visualized using
the horseradish peroxidase–conjugated anti-goat IgG secondary anti-
odies (1/1,000 dilution; Santa Cruz Biotechnology). Sections were
incubated with the anti–cleaved caspase 3 (Asp73) antibody; LSAB2
system (DAKO, Carpinteria, CA) was used to visualize the immune
complexes and incubated with 0.5 mg/mL diaminobenzidine and 0.03%
(v/v) H2O2 in PBS for 10 minutes. Finally, sections were
counterstained with hematoxylin, mounted and observed under a light
microscope at a magnification of ×400, and photographed.

Statistics. Data were expressed as mean ± SD or SE. Statistical
significance was analyzed by Student’s t test. The survival analysis was
done using the Kaplan-Meier method. P < 0.05 was considered to be
statistically significant.

Results

Cytotoxic effects of chloroform extract of A. sinensis on tumor
cell lines and other cell lines. The growth inhibitory effects of
AS-C on cells from GBM, neuroblastoma, leukemia, breast
cancer, and hepatocellular carcinoma, as well as normal
fibroblast and vascular endothelial cells, were determined.

The IC_{50} values of AS-C after 48-hour incubation to brain
tumor cell lines (IC_{50} = 30-47 µg/mL) were significantly lower
than the values for other tumor cells (IC_{50} = 140-175 µg/mL;
P < 0.0001) or normal cells (IC_{50} = 86-300 µg/mL, P < 0.01;
Table 1). For the two normal cell types, vascular endothelial
cells (IC_{50} = 86.2 ± 0.1 µg/mL) were more sensitive to AS-C
than fibroblast cells (IC_{50} > 300 µg/mL, P < 0.05). Two
chemotherapeutic agents currently in use, BCNU and Taxol,
were also tested on GBM cells. GBM cells were not sensitive, or
have low sensitivity, to Carmustine (IC_{50} = 55 to >100 µg/mL);
however, both GBM cell lines (e.g., DBTRG-05MG and GST/
VGH) were sensitive to Taxol (IC_{50} for DBTRG-05MG = 61.0 ±
3.3 µg/mL and IC_{50} for GST/VGH < 0.1 µg/mL). Although
Taxol showed significant cytotoxicity with GBM cells, it also
showed very high cytotoxicity with vascular endothelial cells
(IC_{50} < 0.1 µg/mL). Briefly, these results showed that AS-C
could induce high cytotoxicity to brain tumor cells but low or
no cytotoxicity with normal cell lines.

After AS-C treatment, the morphology of GBM tumor cells
gradually detached from the bottom of culture plates (12-72
hours). The viabilities of detached cells were verified with
TUNEL staining and the results showed that the AS-C–induced
GBM detached cells were in the process of apoptosis at 72 hours
(Fig. 1A and B).

The effects of chloroform extract of A. sinensis on the cell cycle
of glioblastoma multiforme cells. Cell cycle analysis with GBM
cells showed that 70 µg/mL AS-C treatment resulted in cell cycle
arrest at G_0-G_1 phase (>90%; Fig. 1C and D). AS-C induced
a significant proportion of cells to arrest at G_0-G_1 phase
accompanied by a concurrent decrease of S phase from 12 to
24 hours (P < 0.05). In addition, AS-C also decreased the
proportion of the DBTRG-05MG cells that entered the G_2-M
phase from 24 hours after treatment but AS-C increased the
proportion of the RG2 cells that entered the G_2-M phase from
24 hours after treatment (Fig. 1C and D). The results for the
changes in cell cycle for 48 and 72 hours after AS-C treatment
were as similar as the results at 24 hours (data not shown).

Apoptotic pathways in glioblastoma multiforme cells induced by
chloroform extract of A. sinensis. To investigate apoptotic
pathways induced by AS-C treatment, protein expression of Fas
and Fas-L in treated GBM detached cells was first evaluated by
immunoblotting, which showed that AS-C significantly increased Fas expression (from 1- to 159-fold
versus 1-2-fold in DBTRG-05MG and RG2 cells) but not Fas-L

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expression. In addition, the activation of death receptor–induced, apoptosis-related proteins were monitored. Levels of procaspase 8 were slightly increased in GBM cells (1.3- and 1.4-fold in DBTRG-05MG and RG2 cells) at 6 hours and gradually decreased (Fig. 2A) after AS-C treatment, whereas levels of activated caspase 8 had a 21.4-fold increase at 6 hours in DBTRG-05MG cells and a 4.2-fold increase at 12 hours in RG2 cells after AS-C treatment (Fig. 2A). The activation of procaspase 9 and procaspase 3 was also determined (Fig. 2A). Both procaspase 9 and procaspase 3 were highly activated in DBTRG-05MG and RG2 cells after AS-C treatment.

Phosphorylation of p53 and Rb proteins were studied; AS-C caused levels of phosphorylated p53 protein to increase (2.7-fold) at 6 hours after treatment (Fig. 2B) and total p53 protein was also increased (2.2-fold) at 6 hours, gradually decreasing afterward in DBTRG-05MG cells. In contrast, p53 gene was impaired and p16 gene showed homologous deletion in RG2 cells; therefore, their expressions were undetectable in immunoblotting analysis (Fig. 2B). However, levels of phosphorylated Rb proteins (phosphorylation at 795) were decreased (0.4-fold) at 6 hours, with phosphorylated proteins undetectable as soon as 12 hours after AS-C treatment in DBTRG-05MG cells; in RG2 cells, levels of phosphorylated Rb proteins were decreased (0.9-fold) at 12 hours, with phosphorylated proteins undetectable as soon as 24 hours after AS-C treatment (Fig. 2B). The levels of phosphorylated Rb proteins (phosphorylation at 807 and 811) were also detected. However, phosphorylated Rb proteins were not detected in DBTRG-05MG cells, whereas similar decreases in protein expression were observed in RG2 cells.

The major cell cycle checkpoint machineries were also determined. Levels of p16, p21, and Bax proteins in AS-C–treated GBM cells were consequently measured. Levels of all three proteins increased in DBTRG-05MG cells, and the similar results of p21 and Bax were detected in RG2 cells after treatment with AS-C (Fig. 2C). The p16 protein was not detectable in RG2 because p16 gene is depleted in RG2 cells.

Therapeutic effects of chloroform extract of A. sinensis on survival of animals bearing s.c. glioblastoma multiforme tumor. To verify AS-C antitumor activity, in vivo animal experiments were carried out. There was a significant inhibitory effect on tumor (RG2) growth for the AS-C–treated group compared with the control group (P < 0.0001; Fig. 3A). Average tumor size at day 26 was 20.7 ± 1.5 cm³ for the control group compared with only 11.5 ± 0.7 cm³ for the AS-C–treated group. Survival of rats in the AS-C treatment group was significantly prolonged compared with survival in control group (30 ± 2.1 days versus 40 ± 2.7 days, P < 0.0001; Fig. 3B). There were remarkable decreases in the expressions of Ki-67 protein, increases of cleaved caspase 3 protein, and apoptosis of tumor cells at day 18 after AS-C treatment compared with control group in vivo (Fig. 3C).

With a 500 mg/kg s.c. injection of AS-C, no significant drug-related toxicity was observed in animals treated with AS-C based on body weights and histologic analysis of organs (data not shown).

Therapeutic effects of chloroform extract of A. sinensis on rat in situ glioblastoma multiforme tumor. To investigate AS-C antitumor effects with rat in situ GBM tumors, F344 rats were implanted i.c. (striatum) with 5 × 10⁴ RG2 cells and were treated with s.c. AS-C (500 mg/kg/d) on days 4, 5, 6, 7, and 8. There were significant declines in tumor volume for the treated group compared with the untreated group (P < 0.05; Fig. 4B).

### Table 1. The IC₅₀s of different tumors by AS-C treatment

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor type</th>
<th>AS-C IC₅₀ (µg/mL)</th>
<th>Carmustine IC₅₀ (µg/mL)</th>
<th>Taxol IC₅₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Brain tumors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBTRG-05MG</td>
<td>Human GBM cell</td>
<td>46.5 ± 10.9*</td>
<td>100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>G5T/VGH</td>
<td>Human GBM cell</td>
<td>43.7 ± 10.9*</td>
<td>100</td>
<td>61.0 ± 3.3</td>
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<tr>
<td>GBM8401</td>
<td>Human GBM cell</td>
<td>30.8 ± 2.4*</td>
<td>55.6 ± 9.6</td>
<td>ND</td>
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<tr>
<td>GBM8901</td>
<td>Human GBM cell</td>
<td>33.7 ± 1.7*</td>
<td>56.5 ± 1.7</td>
<td>ND</td>
</tr>
<tr>
<td>RG2</td>
<td>Rat GBM cell</td>
<td>30.0 ± 11.5*</td>
<td>&gt;100</td>
<td>ND</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td>Human neuroblastoma</td>
<td>40.5 ± 1.9*</td>
<td>&gt;100</td>
<td>ND</td>
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<td>N18</td>
<td>Mouse neuroblastoma</td>
<td>35.1 ± 5.6*</td>
<td>&gt;100</td>
<td>ND</td>
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<td><strong>Other tumors</strong></td>
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<td></td>
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<tr>
<td>BCM</td>
<td>Human breast cancer</td>
<td>142.8 ± 1.2</td>
<td>ND</td>
<td>&gt;100</td>
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<tr>
<td></td>
<td>mucin-like cell</td>
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<tr>
<td>HL-60</td>
<td>Human leukemia cell</td>
<td>173.8 ± 1.2</td>
<td>ND</td>
<td>&gt;100</td>
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<tr>
<td>J5</td>
<td>Human hepatocellular carcinoma</td>
<td>143.9 ± 0.2</td>
<td>ND</td>
<td>&gt;100</td>
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<td><strong>Normal cells</strong></td>
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<tr>
<td>SVEC</td>
<td>Mouse vascular endothelia cell</td>
<td>86.2 ± 0.1</td>
<td>ND</td>
<td>&lt;0.1</td>
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<tr>
<td>Balb/3T3</td>
<td>Mouse fibroblast cell</td>
<td>&gt;300</td>
<td>&gt;100</td>
<td>ND</td>
</tr>
</tbody>
</table>

**NOTE:** Values are mean ± SD IC₅₀ (µg/mL) in day 2. Abbreviation: ND, not done.

*Significant difference from the brain tumors versus other tumors of AS-C treatment (P < 0.0001).

Significant difference from the brain tumors versus normal cells of AS-C treatment (P < 0.001).

Significant difference from the AS-C versus Carmustine treatment in brain tumors (P < 0.001).
Mean tumor volume at days 14 and 16 were 70 ± 4.8 and 126.4 ± 11.1 mm³ for control group versus 46.2 ± 3.6 and 99.5 ± 9.5 mm³ for the AS-C–treated group. Magnetic resonance imaging data showed that the in situ tumor volumes in the AS-C–treated group were smaller than those in the control group (Fig. 4A). The immunohistochemistry results showed that the Ki-67 proteins were decreased, the cleaved caspase 3 proteins were increased, and apoptosis was increased in tumor cells at day 16 after AS-C treatment compared with control group in vivo (Fig. 4C), which is similar to the results shown in the s.c. RG2 tumor model.

Therapeutic effects of chloroform extract of A. sinensis on xenograft tumor growth. To determine whether AS-C can suppress human GBM tumor growth, nude mice were inoculated s.c. with human DBTRG-05MG cells and treated s.c. or i.p. with a single dose of AS-C (500 mg/kg) on day 5. There was significant suppression of tumor growth in the AS-C IP500 and AS-C SC500 treatment groups compared with the untreated group (P < 0.005; Fig. 5A). Mean values for tumor size at day 38 were 849.9 ± 150.1 mm³ in the control group, 295.5 ± 25.3 mm³ in the AS-C IP500 treatment group, and 155.1 ± 56.4 mm³ in the AS-C SC500 treatment group. Figure 5B shows suppression of human GBM tumors for the AS-C IP500 treatment group and the AS-C SC500 treatment group at day 40.

Cytotoxic activity of chloroform extract of A. sinensis on human glioblastoma multiforme tumor in vivo. To verify whether AS-C can induce human GBM tumor cell death in vivo, we examined the cytotoxic activity of AS-C for human GBM tumor in a nude mouse model. Histologic analysis showed that a significant proportion (~30-50% in AS-C–treated versus 0% in control groups) of cells in the AS-C–treated tumor mass died. In contrast, AS-C–induced cytotoxic effects were not seen in the control tumor mass (Fig. 5C). The human GBM tumor tissues with AS-C treatment in vivo also displayed decrease in Ki-67 expressions, increases in cleaved caspase 3 protein expressions, and tumor cell apoptosis at day 10 after AS-C treatment compared with control group in vivo (Fig. 5D).

Discussion

A. sinensis has been shown to have a broad spectrum of biological activities, including regulation of the immune system, alleviation of menopausal symptoms, and improvement in myocardial blood flow based on its long history in traditional Chinese medicine (5, 15–19). Very few drugs are in development for malignant brain tumors, in part because of the difficulty in drug passage through the blood-brain barrier; the current study is the first to show that AS-C has strong activity against GBM in vitro and in vivo.

In vitro cytotoxic assays showed that not only brain tumor cells but also other tumor cells were sensitive to AS-C extract;
however, the same experiments showed that normal fibroblast cells were resistant to AS-C. No evidence of AS-C–induced cytotoxic effects was found in liver or kidney after a single dose of 500 mg/kg (either i.p. or s.c.). Furthermore, the anticancer efficacy of AS-C in this study was better than that of Carmustine (BCNU). As the cytotoxic efficacy of AS-C was lower than that of Taxol, the cytotoxicity of AS-C to normal cells was also lower than that of Taxol (Table 1). In clinical studies, current antitumor drugs, such as Temozolomide, only slightly prolong survival for patients with GBM (1–9). In the current research, AS-C had a proportionately greater cytotoxic effect on GBM cells than other tumor cells based on in vitro cytotoxic results. In vivo, AS-C inhibited rat GBM tumor growth (Fig. 3A) and prolonged survival in a s.c. tumor model (Fig. 3B), and it especially reduced tumor volume of rat GBM in situ (Fig. 4). These results suggest that AS-C should penetrate the blood-brain barrier to affect GBM cells. Thus, AS-C could inhibit GBM tumor cell growth and induce GBM tumor cell apoptosis in vitro and in vivo. The AS-C extract is oil-like and is difficult to dissolve into water and, therefore, the penetrating ability of AS-C may be due to its hydrophobic (lipid-like) nature. In addition, human GBM cells may have a different metabolism compared with rat GBM cells, resulting in different susceptibility to AS-C extract. Figure 5 indicates that AS-C treatment can also suppress human GBM tumor growth in a s.c. tumor model. These in vivo cytotoxic results reveal the possibility that AS-C extract can be developed as an effective and safe anti-GBM drug.

The human GBM cell line DBTRG-05MG was established from a patient with GBM who had been treated with local brain irradiation and multidrug chemotherapy. Researchers working with the cell line have verified there are no losses of heterozygosity in p53 and Rb tumor suppressor genes (33). Therefore, DBTRG-05MG cells may have multidrug resistance. The RG2 cell line (rat GBM) that was used in this study has impaired p53 expression and homozygous deletions of the p16/Cdkn2a/Ink4a gene locus (34, 35). Our results also showed
that both brain tumor cell lines were resistant to Carmustine (BCNU), an alkylating agent that is one of the main chemotherapeutic agents for treatment of brain tumors (Table 1; refs. 36, 37). In contrast, AS-C impressively suppressed growth of the two GBM tumors in vitro and in vivo in this study. Thus, we conclude that AS-C could induce GBM cell apoptosis by both p53-dependent and p53-independent pathways and may have significant therapeutic effect for tumors than BCNU in certain tumors. In addition, in vitro and in vivo cytotoxic experimental results showed that no severe side effects were found after high doses or high frequency of AS-C treatment (Figs. 3-5; Table 1). These results suggest that AS-C has potential as a potent antitumor drug for malignant tumors with multidrug resistance.

To explore mechanisms that might account for the effects of AS-C on GBM tumor cells, cell cycles were monitored and results showed that inhibition of tumor growth is due to cell cycle arrest at G0-G1 phase (>90%) with induction of multiple apoptosis molecules resulting in apoptotic cell death after AS-C treatment. AS-C induced high levels of expression of p21 and p16 and decreased phosphorylation of Rb protein 6 hours after treatment (Fig. 2B and C). These results indicate that AS-C...
arrests cell cycle at G₀-G₁ phase through regulation of gene expression involving the cyclin/CDK/CKI system. Both p16 and p21 are cdk inhibitors that bind to and negatively regulate cdk or cyclin/cdk complexes (38). The p16 protein, a member of the INK4 family, binds to cdk4 or cdk6 to inhibit kinase activity at mid-G₁ phase (39). The p21 protein binds to cyclin/cdk complexes resulting in inhibition of the G₁-S phase transition (40) by inhibiting cyclin/CDK complex phosphorylation of Rb protein. Therefore, the decrease of phosphorylated Rb proteins should be due to AS-C–triggered cdk inhibitor expression, decreasing CDK activity and resulting in a decrease of phosphorylated Rb proteins. Similar results were also found for monoterpenoid activity, an essential oil of plants and likely the AS-C anticancer compound that has been shown to cause G₁ arrest and lead to an increase in p21 expression (41).

Another compound, aragusterol A, isolated from marine sponges, is a potent anticancer marine steroid, and it has also been shown to cause G₁ arrest by down-regulation of Rb phosphorylation (42).

According to the results of TUNEL assay and immunoblotting, the possible mechanisms of apoptosis induced by AS-C in GBM tumor cells were inferred. First, the p53-dependent apoptosis pathway may involve in the mechanisms of AS-C–induced apoptosis. AS-C treatment could promote phosphorylation of p53 in the DBTRG tumor cells and increase p53 expression, with an expression peak at 6 hours (Fig. 2B).
Phosphorylation at the NH2-terminal region of p53 protein will abolish the Mdm2 inhibition, which causes an increase in Bax transcription (Fig. 2C showed that Bax proteins were increased) and inhibition of Bcl-2 transcription. Because of Bax/Bcl-2 imbalance, Bax can form an active homodimer to trigger cleavage of procaspase 9 (Fig. 2A showed that caspase 9 was increased) and sequentially result in procaspase 3 activation (Fig. 2A also showed that caspase 3 was increased) and apoptosis. DNA damage has been shown to induce phosphorylation of p53 at the Ser15 site and increase and apoptosis. Second, the Fas-FasL–induced apoptosis pathway may also be involved in AS-C–induced apoptosis. After AS-C treatment, increasing expression of Fas was observed (Fig. 2A). Fas is the death receptor responsible for signaling from the cell membrane and triggers activation of procaspase 8 (Fig. 2A showed caspase 8 was increased), subsequently promotes procaspase 3 activation (Fig. 2A), and results in apoptosis. Therefore, AS-C–induced apoptosis of GBM tumor cells might be mediated, at least partially, through mitochondrial and death receptor apoptosis pathways. For RG2 cells, the cell has impaired p53 expression and homozygous deletions of the p16/Cdkn2a/Ink4a gene locus (34, 35). Therefore, the AS-C–induced apoptosis should be caused through the p53-independent pathway (Fas-induced apoptosis pathway) but not the p53-dependent pathway. However, although RG2 cells were impaired for p53 and p16, the caspase 9 and p21 expressions induced by AC-S were still displayed in these cells. Thus, certain mechanisms of p53-independent activation of procaspase 9 and p21 induction may be involved in the AS-C–induced cell apoptosis and cell growth inhibition.

Two major components have been identified in AS-C, K1 and K2. Their molecular weights are ~188.22 and 190.23. Whether one or both of these components is an active antitumor component is yet to be determined. Although the inhibitory effects of AP (extracts from A. sinensis) on invasion and metastasis of hepato-cellular carcinoma cells in vitro have been reported (44), reports indicate that the responsible component is polysaccharide, which is very different in nature from that of the active component in our AS-C extract. The AS-C that we have used has two properties, oil-like (nonpolar) and a smaller molecular weight, which may be the basis for passage through the blood-brain barrier with resulting reduction in tumor volume in the brain.

In conclusion, pronounced antitumor activity in vitro and in vivo suggests that AS-C has potent anticancer effects and causes both cell cycle arrest and apoptosis. The results of anti-GBM treatment with AS-C are significant, providing new hope for effective chemotherapy for such malignant brain tumors. Isolating the specific active compounds of AS-C and examining their mechanisms of action are in process; this work may lead to new therapeutic options and improved understanding of the interaction of phytochemicals with gene regulation in brain cancer cells.

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