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Delayed growth of glioma by *Scutellaria* flavonoids involve inhibition of Akt, GSK-3 and NF-κB signaling

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Abstract

Plants of the genus *Scutellaria* constitute one of the common components of Eastern as well as traditional American medicine against various human diseases, including cancer. In this study, we examined the in vivo anti-glioma activity of a leaf extract of *Scutellaria ocmulgee* (SocL) while also exploring their potential molecular mechanisms of action. Oral administration of SocL extract delayed the growth of F98 glioma in F344 rats, both in intracranial and subcutaneous tumor models. Immunohistochemistry revealed inhibition of Akt, GSK-3α/β and NF-κB phosphorylation in the subcutaneous tumors following treatment with *Scutellaria*. The SocL extract as well as the constituent flavonoid wogonin also showed dose- and time-dependent inhibition of Akt, GSK-3α/β and NF-κB in F98 cell cultures in vitro, as determined by western blot analysis. Pharmacologic inhibitors of PI3K and NF-κB also significantly inhibited the in vitro proliferation of F98 glioma cells, indicating the key role of these signaling molecules in the growth of malignant gliomas. Transfection of F98 cells with constitutively active mutant of AKT (AKT/CA), however, did not significantly reverse *Scutellaria*-mediated inhibition of proliferation, indicating that *Scutellaria* flavonoids either directly inhibited Akt kinase activity or acted downstream of Akt. In vitro Akt kinase assay demonstrated that the SocL extract or wogonin could indeed bind to Akt and inhibit its kinase activity. This study provides the first in vivo evidence and mechanistic support for anti-glioma activity of *Scutellaria* flavonoids and has implications in potential usage of *Scutellaria* flavonoids in adjuvant therapy for malignant tumors, including gliomas.

Keywords

Glioma; *Scutellaria*; Flavonoids; Wogonin; Akt; GSK-3; NF- κ B

Introduction

High-grade gliomas are the most common primary tumor of the brain and one of the most lethal malignant tumors. In spite of progress in surgery, radiotherapy and chemotherapy, prognosis for malignant gliomas still remains dismal [1, 2]. Therefore, the need for developing novel adjuvant therapy cannot be overstated.

Plants of the genus *Scutellaria* constitute one of the common components of Eastern as well as traditional American medicine against myriads of human ailments, including cancer. Extracts, or isolated flavonoids of *Scutellaria* have anti-inflammatory [3, 4], anti-oxidative [3, 4], anxiolytic [5, 6], anti-viral [4] and anti-tumor [7–11] activities. We have recently reported a comprehensive analysis of the leaf, stem and root extracts obtained from thirteen different species of *Scutellaria* for their flavonoid content as well as for their mechanism of anti-cancer activity using glioma, breast carcinoma and prostate carcinoma cell lines in vitro [12]. Among the thirty-nine extracts examined, the leaf extracts of *Scutellaria angulosa* (SanL), *S. integrifolia* (SinL), *S. ocmulgee* (SocL) and *S. scandens* (SscL) showed consistent, dose-dependent anti-proliferative and pro-apoptotic activities against various malignant cell lines. Leaf extract of *S. ocmulgee* (SocL) was the most efficacious among the four. It was, therefore, selected for further studies.

Biological activities of *Scutellaria* have been attributed mainly to the constituent flavonoids [13]. Our previous study had demonstrated the flavonoid wogonin to be a prominent constituent of SocL extract [12]. There have been some studies on the anti-tumor activities of wogonin [8, 14–17], but the molecular mechanism of its activity is not yet clear.

In our earlier study, *Scutellaria* extracts as well as the flavonoids significantly inhibited the *in vitro* growth of malignant brain (U87-MG glioma) and breast cancer (MDA-MB-231) cells without affecting the growth of corresponding non-malignant normal human astrocyte (NHA) and human mammary epithelial (HMEC) cells [12]. These results showed that *Scutellaria* extracts or individual flavonoids target molecular mechanisms that are specific to the malignant phenotype. This study is an endeavor to examine the *in vivo* efficacy of *Scutellaria* against malignant glioma and to explore the molecular anti-tumor mechanisms of *Scutellaria* extract and the constituent flavonoid wogonin focusing on the signaling molecules Akt and NF- κ B.

Using a syngeneic rat glioma tumor model, comprising orthotopic and subcutaneous transplantation of F98 glioma cells into F344 rats, we report delayed growth of tumor following administration of *Scutellaria* extract. The delayed tumor growth was associated with decreased activity of Akt, GSK-3 and NF- κ B signaling molecules *in vivo* as well as *in vitro*.

Materials and methods

Cell lines and flavonoids

Rat malignant glioma cell line F98 was purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM (low glucose) supplemented with 5% FBS. Wogonin was purchased from Wako Chemicals (Richmond, VA).

Cultivation of *Scutellaria* plants and preparation of leaf (SocL) extracts

S. ocmulgee plants were cultivated at the 'Specialty Plants House' as described earlier [12]. The leaves were harvested and shade dried at room temperature until they lost 70% moisture. The dried leaves were then ground and extraction was performed using an ASE apparatus (Dionex Corporation, Sunnyvale, CA) as described earlier [12].

In vivo studies

F98 rat glioma cells (5×10^4 cells in 5 μ l PBS) were stereotactically injected into the right basal ganglia of twelve F344 rats following a published protocol [18]. Five days after tumor transplantation, animals were randomly divided into two groups. One group of six animals received SocL extract (100 mg/kg) via oral gavage in 500 μ l saline, once a day, 5 days a week, for 2 weeks. Tumor volume was estimated following gadolinium enhanced T1-weighted MRI (GE Medical Systems) on day 19 (after 2 weeks of treatment). Number of voxels in each slice (slice thickness 1 mm, total 12 slices) were counted using ImageJ software. Volume = Total number of voxels $\times$ 0.015625 mm³.

Another group of six animals was transplanted with 1×10^6 F98 cells subcutaneously, in the right flank. After 5 days, when the tumor was palpable, the animals were divided into two groups. SocL extract and saline (control group) were administered as described for the intracranial tumor model. On day 29 after tumor transplantation, the animals were euthanized and the subcutaneous tumors were excised and then fixed in 4% PFA-PBS for immunohistochemistry.

Cell proliferation assay

Cells were seeded in 96-well flat-bottom plates (2×10^4 cells/well), and cultured in presence of *Scutellaria* extracts or flavonoids as described elsewhere [12], with some modifications. Cell proliferation was assayed by using the CellTiter-Glo[®] Luminescent Cell Viability Assay kit (Promega), which determines the number of viable cells in a culture by quantification of ATP. At the end of culture period, 100 μ l of the CellTiter-Glo[®] reagent mix was added to 100 μ l of culture volume and then the luminescence measured with integration time set for 0.25 to 1 s using an Omega imaging system (UltraLum Inc., Claremont, CA). The cell proliferation was expressed as a percentage value of control cells cultured with medium alone.

Immunohistochemistry

The paraffin-embedded tumor specimens were cut into 5 μ m tissue sections, deparaffinized and hydrated to PBS (pH 7.4). The sections were pretreated with hydrogen peroxide (3%) for 10 min to remove endogenous peroxidase, followed by antigen retrieval via steam bath for 20 min in Sodium-citrate buffer (pH 6.0). Appropriate primary antibodies (Cell signaling Technology, Danvers, MA) were applied, followed by washing and incubation with the biotinylated secondary antibody (Vector Labs, Burlingame, CA) for 30 min at room temperature. Detection was performed with diaminobenzidine (DAB) and counterstaining with Mayer hematoxylin followed by dehydration and mounting. The sections were analyzed under a fluorescent microscope equipped with a digital camera (Olympus BX51).

Quantification of the cell proliferation index via Ki67 immunostaining

To determine the cell proliferation index by Ki67 immunostaining, the number of cells with distinct nuclear staining was recorded after counting 100 cells in consecutive high power fields in the most reactive areas of the slides. Cells with questionable nuclear staining were discounted. Necrotic or thick areas and severely overlapping tumor cells were also excluded.

The cell proliferating index (PI) was calculated as percentage of Ki67-positive cells to the total number of cells counted.

Transfection of cells with constitutively active (CA) and dominant negative (DN) mutants of AKT

Plasmid vectors containing myristoylated AKT (AKT/CA) and AKT/DN mutants were kind gifts from Dr. Kenneth Walsh, University of Boston, MA [19, 20]. The plasmids were expanded after transforming into competent *E. coli* strain DH5 α , grown in liquid LB medium containing ampicillin, and isolated using a commercial kit (Quiagen). The DNA sequence of the plasmid insert was verified at our DNA Sequencing Core Facility. F98 cells were transfected with the plasmid constructs using lipofectamine transfection reagent (Invitrogen). The stably transfected cells were selected and continuously maintained in medium containing 400 μ g/ml G418.

Immunoprecipitation

Antibodies for immunoprecipitation and the cell lysis buffer were purchased from Cell Signaling Technology (Danvers, MA). Glioma cells, with or without treatment with *Scutellaria* extract and wogonin, were washed with PBS and incubated with the lysis buffer on ice for 5 min. The cell lysates were spun at 20,800 $\times$ g at 4°C for 10 min, the supernatants were isolated and then the protein concentrations determined using Bradford's reagent (Bio-Rad Laboratories, Hercules, CA). Appropriate concentrations of the antibodies were added to the lysate along with 20 μ l of agarose bead slurry (Cell Signaling Technology). After incubation at 4°C for 12 h, the lysates were washed three times and eluted in non-denaturing sample buffer. Following protein estimation, the immunoprecipitate were subjected to western blotting or in vitro kinase assay, as described below.

Western blot analysis

Western blot analysis of the protein samples were performed as described elsewhere [21] with some modifications. Briefly, 20–30 μ g aliquots of total protein were electrophoresed, transferred onto PVDF membrane and probed with specific antibodies against phosphorylated and non-phosphorylated forms of signaling/transcription molecules (Cell signaling Technology, Danvers, MA). Detection of HRP-conjugated Abs was performed using SuperSignal (Pierce, Rockford, IL) and chemiluminescence recorded using an Omega imaging system (UltraLum Inc., Claremont, CA).

In vitro kinase assay

Non-radioactive kinase assay kit for Akt was purchased from Cell Signaling Technology (Danvers, MA). In vitro kinase assay was performed as described earlier [22]. Briefly, a 20 μ l aliquot of the immunoprecipitated Akt was incubated with the substrate protein glycogen synthase kinase-3 (GSK-3) and 1 μ M ATP, in the presence of SocL extract or wogonin, for 30 min. Phosphorylation of GSK-3 was then analyzed by western blotting.

Statistical analysis

A Wilcoxon's log-rank test was performed to determine the statistical difference between various experimental and control groups, using the SPSS package (SPSS Inc, Chicago, IL) [23]. A 'P' value less than 0.05 was considered significant.

Results

Administration of *Scutellaria* extract inhibits tumor growth and enhances survival of glioma-bearing rats

F98 glioma cells (5×10^4 cells) were transplanted intracranially into twelve F344 rats. Six rats were administered with 100 mg/kg SocL extract, while the six control rats received saline only. The median survival of untreated animals was only 14.5 days, while the median survival of SocL treated animals was 24 days, with some animals surviving up to 29 days (Fig. 1a). This indicated a substantial delay in growth of glioma by *Scutellaria*. MRI analysis on day 19 after tumor transplantation showed that the tumor volume in the untreated rat was approximately four times greater than in the rat receiving SocL administration (Fig. 1b).

Similarly, the growth inhibitory effect of SocL extract on gliomas was very pronounced in our subcutaneous tumor model (Fig. 1c). In this model, six F344 rats were injected with 1×10^6 F98 glioma cells in the right flank. Three rats received SocL extracts as described above, while the other three served as controls. By day 29, the three control rats showed signs of excessive tumor burden, whereas the SocL-treated rats did not. Measurement of subcutaneous tumors following excision on day 29 revealed that the size and weight of tumor in SocL treated rats were approximately one-third of that in the control group (Fig. 1c).

Dose-dependent inhibition of F98 glioma cell proliferation by SocL extract and wogonin

SocL extract and wogonin dose-dependently inhibited the proliferation of malignant glioma (F98) cells in vitro. A 50% inhibition of proliferation (IC_{50}) was achieved at 96 h with 125–250 μ g/ml SocL extract (Fig. 2). On the other hand, the IC_{50} value for wogonin was between 50 and 100 μ M concentration (Fig. 2).

Administration of SocL extract inhibits Akt, GSK-3 α/β and NF- κ B phosphorylation in subcutaneously transplanted gliomas

F344 rats were subcutaneously transplanted with 1×10^6 F98 glioma cells. Animals were administered with SocL extract as described earlier. After 29 days of transplantation, animals were euthanized; the tumor was resected and processed for immunohistochemistry. Profuse immunostaining was observed mainly at the periphery of tumor sections obtained from control animals treated with saline, indicating high phosphorylation of Akt, GSK-3 and NF- κ B in the areas with high proliferative activity (Fig. 3, left panel). On the other hand, phosphorylation of Akt, GSK-3 and NF- κ B were significantly inhibited in tumors from animals which received the SocL extract (Fig. 3, right panel). Ki67 staining of the control as well as the SocL treated tumors demonstrated that the phospho-proteins in the tumor borders co-localized with Ki67-positive cells indicating high proliferative activity along the periphery. The cell proliferation index, as measured by Ki67 immunostaining, amounted to 38% in the control tumors (Fig. 3, bottom row, left panel), while in the SocL treated tumors, it was only 6% (Fig. 3, bottom row, right panel), which showed reduced proliferative activity following treatment with SocL extract.

Dose and time-dependent inhibition of Akt phosphorylation by SocL extract and wogonin

F98 glioma cells were cultured in presence of indicated doses of SocL extract or wogonin for various lengths of time (Fig. 4). The cell lysate was prepared and western blot analysis was performed to detect both phosphorylated and non-phosphorylated forms of Akt. Akt was constitutively phosphorylated in F98 glioma, which was significantly inhibited by the SocL extract in a dose- and time dependent manner (Fig. 4a, b, respectively). The flavonoid

wogonin also inhibited the phosphorylation of Akt in a dose-dependent manner (Fig. 4c). These data indicate that SocL extract and wogonin target Akt kinase pathway in F98 glioma cells.

Dose-dependent inhibition of GSK-3 α/β and NF- κ B phosphorylation by SocL extract and wogonin

GSK-3 is a downstream target molecule of Akt. We tested whether SocL extract and wogonin mediated Akt inhibition could lead to inhibition of GSK-3 activity. Similar to Akt, GSK-3 was also constitutively phosphorylated in F98 gliomas, and was significantly inhibited by both SocL extract and wogonin (Fig. 5a). Cross-talk between Akt and NF- κ B pathways has been reported in tumor cells. Western blot analysis also confirmed constitutive phosphorylation of NF- κ B in glioma cells, which was significantly inhibited by SocL extract and wogonin in a dose-dependent manner (Fig. 5b).

Further analysis of major signaling pathways regulating the proliferation of glioma cells

F98 glioma cells were cultured with pharmacologic inhibitors specific to signaling molecules and cell viability was analyzed as described above. Both the signaling inhibitors, LY294002 (PI3K), and IMD-0354 (NF- κ B) dose-dependently inhibited the proliferation of glioma cells (Fig. 6a). Inhibitor of NF- κ B however showed greater inhibition of proliferation compared to that of Akt (Fig. 6a).

Treatment of F98 cells with signaling inhibitors in the presence of SocL extract (250 μ g/ml) resulted in greater inhibition of proliferation compared to treatment with either agent alone (Fig. 6b). On the other hand, inhibition of proliferation by co-treatment with wogonin (100 μ M) and PI3K-inhibitor was only modestly higher compared to the inhibition afforded by either agent alone (Fig. 6b).

In the next experiment, F98 cells, transfected with constitutively active (CA) and dominant negative (DN) AKT plasmids were cultured with SocL extract (250 μ g/ml) and wogonin (100 μ M). F98 cells transfected with dominant negative AKT (F98/AKT-DN) showed significantly reduced proliferation compared to F98/Control or F98/AKT-CA cells (Fig. 6c). However, treatment with SocL extract or wogonin similarly inhibited the proliferation of all cell types irrespective of the associated plasmid transfection (Fig. 6c).

Direct inhibition of Akt kinase activity by SocL extract and wogonin

Akt was immunoprecipitated from F98 cell lysate and its kinase activity was determined by measuring the phosphorylation of the downstream mediator GSK-3 in the presence of indicated doses of SocL extract and wogonin. SocL extract, at a dose 25 μ g/ml or higher, completely inhibited the phosphorylation of GSK-3 by Akt. The flavonoid wogonin also showed dose-dependent inhibition of Akt kinase activity (Fig. 7).

Discussion

In this study, we observed that oral administration of *Scutellaria* extract could significantly delay the in vivo growth of gliomas in both intracranial and subcutaneous tumor models. Administration of SocL extract also prolonged the survival of animals in the intracranial tumor model (median survival 24 days in SocL versus 14.5 days in the control group). The inhibition of tumor growth was relatively less pronounced in the orthotopic (intracranial) model compared to the subcutaneous one. This was probably because the tumor grew very aggressively inside the cranium due to lack of immune response from the host (since the brain is an immune-privileged organ). Moreover, it may also be because of lesser bioavailability of the flavonoids in the brain compared to the periphery, as has been reported

in some studies [24, 25]. A separate study is underway to analyze bioavailability of the flavonoids in various organs, including the brain at different time points following administration of the extract and isolated flavonoids. However, considering the extremely aggressive nature of the tumor in our orthotopic model, even a modest enhancement of survival by *Scutellaria* flavonoids is very encouraging. These results demonstrate that treatment with *Scutellaria* does significantly slow down in vivo growth of malignant gliomas, at least during the initial stages, implying that an adjuvant therapy with *Scutellaria* flavonoids may have potential therapeutic benefits especially in the immediate post-surgical phase when the tumor burden is low.

In our earlier study, SocL extract was shown to contain the flavonoid wogonin [12]. Therefore, wogonin was included in current studies to determine the mechanism of anti-tumor activity of *Scutellaria*. We observed a dose- and time-dependent inhibition of proliferation of F98 glioma cells by the SocL extract and the constituent flavonoid wogonin in vitro. The doses of SocL extract and wogonin required for approximately 50% inhibition of proliferation (IC_{50}) of F98 cells was lower than the IC_{50} doses for U87-MG cell line reported earlier [12]. F98 cells are more aggressive with a much shorter in vitro doubling time (<24 h) compared to U87-MG cells (doubling time >48 h). Moreover, *Scutellaria* extracts and constituent flavonoids had inhibited the proliferation of malignant tumor cells without significantly affecting the normal or non-malignant cells [12]. Together, these data show that *Scutellaria* flavonoids could inhibit proliferation of gliomas by specifically targeting molecules involved in regulation of malignant phenotype. Moreover, it indicates that cells with higher proliferative activity are potentially more susceptible to the cytolytic/cytostatic activity of *Scutellaria* flavonoids.

The immunohistochemistry data demonstrated that the delayed growth of subcutaneously transplanted F98 glioma in animals administered with SocL extract was associated with an inhibition of phosphorylation of key signaling molecules Akt, GSK-3 α/β and NF- κ B. The signaling activity co-localized with intense staining for the proliferation marker Ki67 in the border of the control tumor, suggesting high proliferative activity along the periphery. Moreover, reduced phosphorylation of the signaling molecules in SocL-treated tumors correlated with reduced proliferative index, indicating involvement of Akt/GSK-3/NF- κ B signaling during inhibition of tumor growth by *Scutellaria* flavonoids. Our in vitro studies also demonstrated dose- and time-dependent inhibition of phosphorylation of Akt by SocL extract and wogonin. Similarly, SocL extract as well as wogonin dose-dependently inhibited the phosphorylation GSK-3 α/β . Akt, also known as protein kinase B (PKB), was originally discovered as an oncogene transduced by the acute transforming retrovirus (Akt-8), which was isolated from AKR thymoma [26]. The PI3K/Akt signaling pathway is arguably the major pathway that regulates tumor cell apoptosis, cell proliferation, invasion and angiogenesis in malignant tumors, including gliomas [27–30]. The downstream signaling molecule GSK-3 is also constitutively active in gliomas and can play an important role in mediating tumor-promoting activities of Akt in malignant tumors such as infiltrating gliomas [31, 32]. Studies from other groups have shown that wogonin can inhibit the growth of various breast cancer cell lines in vitro as well as in mouse xenograft models. The growth inhibition was associated with a down-regulation of Akt-mediated canonical Wnt signaling activity [8]. Another study has demonstrated that inhibition of GSK-3 could induce glioma cell death through c-Myc, NF- κ B, and glucose regulation [33]. The current study demonstrated that NF- κ B was indeed constitutively active in F98 glioma cells, and was significantly inhibited by SocL extract and wogonin in a dose-dependent manner. Overall, these data suggest that Akt/GSK-3/NF- κ B signaling pathway constitute a major target for anti-tumor activity of *Scutellaria* flavonoids.

The *in vitro* studies also showed significant dose-dependent inhibition of F98 cell proliferation by specific inhibitors of PI3K as well as NF- κ B, confirming important roles for these signaling molecules in glioma survival and proliferation. Inhibition of proliferation afforded by co-treatment with SocL extract and specific inhibitors of PI3K or NF- κ B was significantly higher compared to the inhibition afforded by either treatment alone. This suggests that flavonoids in SocL extract may target molecules in addition to those in the PI3K/Akt and NF- κ B signaling pathways. On the other hand, inhibition of proliferation by co-treatment with wogonin and PI3K-inhibitor was only modestly higher than the one afforded by the signaling inhibitor alone, indicating once again that inhibition of Akt activity may constitute major anti-tumor mechanism of the flavonoid wogonin.

In order to further explore the role of Akt during inhibition of F98 cell proliferation by *Scutellaria* flavonoids, we used F98 cells transfected with constitutively active (CA) and dominant negative (DN) Akt plasmids. As expected, F98 cells transfected with dominant negative AKT (F98/AKT-DN) showed significantly reduced proliferation compared to the F98/control or F98/AKT-CA cells, further confirming the critical role of Akt during the growth of gliomas. However, expression of constitutively active form of Akt did not significantly reverse the inhibition of proliferation afforded by SocL extract or wogonin. This indicated that *Scutellaria* flavonoids inhibit Akt signaling either by directly binding to the molecule or by acting on downstream signaling molecules in the Akt pathway. Results of the *in vitro* Akt kinase assay revealed that *Scutellaria* flavonoids could indeed directly bind to Akt and inhibit phosphorylation of downstream signaling molecule(s). As PI3K/Akt pathway has been shown to regulate NF- κ B activity in malignant tumors, including gliomas [33–35], inhibition of NF- κ B by *Scutellaria* flavonoids could be a result of their inhibition of Akt kinase activity, at least partially. Since Akt is known to regulate several cellular processes via modulation of various signaling pathways, *it is plausible that direct inhibition of Akt kinase activity by Scutellaria flavonoids may play a major role in their anti-cancer activity*. However, the present study does not rule out the involvement of other Akt-independent mechanisms on tumor inhibition by these flavonoids. Extracts of *Scutellaria* sp. or select isolated flavonoids have also been shown to have varying effects on other signal transduction pathways such as cAMP/PKA and PKC, depending on the cell/tissue type and the experimental design. Studies are in progress in our laboratories to further elucidate the molecular mechanisms of anti-tumor activity of *Scutellaria* flavonoids.

In summary, *Scutellaria* flavonoids substantially delay the *in vivo* growth of malignant glioma and the molecular mechanisms involve direct and indirect inhibition of Akt/NF- κ B signaling pathway. *To the best of our knowledge, this report provides the first in vivo evidence and mechanistic support for anti-glioma activity of Scutellaria flavonoids*. This study has potential implications in the development of adjuvant therapeutic strategies using the flavonoids in conjunction with established chemoradiation regimens.

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Abbreviations

CA	Constitutively active
DN	Dominant negative (mutant)

GSK	Glycogen synthase kinase
NF-κB	Nuclear factor-κB
SocL	<i>Scutellaria ocmulgee</i> leaf (extract)

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Figure 1.

Scutellaria inhibits tumor growth and enhances survival of glioma-bearing rats. **a** F344 rats were *intracranially* transplanted with 5×10^4 F98 glioma cells. After 5 days, one group was administered with SocL extract (100 mg/kg), 5 days a week for 2 weeks. Rats were euthanized when they showed signs of excessive tumor burden, as described. **b** Nineteen days after tumor transplantation, gadolinium-enhanced MRI was performed and tumor volume measured as described in the “Materials and methods” section. **c** Another group of F344 rats were *subcutaneously* transplanted with 1×10^6 F98 glioma cells on the right flank.

SocL administration was performed as described above. Rats were euthanized on day 29 and the subcutaneous tumor was excised and measured

Figure 2.

Dose-dependent inhibition of F98 glioma cell proliferation by SocL extract and wogonin. F98 glioma cells were cultured 96 h in the presence of indicated doses of SocL extract and wogonin. Cell proliferation/viability was analyzed using Cell-glo viability assay kit (Promega) as described in the “Materials and methods” section. Experiments were performed in triplicates and were repeated three times. Data are expressed as % proliferation compared to medium control

Figure 3.

Inhibition of Akt, GSK-3 α/β and NF- κ B phosphorylation, and reduced Ki67 staining in subcutaneously transplanted gliomas following treatment with SocL. F344 rats were *subcutaneously* transplanted with 1×10^6 F98 glioma cells on the right flank. SocL administration was performed as described. Rats were euthanized on day 29; the tumor was resected and processed for immunohistochemistry as described in the “Materials and methods” section. The areas of tumor margin, where most staining is observed, are marked with *black arrows*

Figure 4.

Dose- and time-dependent inhibition of Akt phosphorylation by SocL extract and wogonin. F98 glioma cells were cultured for **a** 12 h with various doses of SocL extract, **b** with 250 $\mu\text{g/ml}$ SocL extract for indicated time, and **c** 12 h with various doses of wogonin. The cells were lysed, electrophoresed and subjected to western blotting. Greyscale density was analyzed using ImageJ software and values for phosphorylated Akt (Ser473) were normalized against those for total Akt. Data are representative of more than two experiments performed with similar results. "*" Denotes $P < 0.05$ versus untreated (medium) control

Figure 5.

Dose-dependent inhibition of GSK-3 and NF- κB activities by SocL extract and wogonin. F98 glioma cells were cultured for 12 h with various doses of SocL extract and Wogonin. The cells were lysed, electrophoresed and subjected to western blotting. Greyscale density was analyzed using ImageJ software and values for **a** phosphorylated GSK-3 α/β (Ser21/9) and **b** phosphorylated NF- κB p65 (Ser536) were normalized against those for **a** total GSK and **b** β -actin, respectively. Data are representative of more than two experiments performed with similar results. '*' Denotes $P < 0.05$ versus untreated (medium) control

Figure 6.

Role of Akt and NF-κB during inhibition of tumor cell proliferation by SocL extract and wogonin. **a, b** F98 glioma cells were cultured with indicated doses of specific signaling inhibitors at indicated doses in the presence of SocL extract (250 μg/ml) and wogonin (100 μM); **c** F98/control, F98/AKT-CA and F98/AKT-DN cells were cultured with SocL extract (250 μg/ml) and wogonin (100 μM). After 96 h, cell viability/proliferation was analyzed as described in the “Materials and methods” section. Experiments were performed in triplicates and were repeated twice. Data are expressed as % proliferation compared to medium control. ‘*’ Denotes $P < 0.05$ versus untreated (medium) control; ‘#’ Denotes $P < 0.05$ versus respective signaling inhibitor control (without SocL or wogonin); ‘^a’ Denotes $P < 0.05$ versus respective cell/plasmid types without SocL or wogonin

Figure 7.

Direct inhibition of Akt kinase activity by SocL extract and wogonin. Akt was immunoprecipitated from F98 glioma cell lysate with anti-Akt antibody, as described. For in vitro Akt kinase assay, 10 μg aliquots of the Akt protein was incubated with 10 μg fusion GSK substrate and 1 μM ATP, in presence of indicated doses of SocL extract and wogonin for 30 min. Phosphorylated GSK was analyzed by western blotting, as described. Data are representative of two experiments performed with similar results