Possible mechanism of growth inhibition by *Scutellaria baicalensis* in an estrogen-responsive mouse tumor cell line

TERUKO MURASHIMA¹,²,³, HIROSHI KATAYAMA¹,², KIMURA SHOJIRO³ and YASUKO NISHIZAWA¹,²

¹Department of Pathology, Research Institute, Osaka Medical Center for Cancer and Cardiovascular Diseases, 1-3-3 Nakamichi, Higashinari 537-8511; ²Laboratory of Clinical and Experimental Pathophysiology, Graduate School of Pharmaceutical Science, Osaka University, Suita 565-0871; ³Department of Radiochemical Laboratory, Osaka University of Pharmaceutical Sciences, Takatsuki 569-1094, Osaka, Japan

Received December 15, 2010; Accepted February 2, 2011

DOI: 10.3892/or.2011.1196

**Abstract.** We have studied the effects of Saiboku-to, a traditional Chinese medicine having suppressive activities for leukotriene production and release, on the proliferation of the estrogen-responsive mouse Leydig tumor cell line B-1F. In our previous reports, it is shown that Saiboku-to promotes, but *Scutellaria baicalensis*, one of the components (herbs) of Saiboku-to, significantly inhibits the proliferation of B-1F cells in vitro and in vivo, and induces DNA fragmentation and morphological changes such as nuclear aggregation and fragmentation. In this study, we examined telomerase activity, cell cycle, polyunsaturated fatty acid metabolism and expression of nuclear factor κB (NF-κB) in order to determine the mechanism of growth inhibition in B-1F cells treated with *Scutellaria baicalensis*. Telomerase activity was decreased in a dose-dependent manner in treated B-1F cells. Cellular populations in the sub-G0/G1 and G2/M phases were increased, but those in M phase had no change. Although cyclin D1 mRNA was highly expressed in the presence of estradiol (E2), cyclin A and E mRNA levels did not significantly change. When B-1F cells were treated with *Scutellaria baicalensis*, expression of cyclin D1 was suppressed and that of p21 was inversely increased. Moreover, *Scutellaria baicalensis* influenced arachidonic and linoleic acid metabolism, and increased production of 13(S)-HODE. In the presence of E2 *Scutellaria baicalensis* decreased expression of NF-κB p65 to 0.71-fold in B-1F cells. These results show that *Scutellaria baicalensis* might induce cell cycle arrest at G1 phase and apoptosis via inhibition of telomerase activity, changes of enzymatic activities in polyunsaturated fatty acid metabolism and suppression of NF-κB.

**Introduction**

Traditional Chinese medicines have been used for treatments of acute and chronic diseases. A traditional Chinese medicine is composed of many components (herbs) and shows various pharmacological actions, mechanisms of which are not fully understood. Also many herbs contained flavonoids which are known to have anti-inflammatory, antioxidant, antibacterial, antiviral and antitumor activities (1-5). Therefore, anti-carcinogenic action and induction of apoptosis by herbs and their constituents are interesting topics of research. In the studies on the effects of Saiboku-to on cell proliferation, we have found that *Scutellaria baicalensis*, one component of Saiboku-to, suppresses the proliferation of estrogen-responsive mouse tumor cell line B-1F. *Scutellaria baicalensis* also contains many flavonoids (6). Recent studies suggest that *Scutellaria baicalensis* effectively arrests cell cycle and induces apoptosis of tumor cell lines (3,7,8).

Telomerase is a ribonucleoprotein (RNP) complex and is composed of telomerase reverse transcriptase (TERT), telomerase RNA component (TERC) and regulating factor of telomerase enzyme activity. Telomerase protects cells from apoptosis by the maintenance of genomic integrity following the stabilization of telomeres (9). Inhibition of telomerase activity has no significant influence on normal cells, but it induces cell death such as apoptosis or senescence in tumor cells.

Arachidonic and linoleic acid metabolites play important roles in carcinogenesis (10,11). When the proliferation of estrogen-responsive B-1F cells is promoted by estradiol (E2), 5-LOX activity is changed and leukotriene (LT) D4 induces apoptosis of B-1F cells (3-9). 13(S)-HODE and 15(S)-HETE (products of 15-LOX-1 and -2, respectively) is known to promote differentiation, growth inhibition and induction of apoptosis. On the other hand, 12(S), 12(R)-HETE, 8(S)-HETE, LT B4 and prostaglandin (PG) E2 (products of 12(S)-LOX, 12(R)-LOX, 8-LOX, 5-LOX and COX-2, respectively) stimulate proliferation, angiogenesis and metastasis.

Nuclear factor κB (NF-κB) forms a complex with an inhibitor of κB (IκB). IκB bound to NF-κB dissociates from the complex by phosphorylation, and NF-κB stimulates gene transcription by binding to DNA. It has been reported that some constituents of traditional Chinese medicines inhibit NF-κB and COX-2.
In cell growth, was dissolved in ethanol to 1 mg/ml and its constituents. day (day 0), the medium was changed to HMB or HMBe medium 2.5x10^6 cells/100-mm dish. 

Cell cycle analysis. B-1F cells were seeded in four well chamber slides (Falcon) at a density of 0.5-1x10^5 cells/well in HMB medium. the following day (day 0), the medium was changed to fresh HMB or HMBe medium with or without 25 µg/ml of Scutellaria baicalensis. on day 2 or 3, floating and attached cells were collected together, resuspended in PBS and fixed overnight in 70% ethanol at 4°C. The cells were washed with PBS, resuspended in 40 µl of the buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, pH 7.0) and incubated for 30 min. After washed, the cells in 100 µl of PBS were incubated with RNase inhibitor at 37°C for 30 min, washed and resuspended in 1 ml of PBS containing 50 µl/ml of propidium iodide, followed by incubation in the dark for 30 min at room temperature. Thereafter, cell cycle was analyzed by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

We examined expression of cyclin and CDK inhibitors in B-1F cells treated with or without 50 µg/ml of Scutellaria baicalensis. The method for RNA isolation was the same as previously published (14). RT-PCR was performed as directed in the manual from the supplier (Ready-To-Go™ RT-PCR Beads). First strand cDNA was synthesized at 42°C for 30 min using 1 µg of total RNA and specific primers. PCR conditions for cyclin A were 95°C for 30 sec, 56°C for 1 min and 72°C for 2 min for 30 cycles, for cyclin D1 were 94°C for 1 min, 60°C for 1 min and 72°C for 2 min for 40 cycles, for cyclin E were 94°C for 45 sec, 58°C for 45 sec and 72°C for 90 sec for 30 cycles, for p21 were 94°C for 45 sec, 62°C for 45 sec and 72°C for 1 min for 27 cycles and for GAPDH were 94°C for 45 sec, 55°C for 45 sec and 72°C for 2 min for 35 cycles. After PCR amplification, all products were incubated for 10 min at 72°C. Amplified products were separated on 2% agarose gel containing ethidium bromide and visualized under UV light. Mouse cyclin A primers are 5'-CCTCTCGAC ATTAGTCTCTGTGGGG-3' (forward) and 5'-GGCCGGTATGGCGACCTCACGGCATTCC-3' (reverse), and RT-PCR product is 1277 bp. Mouse cyclin D1 primers are 5'-CGCTTCTGGTTCTTACTTCA-3' (forward) and 5'-AAC TTCTCGGCAGTCAGGGGA-3' (reverse), and RT-PCR product is 250 bp. Mouse cyclin E primers are 5'-CTGGCTGTCAGGGAGCTTC-3' (forward) and 5'-CTTGGCTGGTGGCTTGTC-3' (reverse), and RT-PCR product is 386 bp. Mouse p21 primers are 5'-AGCTGAGACTTGGG-3' (forward) and 5'-AAAGTCCACGGAGCTTC-3' (reverse), and RT-PCR product is 228 bp. Mouse GAPDH primers are 5'-ATCTTCAAGGAGAAGCCAAA-3' (forward) and 5'-CTGACATGCTTCATG-3' (reverse), and RT-PCR product is 898 bp.

Assay of polyunsaturated acid metabolites and enzymatic activities. Assays of arachidonic acid metabolites and enzymatic activities were carried out as previously reported (15,16). The level of 13(S)-HODE was measured by 13(S)-HODE enzyme immunoassay kit. B-1F cells were seeded at a density of 0.5-1x10^6 cells/60-mm dish in HMB medium. the following day (day 0), the medium was changed to HMB or HMBe medium with or without 50 µg/ml of Scutellaria baicalensis. On day 2, the medium was replaced with fresh medium containing the same compounds and the cells were incubated for 7 h. After incubation, the cells and medium were collected. The cells were washed with PBS, scraped into lysis buffer (10 mM Tris-HCl, pH 7.4, 400 mM NaCl, 1 mM EDTA, 1.0% SDS) and incubated for 15 min at 4°C. Cell lysates were centrifuged at 15000 x g for 15 min at 4°C. The
2 min and at 95°C for 10 min, 40 cycles at 95°C for 15 sec and at 60°C for 1 min, and 1 cycle at 95°C for 15 sec, at 60°C for 1 min and at 95°C for 15 sec. The data were analyzed using ABI 7500 software.

Analysis of estrogen binding sites. The whole cell binding assay was used to study the binding parameters of estrogen binding sites in B-1F cells as previously published (1). Briefly, B-1F cells (2x10⁶ cells) were incubated with 5 nM [2,4,6,7-3H(N)] estradiol (1H[E2]) in the presence of various concentrations of major constituents of Scutellaria baicalensis for 60 min at 37°C. The cells washed with ice-cold HMB medium were transferred to scintillation vials for the determination of the radioactivity specifically bound to the cells.

Materials. The materials were purchased as described previously (13,14). TRAPEze telomerase detection kit was purchased from Chemicon International (CA, USA); 13(S)-HODe enzyme immunoassay kit from Assay Designs, Inc. (MI, USA); Mouse NF-κB factor, p65, Primer set kit and real-time PCR kit for mouse β-actin gene expression from Maxim Biotech. Inc. (San Francisco, USA); Power SYBR-Green PCR master mix from Applied Biosystems (UK); propidium iodide and powder forms of constituents of Scutellaria baicalensis from WAKO (Osaka, Japan).

Statistics. The data were expressed as the mean ± standard error (SE). Data were analyzed by ANOVA with Dunnett’s or Bonferroni’s corrections for multiple comparisons, as appropriate. Calculated p-values of <0.05 were considered to be significant.

Results

Suppression of telomerase activity in B-1F cells by Scutellaria baicalensis. Effects of Scutellaria baicalensis on telomerase activity are shown in Fig. 1. Although we examined telomerase activity on days 2 and 3, only results on day 2 are shown, because similar results were obtained on both days. When B-1F cells were treated with Scutellaria baicalensis, telomerase activity was inhibited under both conditions with and without E2.

Cell cycle arrest of B-1F cells by Scutellaria baicalensis. After the treatment with 25 or 50 µg/ml Scutellaria baicalensis, cell cycle was analyzed by flow cytometry on day 1. The result is shown in Fig. 2A. Scutellaria baicalensis increased cellular populations in sub-G0/G1 phase and G2/M phase. As shown in Fig. 2B, cyclin A and E mRNA did not significantly change with the treatment. In contrast, cyclin D1 mRNA was expressed in the presence of E2, but when B-1F cells were treated with Scutellaria baicalensis, it was suppressed. Expression of p21, CDK inhibitor, was increased. These results show that Scutellaria baicalensis might arrest cell cycle at G1 phase by changing the expression of cyclin D1 and p21.

Effects of Scutellaria baicalensis on polyunsaturated acid metabolism in B-1F cells. B-1F cell proliferation stimulated by E2 is concurrent with decreased 5-LOX activity and then decreased production of LT. At first, we examined effects of

---

**Figure 1.** Telomerase activity in B-1F cells. B-1F cells were seeded onto 100-mm dishes (2.5x10⁴ cells/dish) and treated with or without 25 µg/ml of Scutellaria baicalensis in the presence (E2) or absence (none) of E2. Telomerase activity was examined using TRAPEze telomerase detection kit (S7700). Amplified products were subjected to electrophoresis on 2% agarose gel containing ethidium bromide and visualized under UV light. Lanes with concentration of Scutellaria baicalensis + [ ] are heat-inactivated samples. The other three separate trials gave similar results.
Scutellaria baicalensis on arachidonic acid metabolism in B-1F cells. The results are shown in Fig. 3A and B. Although, activities of 5-LOX and 12-LOX decreased, those of COX-1 and COX-2 increased. 15-LOX activity did not change. Activities of COX-1 and COX-2 increased ~2-fold and 12-LOX activity decreased to >20% in B-1F cells treated with Scutellaria baicalensis. In the treated cells and their conditioned medium, 5-HETE and 12-HETE decreased. In contrast PGD2 and PGE2 increased, and 15-HETE did not significantly change.

Next, we examined 13(S)-HODE production (final product from linoleic acid by 15-LOX-1), in B-1F cells using enzyme immunoassay kit. As shown in Fig. 4, in both cellular fraction and the medium 13(S)-HODE increased (2-fold increase), when the cells were treated with Scutellaria baicalensis.

Expression of NF-κB p65 in B-1F cells treated with Scutellaria baicalensis. NF-κB is a well-known transcription factor to play important roles in immune response and carcinogenesis.
We examined the expression of NF-κB p65 mRNA in B-1F cells treated with *Scutellaria baicalensis* (Fig. 5). *Scutellaria baicalensis* decreased NF-κB p65 expression to 0.71-fold in the presence of E2.

**Effects of major constituents of Scutellaria baicalensis on B-1F cells.** Effects of baicalin, baicalein and wogonin, major constituents of *Scutellaria baicalensis*, on the cell proliferation are shown in Fig. 6. Wogonin effectively inhibited B-1F cell proliferation in the absence of E2. Baicalein showed two-phase effects. Low dose (0.1-1.0 µg/ml) of baicalein increased the proliferation, but higher dose of baicalein inhibited. The most potent growth inhibition was observed in B-1F cells treated with wogonin.

Each constituent of *Scutellaria baicalensis* did not compete with E2 for estrogen receptor-binding in B-1F cells (Fig. 7), showing that the above constituents do not act directly via estrogen binding sites in B-1F cells, as well as Saiboku-to
and *Scutellaria baicalensis*. When the expression of cleaved caspase-3, using immunohistochemistry, was examined in B-1F cells treated with 2.5 µg/ml of each constituent (Fig. 8), it was increased by the above constituents (the most prominent in wogonin-treated cells). These results indicate that some of constituents induce apoptosis in B-1F cells.

**Discussion**

We have demonstrated that *Scutellaria baicalensis* induces cell cycle arrest and apoptosis in B-1F cells. Telomerase is necessary to maintain cell proliferation and is important for tumorigenesis (9,20,21). Its inhibition leads to suppressive
cell proliferation, damage of chromosome and apoptosis by up-regulation of p16, p53, p73 and p63 in addition to FasL, Fas, caspase-8, -7 and -3 (22). Further, a cyclin-dependent kinase inhibitor p21 and p53 are closely linked to cell cycle and up-regulation of p21 is caused by inhibition of telomerase activity (22). In B-1F cells treated with Scutellaria baicalensis telomerase activity was suppressed and cell cycle was arrested at G1 phase with up-regulation of p21 and down-regulation of cyclin D1. One possible explanation for induction of apoptosis and cell cycle arrest in treated B-1F cells is inhibition of telomerase activity.

In order to understand the relationship between poly-unsaturated fatty acid metabolism and carcinogenesis, we have investigated mechanism of estrogen-responsive B-1F cell proliferation and have reported that B-1F cell growth stimulated by E2 is concurrent with decreased LT production as a result of decreased 5-LOX activity (14-16,23-26). Treatment with Scutellaria baicalensis inhibited some enzymatic activities in arachidonic acid metabolism (5-LOX and 12-LOX). Baicalein, one of constituents in Scutellaria baicalensis, is known as a 12-LOX inhibitor. On the other hand COX-1 and COX-2 activities increased 2-fold. Among arachidonic acid metabolites production of 5-HETE and 12-HETE was inhibited, but that of PGD2 and PGE2 was increased. The production of an arachidonic acid metabolite 15-LOX, a product of 15-LOX-2, did not change, but a linoleic acid metabolite 13(S)-HODE, a product of 15-LOX-1, was increased by the treatment. Although 15-LOX activity examined by incubation with arachidonic acid showed no significant change, and 15-LOX-1 activity was not directly measured in this report, increment of 15-LOX-1 activity could be estimated from increased 13(S)-HODE production in B-1F cells. Since 13(S)-HODE is known to promote differentiation, growth inhibition and apoptosis induction, 13(S)-HODE also plays an important role in the inhibition of proliferation of B-1F cells treated with Scutellaria baicalensis.

NF-κB forms a complex with an inhibitor of κB (IκB) and is located in cytoplasm when it is under inactive conditions. IκB bound to NF-κB dissociates from the complex by phosphorylation following influences of drug, oxidative stress and so on. Then NF-κB moves into nucleus and stimulates gene transcription by binding to DNA. NF-κB regulates expression of many genes (for example, IκB-α, bcl-2, IL-6 and cyclin D1). Suppression of NF-κB activation is known as a key to induction of apoptosis in many cell lines (3,12,17,27,28). In our experiments Scutellaria baicalensis slightly inhibited expression of NF-κB p65 in B-1F cells in the presence of E2. Moreover, Scutellaria baicalensis contains many flavonoids and its major constituents are baicalin, baicalein, wogonin, neowogonin and skullcapflavone (6). Baicalein and wogonin inhibited B-1F cell proliferation and induced apoptosis. Scutellaria baicalensis and baicalein have been reported to induce apoptosis by blocking IκB-α phosphorylation (3). Although NF-κB expression alone was examined in this study, similar mechanism probably work in B-1F cells. In this study Scutellaria baicalensis increased NF-κB expression in the absence of E2, by a mechanism which remains unknown.

In conclusion, we have shown that Scutellaria baicalensis induces cell cycle arrest and apoptosis via inhibition of telomerase activity, down-regulation of NF-κB, and changes of enzymatic activities in polyunsaturated fatty acid metabolism. Scutellaria baicalensis or its constituents might be candidates for the treatment of cancer. For example, they possibly target the restoration of 15-LOX-1 activity for the treatment of colorectal cancer. Shureiqi et al have reported that the loss of 15-LOX-1 expression is the possible dominant change in LOX metabolism during colorectal tumorigenesis (29). Further study is necessary to make clear the roles of Scutellaria baicalensis and its constituents in cancer therapy, and also those of other constituents of Saiboku-to such as Saikosaponin.
Acknowledgments

The technical assistance of Mrs. Y. Saeki and Y. Tanigaki is gratefully acknowledged.

References


