The Effect of Scutellaria Baicalensis on the Signaling Network in Hepatocellular Carcinoma Cells

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Scutellaria baicalensis is an anti-inflammatory and antineoplastic Chinese herbal therapy. We have previously shown that S. baicalensis can inhibit hepatocellular carcinoma (HCC) cell growth in vitro. In this study, we sought to determine the effect of S. baicalensis on the cell signaling network using our newly developed Pathway Array technology, which screens cell signaling pathways involved in cell cycle regulation. The HCC cell line (HepG2) was treated with S. baicalensis extract in vitro. The effect on the cell cycle was analyzed by flow cytometry, and the expression of various signaling proteins was assayed with Pathway Array. Our results indicate that S. baicalensis exerts a strong growth inhibition of the HepG2 cells via G2/M phase arrest. The Pathway Array analysis of 56 proteins revealed a total of 14 differentially expressed proteins or phosphorylations after treatment. Of these, 9 showed a dose-dependent decrease (p53, ETS1, Cdc25B, p63, EGFR, ERK1/2, XIAP, HIF-2α, and Cdc25C) whereas one demonstrated a dose-dependent increase (Cyclin E) after treatment with 200 μg/ml of S. baicalensis. Using computer simulation software, we identified additional hubs in the signaling network activated by S. baicalensis. These results indicate that S. baicalensis exerts a broad effect on cell signaling networks leading to a collective inhibition of cell proliferation.

INTRODUCTION

According to the report of the International Agency for Research on Cancer, approximately 442,000 men and 417,000 women were afflicted with liver cancer worldwide in 2002. The most prevalent type of liver cancer is hepatocellular carcinoma (HCC), which is the fifth most common cancer in the world and the third most common cause of death by cancer (1). In some areas of Asia and the Middle East, HCC ranks as the most common cause of cancer-related death (2). In the United States, it was expected there would be an estimated 21,370 new cases and 18,410 deaths in 2008 (3). As of now, treatment options are limited for patients diagnosed with HCC. Conventional resection is only possible if the neoplasm is small and isolated within one lobe of the liver. Radiation therapy is not recommended because it easily damages the sensitive liver tissue, and chemotherapy is only effective if applied regionally. Therefore, a great deal of research is currently devoted to chemoprevention and detection of early stage liver cancers (4).

The exact mechanism and chronological sequence of HCC pathogenesis is not fully understood (5). However, histopathologically, it is clear that the initial alterations in hepatocytes occur in a cirrhotic liver in response to various insults such as viral hepatitis. The phenotypically altered hepatocytes subsequently develop into dysplastic hepatocytes that form foci and nodules, which eventually become a larger mass. Genomically, it is also clear that DNA damage and instability in the hepatocytes (i.e., aberrant DNA methylation, microsatellite instability,
allelic deletions/loss of heterozygosity) are also increased with the histological progression of HCC. At the molecular level, important signaling pathways (e.g., RB, PT53, BRCA, β-catenin, WT1) are also dysregulated sequentially in cirrhosis, dysplasia, and HCC (5,6). Therefore, the development of target-specific treatment of HCC based on the understanding of the molecular alteration of complex signaling pathways has become very attractive but extremely challenging (4).

*Scutellaria baicalensis* Georgi (East Asian skullcap plant, Huang Qin) is a traditional Chinese herb that has been used alone or in combination with other herbs [Xiao Chai Hu Tang (Chinese) or Shosaiko-to (Japanese)] for HCC prevention and treatment (7). Our studies and others have shown that *S. baicalensis* can inhibit the growth of hepatocellular cells in vitro (8,9). In a prospective randomized trial in which oral Shosai-koto (containing 3 gm of *S. baicalensis*) was administered daily to patients with liver cirrhosis for 5 yr, the incidence of hepatocellular carcinoma was lower than those without treatment in the control group (*P* = 0.071) (7). For HBsAg-negative patients, the difference was significant (*P* = 0.024), suggesting that *Scutellaria baicalensis* may be effective in the chemoprevention of hepatocellular carcinoma among HBsAg negative patients with cirrhosis. Given the promising results of these studies, it is important to understand the molecular mechanisms for *S. baicalensis*-related anticancer activities for the planning of future clinical trials.

*S. baicalensis* contains several major flavonoids, including baicalin, baicalein, wogonoside, wogonin, oroxylin A 7-O-glucuronopyranoside (or oxypurinol A 7-O-glucUA), orxyolin A, and more than 40 other components in trace amounts (10). The molecular mechanism of actions of *S. baicalensis* remains unclear, but some studies have shown that it downregulates cyclin D expression (11), suppresses MAPK activity (12), and inhibits cyclooxygenase-2 (COX-2) (13). Furthermore, it has been shown that different components of *S. baicalensis* have different biological effects, indicating that each component acts on different molecular pathways in a cell, causing collective inhibition of cell growth (13). For example, baicalin inhibits the androgen receptor signaling pathway (14), and baicalin enhanced the expression of p27kip1 (15). Oxyrinx A inhibited the expression of LPS-induced iNOS and COX-2 proteins (16). Therefore, an innovative technology is required to understand the global effect of the many components of *S. baicalensis* on the complex cellular signaling network.

Recently, we developed a Pathway Array technology, which is an innovative proteomic assay that allows global screening of changes in protein expression and phosphorylation. The focus of Pathway Array is to evaluate the activation of various components of the signaling network related to oncogenesis. We focused on the proteins and phosphorylation sites altered in cancer cells and functionally linked to proliferation, apoptosis, cell cycle regulation, DNA repair, signaling, and transcription activity. In this study, we applied Pathway Array technology to understand the effect of *S. baicalensis* on the cell signaling network in hepatocellular carcinoma cells (HepG2) by comparing protein expression before and after treatment with *S. baicalensis*.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Drugs**

The powder form of *Scutellaria baicalensis* extract was obtained from E-Fang Pharmaceutical Company (Guangdong, China), which was prepared by boiling the dried root of the plant in water followed by a spray-drying treatment. The extract was dissolved in culture medium to 20 mg/ml, vortexed at room temperature for 1 min, and incubated at 37°C for 1 h. This solution was centrifuged at 5,000 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 20 to 800 µg/ml of *Scutellaria baicalensis* extract. For consistency, the same lot was used throughout the entire study, and the study was conducted before the expiration date for this lot.

**Cell Culture**

HepG2 cell line (American Type Culture Collection, Manassas, VA) was derived from liver hepatocellular carcinoma. The cells were incubated at 37°C with a 5% CO₂ atmosphere in RPMI-1640 medium containing 1% penicillin/streptomycin, and 10% fetal bovine serum.

**Cell Growth Inhibition Assay**

Growth inhibition of *Scutellaria baicalensis* was determined by an 3,4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) assay to measure viable cells. Approximately 5 × 10³ cells were seeded in each well of a 96-well plate and incubated for 24 h. The cells were then treated with various concentrations of *Scutellaria baicalensis*. The cells were then cultured for 3 days at 37°C. After treatment, 10 µl of MTT at a concentration of 5 mg/ml was added to each well. The cells were incubated for an additional 3 h, and the supernatant was discarded. Finally, 100 µl of DMSO was added to the wells to dissolve the precipitate. Optical density (OD) was measured at a wavelength of 570 nm using an EL₃₈₀ (Bio-Tek Instruments, Inc., Winooski, VT).

**Cell Cycle Analysis**

1.5 × 10⁵ cells/well were plated onto 6-well plates and incubated for 24 h at 37°C. Different concentrations of *Scutellaria baicalensis* were added to the wells and incubated for an additional 3 days. The cells were then washed, pelleted, fixed with cold 70% ethanol for at least 30 min, and incubated with 100 µg/ml rNase A and 50 µg/ml propidium iodide in phosphate-buffered saline (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 Cell Quest Pro software (Becton Dickinson, Franklin Lakes, NJ).

Pathway Array

After HepG2 cells (1.0 × 10⁶ cells/10-cm dish) were treated with S. baicalensis at 100 µg/ml or 200 µg/ml for 24 h; total cellular protein was extracted from the cells using a lysis buffer containing 20 mmol/l Tris-HCl (pH 7.5), 20 mmol/l sodium pyrophosphate, 40 mmol/l β-glycerophosphate, 30 mmol/l sodium fluoride, 2 mmol/l EGTA, 100 mmol/l NaCl, 0.5% NP-40, and 1 × proteinase inhibitor cocktail (Roche Applied Science, Indianapolis, IN). After washing with cold PBS twice and then adding 300 microliters of the lysis solution per dish, the cell monolayer was removed from the dish using a scraper. The lysate was sonicated 3 times for 15 s each time and then centrifuged at 14,000 rpm for 30 min at 4°C. The protein concentration was determined with the BCA Protein Assay kit (PIERCE, Rockford, IL). 300 µg of lysated protein was loaded in one well across the entire width of 10% SDS polyacrylamide and separated by electrophoresis (17). After electrophoresis, the proteins were transferred electrophoretically to a nitrocellulose membrane that was then blocked for 1 h with blocking buffer either including 5% milk or 3% BSA in 1× TBST containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween-20. Next, the membrane was clamped on a Western blotting manifold (Mini-PROTEAN II Multiscreen apparatus, Bio-Rad, Hercules, CA) that isolates 20 channels across the membrane. Two sets of antibodies (a total of 56 protein-specific or phosphorylation site-specific antibodies) were individually used for each membrane, and all of the antibodies (from various companies) were validated independently before inclusion in Pathway Array. For the first set of antibodies (28 total), a mixture of one or two antibodies in the blocking buffer were added to each channel and then incubated at 4°C overnight. The membrane was then washed with 1× TBST and was further incubated with secondary antirabbit or antimouse antibody conjugated with horseradish peroxidase (Bio-rad) for 1 h at room temperature. The membrane was developed with chemiluminescence substrate (Immun-Star HRP Peroxide Buffer / Immun-Star HRP Luminol Enhancer) (Bio-Rad), and chemiluminescent signals were captured using the ChemiDoc XRS System (Bio-Rad). The same membrane was also analyzed with densitometric scanning (Quantity One software package, Bio-rad) and normalized using internal standards (i.e., β-actin).

RESULTS

Scutellaria Baicalensis Inhibits Cell Growth

To determine the antiproliferative effect of S. baicalensis extract, we performed an in vitro growth inhibition assays on HepG2 cells. The rate of inhibition was determined as a percentage of viable treated cells compared with viable control cells. S. baicalensis displayed a dose dependent (Fig. 1) growth inhibition in HepG2 cells, with a 50% inhibition concentration (IC₅₀) of 360 µg/ml (Fig. 1) after 72 h of incubation with S. baicalensis. These results were averaged from 6 experiments of HepG2 treated with S. baicalensis.

We also analyzed the effect of Scutellaria baicalensis on cell cycle. Our results show that S. baicalensis caused a significant G2/M phase arrest with concurrent decrease of G0/G1 phase in S. baicalensis-treated HepG2 cells as compared with the untreated cells after 72 h (Fig. 2). The percentages of cell arrest at G2/M phase increased from 6.27% in control cells to 21.45% in cells treated with 400 µg/ml of S. baicalensis. Conversely, the numbers in G0/G1 phase decreased from 89.87% in control cells to 71.88% in cells treated with 400 µg/ml of S. baicalensis. These data indicate that S. baicalensis inhibits HepG2 cell growth at G2/M phase.

S. Baicalensis Alters Signaling Proteins

To better understand the molecular mechanisms of S. baicalensis' effect on cell growth, we performed a global screening of signaling pathways using Pathway Array technology. The Pathway Array technology is a powerful tool for analyzing changes in intracellular protein expression and phosphorylation in major cell signaling pathways including cell proliferation, apoptosis, and stress response. The Pathway Array analysis used two sets of 56 protein- and phospho-specific antibodies on the extract from cells treated with S. baicalensis for 24 h (note that our preliminary studies indicated that at 24 h of treatment, the changes in both protein expression and phosphorylations were able to be detected). Our results revealed that 30 out of 56 proteins/phosphorylations were expressed in HepG2 cells, including XIAP, cdk2, cdk4, p-β-catenin (Ser33/37/Thr41), cPKCa, TRAP, p-edc2 (Tyr15), p-RB (Ser780), ETS1, Hsp90, p-PKC

FIG. 1. Dose-dependent growth inhibition of HepG2 cells after treatment with S. baicalensis for 72 h. Results represent mean values ± SD (bars) of 6 independent experiments performed in triplicate. Student t-test was used to analyze the data. *, P < 0.05. **, P < 0.001.
FIG. 2. Cell cycle distribution after treatment with *S. baikalensis*. HepG2 cells were treated with *S. baikalensis* at 100 µg/ml, 200 µg/ml, and 400 µg/ml, respectively, for 72 h and subjected to flow cytometry analysis. Representative graphs are presented here. The values in the insert represent mean values of 5 independent experiments.

α/β II (Thr638/641), and ERK 1/2 (Fig. 3). After treatment with *S. baikalensis*, 7 of them decreased more than twofold at a dose of 100 µg/ml, whereas 9 decreased and 1 increased at the dose of 200 µg/ml (Table 1). Furthermore, 9 of them showed a dose-dependent decrease (p53, ETS1, cdc25B, p63, EGFR, ERK1/2, XIAP, HIF-2α and cdc25C) and 1 dose-dependent increase (cyclin E) after treating with *S. baikalensis* (Fig. 4). In addition, 5 proteins or phosphorylations (β-catenin, p-GSK-3α/β (Ser21/9), cyclin D1, p-cdc2 (Tyr15), and Hsp90) showed an initial decrease at 100 µg/ml and then increase at 200 µg/ml of *S. baikalensis*. These data suggest that *S. baikalensis* has a broad effect on the cell signal pathways and that the response is dose dependent.

**Effect of *S. Baicalensis* on Cell Signaling Network**

To understand the protein-protein interaction network in HepG2 cells as well as the effect of *S. baikalensis* on this network, we placed those proteins with more than twofold changes in the context of a signaling network using Genes2Networks software (http://actin.pharm.mssm.edu/genes2networks). Genes2Networks integrates the content of 10 mammalian, literature-based, interaction network data sets and can be used to extract relevant subnetworks based on our “seed” list of proteins that were identified in Pathway Array. The output includes a dynamic linkable 3-color, Web-based network map, with a statistical analysis report that identifies significant intermediate nodes used to connect the proteins on seed list to one another. The system can be used to find relationships between nodes from the seed list as well as to predict novel nodes that may play a key role in a signaling network.

Based on this analysis, we were able to predict the network of protein-protein interaction that is affected by *S. baikalensis* treatment (Fig. 5). Among the 15 proteins (Fig. 4) that showed a twofold or greater change after treatment with *S. baikalensis*, 13 proteins (except for XIAP and p63) were found in the database and were used as seed proteins. Genes2Networks identified 38 intermediate components with a total of 138 interactions.
<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Description</th>
<th>Expression Relative to Control (Fold Change)</th>
<th>100 µg&lt;sup&gt;i&lt;/sup&gt;</th>
<th>200 µg</th>
</tr>
</thead>
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<tr>
<td>14-3-3β</td>
<td>A multifunctional regulator involved in cell cycle control and signal transduction</td>
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<td>A cell adhesion protein involved in Wnt-stimulated signaling</td>
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<td>BRCA1</td>
<td>A tumor suppressor that activates DNA repair</td>
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<td>−2.74</td>
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<tr>
<td>Cdc2</td>
<td>Kinase that initiates entry into mitosis with cyclin B</td>
<td></td>
<td>−1.61</td>
<td>−1.70</td>
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<tr>
<td>Cdc25B</td>
<td>A mitotic activator that phosphorylates Cdc2</td>
<td></td>
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</tr>
<tr>
<td>Cdc25C</td>
<td>A phosphatase that activates the Cdc2/Cyclin B complex</td>
<td></td>
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<tr>
<td>Cdk2</td>
<td>A cyclin E-dependent kinase that regulates the G1/S transition</td>
<td></td>
<td>−0.09</td>
<td>−0.04</td>
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<tr>
<td>Cdk4</td>
<td>A cyclin D-dependent kinase that regulates the G1/S transition</td>
<td></td>
<td>0.03</td>
<td>−1.09</td>
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<tr>
<td>Cdk6</td>
<td>A cyclin D-dependent kinase that regulates the G1/S transition</td>
<td></td>
<td>1.24</td>
<td>0.05</td>
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<tr>
<td>Chk1</td>
<td>A protein kinase involved in the G2 DNA damage checkpoint</td>
<td></td>
<td>−0.40</td>
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<tr>
<td>cPKC&lt;sub&gt;α&lt;/sub&gt;</td>
<td>Signal transduction</td>
<td></td>
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<td>−1.18</td>
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<tr>
<td>Cyclin D1</td>
<td>Regulator of G1 progression</td>
<td></td>
<td>−8.17</td>
<td>−0.98</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>Regulator of the G1/S transition</td>
<td></td>
<td>1.03</td>
<td>3.69</td>
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<tr>
<td>EGFR</td>
<td>A growth factor receptor that is overexpressed in many cancers</td>
<td></td>
<td>−1.56</td>
<td>−2.92</td>
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<tr>
<td>ERK</td>
<td>MAP kinase central in cascades signalling cellular</td>
<td></td>
<td>−1.91</td>
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<tr>
<td>ETS1</td>
<td>Transcription factor regulating genes involved in tumor defense, apoptosis, and mitosis</td>
<td></td>
<td>−2.49</td>
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<td>HIF-2α</td>
<td>Transcription factor responsive to hypoxic conditions</td>
<td></td>
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<tr>
<td>HSP90</td>
<td>A molecular chaperone</td>
<td></td>
<td>−4.01</td>
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<tr>
<td>p27</td>
<td>Negative regulator of G1 progression</td>
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<td>p53</td>
<td>A tumor suppressor protein that inhibits the cell cycle</td>
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<td>1.26</td>
<td>−10.00</td>
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<td>p63</td>
<td>A tumor suppressor</td>
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<td>1.31</td>
<td>−5.57</td>
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<td>p-β-catenin (Ser33/37/Thr41)</td>
<td>A cell adhesion protein involved in Wnt-stimulated signaling; when phosphorylated by GSK, it breaks bonds with cadherins, decreasing cell adhesion</td>
<td></td>
<td>−1.40</td>
<td>1.24</td>
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<tr>
<td>p-cdc2 (Tyr15)</td>
<td>Cyclin dependent kinase inhibits cell cycle progression when phosphorylated</td>
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<td>−3.16</td>
<td>1.05</td>
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<td>p-GSK-3α / β (Ser21/9)</td>
<td>Downstream target of Wnt signaling pathway</td>
<td></td>
<td>−2.48</td>
<td>−1.14</td>
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<tr>
<td>p-PKC α / β (Thr638/641)</td>
<td>Signal transduction</td>
<td></td>
<td>−1.10</td>
<td>1.32</td>
</tr>
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<td>A tumor suppressor gene</td>
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<td>−1.27</td>
<td>1.20</td>
</tr>
<tr>
<td>p-RB (Ser807/811)</td>
<td>A tumor suppressor gene</td>
<td></td>
<td>−1.48</td>
<td>1.39</td>
</tr>
<tr>
<td>p-Stat3 (Ser727)</td>
<td>Signals transcription activation</td>
<td></td>
<td>−1.74</td>
<td>1.30</td>
</tr>
<tr>
<td>Trap</td>
<td>Growth inhibitory acid phosphatase</td>
<td></td>
<td>−1.17</td>
<td>−1.73</td>
</tr>
<tr>
<td>XIAP</td>
<td>An antiapoptosis gene</td>
<td></td>
<td>−2.17</td>
<td>−2.62</td>
</tr>
</tbody>
</table>

<sup>i</sup>SB: *Scutellaria Baicalensis*.

Note: (−) indicates fold change in down regulation.
SCUTELLARIA BAICALENSIS AND THE SIGNALING NETWORK IN HEPATOCELLULAR CARCINOMA CELLS

FIG. 3. Representative autoradiographs showing expression and phosphorylation of the signaling-related proteins. HepG2 cells were treated with *S. baicalensis* at 100 µg/ml or 200 µg/ml for 24 h, and the expression and phosphorylation levels of 56 proteins were determined by Pathway Array. The tests were repeated twice, and the results were consistent. A: Control. B: Treatment with 100 µg/ml of *S. baicalensis*. C: Treatment with 200 µg/ml of *S. baicalensis.

Among these components, 3 are membrane receptors (EGFR, Presenilin-1, and Her-2/neu), 42 are central signaling network proteins (e.g., p53, Cyclin D1, ERK1/2, ER-1, AR, Raf-1, p300, MTO1, Chk-1, etc.), 1 apoptosis factor (Bcl-2), and 6 are transcription factors (CREB3, *CTCCB1*, c-Jun, c-Myc, P/CAF and TAF1). Several proteins were identified as key points, or hubs, in this network (Fig. 5). The most central hub to this network was p53, which had 14 links. Other hubs include Cyclin D1 with 13 links; ERK1/2 with 10 links; ER-1 with 11 links; AR with 9 links; and Cyclin E1, Hsp90, Raf-1, and Cdc25 with 7 links each. Additionally, Cyclin A2, p300, MyoD1, PKCα, p107, and 14-3-3β had 6 links each, whereas Chk-1 and GSK-3α had 5 links each. These data suggest that all of these proteins have an important role in regulating *S. baicalensis*-induced cell signaling network changes.

FIG. 4. Differential expression of proteins and phosphorylation. HepG2 cells were treated with *S. baicalensis* at concentrations of 100 µg/ml and 200 µg/ml for 24 h, and the expression levels of these proteins were determined by Pathway Array. Fifteen proteins and phosphorylations were showed to have more than twofold changes as compared with the controls. The results represent the average changes in expression levels of 2 independent experiments. White bars: 100 µg/ml of *S. baicalensis*. Striped bars: 200 µg/ml of *S. baicalensis*.

FIG. 5. The cell signaling network established using Gene2Networks software. The 15 differentially expressed proteins were used as “seed” proteins, but only 13 proteins (black nodes) were connected to one network. The hollow dots indicate intermediate nodes identified by Genes2Networks. The arrows indicate the direction of interaction. TP53: p53; CCND1: Cyclin D1; CTNB1: β-catenin. CCNE1: Cyclin E1. HSP90AA1: Hsp90. EPAS1: HIF-2α. MAPK1: ERK1/2. ERBB2: Her-2/neu. CDH1: E-cadherin. PSEN1: Presenilin-1. MAPT: tau protein. PRKCA: PKCα. YWHAZ: 14-3-3β. CHEK1: Chk-1. CSNK2B: CK2β. CCNA2: Cyclin A2. HERC5: CEBP1. EP300: p300.
DISCUSSION

We have previously demonstrated that *S. baicalensis* can inhibit uncontrolled cell division in many cell lines, including squamous cell cancer cells, prostate cancer cells, colon cancer cells, breast cancer, as well as hepatocellular carcinoma cells (8,13,17). This study further confirmed that *S. baicalensis* can inhibit HepG2 cell growth in vitro with an IC50 of 360 µg/ml (Fig. 1). Our cell cycle analysis data demonstrated that *S. baicalensis* causes HepG2 cell growth arrest at G2/M phase (Fig. 2). Although there was some increase of apoptotic cells, apoptosis is probably not the main mechanism by which *S. baicalensis* causes HepG2 cell death. It is interesting to note that *S. baicalensis* can affect different phases of the cell cycle in different cancer cells. For example, *S. baicalensis* can cause G1 phase arrest in LnCaP cells (17) but G2/M phase arrest for squamous cells and PC-3 (13,17). These results suggested that *S. baicalensis* inhibits cancer cell growth via different mechanisms or different cellular targets in different cell lines.

To determine the molecular mechanism of HepG2 cell growth arrest by *S. baicalensis*, we analyzed a total of 56 proteins related to cell proliferation signaling pathways using multiplex immunoblot (Pathway Array). Our results revealed a total of 15 differentially expressed proteins or phosphorylations. Of these, 9 showed a dose dependent decrease (p53, ETS1, cdc25B, p63, EGFR, ERK1/2, XIAP, HIF-2α, and cdc25C), whereas 1 showed a dose-dependent increase (cyclin E) after treatment with 200 µg/ml of *S. baicalensis*. In addition, 5 proteins or phosphorylations (β-catenin, p-GSK-3-α/β, ERK1/2, XIAP, HIF-2α, and cdc25C), whereas 1 showed a dose-dependent increase (cyclin E) after treatment with 200 µg/ml of *S. baicalensis*. Many of these proteins are involved in carcinogenesis of hepatocellular carcinoma. For example, overexpression of EGFR has been reported in HCC ranging from 4% to 53% (6), and an EGFR inhibitor (Erlotinib) has been shown to significantly inhibit HCC cell growth in vitro as well as in animal studies and human trials. ERK1/2, the downstream effector of EGFR, is also activated in HCC, especially in more severe forms of HCC associated with hepatitis C virus infection, which is correlated with a poorer prognosis (18). GSK-3β is a tumor suppressor involved in the Wnt signaling pathway that phosphorylates β-catenin, which results in the degradation of β-catenin. Overexpression of GSK-3β was found in HCC and was correlated with the accumulation of β-catenin in the nucleus and an increase in expression of genes downstream of β-catenin, such as Cyclin D1 and c-myc (19).

The hypoxia-inducible transcription factor-2alpha (HIF-2α) is important in cell proliferation under hypoxic conditions, which is advantageous for neoplastic growth. The expression of HIF-2α was positive in 69.5% of tissue samples of patients with HCC and correlated with a poorer prognosis (20). ETS-1 is a transcription factor that activates the expression of genes that are involved in cellular proliferation, differentiation, development, transformation, and apoptosis (21), and its expression is increased in hepatocellular carcinoma (22). X-linked inhibitor of apoptosis (XIAP) potently inhibits the enzymatic activity of caspase cascade including caspase-9, caspase-3, and caspase-7 (23). Downregulation of these proteins by *S. baicalensis* may potentially promote apoptosis and inhibit the growth of HepG2 cells, although we do not believe this to be the primary effect of *S. baicalensis* on HepG2 cells.

However, the majority of proteins or phospho-proteins that were affected by *S. baicalensis* were nuclear proteins and cell cycle regulatory factors including p53, p63, ETS1, HIF2α, Cdc25C, Cdc25B, Cdc2, Cyclin D1, and Cyclin E. The effect of *S. baicalensis* on these factors may be direct or indirect (i.e., via the downregulation of upstream effectors, such as EGFR, or transcription factors, such as ETS1). Cdc2, Cdc25C, and Cdc25B are important cell cycle regulators and control the cell cycle progression from G2 to M phase. Both p53 and p63 are transcription factors and are also involved in the cell cycle regulation (24). An upregulation of p53 and Cyclin E expression and downregulation of Cyclin D2 and Cyclin A expression was also observed in rat heart endothelial cells after treatment with baicalein, which caused cell growth arrest at G2 phase (11). It is worthy to note that the downregulation of tumor suppressors p53 and p63 and the upregulation of oncogenes Cyclin E and Cyclin D1 is unexpected in an antineoplastic treatment. This may be reflective of the complex cellular response to the various synergistic chemical components found in *S. baicalensis* extract. Again, the dose-dependent changes in some of the proteins and phosphorylations (Fig. 4) may also be attributable to the complicated intracellular signaling response. Therefore, it is conceivable that the sum of the changes in cell cycle-related protein expression and activity may lead to the cell cycle arrest at G2/M phase of HepG2 cells caused by *S. baicalensis*.

It is important to note that the proteins identified in this study do not act independently to inhibit cell proliferation but rather as part of a complex signaling network. Genes2Networks analysis identified 38 components with a total of 138 interactions in a complex signaling network (Fig. 5). The most important hubs in this network include p53 (14 links), Cyclin D1 (13 links), ERK1/2 (10 links), ETS1 (9 links), AR (9 links), Cyclin E1 (7 links), Hsp90 (7 links), Raf-1 (7 links), MyoD1 (6 links), PKCα (6 links), p107 (6 links), 14-3-3β (6 links), Chk-1 (5 links), and GSK-3α (5 links). These results indicate that *S. baicalensis* exerts a broad effect on the cell signaling networks, but it especially acts on the key hubs identified here. Many of these proteins are important elements of the signaling network that are dysregulated in neoplastic cells, causing unregulated cell growth. *S. baicalensis* may target many components of these dysregulated networks, leading to a collective inhibition of cell proliferation. Additionally, these proteins may be useful targets for the development of effective therapy for HCC.

*Scutellaria baicalensis* contains 4 major flavonoids in addition to other compounds found in trace amounts: baicalin (80%), wogonoside (16%), baicalein (2%), and wogonin (1%) (10,25). It is possible that different components may act on the
different targets in this complex signaling network, resulting in a synergistic antiproliferative effect. This study supports the traditional use of raw herbs in decoction at the molecular level. Future study of individual components of *S. baicalensis* on the complex signaling network is warranted.

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