Inhibitory Effects of Baicalein on the Influenza Virus in Vivo Is Determined by Baicalin in the Serum

Ge Xu,a,### Jie Dou,a,### Lei Zhang,a Qinglong Guo,*b and Changlin Zhou*,a

a School of Life Science & Technology, China Pharmaceutical University; and b Department of Physiology, China Pharmaceutical University; 24 Tong Jia Xiang, Nanjing, Jiangsu 210009, P. R. China.

Received August 28, 2009; accepted October 13, 2009; published online November 10, 2009

Baicalein, an extract from Scutellaria baicalensis, was evaluated for its ability to inhibit the influenza virus in vivo. Oral administration of baicalein to BALB/c mice infected with the influenza A/FM1/1/47(H1N1) virus showed significant effects in preventing death, increasing the mean time to death, inhibiting lung consolidation, and reducing the lung virus titer in a dose-dependent manner. These effects are believed to be due to baicalin, the metabolite of baicalein in the serum. At a concentration of baicalin 2 μg/ml in overlay medium, it showed significant inhibition in the plaque assay, and the mean IC₅₀ value of baicalin was calculated as 1.2 μg/ml in the cytopathic effect assay. Our results showed that baicalein warrants further research as a potential antiinfluenza viral agent.

Key words  baicalein; baicalin; antiviral activity; serum pharmacology

Baicalein (5,6,7-trihydroxyflavone) is a flavonoid derived from the root of Scutellaria baicalensis, a traditional Chinese medicine used for hundreds of years. This flavonoid demonstrates a variety of biological activities, such as antibacterial, antiinflammatory, antioxidant, antitumor, antiproliferative, anticoagulant, and vascular-protective effects.1-3 Baicalein is also an inhibitor of reverse transcriptase of the human immunodeficiency virus (HIV), interferes with the RNA replication of HIV, and induces apoptosis in infected cells in vitro.3 Baicalein is the most potent inhibitor of the human cytomegalovirus (HCMV), displaying an IC₅₀ value of 0.4±0.04 μM in the colorimetric assay and 1.2±0.8 μM in the titer reduction assay. It also significantly reduces the level of early and late proteins of HCMV, as well as viral DNA synthesis.4-5

Influenza, one of the major pandemic diseases worldwide, represents a grave threat to human health. At present, only two types of chemical drug are used clinically in the treatment of influenza. One type is the M2 proton channel blockers (amantadine and rimantadine), and the other is neuraminidase inhibitors (zanamivir and oseltamivir). However, resistance to these drugs has been reported.5-8 Furthermore, the high price and lack of a sufficient supply prevent oseltamivir (Tamiflu) from being widely used in developing countries. Traditional Chinese medicine, which is widespread in nature with good efficacy, is beginning to play a more important role in this area.

It has been reported that baicalein demonstrates inhibitory effects on viruses and that more than 90% of baicalein is rapidly metabolized into baicalin, which is transported to the mesenteric blood when perfused through the rat jejunum.9,10 However, the mechanism of its antiviral effect is unknown. Here, we report for the first time that baicalein has an inhibitory effect on the influenza virus A/FM1/1/47(H1N1) in vivo and that this effect is mainly due to its metabolite baicalin in serum.

MATERIALS AND METHODS

Reagents  Baicalein was a gift from Prof. Qinglong Guo of the Department of Physiology, China Pharmaceutical University. It was dissolved in 0.5% sodium carboxymethyl cellulose (CMC) for in vivo experiments and in dimethyl sulfoxide (DMSO) for in vitro tests. Ribavirin injection was obtained from the China Pharmaceutical University Pharmaceutical Co., Ltd., Nanjing, China. Reference samples of baicalein and baicalin used in HPLC analysis were obtained from the National Institution for the Control of Pharmaceutical and Biological Products, China. Methanol, acetonitrile, and phosphoric acid (HPLC grade) were purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China.

Virus and Cells  The influenza A/FM1/1/47(H1N1) virus used in this study was preserved in the Department of Microbiology, School of Life Science and Technology, China Pharmaceutical University. The virus was grown in the allantoic cavity of 10-d-old embryonated hen eggs at 37°C for 2 d.11 The allantoic fluid was harvested and filtered with a 0.22-μm cellulose acetate membrane. The filtered liquid was stored in small aliquots at −70°C until further use.

Madin–Darby canine kidney (MDCK) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS). The cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C.

Animals  Ninety-six female BALB/c mice (18—22 g) and 10 Sprague-Dowley (SD) rats were obtained from the Laboratory Animal Center, Nanjing Medical University, China, and treated according to the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health.

Antiviral Effects of Baicalein in Vivo  After a 2-d acclimation period, the mice were slightly anesthetized by the inhalation of diethyl ether and intranasally infected with 8×MLD₅₀ of mouse-adapted influenza A/FM1/1/47(H1N1) virus in 100 μl of phosphate buffered saline (PBS). The mice were treated with various doses of baicalein solution (240, 480, 960 mg/kg/d) by oral gavage twice daily for 4 d beginning 24 h after virus inoculation. Ribavirin (400 mg/kg/d) or sterile sodium chloride (100 μl) was given as a positive control or placebo control, respectively. All doses were determined
in advance in toxicity experiments. Normal controls were treated with sodium chloride. Ten animals per group were observed for 14 d for clinical signs of infection or death.

An additional 6 mice per group were used to assess lung infection parameters on day 5 of infection with the influenza virus. Mice were weighed and killed, and then the lungs were removed and weighed. The lung index and lung index inhibition were calculated as:

\[
\text{lung index} = \frac{A}{B} \times 100\%
\]

\[
\text{lung index inhibition (\%)} = \frac{(C - D)}{C} \times 100\%
\]

where \(A\) is lung weight, \(B\) is body weight, \(C\) is mean lung index of the placebo control group, and \(D\) is mean lung index of the drug-treated group.

Lung consolidation scores ranging from 0 (normal appearance) to 4 (100% of the lung exhibiting a plum color) were assigned. After being fixed in 10% formalin and embedded in paraffin, four lungs per group were sectioned for histologic examination. Pathologic changes were evaluated based on the following aspects: hyperemia and bleeding of the lungs; leukopoesis; bronchiole epithelium cell necrosis; exudate of lung; alveolar interstitial pneumonia; and lung abscess.

The remnant lungs were homogenized in PBS to give a 10% (w/v) suspension, and the homogenate was centrifuged at 2000\(\times g\) for 10 min. The supernatant was serially diluted and inoculated into 10-d-old embryonated hen eggs. Virus titers in the lungs were calculated using the method of Reed and Muench\(^{(2)}\) and egg infective dose (EID) expressed as mean \(\log_{10}\text{EID}_{50}/\text{ml} \pm \text{S.D.}

Preparation of Medicine Serum Ten SD rats were used in this experiment. Six rats were orally administered a baicalein solution at a dose of 500 mg/kg once daily for 5 d. The other 4 rats were treated with saline as controls. On day 5, after 2.5 h of treatment with baicalein, the rats were anesthetized with an intraperitoneal injection of 10% (m/v) amobarbital sodium at a dose of 500 mg/kg, and blood was collected under sterile conditions from the main ventral artery. After standing at 4 °C for 30 min, the serum was obtained by centrifugation at 3000\(\times g\) for 20 min and stored in small aliquots at −20 °C.\(^{(13)}\)

HPLC was used for quantitative analysis of baicalein and its metabolite baicalin in the serum. The LC-2010 Shimadzu HPLC system was employed with a Class VP workstation for data collection. Liquid chromatographic separation was achieved using a Lichrospher C\(_{18}\) analytical column (6.0 mm\(\times 150\) mm, 5 \(\mu m\)) connected to a C\(_{18}\) guard column (Hanbon Science & Technology Co., Ltd., Jiangsu, China). Reference substances of baicalein and baicalin were dissolved in methanol to concentrations of 196 \(\mu g/ml\) and 19.8 \(\mu g/ml\), respectively, for use as internal standards. To prepare for HPLC analysis, 400 \(\mu l\) of methanol–acetonitrile (1:1, v/v) and 100 \(\mu l\) of 0.4% phosphoric acid were added to a 200-\(\mu l\) serum sample. After vortex mixing for 3 min, the mixture was centrifuged for 10 min at 12000\(\times g\). Six hundred microliters of the upper layer was obtained, evaporated under \(N_2\), and reconstituted with 200 \(\mu l\) of the mobile phase prior to HPLC analysis. Blank serum and blank serum with 20 \(\mu l\) of the internal standards were prepared in parallel as controls.\(^{(14,15)}\) The mobile phase consisted of methanol–0.2% phosphoric acid (48:52, v/v) at a constant flow rate of 1.0 ml/min. The column temperature and the detection wavelength were set at 30 °C and 275 nm, respectively. The preparations were analyzed both qualitatively and quantitatively by comparisons of retention times and analyzed peak areas with those of the internal standards.

Antiviral Effects of Medicine Serum in Vitro Antiviral activities of medicine serum were measured in the plaque inhibition assay. MDCK cells (1\(\times 10^5\) cells/ml) were cultured in 24-well plastic plates for 24 h and inoculated with 100 plaque forming units (PFU) per well of influenza A/FM1/1/47(H1N1) virus. After 60 min for virus absorption, the solution was removed and replaced with 1 ml of overlay medium (DMEM medium containing 1% carboxymethyl cellulose, without serum) containing different concentrations of medicine serum. The final concentrations of baicalin in the overlay medium were 0.5 \(\mu g/ml\), 1 \(\mu g/ml\), and 2 \(\mu g/ml\). After incubation at 37 °C for 3 d, the solution was removed and the cells were fixed with 10% formaldehyde solution for 60 min at room temperature. Then the cells were stained with 0.5% (w/v) crystal violet solution for 15 min, and the number of plaques was counted for each well.

Cytopathic Effect Assay To examine whether baicalein can inhibit the cytopathic effect (CPE) of cells infected by influenza A/FM1/1/47(H1N1) virus, a 96-well tissue culture plate was seeded with 100 \(\mu l\) of 1\(\times 10^5\) cells/ml in DMEM with 10% FBS. After cells were incubated for 18—24 h at 37 °C, the solution was removed and the cells were infected with 100 PFU per well of influenza A/FM1/1/47(H1N1) virus at 37 °C for 1 h. The viral inocula were removed and replaced with growth medium (DMEM medium without serum) containing different concentrations of medicine serum. The final concentrations of baicalin in the growth medium were 0.25 \(\mu g/ml\), 0.5 \(\mu g/ml\), 1 \(\mu g/ml\), 2 \(\mu g/ml\), and 3 \(\mu g/ml\). Each treatment was performed in quadruplicate. After incubation for 3 d at 37 °C, the cells were fixed with 100 \(\mu l\) of 10% formaldehyde for 1 h at room temperature. After removal of the formaldehyde, the cells were stained with 0.5% (w/v) crystal violet solution for 15 min at room temperature. The plate was washed and dried, and the intensity of crystal violet staining for each well was measured at 570 nm. The IC\(_{50}\) value of baicalin was calculated from the absorbance values.\(^{(16)}\)

Statistical Analysis Data from the in vivo studies are presented as mean±S.D. Comparisons were made between drug-treated and placebo groups. Differences in the mean day to death, lung consolidation scores, and lung virus titers were analyzed using the two-tailed Mann–Whitney U-test. The probability of survival was estimated with the Kaplan–Meier method, and survival estimates were compared using the log-rank test.\(^{(17)}\)

RESULTS

Antiviral Effects of Baicalein in Vivo Influenza viral infection leads to high mortality, lung virus titers, and pneumonia.\(^{(18)}\) Therefore the efficacy of treatment was evaluated on the basis of survival rate, mean day to death, lung virus titers, and lung parameters including lung consolidation and the inhibition of the lung index. The results showed that baicalein displayed a protective effect on mice infected with influenza A/FM1/1/47(H1N1) virus (Table 1). Oral gavage
with all doses of baikalein (240, 480, 960 mg/kg/d) resulted in protection from death and statistically significant prolonged survival time of mice. A survival rate of 50% was obtained even at the lowest dose of 240 mg/kg/d, while all the placebo control mice died. Compared with the other two doses, treatment with baikalein at the dose of 960 mg/kg/d had the most effect on life protection with a survival rate of 70% and the mean day to death of 12.4±2.6 d. Furthermore, in the 960 mg/kg/d group, mortality occurred on day 8 after infection, which was 3 d later than in the placebo group, 2 d later than in the 240 mg/kg/d group, and 1 d later than in the 240 mg/kg/d group (Fig. 1).

Lung parameter data showed that treatment with baikalein provided a dose-dependent protective effect from viral pneumonia (Table 1). Inhibition of the lung index was detected at 26%, 30%, and 35% with the doses of 240, 480, and 960 mg/kg/d, respectively. Stronger inhibition of the lung index (35%) and less lung consolidation (1.2±0.75) were observed in the mice treated with baikalein 960 mg/kg/d than in those treated with ribavirin (32%; 1.3±0.52). Meanwhile, virus replication was reduced in all treatment groups. The lowest virus titer detected was 3.8±0.8 in 960 mg/kg/d group. The results of the histologic examination were consistent with the lung parameter data (Fig. 2). Protection from bronchitis and interstitial pneumonia was found in all groups treated with baikalein, and the degrees of protection were different depending on the baikalein dose.

**Determination of Baicalin in Serum**

The concentrations of baikalein in serum were measured using a high-performance liquid chromatography method. The concentrations were 5.2±0.9, 6.3±1.0, and 7.4±1.1 μg/ml for the 240, 480, and 960 mg/kg/d groups, respectively. The concentrations were increased in a dose-dependent manner compared to the placebo group (0.5±0.1 μg/ml).

**Table 1. Protective Effects of Baicalein in Mice Infected with A/FM1/1/47(H1N1)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg/d)</th>
<th>Survivors/total (%)</th>
<th>MDD±S.D.</th>
<th>Mean lung parameters</th>
<th>Lung index inhibition %</th>
<th>Virus titerlog10 EID50/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Score±S.D.</td>
<td>Lung index±S.D.</td>
<td></td>
</tr>
<tr>
<td>Baicalein</td>
<td>960</td>
<td>7/10 (70)***</td>
<td>12.4±2.6**</td>
<td>1.2±0.75**</td>
<td>0.71±0.07***</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>480</td>
<td>6/10 (60)***</td>
<td>11.5±3.3**</td>
<td>1.8±0.41**</td>
<td>0.76±0.06***</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>5/10 (50)***</td>
<td>10.6±3.6*</td>
<td>2.8±0.41*</td>
<td>0.81±0.04***</td>
<td>26</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>400</td>
<td>6/10 (60)***</td>
<td>10.7±4.3*</td>
<td>1.3±0.52**</td>
<td>0.74±0.09***</td>
<td>32</td>
</tr>
<tr>
<td>Placebo</td>
<td>—</td>
<td>0/10 (0)</td>
<td>6.8±1.1</td>
<td>3.5±0.55</td>
<td>1.09±0.06</td>
<td>—</td>
</tr>
<tr>
<td>Normal</td>
<td>—</td>
<td>10/10 (100)</td>
<td>14.0±0.0</td>
<td>0.0±0.0</td>
<td>0.71±0.09</td>
<td>—</td>
</tr>
</tbody>
</table>

a) Mean day to death of mice dying prior to day 14; b) log10 EID50/ml. *p<0.05, **p<0.01, ***p<0.001, compared with values in placebo controls.

**Fig. 1. Effects of Baicalein on Mouse Survival**

BALB/c mice were infected with 8×MLD50 of influenza virus (H1N1) and orally treated with baicalein (240, 480, 960 mg/kg/d) twice daily for 4 d, beginning 24 h after infection. Ten mice per group were observed for 14 d for clinical signs of infection or death. The Kaplan–Meier method was used to estimate the probability of survival, which is expressed as a survival distribution function. A value of 1 corresponds to 100% survival.

**Fig. 2. Pathologic Changes in Lungs in Mice Infected with A/FM1/1/47(H1N1) (200×)**

The mice were killed 3 d after infection. The lungs were removed and rinsed with sterile PBS. Afterfixing in 10% formalin and embedding with paraffin, the lungs were sectioned for histologic examinations. Pathologic changes were evaluated based on hyperemia and bleeding of lungs, leukopenesis, bronchiolitis epithelium cell necrosis, exudate of lung, alveolus interstitial pneumonia, and lung abscess.
of baicalein and its metabolite in the serum of rats treated with baicalein (500 mg/kg) were analyzed using HPLC 2.5 h after treatment on day 5 (Fig. 3). The retention times of baicalein and baicalin were 34.2 min and 10.6 min, respectively. Baicalin at the concentration of 17.0 μg/ml was detected in the serum of treated rats, while baicalein was detected at the concentration of 0.8 μg/ml, suggesting that the majority of baicalein had been metabolized into baicalin, and the amount of baicalein remaining was extremely small.

**Antiviral Effects of Medicine Serum *in Vitro***

MDCK cells were infected with influenza A/FM1/1/47(H1N1) virus and incubated in overlay medium with medicine serum containing various concentrations of baicalin for 72 h. It was shown that baicalin inhibited influenza virus replication in a dose-dependent manner (Fig. 4A). At the concentrations of 1 μg/ml and 2 μg/ml, baicalin showed significant inhibitory effect. At 0.5 μg/ml, baicalin showed a slight inhibitory effect. Therefore the concentration of baicalin that effectively reduced the plaque of influenza A/FM1/1/47(H1N1) virus was between 0.5 μg/ml and 1 μg/ml. The level of protection of MDCK cell monolayers provided by baicalin from viral CPE was measured using influenza A/FM1/1/47(H1N1) virus. The ratio of 100 PFU per well of virus was required to produce 90% CPE at 3 d post infection. After staining of the cell monolayers with crystal violet, four sets of absorbance values were averaged to generate a dose-response curve (Fig. 4B). Absorbance values were proportional to the overall health of the cell monolayers, as demonstrated by the reduction in protection afforded by baicalin as the concentration decreased. Protection of the monolayers from the CPE induced by influenza A/FM1/1/47(H1N1) virus was observed at baicalin concentrations from 1 to 3 μg/ml. The IC<sub>50</sub> value of baicalin was calculated as 1.2 μg/ml from the inhibition curve.

**DISCUSSION**

In this paper, the inhibitory activity of baicalein against influenza A/FM1/1/47(H1N1) virus *in vivo* was examined and its mechanism investigated. The oral administration of baicalein showed good effect against influenza A/FM1/1/ 47(H1N1) virus infection in mice, such as increasing survival rate, prolonging survival time, inhibiting lung consolidation, and reducing lung virus titers. Baicalin, with a glucose residue, was difficult to absorb. After oral administration, it was converted into baicalein by β-glucuronidase of intestinal bacteria and absorbed. When transported to the liver, baicalein was transformed into metabolites by UDP-glucuronosyltransferases (UGT) in liver microsomes and exerted activity, including 7-O-glucuronide-5-methoxy-5-hydroxyflavone, 6-O-glucuronide-5,7-dihydroxyflavone, and 7-O-glucuronide-5,6-dihydroxyflavone (baicalin) (Fig. 5). Among them, baicalin was the main metabolite. This mechanism was similar to the activation of sennosides and saikosaponin. Sennosides present in *Senna folium* are metabolized to rhein and rhein anthrone to produce a laxative action.

Saikosaponin extracted from the root of *Bupleurum falcatum* must be metabolized in the intestinal tract to exert anti-inflammatory and antitumor activity.
According to our results, the serum of rats orally administered baikalein for 2.5 h exhibited an obvious inhibitory effect on the duplication of influenza A/FM1/1/47(H1N1) virus in MDCK cells. Since the amount of baikalein detected in the serum was too small to exert that effect and baikalin is the predominant metabolite of baikalein, it was reasonably concluded that baikalin plays an important role in antinfluenza activity. From the data obtained, the IC50 value of baikalein against the influenza virus \textit{in vitro} was 1.2 \( \mu \text{g/ml} \) in the CPE assay, and baikalin also showed significant effects against the virus at the concentration of 2 \( \mu \text{g/ml} \) based on the plaque inhibition assay. According to previous studies, it was reasonable to assume that baikalin possesses a common, nonspecific antiviral mechanism based on its inhibitory effects on a large number of viruses. We will also search for the mechanism through its modulation of the immune system in our future investigations.

Acknowledgments We would like to thank Prof. Wenyuan Liu of the Department of Pharmaceutical Analysis, China Pharmaceutical University, for excellent analytical assistance. This study was sponsored by the Qing Lan Project (2008) from Jiangsu province, and the “111 Project” from the Ministry of Education of China and State Administration of Foreign Expert Affairs of China (No. 111-2-07).

REFERENCES