1. Introduction

*Scutellaria radix* (Huang-Qin in Chinese and Ogon in Japanese), the root of *Scutellaria baicalensis* Georgii, has been used as a traditional remedy for hepatitis, cirrhosis, leukemia, hepatoma, hyperlipemia, arteriosclerosis and inflammatory diseases (Lim, 2003; Nakamura et al., 2003). The active ingredients were found to be baicalin, wogonin and baicalein. It is reported that these flavonoids have diverse pharmacological effects such as cell cycle arrest and metabolism interference in cancer cells (Kimura, 2003; Nakamura et al., 2003). Wogonin (5,7-dihydroxy-8-methoxyflavone, Fig. 1), one of the active flavonoid originated from the *Scutellaria baicalensis* Georgii *radix* (Lim, 2004), is used in the therapy for atherosclerosis and restenosis due to its antioxidant (Gao et al., 1999; Lim et al., 1999), anti-inflammatory (You et al., 1999; Park et al., 2001), and antithrombotic activities (Kimura et al., 1997). Recent studies in several laboratories including ours have demonstrated that wogonin possesses potent anticancer activity both *in vitro* and *in vivo* due to its antiproliferative (Chung et al., 2008), apoptosis-inducing (Lee et al., 2008), angiogenesis inhibition (Lu et al., 2008), cell migration inhibition (Piao et al., 2008), and differentiation-inducing activities (Zhang et al., 2008a, 2008b). However, the side effects and toxicity of wogonin has not been well defined. This limitation retards the approval of clinical applications of this natural product. We previously investigated the acute and subchronic toxicity of wogonin using albino mice and Sprague–Dawley rats, respectively. The result indicated that a long period of treatment with a high dose of wogonin (120 mg/kg) induced reversible heart injury in rats (Qi et al., 2009). Here, we further studied the subchronic toxicity and pharmacokinetic of wogonin using Beagle dogs on the basis of the GLP (Good Laboratory Practice) of the People's Republic of China. It is expected that the results presented will help to establish appropriate dosage, frequency, and treatment duration in clinical applications of this agent.

2. Materials and methods

2.1. Preparation of wogonin

Wogonin was kindly provided by the School of Pharmacy, China Pharmaceutical University (lot# 20040702, purity: >99%). It was...
extracted from *Scutellaria baicalensis* Georgi radix according to the protocols reported previously (Hui et al., 2002) and its purity was determined by chromatography–tandem mass spectrometry (Chen et al., 2002). The extract was processed by gelisccation and dissolved to various concentrations with 0.9% normal saline (NS) before administration.

### 2.2. Animals

Beagle dogs were 7–8 months old and 7.5–9.0 kg for the incipient study. They were provided by the Experimental Animal Center of Nanhai Technological Limited Company (Certificate No. SXK2003-0006) and housed individually in the stainless-steel cages in a controlled environment (temperature 18 ± 3°C, humidity 50 ± 20%, 12 h light/12 h dark cycles). Ventilation was given once every 30 min and quantitative pellet diet was given at fixed time each day (Experimental Condition Certificate No. SYXK2003-0002). During a 2-week acclimatization period, parameters including body weight, temperature, appetite and performance of the dogs were observed and recorded before treatment. Animals passing the required routine tests including blood, urine, manure and electrocardiogram tests were selected for the designed experiments.

### 2.3. Experimental design of subchronic study

In this study, test groups I–IV, 6 male and 6 female dogs each, were treated with 60, 30, 15 mg/kg wogonin and vehicle, respectively, via intravenous infusion once daily for 45 or 90 days. The route of administration was consistent with the clinical administration route and the rate of injection was kept constant (5 ml/min) during each treatment. Twenty-four hours after the last administration of 45-day/90-day treatment, 4 dogs (2 females and 2 males) from each group were euthanized and various parameters were measured and compared with the values obtained prior to treatment. The 4 remaining animals in each group were carefully observed for an additional 30 days withdrawal period during which the dogs were fed with normal diet. All animals were then euthanized and various parameters determined.

### 2.4. Body general parameters

Body weight and average food consumption of the dogs were recorded every week. Their body temperatures were measured before and after administration and in the withdrawal period. The animals were observed carefully throughout the experiment, especially the moment after administration. The objective signs including color pattern, cleanliness, behavior, food intake, urine, manure, psychic states, eye and porous channel secretions were measured and recorded every day.

### 2.5. Blood analysis

Blood samples were collected from peripheral veins and taken into the tubes containing EDTA (1.5 mg) and heparin (0.125 mg) for hematological and biochemical analyses. Plasma was prepared by centrifugation at 2500 rpm for 10 min. The pertinent hematological such as blood red cells, white cells, hemoglobin, platelet, average plasctocyte volume were measured by CA-100 hemagglutinin analyser (SYSMEX; Japan). The plasma biochemical parameters such as glucose, total serum cholesterol, alkaline phos-phatase, alanine aminotransferase triglyceride were determined by HITACHI-7020 automatic biochemistry analyzer (HITACHI, Japan). The ions including natrium (Na⁺), potassium (K⁺), chloridion (Cl⁻) and total calcium ion (TCa) were analyzed by NOVA-10 electrolytes analyzer (NOVA, USA).

### 2.6. Autopsy and histopathological study

Animals were sacrificed by exsanguinations and dissected. During the process of dissecting, the color, texture and lump of parenchymatous organs were carefully examined. The color and integrity of the cavities’ mucosa were also examined. In the meantime, the weight of the brain, hypothalons, lungs, heart, thymus glands, liver, spleen, kidneys, adrenal glands, prostate, testicle, venter and ovaries were measured and recorded. Organ-body index was calculated according to the following formula (Liu et al., 2004): Organ-body index (%) = Wet organ weight/Body weight × 100%

Histopathological investigation was done according to methods described previously (Akdogan et al., 2003; Abd-Elhamid, 2004). Briefly, small organ pieces (3–5 mm thick) were fixed in 10% formal-saline (0.9% NaCl in 10% formaldehyde) for 24 h and washed in running water for another 24 h. Samples were dehydrated by passing through 50, 70, 90, and 100% alcohol over a 2-day period, and then cleared in benzene to remove alcohol until the tissues became transparent. This was followed by staining with haematoxylin–eosin and thorough examination using light microscope. In addition to the organs mentioned above, the pancreas, chorda spinalis, hypophysis, cranial nerve, absorbent gland, bladder, and bone marrow (chest bone) were also tested.

### 2.7. Experimental design of plasma pharmacokinetic study

Pharmacokinetic study was carried out according to methods described previously (Jia et al., 2008). Briefly, dogs were divided into two groups according the gender (3 dogs each). All animals were given the abrosia 12 h before the experiment and administered intravenously with single injection of wogonin (20 mg/kg), and the dose was based on the results of the toxicological studies in rats and dogs. Blood samples (0.4 ml) were collected by puncture from the retroorbital sinus before and 5, 10, 20, 30, 45, 60, 120, 240, 360 and 480 min after drug administration. Several blood samples were also obtained from the vehicle control animals which were given 0.9% NaCl via intravenous infusion served to provide the background control. Plasma was obtained from the blood by centrifugation at 3000g for 10 min then stored at −20°C until analysis.

### 2.8. Pharmacokinetic analysis

The quantitative determination of wogonin in plasma was analyzed by a modified sensitive, specific liquid chromatography–tandem mass spectrometry (LC-MS/MS) method (Feng et al., 2002; Buhrow et al., 2006) using the LC-MS/MS apparatus (FINNIGAN TSQ, USA). Pharmacokinetic parameters were estimated from the plasma concentration–time data by non-compartmental analysis. The elimination half-life ($t_{1/2}$) was determined by linear regression of the terminal portion of the plasma concentration–time curve. The area under the plasma concentration–time curve from zero to the last measurable plasma concentration termed $AUC_{0-t}$ was calculated by the trapezoidal rule and the first-order (Eq. 1) or zero-order (Eq. 2) elimination kinetics.
Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Emesia</th>
<th>Hyperptyalism</th>
<th>Somatathenia</th>
<th>Swollen snout</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Emergence time</td>
<td>Frequency</td>
<td>Rank</td>
<td>Emergence time</td>
</tr>
<tr>
<td>I</td>
<td>D2</td>
<td>6/6</td>
<td>+++</td>
<td>D3</td>
</tr>
<tr>
<td>II</td>
<td>D2</td>
<td>4/6</td>
<td>++</td>
<td>D2</td>
</tr>
<tr>
<td></td>
<td>D55</td>
<td>1/6</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>D19</td>
<td>2/6</td>
<td>+</td>
<td>D19</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: D means the day after the administration began. +++ represents severe; ++ represents general; + represents light.

concentration point (AUC∞) was calculated by the linear trapezoidal method. Extrapolation from time zero to infinity (AUC∞) was calculated as follows: $AUC_\infty = AUC_t + C_t / k_e$, where $C_t$ was the last measurable plasma concentration and $k_e$ was the terminal elimination rate constant. The total body clearance (CL) was calculated as dose/AUC∞. The apparent volume of distribution ($V_d$) was calculated as CL/$k_e$.

2.9. Statistical analysis

Group values for all variables in the studies were analysed by the one-way analysis of variance (ANOVA) method. Statistical evaluations of the data were initially tested by the homogeneity of variances. Data with homologous variances were further treated by Student’s t-test. P-values less than 0.05 were considered significant. The Aspin–Welch statistical analysis was employed to evaluate data sets with non-homologous variances.

3. Results

3.1. Effect of long-term intravenous administration of wogonin on the general behavior of dogs

The symptoms including emesis, hyperptyalism, somastathenia, swollen snout accompanied with scratching behavior and discontinuity urine dripping were observed among animals in wogonin-treated groups, especially in group I (60 mg/kg). As shown in Table 1, the grade and frequency of the symptom were in a dose-dependent manner. Animals in group III (15 mg/kg) displayed normal physical behavior compared with those in the control group. The symptoms described above disappeared in the 30-day withdrawal period. No significant difference in the body weigh of the animals treated with wogonin was noted compared with those of the controls during the corresponding period (Fig. 2). The body temperature and food intake and water consumption of the animals treated did not change compared with the control group (data not shown).

3.2. Effect of long-term intravenous administration of wogonin on the hematological and biochemical parameters of dogs

The effect of long-term intravenous administration of wogonin on the hematological and biochemical parameters is presented in Table 2. When dogs were treated for 45 day, the levels of average plastocyte volume (MPV) and plastocyte volume disposition width (PDW) in groups I and II were higher than those of controls, while the changes vanished after the withdrawal period. When treatment extended to 90 days, the MPV and PDW values of the dogs in group I were notably higher ($P < 0.01$) compared with those of controls. Moreover, the level of triglyceride (TG) in animals of group I was higher than those of controls ($P < 0.05$) after the 90th day wogonin treatment. No significant differences were observed for most of other hematological and biochemical parameters between groups ($P > 0.05$). All hematological and biochemical parameters fell within the normal range at the end of the 30-day withdrawal period (data not shown).

3.3. Effect of long-term intravenous administration of wogonin on organs and histopathological changes of dogs

Autopsy study was carried out after the animals were treated for 45 days and 90 days. The organs, including heart, liver, spleen, lung, kidney, adrenal gland, thymus, thyroid gland, brain, uterus, ovary and testis were carefully examined. No noticeable pathologic changes were observed by naked eyes. Moreover, no significant differences in the organ-body weight indices of the organs mentioned above were found (Figs. 3 and 4). Following the 45-day and 90-day wogonin treatment, the histopathological investigations were...
carried out. In the specimens from both batches of animals, inflammatory cell infiltration and cell degeneration were detected in the kidney, lung, bladder, liver, heart, adrenal gland and thymus of few dogs in groups treated with or without wogonin. Other organs including spleen, thyroid gland, brain, uterus, testis, prostate, pancreas, chorda spinalis, typophysis, cranial nerve, absorbent gland, bladder, and bone marrow (chest bone) showed no sign of pathological changes compared with the corresponding organs of the controls. Animals in the withdrawal experiment were sacrificed after the 30 days recovery period. No pathological changes were observed in any organs of these animals (supplementary materials).

3.4. Plasma pharmacokinetic analysis of wogonin in dogs

The time courses of wogonin plasma concentration after intravenous administration to Beagle dogs are shown in Fig. 5, and the pharmacokinetic parameters are summarized in Table 3. Concentration–time curves were typical for wogonin administered via intravenous injection, which were observed incessantly decreasing in 4 h after the treatment. Pharmacokinetic parameters were estimated from the plasma concentration–time data by non-compartmental analysis. After administration of wogonin at the dose of 20 mg/kg, the AUC∞, CL, MRT, Vd and t1/2 of wogonin administered by intravenous injection in dogs were 2137.9 ± 231.4 ng h/ml, 9.45 ± 0.109 L/h/kg, 0.14 ± 0.03 h, 0.68 ± 0.14 L/kg, and 1.51 ± 0.43 h, respectively. Moreover, we observed no gender-based difference in the pharmacokinetic data.

Table 2
Effect of long-term wogonin administered by intravenous infusion on hematological and biochemical parameters in Beagle dogs.

<table>
<thead>
<tr>
<th>Group</th>
<th>WBC (G/L)</th>
<th>RBC (T/L)</th>
<th>MPV (fl)</th>
<th>HGB (G/L)</th>
<th>PDW%</th>
<th>ALP (mmol/l)</th>
<th>T.CHO (mmol/l)</th>
<th>BUN (mmol/l)</th>
<th>TG (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (60 mg/kg) 120 days treatment</td>
<td>11.6 ± 0.6</td>
<td>4.2 ± 0.4</td>
<td>4.4 ± 0.6</td>
<td>4.8 ± 0.3</td>
<td>4.4 ± 0.6</td>
<td>4.8 ± 0.3</td>
<td>4.4 ± 0.6</td>
<td>4.8 ± 0.3</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>Group II (30 mg/kg) 120 days treatment</td>
<td>14.1 ± 0.7</td>
<td>5.0 ± 0.5</td>
<td>5.4 ± 0.7</td>
<td>5.8 ± 0.5</td>
<td>5.4 ± 0.7</td>
<td>5.8 ± 0.5</td>
<td>5.4 ± 0.7</td>
<td>5.8 ± 0.5</td>
<td>5.4 ± 0.7</td>
</tr>
<tr>
<td>Group IV (control) 120 days treatment</td>
<td>18.2 ± 0.9</td>
<td>5.8 ± 0.6</td>
<td>6.2 ± 0.8</td>
<td>6.6 ± 0.6</td>
<td>6.2 ± 0.8</td>
<td>6.6 ± 0.6</td>
<td>6.2 ± 0.8</td>
<td>6.6 ± 0.6</td>
<td>6.2 ± 0.8</td>
</tr>
</tbody>
</table>

Fig. 4. The organ-body weight indices of Beagle dogs after 30-days withdrawal period. The organ-body weight index was calculated according to the following formula: Organ-body weight index (%) = Wet organ weight/Body weight × 100.

Fig. 5. Mean plasma concentration–time profiles of wogonin (20 mg/kg) after intravenous injection to Beagle dogs.

Table 3
Pharmacokinetic parameters of wogonin after single intravenous injection (20 mg/kg) in Beagle dogs (mean ± SD).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Gender group</th>
<th>All (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC∞ (ng h/ml)</td>
<td>2141.3 ± 354.2</td>
<td>2134.5 ± 74.7</td>
</tr>
<tr>
<td>CL (l/h/kg)</td>
<td>9.52 ± 1.68</td>
<td>9.38 ± 0.33</td>
</tr>
<tr>
<td>Vd (L/h)</td>
<td>0.68 ± 0.12</td>
<td>0.67 ± 0.07</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>1.55 ± 0.56</td>
<td>1.45 ± 0.31</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>0.14 ± 0.04</td>
<td>0.14 ± 0.01</td>
</tr>
</tbody>
</table>

AUC∞: area under the curve from zero to infinity; CL: plasma clearance; Vd: apparent volume of distribution; t1/2: elimination half life; MRT: mean residence time.
4. Discussion

Polyphenolic compounds are widely distributed in plants and human diets (Lee et al., 2002). Flavonoids are one type of polyphenols and which include flavones, isoflavones, and flavanones. In contrast to the beneficial effects, flavonoids have also been found to be harmful (Uhl et al., 2003; Ammar et al., 2008). Wogonin, one of the flavonoid compound, extracted from the root of Scutellaria baicalensis Georgi, was addressed extensive pharmacologically activity in practice, especially the potent anticancer activity demonstrated recently.

It was reported that little wogonin was detected in rat plasma after by intragastric administration (Yan et al., 2007). Due to the low oral absorption of wogonin, the animals were treated by intravenous administration for the present studies. Based on the clinical administration method, intravenous infusion was chosen as the way of the administration and it is the most appropriate way to maintain constant maternal plasma concentration. Moreover, we have shown that a long period of treatment with a high dose of wogonin (120 mg/kg) could induce heart injury in rats according to the subchronic toxicological studies using Sprague–Dawley rats (Qi et al., 2009). Based on this knowledge, 15, 30 and 60 mg/kg were chosen as the low, middle and high dosage in the present study. The dosages used were 18–72 times (body weight) or 9.6–38.5 (body surface area) higher than the human therapeutic dosage, which are within the acceptable range for dog models (Means et al., 1989; Bruchim et al., 2006).

Beagle dogs were treated with wogonin via intravenous infusion every day for 90 days. Typical toxicological responses such as emesis, hyperpalsia, somatesthesia, swollen snout accompanied with the scratching and the discontinuity urine dripping were observed among animals in treatment groups, especially in group I (60 mg/kg). The grade and frequency of the symptom were in a dose-dependent manner, which indicated that wogonin could affect the normal physiological status of the animals. Since body weight loss has been used as an indicator of adverse effects of drugs and chemicals (Tofovic and Jackson, 1999), the results suggested that the toxic effects of wogonin were transient at the doses used in this study. There were some changes on the level of average plasctocyte volume, plasctocyte volume disposition width and triglyceride of the dogs treated with wogonin (Table 2). However the diversification of these parameters was within normal fluctuation according to the standards in the Guide for Chemicals Long-term Toxicity Test of China and the values had no correlation with the doses, and therefore had no clinical significance. We have previously reported that long period of treatment with a high dose of wogonin (120 mg/kg) induced reversible heart injury in rats. Therefore, finding the target organs of wogonin in dogs is prominent. Results of autopsy study showed that notable change caused by long period wogonin treatment in organs. As for the histopathological examination, no parenchymatous changes were detected except for some inflammatory cell infiltration and cell degeneration in the organs of the animals from both 45-day and 90-day treatment batches as well as the animals in corresponding control groups, which may caused by the individual difference on account of the breeding and circumstance. Moreover, parameters of the electrocardiogram of the wogonin-treated dogs were quite normal (data not shown). Collectively, our data demonstrated that long period of wogonin treatment caused no organs injury in dogs and addressed the clear evidence of the safety.

As to the pharmacokinetic study in Beagle dogs, plasma concentration–time profiles were obtained and these results helped to characterize the systemic exposure to wogonin via intravenous infusion. Moreover, no difference was detected between the genders. The data of this study will help to establish appropriate dosage, frequency, and treatment duration in clinical applications of this promising anticancer drug.

5. Conclusions

In the present study, we carried out the subchronic toxicity and plasma pharmacokinetic studies to assess the safety of wogonin with Beagle dogs. Wogonin was administered via intravenous infusion at dosages of 60, 30 and 15 mg/kg per day for 90 days followed by general body parameters, hematochemical, plasma biochemical, histopathological, and viscera examinations. The weight of animals in the treatment groups remained the same as the control group. And wogonin did not affect the levels of plasma biochemical parameters including plasma aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and creatinine kinase. In pathological examination, there were no morphological changes in the organs of the wogonin-treated animals. Our results demonstrate that wogonin offers a wide margin of safety and has no organ toxicity for the long time intravenous administration in dogs. A non-organ-toxic dosage of wogonin administered via intravenous administration is 60 mg/kg. It is approximately 72.0 (body weight) or 38.5 (body surface area) times higher than that of the dose (50 mg/60 kg) used for human trials. The AUC(0-24) and t1/2 of wogonin administered by intravenous injection in dogs were 21373±231.4 ng.h/ml and 1.51 ± 0.43 h, respectively. It is anticipated that the results presented here provide a foundation for the further clinical research and evaluation of this promising anticancer agent.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.jep.2009.04.031.

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