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Developmental toxicity and genotoxicity studies of wogonin

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ABSTRACT

We studied the developmental toxicities and genotoxic potency of a widely bioactive plant medicine-wogonin *in vivo* and in vitro. In the *in vivo* developmental experiments, high dose of wogonin (40 mg/kg, intravenous injection) significantly induced the maternal weight gains and affected fetus including body-weight, resorptions, live birth index and fetal skeletal alterations. In Ames test, no concentration-dependently increased TA98, TA100, and TA102 revertants were detected in wogonin groups whether in presence of metabolic activating enzymes or not. In the chromosome aberration test, wogonin dose-dependently increased structural chromosomal aberrations in CHL cells both with and without S9, even the effect was all judged (−). In micronucleus assay, no significant changes of MNPCE/PCE and PCE/NCE were found on mouse bone marrow micronucleus in wogonin groups. We concluded that wogonin induced developmental toxicities on pregnant mice and fetus, and the genotoxicities were positive. However no significant malformation was observed and only in vitro potency of chromosome aberration was weak, which suggested us wogonin could be a relatively safe drug in clinic.

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1. Introduction

Scutellaria baicalensis Georgi is a traditional Chinese medicinal herb mainly harvested from the northern part of China. *S. baicalensis* Georgi radix has been used as a traditional drug in oriental countries for various purposes (Lim et al., 2003). One of the active components in the plant is 5,7-dihydroxy-8-methoxyflavone, also known as wogonin (Fig. 1). Wogonin is a flavonoid and has been shown to exert antioxidant (Gao et al., 1999), antiviral (Ma et al., 2002; Guo et al., 2007), antithrombotic (Kimura et al., 1997) and anti-inflammatory (Park et al., 2001) activities. Recently, more and more studies focused on its antiproliferative and apoptosis inducing activity in human tumor cells (Chow et al., 2008; Lee et al., 2008; Li-Weber, 2009). In addition, it's differentiation inducing effect (Zhang et al., 2008) and potential effect of overcoming multidrug resistance (Lee et al., 2009) were reported. Based on the wide use and pharmacological research, the acute and sub-chronic toxicities and plasma pharmacokinetic of wogonin were determined (Peng et al., 2009; Qi et al., 2009) in our lab. The LD50 of 286.15 mg/kg on mice and some heart injury in rats by long period of wogonin treatment were shown. Besides, a wide margin of safety and no significant organ toxicity in dogs for a long time intravenous administration was detected. For advanced studies on toxicities of wogonin, the developmental toxicities and genotoxicities were investigated in this paper. The studies were followed and conducted in accordance with 'Chemical Toxicity Test Technique Guideline' issued by State Food and Drug Administration (SFDA of China). We hope the results presented in this paper should be helpful to establish the safe dosage, frequency and therapeutic duration in clinical applications of this natural product.

2. Materials and methods

2.1. Test material

Wogonin was prepared at China Pharmaceutical University, Nanjing, China (purity:>99%). It was dissolved at various concentrations in DMSO or physiological saline before use. For *in vivo* experiments, wogonin was administrated intravenously (*i.v.*) once a day.

2.2. Chemicals

Fetal bovine serum (FBS), penicillin/streptomycin, RPMI-1640, and trypsin were obtained from Gibco BRL (Grand Island, NY). 3-methylcholanthrene, 4-nitroquinoline-N-oxide (4-NQO), 2-aminoanthracene (2AA), sodium azide, colcemid and 2-aminofluorene

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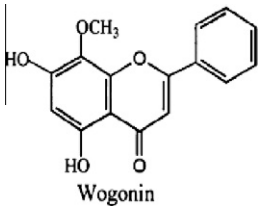

Fig. 1. Chemical structure of wogonin. Molecular formula: $C_{16}H_{12}O_5$; molecular weight: 284.26.

(2-AF) were obtained from Sigma Chemical Co. Ltd. Cyclophosphamide (CP), mitomycin c (MMC), daunorubicin, acrichine was supplied by Jiangsu Heng Rui medicine Co., Ltd. (China).

2.3. Animal husbandry

For developmental toxicity study, female Sprague–Dawley (SD) rats weighing 230 ± 20 g and male SD rats weighing 300–400 g were obtained from Shanghai Sipper-bk Animal Co., Ltd. (China). They were maintained in pathogen-free stainless steel cages at 24 ± 2 °C with a standard 12 h light/12 h dark cycle and allowed free access to tap water and food.

For Ames test, Wistar rats (200 ± 20 g) were used for preparation of the liver microsomal (S9) fraction. For the erythrocyte micronucleus studies, healthy Kunming albino mice of both sexes with body weight of 25 ± 1 g were used. All the Wistar rats and Kunming albino mice were provided by Animal Facility at China Pharmaceutical University. They were maintained under the same conditions as described above and were allowed free access to tap water and food.

All the housing conditions and test operations were carried out according to GLPs of the People's Republic of China.

2.4. Dose level selection

A pilot developmental toxicity study was conducted to assist in setting dose levels for the main developmental study. Three groups of pregnant female rats were exposed to concentrations of 80, 60 and 40 mg/kg of wogonin over days 6–15 of gestation, two rats in every group; the day of confirmed mating was designated as gestation day 0 (GD0). Except for slightly reduced weight gains, no adverse effects were observed at 40 mg/kg. At 80 mg/kg, one animal died at the 11th day of pregnancy (GD11). Significant maternal toxicities such as increased number of resorptions and reduced live fetuses were observed at 60 mg/kg. So the concentrations of 40, 13.3 and 4.4 mg/kg were selected for the developmental toxicity study.

For genotoxicity studies in vitro (Ames test), dose levels of wogonin in preliminary test were 5000, 2500, 1250, 625, 313, 156, and 0 μg/plate. In the groups of 5000 and 2500 μg/plate, the significant bacteriostatic actions were observed (data were not shown). Based on the results, dose levels of 1250, 125, 12.5, 1.25 and 0.125 μg/plate were used.

For chromosomal aberration test, Chinese hamster lung fibroblast (CHL) cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS. In the direct method, cells were seeded at a density of 2.2×10^4 cells per dish on the third day, the cells were treated with wogonin for 48 h. An IC50 of 15.697 μg/ml was calculated by probit analysis. Based on the results, the high dose was determined as 16 μg/ml, the middle and low doses were 8 and 4 μg/ml.

For micronucleus studies, two groups of mice, with 10 each (5 male and 5 female) were exposed to concentrations of 143.0 and 71.5 mg/kg of wogonin once by intravenous injection. The high dose was determined as the half of the LD50 value of wogonin on mice (Qi et al., 2009). In 12–72 h after injection, at every time point (12, 18, 24, 48 and 72 h), 2 mice (1 male and 1 female) were sacrificed. No frequency of micronuclei in polychromatic was detected. So the time point of 24 h and the doses of 143.0, 71.5 and 35.8 mg/kg were selected.

2.5. Experimental procedures

2.5.1. Developmental toxicities in rats

Female rats were mated overnight at the proportion of three females to each male. Vaginal smears were collected daily and examined in optics microscopy for the presence of sperm. The day of sperm detected in vaginal smears was designated as gestation day 0 (GD0). The mated females were randomly assigned to different experimental groups (15 rats per-group) and treated by 40, 13.3 and 4.4 mg/kg of wogonin and vehicle alone once everyday by *i.v.* in a volume of 2 ml/kg from day 6 to day 15. The maternal body weights were measured on 0, 3, 7, 10, 13, 16 and 20 days of pregnancy gestation. Food consumption was measured on days 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, and 20 of gestation. Individual clinical observations were recorded each morning and each afternoon of the exposure period.

At gestation day 20, the rats were killed and the fetuses were removed immediately. The following data were recorded: total litter size, birth weights, live birth index [(number of live offspring/number of offspring delivered) × 100%], sex ratio and external alterations (Muller et al., 2009). Approximately one-half of the live fetuses were decapitated prior to dissection and fixed in Bouin's solution and subsequently examined for visceral alterations using a free-hand sectioning technique (Wilson, 1965). The remaining fetuses were fixed in ethanol, eviscerated, macerated in 1% aqueous potassium hydroxide solution, stained with alizarin red S, and examined for skeletal alterations (Staples and Schnell, 1964).

2.5.2. S9 fraction preparation

Rat liver S9 used for metabolic activation was prepared as described previously (Maron and Ames, 1983). To obtain the liver microsomal fraction, each Wistar rats was administered by intraperitoneal injection with 3-methylcholanthrene (30 mg/kg) every day, and 4 days later the rats were killed by cervical dislocation. The livers were homogenated, diluted 1:4 with 0.15 M KCl, and centrifuged for 10 min at 9000*g*. The supernatant was pulled and diluted (giving a protein concentration of 30 mg/ml), frozen in small aliquots, and stored at −196 °C until use.

The final preparation of the metabolizing system (S9 mixture) was made in accordance with the protocol of (Ames et al., 1975). The composition and final concentrations of the S9 mix were as follows: glucose-6-phosphate, 4.4 mM; nicotinamide-adenine dinucleotide phosphate (NADP), 0.84 mM; KCl, 30 mM; $NaHCO_3$, 0.032%; and S9 fraction, 10% (Cheng et al., 2004).

2.5.3. In vitro genotoxicity studies (Ames test)

The method followed the recommendations of Maron and Ames (Maron and Ames, 1983). The *Salmonella typhimurium* bacteria and histidine auxotrophic strains TA97, TA98, TA100, and TA102 were obtained from Shanghai Institute for Drug Control and grown for 14 h at 35 ± 2 °C with continuous shaking. Bacteria were grown to a density of 2×10^9 cells/ml with OD600 absorbance of 0.2–0.3. Top agar, containing 2 ml of heated agar, 0.1 ml of test chemical, 0.1 ml of bacteria, and 0.5 ml of S9 solution, was mixed up and added to three different minimal glucose agar plates. All plates were incubated at 37 °C for 48 h, and the number of bacteria colonies was determined. The entire experiment was replicated again on a different day with a total of six plates for each concentration of wogonin with and without S9. S9 liver cell extracts contain

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