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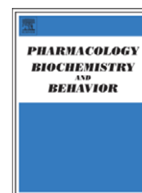

Dopamine transporter inhibitory and antiparkinsonian effect of common flowering quince extract

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ABSTRACT

Common flowering quince (FQ) is the fruit of *Chaenomeles speciosa* (Sweet) Nakai. FQ-containing cocktails have been applied to the treatment of neuralgia, migraine, and depression in traditional Chinese medicine. The present study assessed whether FQ is effective in dopamine transporter (DAT) regulation and antiparkinsonism by utilizing *in vitro* and *in vivo* assays, respectively. FQ at concentrations of 1–1000 μg/ml concentration-dependently inhibited dopamine uptake by Chinese hamster ovary (CHO) cells stably expressing DAT (D8 cells) and by synaptosomes. FQ had a slight inhibitory action on norepinephrine uptake by CHO cells expressing the norepinephrine transporter and no inhibitory effect on γ-aminobutyric acid (GABA) uptake by CHO cells expressing GABA transporter-1 or serotonin uptake by the serotonin transporter. A viability assay showed that FQ mitigated 1-methyl-4-phenylpyridinium-induced toxicity in D8 cells. Furthermore, in behavioral studies, FQ alleviated rotational behavior in 6-hydroxydopamine-treated rats and improved deficits in endurance performance in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. Furthermore, immunohistochemistry revealed that FQ markedly reduced the loss of tyrosine hydroxylase-positive neurons in the substantia nigra in MPTP-treated mice. In summary, FQ is a selective, potent DAT inhibitor and has antiparkinsonian-like effects that are mediated possibly by DAT suppression. FQ has the potential to be further developed for Parkinson's disease treatment.

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1. Introduction

FQ is the dried, nearly ripe fruit of *Chaenomeles speciosa* (Sweet) Nakai (Family Rosaceae) that is mainly distributed in China, with centers of origin in Anhui, Yunnan, Shanxi, Gansu, Guizhou, Sichuan, and Guangdong Provinces. The nutritional and edible fruit has been widely used for its beneficial health effects for hundreds of years in China by processing it into preserved fruit, canned food, wine, vinegar, syrup, and hair- and skin-care products. Clinically, FQ is commonly used as a therapeutic agent for the treatment of rheumatism, cholera, enteritis, and beriberi. In addition, FQ-containing prescriptions (oral herbal cocktails) contain 9–30 g of FQ and have been widely used in folk medicine for the treatment of neuralgia, migraine (He and Jiang, 2006), stroke (Wang, 2006), depression (An and Cheng, 2007), and other diseases with symptoms of limb tremors or spasms (dyskinesias) resembling symptoms of Parkinson's disease. Such therapeutic applications suggest that FQ may provide significant antiparkinsonian benefit.

Parkinson's disease is a chronic neurodegenerative disorder characterized by massive and progressive degeneration of the nigrostriatal dopaminergic system (Park et al., 2003), resulting in abnormal motor behaviors such as muscular rigidity, postural abnormality, and tremor (Blum et al., 2001). However, the etiopathogenesis of dopaminergic loss in Parkinson's disease remains unclear (Sulzer, 2007; Rosner et al., 2008). Current evidence points to the presence of ongoing oxidative stress and the generation of radical oxygen species due to inhibition of complex I selectively in the substantia nigra leading to aggregation of α-synuclein and subsequent loss of dopamine neurons (Chiueh et al., 2000; Dawson and Dawson, 2003; Eberhardt and Schulz, 2003). L-DOPA administration is the most commonly employed treatment (Agnati et al., 2004), but it only alleviates clinical symptoms in the early stages of the disease and is less effective as the disease progresses (i.e., when side effects of on–off fluctuations and dyskinesias occur) (Ahlskog and Muenter, 2001; Hurtig, 1997). No therapy has yet been made available that slows or halts the neurodegeneration associated with Parkinson's disease (Ravina et al., 2003).

The dopamine transporter (DAT) is an important regulating protein involved in dopaminergic transmission. The membrane protein belongs

Abbreviations: FQ, common flowering quince; i.p., intraperitoneal; i.g., intragastric; s.c., subcutaneous; DAT, dopamine transporter; NET, norepinephrine transporter; SERT, serotonin transporter; GABA, γ-aminobutyric acid; GAT-1, γ-aminobutyric acid transporter; CHO, Chinese hamster ovary cells; 6-OHDA, 6-hydroxydopamine; GBR12,935, (1-[2-(diphenylmethoxy)ethyl]-4-[3-phenylpropyl]-piperazine); MTT, methyl thiazolyl tetrazolium acid; HBSS, Hank's balanced salt solution; PBS, phosphate-buffered saline; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP^+, 1-methyl-4-phenylpyridinium; TH, tyrosine hydroxylase.

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to Na^{+}/Cl^{-}-dependent neurotransmitter transporters, is expressed mainly in the extrapyramidal system, and plays crucial roles in limiting dopamine activity in the central nervous system (Hersch et al., 1997; Nelson, 1998). Compounds that directly (Hansard et al., 2002b) or indirectly (Joyce et al., 2004) inhibit DAT activity may be useful in the treatment of the motor symptoms of Parkinson's disease. Some DAT inhibitors have been shown to have antiparkinsonian-like effects in animal models (Nutt et al., 2004) via binding to presynaptic DAT, and thereby blocking dopamine reuptake back into the synaptic cleft and enhancing dopaminergic neurotransmission by activating dopamine receptors (Torres et al., 2003). Previous studies have shown that DAT inhibitors such as amphetamine (Parkes et al., 1975), nomifensine (Teychenne et al., 1976; Park et al., 1981), bupropion (Goetz et al., 1984), and mazindol (Delwaide et al., 1983) have beneficial effects in patients with advanced Parkinson's disease.

We therefore hypothesized that FQ may have an inhibitory effect on DAT and a regulatory effect on the abnormal extrapyramidal system manifested in an animal model of Parkinson's disease. In this study, we screened hundreds of Chinese herbs by a transgenic cell-line screening system and discovered that FQ indeed had an inhibitory effect on DAT and an antiparkinsonian-like effect in two Parkinson's disease models.

2. Materials and methods

2.1. Preparation of aqueous extract of FQ

FQ, a dried fruit of *C. speciosa* (Sweet) Nakai, was bought from Anhui Province, China, and was identified and authenticated by an expert herbalist at the Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The voucher specimen (no. 20050708) was deposited at the Herbarium of Cell-Star Bio-Technologic Co., Ltd. (Shanghai, China). The specimen was washed, crushed, and homogenized. The crushed aggregate was diluted in distilled water (weight:volume, 1:10) and heated at 100 °C for 1 h. The supernatants were collected, and the remaining portion was diluted and heated further for 1 h to yield additional extract. The two extract solutions were mixed, filtered, and centrifuged (2000 ×*g* at 20 °C) for 10 min to remove any water-insoluble components. The supernatants were lyophilized in a freeze dryer. The dried powder (yield rate 20.9%) was collected and stored at −20 °C for experimental use. Prior to experimentation, FQ extract powder was redissolved in 0.9% normal saline or distilled water at different concentrations for *in vivo* or *in vitro* study, respectively.

2.2. Cell culture and DAT transfection

CHO cells expressing rat DAT, rat serotonin transporter (SERT), mouse GABA transporter (GAT-1), or human norepinephrine transporter (NET) were used in this study, and the transgenic methods have been described previously (Liu et al., 2001; Xu et al., 2006). Rat DAT-pCDNA3 was transferred to CHO cells through electroporation. Selection of the transfected cells was conducted in culture by the addition of Geniticin (G418). CHO cells stably expressing DAT then were subcloned by limiting dilution methods. Several subclones were selected using a $[^3H]$dopamine uptake assay. The cell clone with the highest uptake, designated as D8 cells, was chosen for further experimentation. Similarly, a clone highly expressing GAT-1, SERT, or NET (designated as G1 cells, S6 cells, and N1 cells, respectively) was obtained. Fig. 1 shows 20- to 30-fold enhancement of neurotransmitter uptake after the CHO cells were transfected with the corresponding neurotransmitter transporter. GBR12,935 (selective DAT reuptake inhibitor), desipramine (NET inhibitor), fluoxetine (SERT inhibitor), and tiagabine (GAT-1 inhibitor), at concentrations of 1, 10, 10, and 10 μM, respectively, significantly inhibited the enhanced uptake, thus confirming the validity of the screening model.

2.3. $[^3H]$Dopamine uptake inhibitory assay in vitro

D8 cells were grown in RMPI1640 medium (Gibco BRL Life Technologies) containing 10% fetal bovine serum (Gibco BRL Life Technologies) to near confluence in 48-well tissue culture plates (Costar) (approximately 60,000 cells per well). D8 cells then were rinsed once with phosphate-buffered saline (PBS) and pre-incubated in 100 μl Hank's balanced salt solution (HBSS) at room temperature for 10 min. $[^3H]$Dopamine (8.8 Ci/mmol, Amersham Pharmacia Biotech), ascorbic acid, and pargyline were added to final concentrations of 0.1, 100, and 100 μM, respectively. Cells then were incubated at room temperature for another 20 min. The reaction was terminated by aspiration of the HBSS, and the cells were washed three times rapidly (10 s/wash) with ice-cold PBS followed by solubilization in 2 N NaOH. An aliquot was measured by a liquid scintillation counting analyzer (TRI-CARB2900TR, Pakard) to quantify $[^3H]$dopamine uptake. For the study of the inhibitory effect of FQ, different concentrations of FQ and GBR12,935 solutions were added at the beginning of the uptake assay. For serotonin and norepinephrine uptake assays, the procedures were similar to that for dopamine uptake assay in D8 cells, with the exception that 50 nM $[^3H]$serotonin (111 Ci/mmol, Amersham Pharmacia Biotech) or 25 nM $[^3H]$norepinephrine (40 Ci/mmol, Amersham Pharmacia Biotech) was used instead of $[^3H]$dopamine for S6 cells or N1 cells, respectively. For the GABA uptake assay, 50 nM $[^3H]$GABA (88 Ci/mmol, Amersham Pharmacia Biotech) was used instead of $[^3H]$dopamine and ascorbic acid and pargyline in the system for G1 cells. The concentration–effect curve was established to measure IC_{50} values (concentration required to inhibit specific dopamine uptake by D8 control cells by 50%), and E_{max} values (maximal effect on dopamine uptake inhibition) and EC_{50} values (effective concentration to reach 50% of E_{max}) were analyzed by nonlinear aggression. Absolute inhibition (%) = [(DPM value of vehicle-treated D8 cells − DPM value of drug-treated D8 cells)/(DPM value of vehicle-treated D8 cells − DPM value of background)] × 100%, where DPM indicates the disintegrations per minute.

2.4. Uptake of dopamine by striatal synaptosomes

The procedure has been described elsewhere (Kokoshka et al., 1998) with some modifications. Briefly, male Sprague–Dawley rats (200–250 g) were sacrificed by decapitation, and the striatum was dissected out. Fresh striatal tissues were homogenized with a glass homogenizer with 10 to 20 strokes in ice-cold 0.32 M phosphate-buffered sucrose and centrifuged (1000 ×*g* at 4 °C) for 10 min. The supernatants (S1) then were

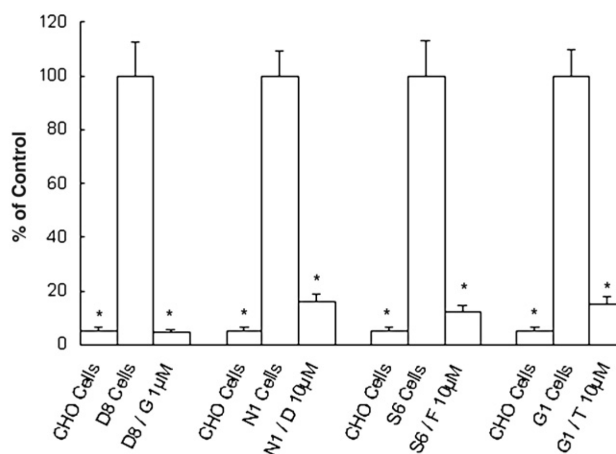

Fig. 1. Functional confirmation of *in vitro* screening systems. Dopamine, norepinephrine, serotonin, and GABA uptake assays were conducted in transgenic CHO cells expressing DAT (D8 cells), NET (N1 cells), SERT (S6 cells), or GAT-1 (G1 cells), respectively. G (GBR12,935), D (desipramine), F (fluoxetine), and T (tiagabine) are selective inhibitors of DAT, NET, SERT, and GAT-1, respectively. $*P<0.001$ compared with control group (D8, G1, S6, and N1 cell groups). Values are expressed as mean ± SEM of triplicate cell samples.

centrifuged (22,000 ×g at 4 °C) for 15 min, and the resulting pellets (P2) were resuspended in ice-cold 0.32 M phosphate-buffered sucrose solution. Aliquots (resuspended P2) were preincubated in Na^+-Krebs Ringer Henseleit medium (pH 7.4) containing NaCl 103 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM, KH_2PO_4 1 mM, $NaHCO_3$ 27 mM, and glucose 5.4 mM at 37 °C for 5 min. The incubation was continued in the same medium containing 100 μM pargyline, 100 μM ascorbic acid, and 100 nM [^{3}H] dopamine (100 μl final volume) for an additional 10 min. The reaction was stopped by dilution with 1.2 ml of ice-cold Li-Krebs Ringer Henseleit medium (in which NaCl was substituted by LiCl) and centrifuged (12,000 ×g at 4 °C) for 10 min. Pellets were resuspended with 1.2 ml of ice-cold Li-Krebs Ringer Henseleit medium and centrifuged again as described above. The pellets then were decomposed by adding 2 N NaOH 100 μl, and an aliquot was used for determination of radioactivity. The specific uptake of dopamine was defined as the difference between total uptake in the Na-Krebs Ringer Henseleit medium, and nonspecific accumulation was determined in the Li-Krebs Ringer Henseleit medium. To detect the effect of FQ on dopamine uptake by striatal synaptosomes, different concentrations of FQ and GBR12,935 were added at the beginning of uptake. EC_{50} then was determined. Absolute inhibition (%)=[(dopamine uptake produced by vehicle−dopamine uptake produced by drug)/(specific dopamine uptake produced by vehicle)]×100%.

2.5. Effect of FQ on D8 cell viability after MPP^+ (1-methyl-4-phenylpyridinium) administration

D8 cells were cultured in RMPI1640 medium and inoculated in 96-culture wells (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The cells were grown to near confluence at about 20,000 per well after 24-h incubation. FQ at various concentrations then was added to the wells and incubated for 30 min. MPP^+ subsequently was added into the wells to a final concentration of 33.3 μM with a total volume of 200 μl. The same volumes of PBS and GBR12,935 (1 μg/ml) were used as blank control and positive control, respectively. Later, the plate cells went through a 24-h incubation followed by the addition of methyl thiazolyl tetrazolium (MTT) reagent with a final concentration of 0.5 mg/ml. The dyeing reaction continued for 4 h until purple precipitates were visible. Finally, the cells were solubilized by 100 μl dimethylsulfoxide following a 30-min incubation at room temperature, and optical density (OD) values were recorded at 570 nm in Multiscan MK3 (Nova and Technology Development Co. Ltd., Beijing, China). EC_{50} values for FQ and GBR12,935 were defined as the effective concentration to reach 50% of E_{max}. Protection rate=[(OD value of FQ-treated toxic cells−OD value of toxic cells)/(OD value of control cells−OD value of vehicle-treated toxic cells)]×100%.

2.6. Animals

Adult male Sprague–Dawley rats (weighing 200–220 g, 2 months of age) and male C57BL/6 mice (weighing 30–35 g, 14 months of age) were purchased from the Laboratory Animal Center of the Chinese Academy of Science (Shanghai, China). Animals were housed at room temperature (22±3 °C) under standard 12-h light/dark cycles (lights on at 07:00 AM) with unlimited access to food and water. Animals were housed for 1 week prior to the experiments. The experimental protocols were approved by the Laboratory Animal Center of the Chinese Academy of Science. All procedures were in accordance with the National Institutes of Health's guidelines regarding the principles of animal care.

2.7. Effect of FQ on behavioral abnormality in 6-hydroxydopamine (6-OHDA)-lesioned rats

Rats were anesthetized with chloral hydrate [0.5 g/kg, intraperitoneal (i.p.)] and given a unilateral stereotaxic injection of 12 μg 6-OHDA (to elicit mild parkinsonian symptoms) or 20 μg 6-OHDA (to elicit moderate parkinsonian symptoms) in a volume of 4 μg/μl with 0.2 mg/ml L-ascorbic acid into the right striatum (coordinates from bregma: anterior/posterior 0.7 mm, medial/lateral 2.6 mm, dorsal/ventral 4.5 mm). The injection rate was 1 μl/min using a 26-gauge Hamilton microsyringe. After surgery, the animals were returned to their home cages for 2 weeks. Rotational behavior then was tested, and the number of complete 360° turns (contralateral to the lesioned side) per 30 min after apomorphine challenge [0.5 mg/kg, subcutaneous [s.c.]) was recorded. Rats that exhibited 60 to 120 turns per 30 min (mild parkinsonism) and 150 to 250 turns per 30 min (moderate parkinsonism) were screened for comparative pharmacologic evaluation using the two distinct lesion models. Rats in each parkinsonian model were randomly split into two groups: model group [n=10; normal saline, intragastric (i.g.)] and treatment group (n=10; FQ 0.5 g/kg body weight, i.g.). After group assignment, the animals received once-daily administration of FQ or normal saline for 8 weeks (mild model) or 14 weeks (moderate model). Apomorphine-induced rotational behavior was rated by observers blind to treatment every 1 week (mild model) or 2 weeks (moderate model) after treatment onset. The net number of rotations (contralateral minus ipsilateral) was recorded over 30 min.

2.8. Effect of FQ on behavioral and neurodegenerative changes in MPTP-treated mice

2.8.1. Rotarod test

The rotarod consisted of a rotating spindle (6 cm diameter) and five individual compartments. Prior to the experiment, C57BL/6 mice were trained twice daily for two successive days to acclimate to the rotarod system. Briefly, animals were placed on the rod, and the rod was turned such that the mice were required to run at a speed of 12 rotations per minute for 120 s. For the last training session, retention time (i.e., duration of time from beginning rod rotation to the mouse falling from the rod; Rozas et al., 1998) was measured three times within a span of 300 s. The mean of the three measurements represented overall performance. Mice with a retention time of more than 200 s were randomly divided into five groups: Group A (vehicle control), Group B (model), Groups C and D (treatment groups), and Group E (reference group) (n=8). Drugs then were administered to each group as follows. Mice in Groups B, C, D, and E were treated with the same volume of 0.9% normal saline (i.g.), FQ 0.25 g/kg or 1 g/kg (i.g.), or GBR12,935 5 mg/kg (i.p.), respectively, 30 min prior to i.p. injection with MPTP (25 mg/kg). Group A was administered saline and MPTP. All mice received once-daily treatment with drugs and MPTP for three successive weeks. Six hours after the last administration (Day 21), the mice were subjected to rotarod assessment as described above.

2.8.2. Tyrosine hydroxylase (TH) immunostaining

Mice were decapitated, and brains were removed from the cranium immediately after the behavioral evaluation. For immunohistochemistry, brains were washed in vials containing PBS and placed in paraformaldehyde solvent for 24-h fixation and then immersed in a phosphate-buffered sucrose solution for storage at 4 °C for at least 24 h prior to sectioning. Brains were frozen using a cryostat and sectioned into 40 μm-thick sections by a microtome. A polyclonal anti-TH antibody (Chemicon International, Temecula, CA, USA) and a Vectastain elite ABC kit (Vector Laboratories, Burlingame, CA, USA) were used to process immunohistochemistry for detection of TH protein. In brief, the sections were incubated in phosphate-buffer solution mixed with 3% goat serum and 0.2% Triton X-100 for 12 h, and then in rabbit anti-mouse TH antibody solvent at a dilution of 1:3000 at 4 °C for 48 h. Afterward, the sections were washed in 0.01 M PBS (pH 7.5) three times (5 min each). Sections then were incubated with a secondary antibody (biotinylated anti-rabbit IgG, Vector Laboratories, Burlingame, CA, USA) at 37 °C for 1.5 h and with ABC solution for an additional 1 h. After being washed in PBS (three times) and Tris buffer (once), the sections were reacted with 0.05 M Tris buffer containing 0.02% diaminobenzidine and 0.003% hydrogen peroxide (H_2O_2) for 5 min. The dyeing sections were rinsed with PBS buffer, mounted on gelatin-coated slides, air-dried,

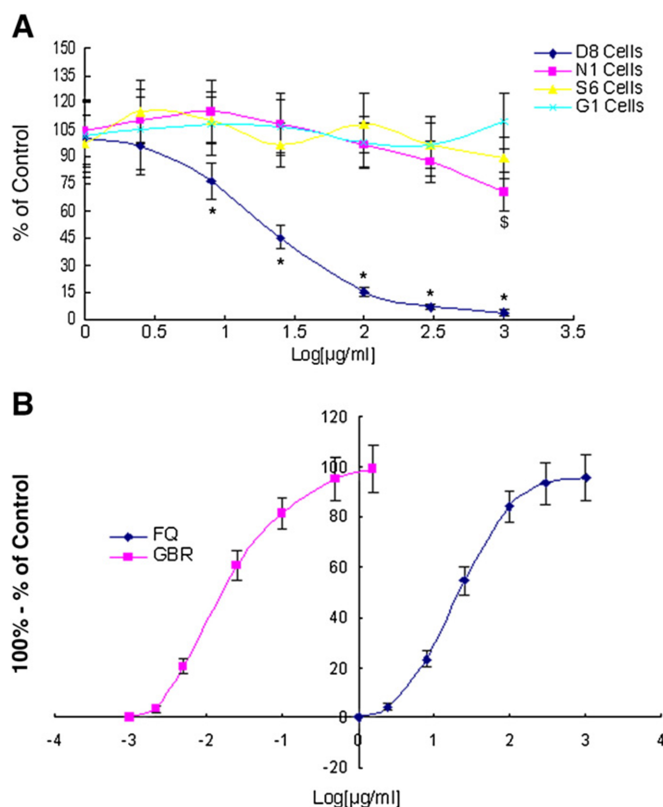

Fig. 2. Selective inhibitory effect of different concentrations of FQ on [^{3}H]dopamine uptake by D8 cells. Uptake assays were conducted after D8, N1, S6, and N1 cells were incubated with different concentrations of FQ. The results showed that FQ significantly and specifically inhibited DAT (A), wherein *Y*-axis represents percentage of uptake in the four types of control cells. The significant differences in DPM values between groups were analyzed by ANOVA. $^{\$}P<0.01$, $^{*}P<0.001$ compared with D8 and N1 control cells, respectively. (B) The different concentration–effect curves between FQ and GBR12,935. The absolute inhibition on the *Y*-axis was obtained by 100% (normalized uptake by control cells) minus percent of control (normalized uptake by drug-treated cells). EC_{50} and E_{max} values are summarized in Table 1. Values are expressed as mean ± SEM of three experiments performed in triplicate.

dehydrated, and coverslipped using mounting fluid. For each animal, mesencephalic sections were examined at four similar coronal levels of the medial mammillary nucleus, posterior part, in each animal, and TH neuron counting was performed by experimenters blind to treatment condition and manually by light microscopy using a superimposed grid to facilitate the procedure. The number of TH-positive neurons of each mouse was expressed as the average counts obtained from the representative sections.

2.9. Data analysis

Data were analyzed using SPSS software v.13.0 (Chicago, IL, USA). Values are expressed as mean ± SEM. Analysis of variance (ANOVA) followed by least significant difference (LSD) *post hoc* tests was used to examine differences between groups, with the exceptions that behavioral changes in 6-OHDA-lesioned rats and differences in EC_{50} and E_{max} values between the FQ cell group and GBR12,935 cell group in the *in vitro* study were analyzed by Student's independent *t*-test. $P<0.05$ was considered statistically significant. pEC_{50} and E_{max} were analyzed by GraphPad Prism software v.4.01 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. FQ selectively inhibited DAT

The effects of FQ on neurotransmitter transporters were studied by measurement of isotope-labeled transmitter uptake by corresponding transgenic CHO cells. FQ at concentrations of 1–1000 μg/ml (added cumulatively in increments of 0.5 log units) significantly and concentration-dependently inhibited dopamine uptake by D8 cells compared with vehicle control ($P<0.01$ and $P<0.001$, respectively). The generated concentration–effect curve revealed an FQ IC_{50} value of 21.4 ± 3.64 μg/ml by linear regression analysis ($r^2=0.964$; $y=39.7x-3.08$; unit of x: log [μg/ml]). To detect the specificity of FQ for dopamine uptake inhibition, the effects of FQ on norepinephrine, serotonin, and GABA transport into transgenic CHO cells were examined. The results showed that FQ at concentrations of 1–1000 μg/ml had no significant effect on serotonin uptake by S6 cells or GABA uptake by G1 cells. FQ at concentrations up to 1000 μg/ml exhibited only mild suppressive effects on norepinephrine uptake by N1 cells (Fig. 2A). The DAT-selective inhibitory effect was thus produced after FQ was added to the *in vitro* screening platform (Fig. 2A). To compare bioactivities of FQ and GBR12,935, different concentrations of the two drugs were added to the D8 cell reaction system, and concentration–effect curves were generated. As shown in Fig. 2B, the FQ curve was flatter than the GBR curve, with about a 3 log-unit difference in maximal effects between the two drugs. The EC_{50} and E_{max} values of FQ for dopamine inhibition were 21.5 ± 3.34 μg/ml and 92.3 ± 7.15%, respectively. The reference compound GBR12,935 (0.001–1.6 μg/ml) gave EC_{50} and E_{max} values of 0.0085 ± 0.00016 μg/ml and 97.1 ± 5.22%, respectively. The EC_{50} value of FQ was much greater than that of GBR12,935 ($P<0.001$), whereas no significant difference in E_{max} was found between FQ and GBR12,935 (Table 1).

An inhibitory effect of FQ also was found on dopamine transport into dopaminergic synaptosomes freshly harvested from rat striatum. Dopamine uptake by synaptosomes incubated with FQ (10, 33, 100 μg/ml) was concentration-dependently downregulated compared with control ($P<0.01$ at 10 μg/ml, $P<0.001$ at 33 and 100 μg/ml) (Fig. 3A). The shape difference in the concentration-effect curves for FQ and GBR12,935 was similar to that exhibited in the D8 cell system (Fig. 3B). In addition, the E_{max} and EC_{50} values of the two drugs for inhibition in synaptosomes were similar to those displayed in D8 cells (Table 1).

3.2. Effect of FQ on cell viability after MPP^+ administration

The MTT test showed that viability of the D8 cells incubated with MPP^+ only (control) for 24 h was decreased compared with MPP^+-free

Table 1
Summary of EC_{50} and E_{max} of FQ for dopamine uptake inhibition and cell lesion protection

		Inhibition of dopamine uptake by D8 cells	Inhibition of dopamine uptake by synaptosomes	Protection rate for MPP^+ induced cell lesion
EC_{50} (μg/ml)	FQ	21.5 ± 3.34*	25.2 ± 5.53*	38.1 ± 4.49*
	GBR12,935	0.0085 ± 0.00016	0.0075 ± 0.0018	0.019 ± 0.0062
E_{max} (%)	FQ	92.3 ± 7.15	94.8 ± 7.33	71.4 ± 7.89
	GBR12,935	97.1 ± 5.22	95.9 ± 10.1	75.2 ± 9.72

Significant difference in EC_{50} for inhibition of dopamine uptake or protection against cell lesion was produced between FQ-treated and GBR12,935-treated group, but no difference in E_{max} (obtained in the three reaction systems) were observed between the two groups. $^{*}P<0.001$ compared with GBR12,935-treated cell group (Student's independent *t*-test). Values are expressed as mean ± SEM of three independent experiments.

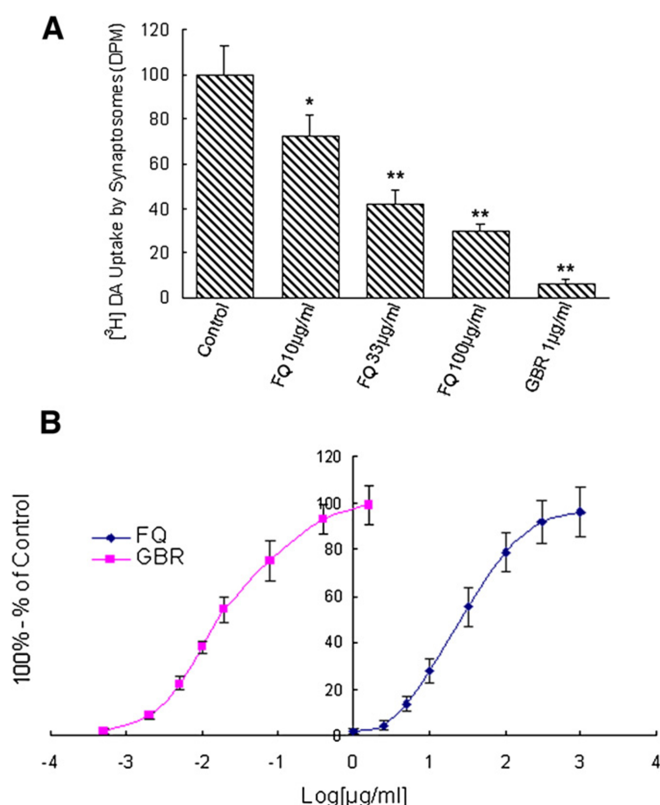

Fig. 3. Effect of FQ on [^{3}H]dopamine uptake by synaptosomes from rat striatum. (A) Synaptosomes were treated with the same volume of vehicle solvent (control), different concentrations of FQ (1–1000 μg/ml) and GBR12,935 (0.0005–1.6 μg/ml). The results showed that FQ concentration-dependently inhibited dopamine uptake by synaptosomes. *$P<0.01$, **$P<0.001$ compared with control group. (B) A comparative study showed different shapes of the concentration–inhibition curves between FQ and GBR12,935. EC_{50} and E_{max} values are summarized in Table 1. Values are expressed as mean ± SEM of three experiments in quintuplicate.

D8 cells ($P<0.01$). However, FQ (10, 33,100 μg/ml) induced a significant increase in D8 cell viability when co-incubated with MPP^+ solution compared with control ($P<0.001$). Treatment with GBR12,935 (1 μg/ml) also improved MPP^+-induced cell lesion. FQ at varying concentrations had no effect on viability of MPP^+-free D8 cells (Fig. 4A). As shown in Fig. 4B, FQ (1–1000 μg/ml) and GBR12,935 (0.001–1.6 μg/ml) generated different concentration–effect curves in protection against toxicity in D8 cells, similar to the curves generated by FQ and GBR12,935 in the uptake assays. Nonlinear regression produced different EC_{50} and E_{max} values (%, the difference in OD values between vehicle control and MPP^+ lesioned control) (FQ, 38.1 ± 4.49 μg/ml and 71.4 ± 7.89%; GBR12,935, 0.019 ± 0.0062 μg/ml and 75.2 ± 9.72%, respectively). Although the EC_{50} value of FQ was much greater than that of GBR12,935, the two drugs possessed similar E_{max} values (Table 1).

3.3. Effect of FQ on unilateral turns in 6-OHDA-lesioned rats

Two Parkinson's disease models in 6-OHDA-lesioned rats (mild and moderate models, chosen based on different levels of rotational asymmetry) were used to explore the effect of FQ on parkinsonian-like symptoms. As shown in Fig. 5, FQ administration produced antiparkinsonian effects in the two models to differing degrees. Behavioral measurement of apomorphine-induced rotational turning in mild model rats (Fig. 5A), performed before and every week after treatment onset, showed that FQ significantly and time-dependently reduced abnormal turns (1 to 8 weeks) compared with time-matched controls ($P<0.05$ at 1 and 2 weeks, $P<0.001$ at 3–8 weeks). In contrast, FQ treatment in moderate model rats that were tested before and every 2 weeks after treatment onset showed no significant difference between model and treatment groups during the first 2-week treatment, whereas unilateral turns in the FQ group gradually decreased from week 4 to week 14 compared with time-matched controls ($P<0.05$ at 4–6 weeks, $P<0.001$ at 10–14 weeks). In addition, the behavioral decrement in the FQ group was significantly attenuated 8 weeks after treatment onset in the mild model ($P<0.05$) and 14 weeks after treatment onset in the moderate model ($P<0.001$) compared with pretreatment.

3.4. Effect of FQ on retention time in MPTP-treated mice

We conducted another behavioral study using a model of MPTP-induced dopaminergic depletion to further evaluate the antiparkinsonian effects of FQ. C57BL/6 mice treated with MPTP showed spontaneous motor deficits such as immobility and tremble. Retention time of mice in the model group [normal saline (NS)/MPTP] significantly decreased compared with the normal group (NS/NS; $P<0.001$) after 3-week treatment, indicating the validity of this Parkinson's disease model (Fig. 6). MPTP-treated mice administered with FQ (0.25, 1 g/kg body weight) showed a significant increase in

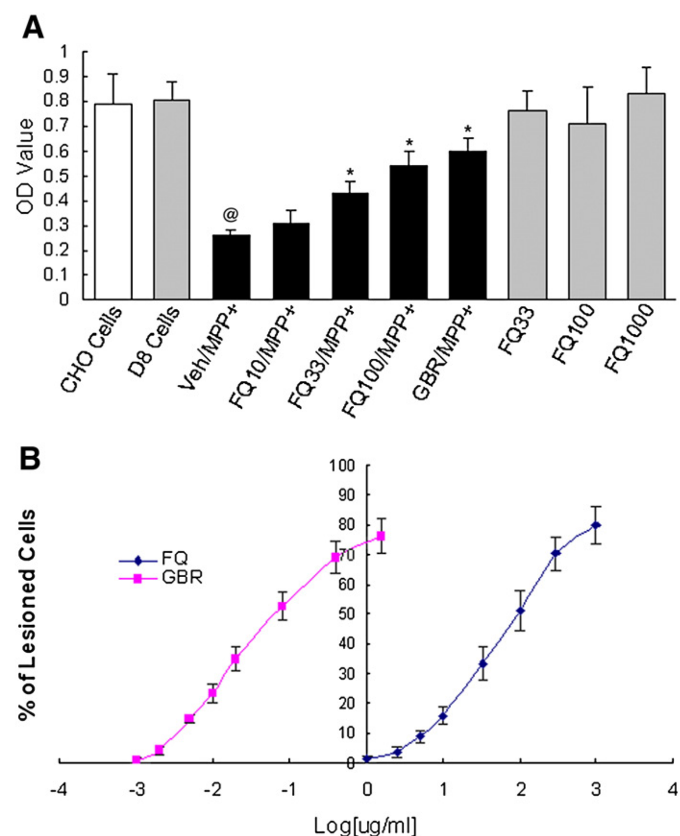

Fig. 4. Effect of FQ on MPP^+-induced D8 cell toxicity. (A) Solid black bars represent D8 cells that were co-incubated with MPP^+ and FQ or GBR12,935. FQ at concentrations (33 and 100 μg/ml) and GBR12,935 (1 μg/ml) produced significant protective effects on cell lesions induced by MPP^+. Both CHO (open bar) and D8 cells (the first solid gray bar) were treated with vehicle solvent only. Veh/MPP^+ (the first solid black bar) is the control group treated with vehicle solvent and MPP^+. The last three gray bars represent cell groups treated with FQ at concentrations of 33, 100, and 1000 μg/ml (FQ33, FQ100, and FQ1000), respectively. $F(5,18)=64.8$, $P<0.001$; $^{@}P<0.001$ compared with D8 cell group; *$P<0.001$ compared with Veh/MPP^+ cell group. Values are expressed as mean ± SEM of quadruplicate samples. FQ at concentrations of 33, 100, and 1000 μg/ml had no effect on D8 cell viability [$F(3,12)=1.42$, $P=0.286$]. (B) Y-axis represents protection rate. The shape of the concentration–protection curve produced by FQ is different than that produced by GBR12,935. EC_{50} and E_{max} value for both FQ and GBR12,935 for protection against D8 lesion are shown in Table 1.

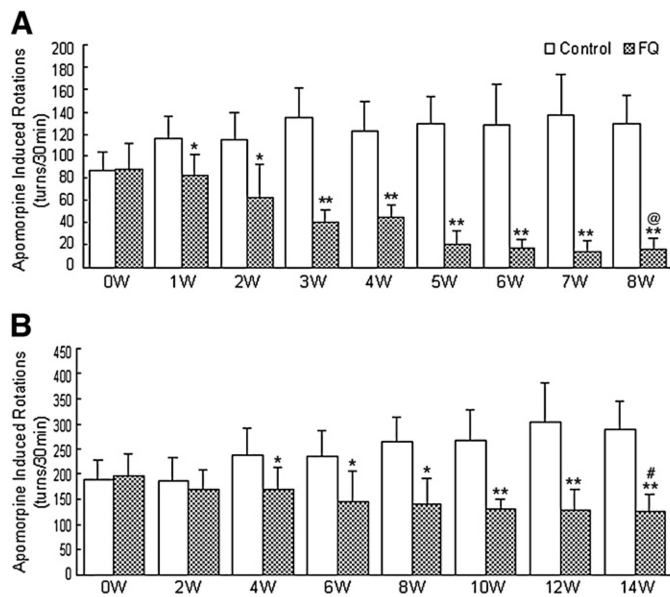

Fig. 5. Effect of FQ on hemi-parkinsonian behavior induced by injection of 6-hydroxydopamine (6-OHDA) into rat striatum. The rotations of mild Parkinson's disease rats (A) were tested before (0 W), and 1 week (1 W), 2 weeks (2 W), 3 weeks (3 W), 4 weeks (4 W), 5 weeks (5 W), 6 weeks (6 W), 7 weeks (7 W), and 8 weeks (8 W) after treatment onset. The rotations of the moderate model (B) were tested before (0 W), and 2 weeks (2 W), 4 weeks (4 W), 6 weeks (6 W), 8 weeks (8 W), 10 weeks (10 W), 12 weeks (12 W), and 14 weeks (14 W) after treatment onset. Control (model group), treated with vehicle; FQ group, treated with FQ 0.5 g/kg body weight. #$P<0.05$, @$P<0.001$ compared with before treatment; *$P<0.05$, **$P<0.001$ compared with model group (paired- or Student's independent-sample *t*-test). Values are expressed as mean ± SEM of 10 rats.

retention time compared with the model group ($P<0.001$). The positive control group (GBR/MPTP) treated with GBR12,935 (5 mg/kg) likewise exhibited significant enhancement of retention time ($P<0.001$). No remarkable difference was observed between the group treated with 1 g/kg FQ and the group treated with 5 mg/kg GBR12,935 ($P=0.59$).

3.5. Effect of FQ on TH neuron immunostaining in MPTP-treated mice

Evaluation of morphologic alterations in dopaminergic neurons of MPTP-treated mice was performed after the rotarod test. TH immunohistochemistry revealed that injection of MPTP produced a significant reduction of dopaminergic neuronal population in the substantia nigra of C57BL/6 mice. The number of TH-positive neurons in the substantia nigra of the model group showed a reduction of approximately 70% of the normal group ($P<0.001$). However, the number of TH-positive neurons significantly increased in the substantia nigra of the FQ group compared with the model group ($P<0.005$). The significant improvement also was found in the reference group (GBR/MPTP) compared with the model group ($P<0.001$). No difference in TH-positive neurons was observed between the group treated with 1 g/kg FQ and the group treated with 5 mg/kg GBR12,935 ($P=0.76$) (Fig. 7).

4. Discussion

Previous pharmacological studies have shown that FQ has analgesic, anti-rheumatic, and anti-inflammatory effects (Chen and Wei, 2003). Using a transgenic cell-line system, we screened DAT targeting of medicinal materials from hundreds of Chinese herbs that have been used clinically in the treatment of nervous system diseases. Using a high throughput *in vitro* screening system, the results of our present investigation confirm that FQ is a potent inhibitor of DAT. A particularly interesting finding was that the FQ extract was found to be highly selective for DAT and had no effect on GAT-1 or SERT and little effect on NET at concentrations up to 1000 μg/ml. An *ex vivo* study showed that FQ reduced dopamine transport into striatal synaptosomes, providing additional support for the inhibitory effect of FQ on DAT.

The IC_{50} of FQ for selective inhibition in D8 cells was 21.4 ± 3.64 μg/ml, and the EC_{50} of FQ for DAT inhibition in D8 cells or synaptosomes was much greater than that of GBR12,935. FQ inhibited the DAT with relatively moderate potency. Despite having efficacy (E_{max} value) for DAT inhibition similar to the potent DAT antagonist GBR12,935, additional evidence is required to verify the potential efficacy of FQ *in vivo*. Possible competing toxic or side effects on D8 cells by FQ at higher concentrations cannot be summarily ruled out.

The moderate potencies of FQ for uptake inhibition in D8 cells and synaptosomes led to the assumption that some unknown active ingredients, with markedly lower content in FQ extract, may contribute to its inhibitory efficacy. Phytochemistry studies by other laboratories have shown that FQ contains several organic acids such as manifold amino acids, malic acid, oleanolic acid, betulinic acid, 3-*O*-acetyl pomolic acid, ethyl chlorogenate, protocatechuic acid, gallic acid, kojic acid, tartaric acid, citric acid, glucosides, vitamins, and pectin (Dai et al., 2003; Yin et al., 2006; Wu et al., 2004), all of which are beneficial to the human body. Of these compounds, fumarate, oleanolic acid, DL-malic acid, citric acid, and glucosides were evaluated in uptake assays to determine whether they possess activity on monoamine transporters. The results showed that these compounds at concentrations up to 100 μg/ml had no effect on DAT or other transporters (data not shown). The inhibitory action of FQ, therefore, is hypothesized to occur through the action of some other compound with a different chemical structure. Thus, isolation of the trace chemicals may need to be considered.

The MTT test showed that treatment with FQ at concentrations up to 1000 μg/ml did not affect D8 cell viability, which further confirmed the inhibitory effect and specificity of FQ on dopamine uptake. FQ did not elicit cell toxicity when uptake by D8 cells occurred. As expected, FQ significantly mitigated MPP^+-induced toxicity of D8 cells. DAT inhibitors have been shown to be neuroprotective because they inhibit toxic ligands such as MPP^+ and 6-OHDA from being transported through DAT (McKinley et al., 2005), supporting the hypothesis that FQ might have dopaminergic neuroprotective effects through DAT inhibition. In addition, a comparative study of FQ and GBR12,935 showed a higher EC_{50} for FQ than for GBR12,935 and similar E_{max}

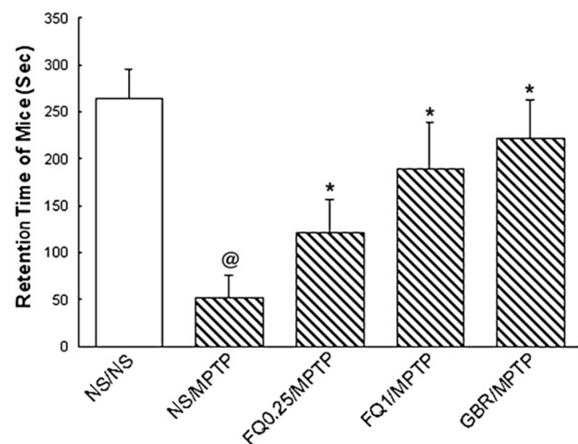

Fig. 6. Effect of FQ on parkinsonian behavior in MPTP-treated mice. Mice in the normal saline/normal saline (NS/NS) group were administered with 0.9% normal saline only. NS/MPTP is the model group treated with 0.9% normal saline and MPTP. FQ0.25/MPTP, FQ1/MPTP, and GBR/MPTP represent treatment with MPTP 30 min following administration of 0.25 and 1 g/kg of FQ and 5 mg/kg of GBR12,935, respectively. $F(4,45)=53.3$, $P<0.001$; @$P<0.001$ compared with NS/NS group; *$P<0.001$ compared with NS/MPTP group. Values are expressed as mean ± SEM of 10 mice.

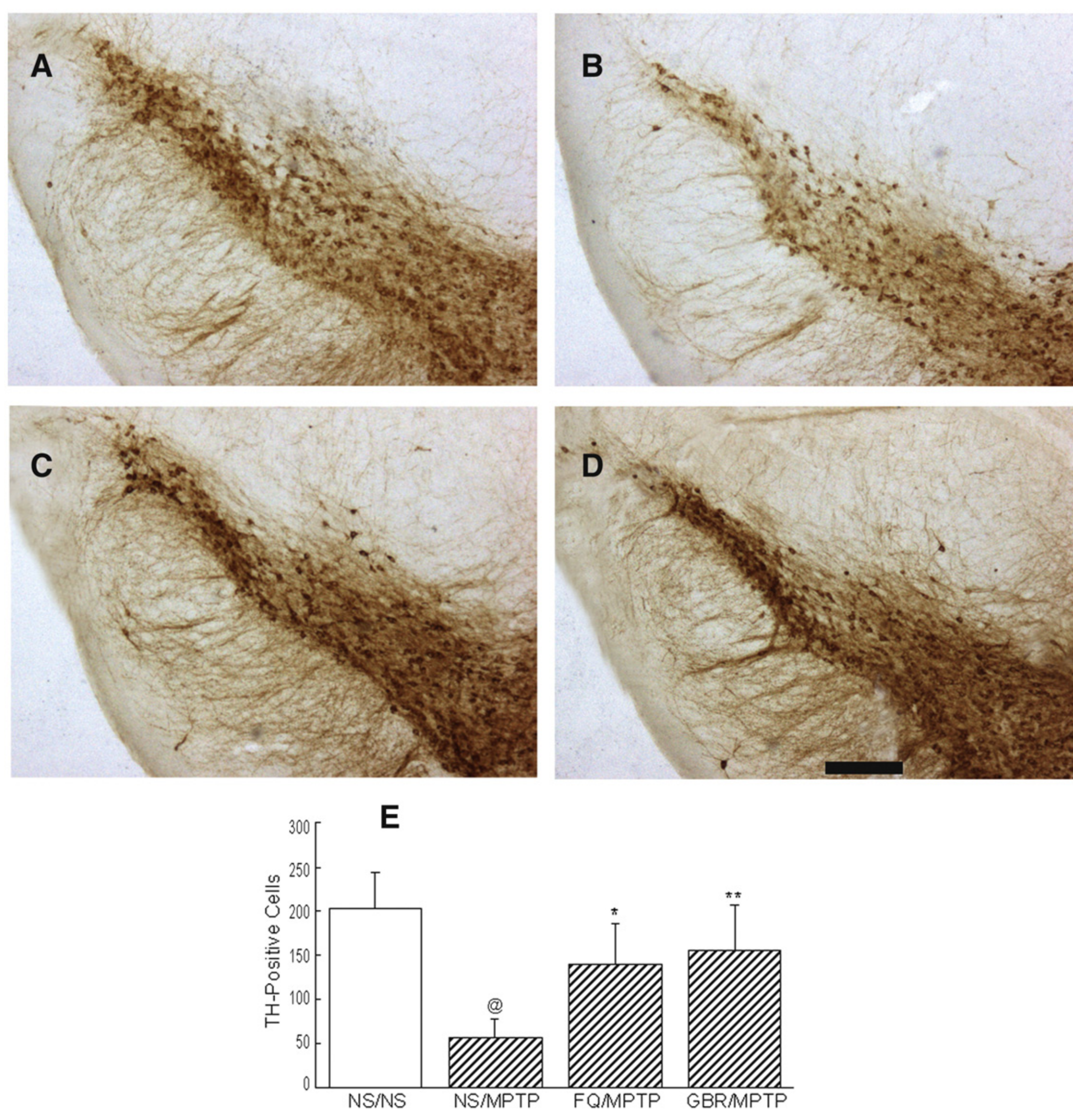

Fig. 7. Immunohistochemical study of the effect of FQ on TH-immunoreactive neurons in the substantia nigra of MPTP-treated mice (treatment design shown in Section 2.8.1). Sections show TH-specific immunohistochemical staining in substantia nigra of mice in the (A) normal saline/normal saline (NS/NS) group treated with 0.9% normal saline, (B) NS/MPTP group treated with 0.9% normal saline and MPTP, (C) FQ/MPTP group treated with FQ (1 g/kg) and MPTP, and (D) GBR/MPTP group treated with GBR12,935 (5 g/kg) and MPTP. Scale bar = 200 µm. (E) Quantification of TH-immunoreactive neurons in the substantia nigra. $F(3,16) = 12.4$, $P < 0.001$; $^{@}P < 0.001$ compared with NS/NS group; $^{*}P < 0.005$, $^{**}P < 0.001$ compared with NS/MPTP group. Mean counting value of the four slices in each animal represents TH-positive neuron number of the brain. Values are expressed as mean ± SEM of neuronal number of five mice.

values, suggesting that FQ may have efficacy in dopaminergic protection similar to GBR12,935.

We established two animal models (6-OHDA-lesioned rats and MPTP-treated mice) to determine whether FQ has pharmacologic antiparkinsonian activity. The neurobehavioral study showed that FQ time-dependently alleviated 6-OHDA-induced hemi-parkinsonian rotations in rats, and FQ exhibited more potent antiparkinsonian action in the mild model than in the moderate model. The antiparkinsonian effect of FQ, particularly in the mild group, suggests its potential for the prevention of early Parkinson's disease. The improvement in abnormal rotation in 6-OHDA-lesioned rats is possibly through a mechanism of DAT inhibition. Dopamine receptor supersensitivity is hypothesized to cause rotational behavior in the unilateral lesion model. The chronic increase in dopamine synaptic content via DAT inhibition may desensitize dopamine receptors, which would thus contribute to balancing the asymmetric function of dopamine receptors and subsequently delaying abnormal rotations. A previous study in our laboratory confirmed that lobeline is a novel DAT inhibitor with moderate potency and can significantly reduce the number of turns in 6-OHDA-lesioned rats (Li et al., 2007), providing additional evidence for the potential mechanism of the antiparkinsonism effect of FQ.

The rotarod test usually is applied to pharmacological evaluations of coordinated movement in animals. It is expressed as retention time and is actually representative of the extrapyramidal functional state. The present neurobehavioral study showed that FQ significantly and dose-dependently delayed the reduction of retention time induced by MPTP toxicity in C57BL/6 mice, which is consistent with reports recorded in ancient medical books such as the *Great Compendium of Materia Medica* (an encyclopedia of Chinese herbs; Li shizhen, 1596) of FQ ameliorating uncontrollable extension and bending of limbs and trunk. FQ has antiparkinsonian effects in the MPTP mouse model, providing additional behavioral evidence confirming the potent action of FQ in antiparkinsonism. The nonselective DAT inhibitor brasofensin (Pearce et al., 2002) and the selective DAT inhibitor GBR12,909 (Hansard et al., 2002a) produced a prolonged antiparkinsonian response in animal models. FQ may act to alleviate parkinsonism through a mechanism of DAT inhibition that subsequently enhances

dopaminergic transmission in substantia nigra-lesion Parkinson's disease models.

Morphological evidence in the present study further revealed the antiparkinsonian effect of FQ. TH immunohistochemistry showed that the loss of TH-positive neurons in the substantia nigra in MPTP-treated mice was significantly reduced by FQ extract administration. The improvement of dopaminergic loss in the lesioned substantia nigra corroborates the results from the MTT experiment in D8 cells and strongly supports the neuroprotective action of FQ. MPP^+ is a metabolite of MPTP, and a possible mechanism for FQ's efficacy may be through inhibition of MPP^+ transport into dopaminergic neurons and subsequently to avoidance of depression of respiratory chain enzymes in mitochondria (Gonzalez-Polo et al., 2001; Watanabe et al., 2005).

Edible FQ, usually called “longevity fruit” because of its beneficial health and anti-aging effects, has been used for food as well as for medicine for centuries by Chinese people. No record exists of FQ toxicity or side effects when chronically orally administered at doses of 9–30 g. The edible fruit was thus listed as one of 87 “pharmafoods” by the Ministry of Health of the People's Republic of China in 2002 (document no. 51). In addition, empirical studies on FQ toxicity showed no mouse deaths or abnormalities in vital organ systems after administration of FQ at doses up to 10 g/kg (i.g.) daily for 4 successive weeks (data not shown), corroborating its safety in application. FQ may have marked potential to be developed into a therapeutic preparation used in the prevention or treatment of the early phase of Parkinson's disease, especially in older patients with the disease (approximately one in every 100 persons over the age of 55; Tanner and Bfsen-Shlomo, 1999). Many researchers have shown that DAT inhibitors can prevent some of the side effects of replacement therapy, such as dyskinesia (Rosenzweig-Lipson et al., 1994; Xu et al., 1994; Pearce, 1996; Pearce et al., 1999; Jenner, 2000). Therefore, future studies will elucidate whether FQ, as a novel antiparkinsonian agent, might also prevent dyskinesia induced by L-DOPA treatment of Parkinson's disease.

In summary, the present pharmacological study demonstrated that the water extract of FQ is a novel DAT inhibitor with moderate potency and a potential antiparkinsonism treatment. For further study, the active compounds from FQ extract will be isolated and identified and may be structurally modified as potentially more robust lead compounds for DAT inhibition and antiparkinsonism.

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References

Agnati LF, Leo G, Vergoni AV, Martinez E, Hockemeyer J, Lluis C, et al. Neuroprotective effect of L-DOPA co-administered with the adenosine A2A receptor agonist CGS 21680 in an animal model of Parkinson's disease. Brain Res Bull 2004;64:155–64.

Ahlskog JE, Muenter MD. Frequency of levodopa-related dyskinesias and motor fluctuations as estimated from the cumulative literature. Mov Disord 2001;16: 448–58.

An CP, Cheng W. Review on mechanisms and symptoms of depression in TCM (Traditional Chinese Medicine). Inf Tradit Chin Med 2007;24:12–4.

Blum D, Torch S, Lambeng N, Nissou M, Benabid AL, Sadoul R, et al. Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. Prog Neurobiol 2001;65:135–72.

Chen Q, Wei W. Effects and mechanisms of glucosides of *Chaenomeles speciosa* on collagen-induced arthritis in rats. Int Immunopharmacol 2003;3:593–608.

Chiueh CC, Andoh T, Lai AR, Lai E, Krishna G. Neuroprotective strategies in Parkinson's disease: protection against progressive nigral damage induced by free radicals. Neurotox Res 2000;2:293–310.

Dai M, Wei W, Shen YX, Zheng YQ. Glucosides of *Chaenomeles speciosa* remit rat adjuvant arthritis by inhibiting synoviocyte activities. Acta Pharmacol Sin 2003;24:1161–6.

Dawson TM, Dawson VL. Molecular pathways of neurodegeneration in Parkinson's disease. Science 2003;302:819–22.

Delwaide PJ, Martinelli P, Schoenen J. Mazindol in the treatment of Parkinson's disease. Arch Neurol 1983;40:788–90.

Eberhardt O, Schulz JB. Apoptotic mechanisms and antiapoptotic therapy in the MPTP model of Parkinson's disease. Toxicol Lett 2003;139:135–51.

Goetz CG, Tanner CM, Klawans HL. Bupropion in Parkinson's disease. Neurology 1984;34:1092–4.

Gonzalez-Polo RA, Mora A, Clemente N, Sabio G, Centeno F, Soler G, et al. Mechanisms of MPP^+ incorporation into cerebellar granule cells. Brain Res Bull 2001;56:119–23.

Hansard MJ, Smith LA, Jackson MJ, Cheetham SC, Jenner P. Dopamine reuptake inhibition and failure to evoke dyskinesia in MPTP-treated primates. Eur J Pharmacol 2002a;451:157–60.

Hansard MJ, Smith LA, Jackson MJ, Cheetham SC, Jenner P. Dopamine, but not norepinephrine or serotonin, reuptake inhibition reverses motor deficits in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated primates. J Pharmacol Exp Ther 2002b;303:952–8.

He SM, Jiang J. Review on TCM treatment of migraine and tension-type headache. Chin Arch Tradit Chin Med 2006;24:1469–71.

Hersch SM, Yi H, Heilman CJ, Edwards RH, Levey AI. Subcellular locallization and molecular topology of dopamine transporter in the striatum and substantia nigra. J Comp Neurol 1997;388:211–27.

Hurtig HI. Problems with current pharmacologic treatment of Parkinson's disease. Exp Neurol 1997;144:10–6.

Jenner P. Factors influencing the onset and persistence of dyskinesia in MPTP-treated primates. Ann Neurol 2000;47(4 Suppl 1):S90–S104.

Joyce JN, Woolsey C, Ryoo H, Borwege S, Hagner D. Low dose pramipexole is neuroprotective in the MPTP mouse model of Parkinson's disease, and down-regulates the dopamine transporter via the D3 receptor. BMC Biol 2004;2:22.

Kokoshka JM, Metzger RR, Wilkins DG, Gibb JW, Hanson GR, Fleckenstein AE. Methamphetamine treatment rapidly inhibits serotonin, but not glutamate, transporters in rat brain. Brain Res 1998;799:78–83.

Li CY, Ji XH, Hui GZ, Zhang JG, Zhou W, Bu XY, et al. Effects of lobeline on dopamine uptake by plasma membrane dopamine transporter. Chin J Neurosurg Dis Res 2007;6:247–9.

Liu Z, Wang Y, Zhao W, Ding J, Mei Z, Guo L, et al. Peptide derived from insulin with regulatory activity of dopamine transporter. Neuropharmacology 2001;41: 464–71.

McKinley ET, Baranowski TC, Blavo DO, Cato C, Doan TN, Rubinstein AL. Neuroprotection of MPTP-induced toxicity in zebrafish dopaminergic neurons. Brain Res Mol Brain Res 2005;141:128–37.

Nelson N. The family of Na+/Cl− neurotransmitter transporters. J Neurochem 1998;71:1785–803.

Nutt JG, Carter JH, Sexton GJ. The dopamine transporter: importance in Parkinson's disease. Ann Neurol 2004;55:766–73.

Park DM, Findley LJ, Hanks G, Sandler M. Nomifensine: effect in Parkinsonian patients not receiving levodopa. J Neurol Neurosurg Psychiatry 1981;44:352–4.

Park HJ, Lim S, Joo WS, Yin CS, Lee HS, Lee HJ, et al. Acupuncture prevents 6-hydroxydopamine-induced neuronal death in the nigrostriatal dopaminergic system in the rat Parkinson's disease model. Exp Neurol 2003;180:93–8.

Parkes JD, Tarsy D, Marsden CD, Bovill KT, Phipps JA, Rose P, et al. Amphetamines in the treatment of Parkinson's disease. J Neurol Neurosurg Psychiatry 1975;38:232–7.

Pearce RK, Jackson M, Britton DR, Shiosaki K, Jenner P, Marsden CD. Actions of the D1 agonists A-77636 and A-86929 on locomotion and dyskinesia in MPTP-treated L-dopa-primed common marmosets. Psychopharmacology 1999;142:51–60.

Pearce RK, Smith LA, Jackson MJ, Banerji T, Scheel-Kruger J, Jenner P. The monoamine reuptake blocker brasofensine reverses akinesia without dyskinesia in MPTP-treated and levodopa-primed common marmosets. Mov Disord 2002;17:877–86.

Pearce RKB. Dopamine D1 receptor agonists and dopamine reuptake blockers: new treatment stratagems for Parkinson's disease. Exp Opin Ther Patents 1996;6: 949–53.

Ravina BM, Fagan SC, Hart RG, Hovinga CA, Murphy DD, Dawson TM, et al. Neuroprotective agents for clinical trials in Parkinson's disease: a systematic assessment. Neurology 2003;2:1234–40.

Rosenzweig-Lipson S, Hesterberg P, Bergman J. Observational studies of D1 and D2 agonists in squirrel monkeys. Psychopharmacology 1994;116:9–18.

Rosner S, Giladi N, Orr-Urtreger A. Advances in the genetics of Parkinson's disease. Acta Pharmacol Sin 2008;29:21–34.

Rozas G, Lopez-Martin E, Guerra MJ, Labandeira-Garcia JL. The overall rod performance test in the MPTP-treated-mouse model of Parkinsonism. J Neurosci Methods 1998;83:165–75.

Sulzer D. Multiple hit hypotheses for dopamine neuron loss in Parkinson's disease. Trends Neurosci 2007;30:244–50.

Tanner CM, Ben-Shlomo Y. Epidemiology of Parkinson's disease. Adv Neurol 1999;80:153–9.

Teychenne PF, Park DM, Findley LJ, Rose FC, Calne DB. Nomifensine in parkinsonism. J Neurol Neurosurg Psychiatry 1976;39:1219–21.

Torres GE, Gainetdinov RR, Caron MG. Plasma membrane monoamine transporters: structure, regulation and function. Nat Rev Neurosci 2003;4:13–25.

Wang LZ. Therapy of apoplectic sequelae by six schemes. Henan Tradit Chin Med 2006;26:31–2.

Watanabe Y, Himeda T, Araki T. Mechanisms of MPTP toxicity and their implications for therapy of Parkinson's disease. Med Sci Monit 2005;11:RA17–23.

Wu H, Wei W, Wu CY. Study on chemical composition and pharmacological activity of chaenomeles fruit. J Anhui Tradit Chin Med Coll 2004;23:62–4.

Xu LF, Chu WJ, Qing XY, Li S, Wang XS, Qing GW, et al. Protopine inhibits serotonin transporter and noradrenaline transporter and has the antidepressant-like effect in mice models. Neuropharmacology 2006;50:934–40.

Xu M, Hu XT, Cooper DC, Moratalla R, Graybiel AM, White FJ, et al. Elimination of cocaine-induced hyperactivity and dopamine-mediated neurophysiological effects in dopamine D1receptor mutant mice. Cell 1994;79:945–55.

Yin K, Gao HY, Li XN, Wu LJ. Chemical constituents of *Chaenomeles speciosa* (Sweet) Nakai. J Shenyang Pharm Univ 2006;23:760–3.