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Research Article

In vitro dopaminergic neuroprotective and *in vivo* antiparkinsonian-like effects of Δ^3,2-hydroxybakuchiol isolated from *Psoralea corylifolia* (L.)

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Abstract. Cocktail recipes containing *Psoralea corylifolia* seeds (PCS) are used to empirically treat Parkinson disease. A PCS isolate Δ^3,2-hydroxybakuchiol (BU) can inhibit dopamine uptake in dopamine transporter (DAT) transfected Chinese hamster ovary (CHO) cells, and dopamine reuptake blockade may provide an alternative approach for ameliorating parkinsonism. Here, we assessed the potential dopaminergic neuroprotective, and antiparkinsonian-like activity of BU. BU sample size was increased by using a scale-up extraction paradigm. Pharmacologically, BU significantly protected SK-N-SH cells from 1-methyl-4-phenylpyridinium (MPP^+) insult, produced striking inhibitory actions on dopamine/norepinephrine uptake and WIN35,428 binding in synaptosomes on *in vivo* administration, and significantly preventing poor performance on rotarod and dopaminergic loss in substantia nigra in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mice. BU acts by protecting dopaminergic neurons from MPP^+ injury and preventing against MPTP-induced behavioral and histological lesions in the Parkinson's disease (PD) model, possibly by inhibiting monoamine transporters. These findings suggest that BU could be meaningful in PD treatment.

Keywords. Δ^3,2-hydroxybakuchiol, scale-up extraction, dopamine transporter, reuptake inhibitor, Parkinson's disease.

Introduction

Psoralea corylifolia L. (Leguminosae) seeds (PCS), i.e. fructus psoraleae, has been adopted in the production of herbal medicines in China for hundreds of years. As a therapeutic plant, PCS is used empirically to prevent brain aging and to treat dementia [1, 2] and, more recently, has been proven to possess antidepressant action [3–5]. Two anecdotal clinical reports showed that this Chinese folklore herb, combined with other herbs when prescribing herb cocktail recipes, can significantly improve parkinsonism [6, 7]. We have previously disclosed that PCS extract [8] and its meroterpenoid isolate, Δ^3,2-hydroxybakuchiol (BU) [9], markedly inhibited monoamine

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transport in monoamine transporter transgenic Chinese hamster ovary (CHO) cells, with selectivity for uptake of dopamine and norepinephrine.

Dopamine transporter (DAT) belongs to members of the Na^+/Cl^-dependent neurotransmitter transporter family, with the property of a 12-transmembrane topological structure, and acts by limiting dopamine (DA) activity by the mechanism of reuptake in the central nervous systems [10, 11]. DAT also plays a pivotal role in selective dopaminergic toxicity of endogenous and exogenous N-methylated heterocylic neurotoxins structurally related to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or its active metabolite, 1-methyl-4-phenylpyridinium (MPP^+). Vulnerability of distinct dopaminergic subregions of brain in idiopathic PD or MPP^+-induced parkinsonism is positively correlated with basal levels of DAT expression across these subregions [12, 13]. As reported in several *in vivo* studies, compounds acting as DAT blockers may be useful in the treatment of the motor symptoms of Parkinson disease (PD) [14, 15]. For example, the selective DA reuptake inhibitor 1-(2-(bis-(4-fluorophenyl)-methoxy)ethyl)-4-(3- phenylpropyl) piperazine) dihydrochloride (GBR12,909) can reverse motor deficits in MPTP-treated primates [15]. Thus, DAT may play a role in the pathogenesis of PD, and DA reuptake blockers could offer a novel approach for early PD treatment or parkinsonism improvement. We hypothesized that the bakuchiol analog BU could possess dopaminergic neuroprotective activity in the PD model. The objective of this study was to evaluate its potential dopaminergic neuroprotective, and antiparkinsonian-like effect by using *in vitro* and *in vivo* neurotoxin-lesioned models, prior to which a scale-up extraction technique (such as using supercritical carbon dioxide extraction and column chromatography methods in combination with activity guidance) was explored for the purpose of increasing BU yield from PCS extract and thereby meeting the requirement of animal study.

Materials and methods

Uptake assay in transporter transgenic CHO cells. CHO cells expressing the rat DAT (rDAT; GI 310097) and human NET (hNET; GI 189257), respectively designated as D8 and N1 cells, were established as described in references [8, 9] and adopted for detecting the activity of each extract and for directing isolation of BU. D8 or N1 cells were grown in RMPI1640 medium containing 10% fetal bovine serum to near confluence in 48-well tissue culture plates (Costar Inc., USA) (approximately 60 000 cells per well). After being rinsed once with phosphate-buffered saline (PBS), the cells were preincubated in 100 µl Hank's balanced salt solution (HBSS) at room temperature for 10 min. [^{3}H]DA or [^{3}H]norepinephrine (NE), ascorbic acid, and pargyline were added to final concentrations of 100 nM, 100 µM, and 100 µM, respectively, and then the cells were incubated at 37 °C for an additional 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 (4 °C). The cells were then solubilized in 2N NaOH, and an aliquot was measured by liquid scintillation (TRI-CARB2900TR, PerkinElmer, Waltham, MA, USA) to quantify uptake of [^{3}H]DA or [^{3}H]NE. To explore the effects of each PCS extract on DA/NE uptake, different concentrations of extracts were added to cell cultures at the beginning of the uptake assay.

BU preparation by supercritical carbon dioxide extraction combined with column chromatography. Previously, BU was isolated from fructus psoraleae petroleum ether extract on a laboratory-scale in our lab, yielding such a low sample quantity that it could not meet the requirements of an animal study. To explore a scale-up extraction technique, supercritical carbon dioxide extraction was adopted to increase its sample size. 40,000 g fructus psoraleae (seeds of *Psoralea corylifolia* L.; Voucher specimen No. 20060611) were purchased from a supplier in Henan Province, China, and identified and authenticated by Prof. Guowei Qin (Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences). This herb source (with a small sample size) was powdered, extracted with 95% ethanol, and standardized on amounts of 6.57% psoralen and 3.57% isopsoralen [using high-pressure liquid chromatography (HPLC); the two reference compounds, with a purity of 99.99%, were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China)]. BU content in this sample was determined as 0.051%. Before extraction, technological parameters for the supercritical carbon-dioxide-extraction system (HA420-40-96-EX, Huaan Supercritical Equipment Co., Ltd, Nantong, China) were determined by means of orthogonal designs, i.e. $L9(3^4)$ and $L4(2^3)$, through which the optimal parameters adopted were 25 Mpa extraction pressure, 60 °C temperature, and 140 kg/h flow rate. Then, under these conditions, all crude powder (E0) went through this system and gave an extract (E1), which was at recovery rate of 84.97% and contained 1.51% BU. Uptake assay showed that E1 exhibited a potent inhibitory action on DA uptake in D8 cells ($IC_{50} = 1.08$ µg/ml) or NE uptake in N1 cells ($IC_{50} = 1.26$ µg/ml), which was generally similar to our previous report on the activity of

Table 1. Purity, IC50, and extraction recovery

Extract	BU Purity (%)	IC_{50} (μg/ml)		Recovery rate (%)		
		DA uptake by D8	NE uptake by N1	Extract	BU	Total (BU)
E0	0.051	nd	nd			
E1	1.51	1.08	1.26		84.97	
E2	9.81	nd	nd	10.02	89.53	
E3	37.11	0.37	0.51	30.03	94.89	
E4	65.11	nd	nd	25.22	65.01	
E5	90.02	nd	nd	59.33	72.07	
E6	95.60	0.19	0.31	90.11	90.11	30.35

E0, fructus psoraleae crude power; E1, supercritical carbon dioxide extract of fructus psoraleae; E2, AB-8 macroporous resin extract; E3, XAD-1600 macroporous resin extract; E4, 200~300 mesh silica gel (petroleum ether/acetone) extract; E5, 200~300 mesh silica gel (chloroform/methanol) extract; E6, H silica gel (chloroform/methanol) extract. D8, DAT transgenic CHO cells; N1, NET transgenic CHO cells. nd, not detected.

petroleum ether extract [8]. Further isolation was performed using AB-8 macroporous resin chromatography [under conditions: sample/resin (1 : 5) as stationary phase, 55–60% ethanol as eluent], and subsequently an extract (E2) was given, with 10.02% recovery for E2 and 89.53% recovery for BU, and with content of 9.81% BU. Afterwards, the E2 went through isolation by XAD-1600 macroporous resin (sample/resin = 1 : 20, eluent: 55–60% ethanol) followed by a subsequent freezing crystallization, which gave an extract (E3) containing 37.11% BU, with 30.03% recovery for E3 and with 94.89% recovery for BU (through activity tracing, $IC_{50} = 0.37$ μg/ml for DAT and 0.51 μg/ml for NET). Furthermore, silica gel column chromatography was used to improve the purity; wherein E3 went through three column chromatographies (the former two on 200~300 mesh silica gel and the third on H silica gel), respectively under conditions: petroleum ether / acetone (15 : 1, 10 : 1, 5 : 1), chloroform / methanol (500 : 0, 500 : 1, 200 : 1, 100 : 1), and chloroform / methanol (500 : 0, 500 : 1, 200 : 1), which gave three corresponding extracts (E4, E5, and E6), containing 65.11%, 90.02%, and 95.60% BU respectively. Recovery for E4, E5, or E6 was 25.22%, 59.33%, or 90.11%, and relevant recovery for BU (followed by each extraction) was 65.01%, 72.07%, or 90.11%, respectively. Total BU recovery from this herb material was 30.35%, and final BU yield was 6160.58 mg.

The purity of BU was analyzed by the normalization method following HPLC. HPLC analytical conditions were as follows: column: ZORBAX SB-C18 reversed-phase column (4.6 mm × 250 mm, 5 μm, id); detector at 260 nm; mobile phase: ethyl hydrazine – H_2O (V/V) (0–40 min: 46/54–80/20), flow rate at 1.0 ml/min. The pure compound BU was verified by uptake assay on transporter transgenic CHO cells as well as by structure identification. Purity, IC_{50}, and recovery rate in every stage are summarized in Table 1. The molecular structure of the final pure sample (BU) was elucidated by ^{1}HNMR(400HZ) and ^{13}CNMR (Varian, Palo Alto, CA, USA).

BU was dissolved in deionized water containing 1% dimethylsulfoxide for *in vitro* study or in refined soybean oil for *in vivo* study, both of which were prepared just before experimentation.

MTT and WST-8 assay with SK-N-SH cells. The effect of BU on cell viability was assessed by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8). SK-N-SH cells were cultured in cell culture flasks containing DMEM supplemented with 10% FCS (10% FCS-DMEM) at 37 °C under 5% CO_2/ 95% humidified air incubator for indicated time. The cells were inoculated in 96 culture wells (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Plate cells were approximately 10 000 per well after 12 h incubation. Increasing concentrations of BU, or its reference GBR12,935, were added into the cell wells and incubated for 4 h. MPP^+ was thereafter added into the wells to a final concentration of 100 μM with a total volume of 200 μl and incubated for an additional 24 h. For MTT test, MTT reagent (with a final concentration of 0.5 mg/ml) was added to the system. The cells were solubilized by 100 μl dimethylsulfoxide following 4 h dyeing reaction, and then the optical density (OD) value was recorded at 570 nm Multiscan MK3 (Nova Technology Development Co. Ltd., Beijing, China). For WST-8 reduction assay, the culture was administered with WST-8 and incubated for 3 h at 37 °C prior to the colorimetry according to the instructions of the cell counting kit-8 (CCK-8) manufacturer (Dojindo Laboratories, Tokyo, Japan). For assessment of the effect of BU itself on cell viability, vehicle solvent was used

instead of MPP^+. Percentage of activity of MTT or WST-8 reduction was represented as the ratio of the testing compound's activity at different concentration points to that at concentration 0 μM (control).

Animals. Adult male Sprague–Dawley rats (weighing 200–220 g and age two months) and male C57BL/6J mice (weighing 30 to 36 g, 13 months old) were purchased from the Laboratory Animal Center of the Chinese Academy of Science (Shanghai, China). The 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 for one week prior to the experimentation. The injection volume per gram weight used to administer compounds was 0.01 ml. The experimental protocol was approved by Laboratory Animal Center of Chinese Academy of Science for the use of animal subjects. All procedures were in accordance with the National Institute of Health's guidelines regarding the principles of animal care.

DA- and NE-uptake test and [^{3}H]WIN35,428 binding assay on synaptosomes. The rats were randomly divided by six groups, each receiving four intraperitoneal (IP) injections (2-h intervals) of vehicle solvent, BU (5.44, 16.32, or 48.96 mg/kg), GBR12,935, or desipramine at 60 min prior to sacrificing (n = 6 per dosing group). The uptake procedure as previously described [9] was used with a slight modification. These pretreated rats were sacrificed by decapitation, and the striatum and hippocampus were dissected. Fresh brain tissues were individually homogenized with an electric homogenizer (300 r.p.m. for 15 s) in ice-cold 0.32 M phosphate-buffered sucrose and centrifuged at $1000 \times g$ for 10 min at 4 °C. The supernatants (S1) were then centrifuged at 22 000 × g for 15 min at 4 °C. The resulting pellets (P2) were resuspended in ice-cold 0.32M phosphate-buffered sucrose solution, followed by standardization using the Bradford assay (on an amount of 0.85 mg/ml protein). Then these samples were incubated in Na^+-Krebs Ringer Henseleit medium (NaCl 103 mM, CaCl2 1 mM, $MgCl_2$ 1 mM, KH_2PO_4 1mM, $NaHCO_3$ 27 mM, and glucose 5.4 mM; pH 7.4) containing 100 μM pargyline, 100 μM ascorbic acid, and 100 nM ^{3}H-labeled DA (for assay on striatal synaptosomes) or ^{3}H-labeled NE (for assay on hippocampal synaptosomes) with final volume of 100 μl at 37 °C for 10 min. The reaction was stopped by dilution with 1.2 ml ice-cold Li^+-Krebs Ringer Henseleit medium (in which NaCl was substituted by LiCl) followed by centrifugation at 12 000 × g at 4 °C for 10 min. The pellets were then decomposed by NaOH (2N, 100 μl), and an aliquot was used for measuring radioactivity. The specific transmitter uptake was defined as the difference between total uptake in Na^+-Krebs Ringer Henseleit medium and the nonspecific accumulation determined in Li^+-Krebs Ringer Henseleit medium.

[^{3}H]WIN35428 (2 nM final concentration) binding assay was conducted in phosphate-buffered 0.32 M sucrose, pH 7.4, on the remaining P2 synaptosomal samples from striatal tissue. Samples in reaction tubes were incubated on ice for 2 h and then centrifuged and decomposed. Measurement of DPM values were performed similarly to uptake assay. The specific binding was defined as the difference between the total and nonspecific binding (determined by the addition of 100 μM cocaine to parallel incubations).

Rotarod test. Behavior training was conducted before formal experimentation. All mice were placed and allowed to learn crawling on an accelerating rotarod using Rotarod System (DigBehav-RRTM, Shanghai JiLiang Science and Technology Co.LTD, China) 10:00 AM to 11:00 AM once daily for three consecutive days. The parameters for accelerating rotarod performance were defined as follows: acceleration rate 8 to 17 rotations per min and time-span 5.5 min. Retention time (latency to fall off the accelerating rod) was recorded by DigBehav-RRTM System. The animals with retention time of more than 240 s were adopted for experimentation. All animals adopted were separated into six groups (n = 10), i.e. normal (A), model (B), treatment (C, D, and E), and positive group (F). Group B, C, D, E and F were treated (IP) with vehicle solvent, BU at doses of 4, 20, and 100 mg/kg, and L-DOPA at 70 mg/kg (combined with 15 mg/kg benserazide; Roche, Shanghai, China; dissolved in 0.9 % saline just before experimentation), respectively, 30 min before subcutaneous injection of 20 mg/kg MPTP (Sigma–Aldrich, St. Louis, MO; dissolved in 0.9 % saline before experimentation). Group A received vehicle solvent and 0.9 % saline instead of BU and MPTP, respectively. The soybean oil was adopted as vehicle solvent in group A and group B, for it had been demonstrated, by a pretest, that retention time in MPTP mice treated with soybean oil was similar to that with 0.9 % saline (vehicle of L-DOPA). All animals were administered once daily for 14 consecutive days.

Retention time of each mouse on rotarod was recorded for 5.5 min at 4 h post-injection of MPTP on experimental day 4, 9, and 14, excepting that a series of measurements were, on the last experimental day, carried out at 1, 2, 4, 6, 8, 10, and 12 h post-administration of MPTP.

Tyrosine hydroxylase (TH) immunostaining. The mice (five adopted from each aforementioned group) were sacrificed by cervical dislocation and

their brains were removed immediately after behavioral experimentation. The brains were washed in vials containing cold PBS, placed in paraformaldehyde solvent for 24-h fixation, and then immersed in a phosphate-buffered sucrose solution for storage (4 °C) for at least 24 h prior to sectioning. Brains were frozen using a cryostat (Leica CM 3050 S; Leica, Nussloch, Germany) 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 detecting TH protein. Briefly, these sections were incubated in PBS 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 an additional 48 h. Afterwards, the sections were washed in 0.01M PBS (pH 7.5) three times (5 min each) and then incubated with a secondary antibody (biotinylated anti-rabbit IgG, Vector Laboratories, Burlingame, CA, USA) at 37 °C for 1.5 h and then with ABC solution for additional 1 h. After being washed with 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. Subsequently, the dyeing sections were rinsed with PBS buffer, mounted on gelatin-coated slides, air-dried, 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. 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 from the representative sections.

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. $P < 0.05$ was considered statistically significant. IC_{50} values were generated by linear regression ($r^2 > 0.95$).

Results

Bioactivity validation. The pure isolate showed a perfect efficacy for transporter inhibitions (IC_{50}: 0.19 μg/ml for DAT and 0.31 μg/ml for NET; see Table 1), generally consistent with the data reported in reference [9].

Structural elucidation and physicochemical properties. The data of ^{1}H-NMR (Table 2) were: H-2 (7.14, d, J = 8.40), H-3 (6.65, d, J = 8.70), H-5 (6.65, d, J = 8.70), H-6 (7.14, d, J = 8.40), H-7 (6.18, d, J = 16.5), H-8 (6.00, d, J = 15.9), H-10 (2.16, d, J = 4.2), H-11 (5.56,m), H-14 (1.19, s), H-15 (1.19, s), H-16 (1.01, s), H-17 (5.87, dd, J = 10.5, 17.4), H-18a (4.96, d, J = 19.8), H-18b (4.98, d, J = 9.6), and data of ^{13}C-NMR (Table 3) were: CH-2(128.74), CH-3(116.74), CH-5(116.74), CH-6(128.93), CH-7(135.92), CH-8(128.74), CH-10(45.81), CH-11(124.44), CH-14(30.53), CH-15(30.53), CH-16(24.50), CH-17(147.70), CH-18(112.87). Its molecular formula was identified as $C_{18}H_{24}O_2$ (MW: 272.17), which is typical of monoterpene structure (Fig. 1). These data confirm that the pure isolate is Δ^3,2-hydroxybakuchiol, i.e. BU.

Physicochemical tests disclosed some common properties of BU. It was a light brown, oily substance at room temperature, dissolvable in chloroform, ethanol, and methanol and insoluble in water. Its redox

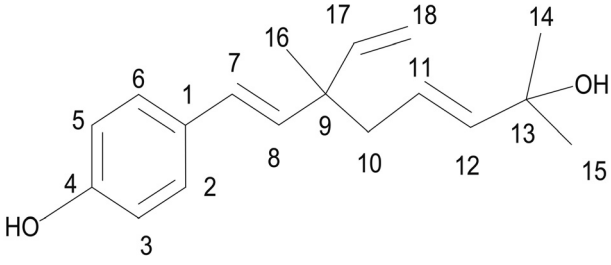

Figure 1. Chemical structure of BU. Numbers in the schematics,1 – 18, refer to Tables 2 and 3.

potential (ORP) was 227 mv. Ultraviolet scanning of BU (dissolved in methanol at concentration of 10μg/ml) showed an absorption maximum at 260 nm. Poor stability was shown after storing it in dilute hydrochloric acid solvent; however, in 0.1M sodium hydroxide solvent or hydrogen peroxide solvent, its stability was maintain for more than 2 h or 5 h, respectively.

Neuroprotection of BU on the MPP^+-insulted SK-N-SH cells. When conducting viability assay using MTT regent, a marker of mitochondrial reduction activity, the viability of SK-N-SH cells decreased to 59.36 % of the control (0 μM BU) at 24 h after the cells were subjected to MPP^+ insult. By contrast, the cells pretreated with BU (1 to 100 μM) or GBR12,935 (1 and 5 μM), after administration of MPP^+ for 24 hours, displayed higher levels of viability (64.58 %, 70.36 %, 83.68 %, and 91.87 % of control for cell groups pretreated with BU at 1, 5, 25, and 100 μM, or 78.11 % and 90.36 % of control for those pretreated with GBR at 1

Table 2. Data in CD3OD, 1HNMR-300HZ NMR spectra

No.	Chemical shift	Chemical shift ®	Hydrogen atom number	peak pattern	peak pattern ®	Coupling constant	Coupling constant ®
16	1.01	1.16	3H	s	s		
14	1.19	1.30	3H	s	s		
15	1.19	1.30	3H	s	s		
10	2.16	2.20	2H	q	d	J = 4.2	J = 5.8
18	4.96	4.99	2H	d	d	J = 19.8	J = 17.4
18	4.98	5.03		d	d	J = 9.6	J = 10.8
11	5.56	5.61	2H	m	m		
12		5.61			m		
17	5.87	5.87	1H	m	dd	J = 10.5, 17.4	J = 10.8, 17.3
8	6.00	6.03	1H	d	d	J = 15.9	J = 16.2
7	6.18	6.23	1H	d	d	J = 16.5	J = 16.2
3	6.65	6.75	2H	d	d	J = 8.70	J = 8.5
5	6.65	6.75		d	d	J = 8.70	J = 8.5
2	7.14	7.21	2H	d	d	J = 8.40	J = 8.5
6	7.14	7.21		d	d	J = 8.40	J = 8.5

R, reference of Labbe et al. (1996) [17].

and 5 μM, respectively) than those treated with MPP^+ alone [$p < 0.05$ or $p < 0.01$, BU (5, 25, or 100 μM) / GBR (1 or 5 μM) compared with MPP^+ -only, respectively)] (Fig. 2A); wherein, most of MPP^+-injured cells in the 100μM-BU- or 10μM-GBR12,935- treated group appeared healthy and had almost normal thickness under the phase contrast microscope (data not shown). For the purpose of confirming this action (i. e. ruling out the possibility that BU may provoke formazan exocytosis by enhancing membrane permeability), another viability assay was conducted by using CCK-8. Similarly, the CCK-8 test also exhibited a significant depressive effect on cell viability following exposure to MPP^+, with average value of 46.46 % of the control (0 μM BU), and also showed a marked prevention of MPP^+-induced cell toxicity when coincubated with this test compound BU or reference GBR12,935 (Fig. 2B). Additionally, both MTT and CCK-8 test showed that there was no clear difference in viability between SK-N-SH cells with BU (1 – 100 μM) itself and those with vehicle (Fig. 2).

Repeated dose administration of BU decreased uptake of plasmalemmal DA and NE and binding of [^{3}H]WIN35428. As assessed *ex vivo* on synaptosomes prepared from BU-treated rats, multiple BU administration produced a dose-dependent decrease of DA uptake in striatal synaptosomes (Fig. 3A) and of NE uptake in hippocampal synaptosomes (Fig. 3B).
Statistically, all dosing groups (5.44, 16.32, or 48.96 mg/kg BU) showed levels of DA uptake significantly different from control group ($p < 0.05$ or 0.01 respectively), and levels of NE uptake in the groups dosed with 16.32 and 48.96 mg/kg BU were also significantly different from that in control group ($p < 0.05$ or 0.01 respectively). The group with 5.44 mg/kg of reference compound GBR12,935 or desipramine also showed a marked decrease of DA- or NE-uptake by corresponding brain synaptosomes ($p < 0.01$ or 0.05 respectively, compared to control group). As with uptake assay, a significant decrease in membrane binding of [^{3}H]WIN35428 to striatal synaptosomes from drug-administered rats was shown in group with 5.44, 16.32, or 48.96 mg/kg of BU, or in that with GBR ($p < 0.05$ or 0.01 respectively, compared to control group) (Fig. 3C).

Effect of BU on retention time in MPTP-treated mice. The C57/BL mice treated with 20 mg/kg of MPTP showed spontaneous motor behavioral deficits such as immobility and tremble, which were typical symptoms of PD. As shown in model group (solvent/MPTP) in Fig. 4A, the retention time was significantly decreased during 14-day administration of MPTP (solvent/NS compared to normal group, $P < 0.01$); however, as seen in treatment groups, a dramatic blunted action on this MPTP-associated behavioral profile was produced by BU administration. The performance improvement in the MPTP mice on experimental day 4 was relatively weaker, with a mild but significant increase of retention time in the group dosed with 100 mg/kg of BU ($P < 0.05$ compared to vehicle); an additional five-day (day 4 to 9) treatment produced a moderate efficacy for increasing retention time ($P < 0.05$ and 0.01, 20 mg/kg or 40 mg/kg compared to

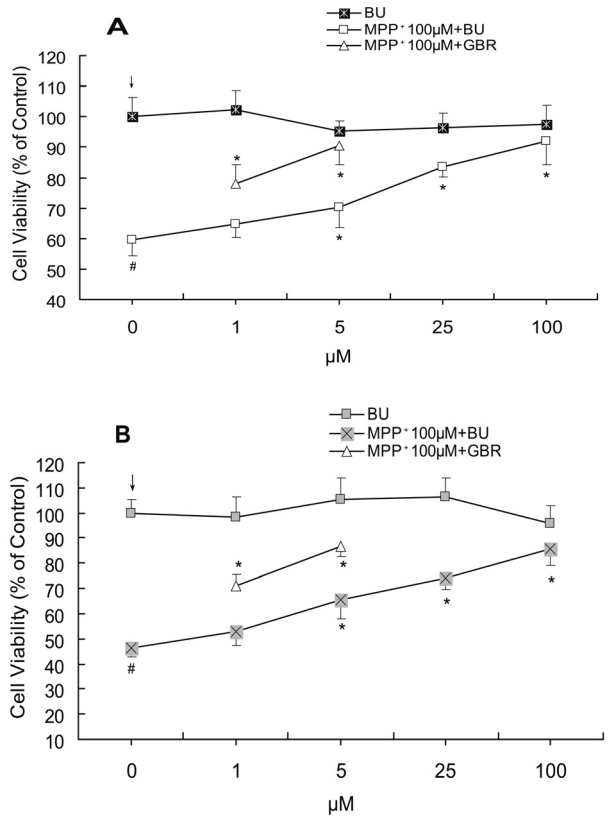

Figure 2. Effect of BU on cell toxicity induced by 1-methyl-4-phenylpyridinium (MPP^+). SK-N-SH cells were preincubated with vehicle or different concentrations of BU for 4 h followed by 24 h coincubation with MPP^+. To assess the effect of BU itself on cell viability, vehicle solvent was added to the wells instead of MPP^+. Selective DAT inhibitor GBR12,935 (GBR) served as positive control. Cell viability was determined by using MTT (*A*) and CCK-8 (*B*) assay and expressed as % of control (*arrow*). Each X axis represents concentrations of BU or GBR, wherein 0 μM is vehicle solvent (double distilled water containing 1 % DMSO). # $p < 0.01$, compared to control (*arrow*); *$p < 0.05$ and **$p < 0.01$, compared to MPP^+-lesioned control cells (# at 0 μM). Values are mean ± S.E.M. of five well samples (one-way ANOVA).

vehicle, respectively); and a high efficacy ($P < 0.05$ for the 4 mg/kg dose, $P < 0.01$ for doses of 20 mg/kg and 100 mg/kg, compared to vehicle respectively) was subsequently observed following the last five-day (day 14) treatment (Fig. 4A). On experimental day 14, the time-response relationship was even more obvious when measuring at a differing time point. As shown in Fig. 4B, in group with 100 mg/kg of BU, an initial 2 h steep curve that declined quickly was followed by a long-lasting, time-dependent activation of performance, until about 6 h, when the levels of retention time were highest (with levels comparable to that seen in L-DOPA or saline group); in the group with 4 or 20 mg/kg of BU, the time-dependent improvement appeared after an initial 4-h plateau phase, with a curve type generally similar to group with 100 mg/kg BU with the exception of an approximately 2-h delay; and a flat curve (the maximal effect) in each BU-treated group was seen around 8 h post-injection of MPTP. Comparatively, animals treated with reference drug L-DOPA (70 mg/kg) showed a similar retention time to the normal group whenever measurement was conducted (Fig. 4A and B).

Effect of BU on TH immunostaining in MPTP-treated mice. As shown in Figure 5A, sections in model (Fig. 5A2) or positive L-DOPA group (Fig. 5A3) showed a sparser TH-positive staining than the normal group (Fig. 6A1). Sections of 4 mg/kg (Fig. 5A4), 20 mg/kg (Fig. 5A5), and 100 mg/kg group (Fig. 5A6) exhibited a relatively dense staining. Quantification of TH-immunoreactive neurons disclosed that the subchronic MPTP insult produced a significant reduction of the TH-positive neuronal population in the substantia nigra (SN), 40.12 ± 6.89 % (normalized by normal control, same below) ($P < 0.01$ compared to normal). However, administration of BU significantly attenuated the loss of TH positive neurons, wherein the mean values are 51.09 ± 8.11 % for the 4 mg/kg group, 71.23 ± 7.89 % for 20 mg/kg group, and 80.13 ± 10.36 % for 100 mg/kg group ($P < 0.05$ or 0.01, each compared to model, respectively) (Fig. 5B). By contrast, TH-neuron number in the L-DOPA group (LDP) was 44.36 ± 5.69 %, and there was no difference in TH neuron number between the L-DOPA- and Vehicle/MPTP- group (Fig. 5B). As shown in Fig. 5C, compared to normal mice, the cell shape in the SN in model group (Fig. 5C2) was prominently changed from a round shape in normal group (Fig. 5C1) to a multi-shape pattern, i.e. irregular, moon-, or triangle-shape with an uneven color, and most of the cells were shrunken with some loss of nucleus and/or cytoplasm. However, BU treatment (Fig. 5 C4, C5, and C6) exhibited relatively pronounced protection from abnormal cell shape and size, and the most striking effect was shown in group with 100 mg/kg of BU (Fig. 5C6), in which the cells in SN were relatively round, intact with clear nucleus and/or cytoplasm, generally resembling those observed in intact animals. Comparatively, the cell shape pattern in L-DOPA group (Fig. 5C3) was generally similar to that in the model group (Fig. 5C2).

Discussion

Previously using our laboratory-scale column chromatographic method, 4 kg of PCS medicinal material (from Sichuan) was used for extraction, and the BU yield was around 1 g [9], which would be far less than the total dosage chronically given in an animal study. In this study, by using the scale-up extraction techni-

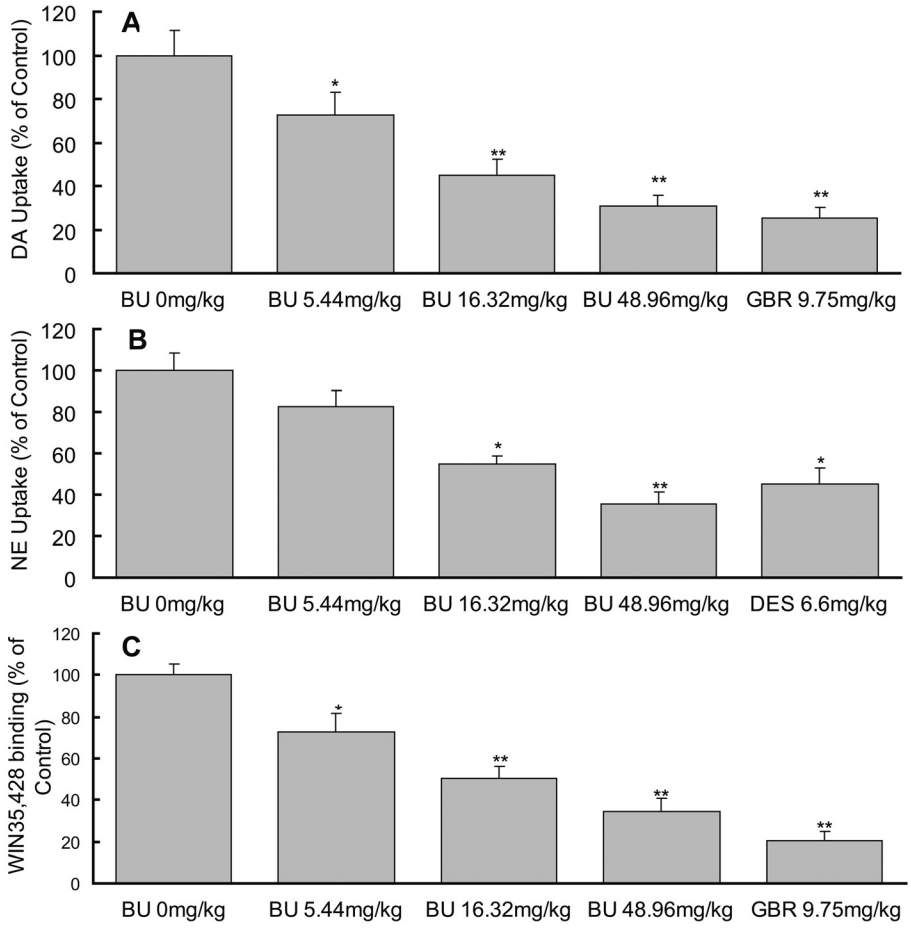

Figure 3. Effect of *in vivo* administration of BU on plasmalemmal [^{3}H]DA and [^{3}H]NE uptake and [^{3}H]WIN35428 binding. Rats (n = 6 per group) received four injections (2-h intervals; IP) of vehicle solvent, BU (5.44, 16.32, or 48.96 mg/kg), GBR12,935 (GBR, 9.75 mg/kg), or desipramine hydrochloride (DES, 6.6 mg/kg) 60 min prior to sacrificing. The striatum and hippocampus were then harvested to prepare synaptosomes. [^{3}H]DA (*A*) or [^{3}H]NE (*B*) uptake was assayed *ex vivo* with striatal synaptosomes or hippocampal synaptosomes, respectively, and [^{3}H]WIN35428 binding (*C*) was measured with striatal synaptosomes. GBR and DES, NET and DAT inhibitors respectively, served as positive control. Compared to the control group, * and **, $p < 0.05$ and 0.01, respectively. Values are normalized by control group (0 mg/kg, i.e. vehicle solvent) and expressed as mean ± SEM of n rats, each with triplicate synaptosomal reaction tubes.

que, i.e. supercritical carbon dioxide extraction technique together with column chromatographies and with activity guidance, 40 kg of this medicinal material (from Henan) can be extracted, and BU was isolated with a yield of around 6 g despite its lower total recovery (30.35 %). The low recovery shown in this study is possibly due to a loss in the frequent column chromatography. Extraction parameters thus need to be optimized in any near future study. Moreover, the relative yield/crude material ratios for the present extraction (≈ 1 g/4 kg) was appreciably less than that for the previous (≈ 6 g/40 kg); such difference could be due to the distinction of BU contents in herbal materials from particular regions [16]. Thus, screening of PCS medicinal material that highly contains BU in a certain producing area also needs to be considered in future technique innovation. As shown in Table 1, although the extraction procedure evoked an increase of BU content, degrees of increase of BU yield did not appear to be proportional to the relevant enhancement of bioactivity by PCS fraction, suggesting that some other known or unknown constituents also possibly hit monoanime transporters, as supported by the evidence that other PCS isolates such as hydroxybakuchiol and Δ^1,3-hydroxybakuchiol also possess potent inhibitory action on DAT and NET [8]. NMR spectrometry (Tables 2 and 3) gave a series of parameters related to hydrogen and carbon atoms, generally similar to data reported by Labbe et. al. [17]. Therefore, the pure compound BU was identified as Δ^3,2-hydroxybakuchiol, which was further verified by data from bioactivity detection (Table 1).

The chemical structure of BU was first identified from fructus psoraleae (*Psoralea corylifolia* L.), an herb used clinically as a health supplement ingredient or as an anti-aging agent in traditional Chinese medicine, in 1996 [17], and it was subsequently proven to have inhibitory activity against antigen-induced degranulation [18]. Our previous investigation showed that this meroterpenoid bakuchiol analog BU is a potent monoamine transporter inhibitor with selectivity for DAT and NET [9]. DAT has been proven to catalyze the reuptake of MPP^+, 1-benzyl-1,2,3,4-tetrahydroisoquinoline derivatives [19], and β-carboline [20], in addition to regulating synaptic DA content. DAT thus may be considered a molecular gate for neurotoxins. The present results showed that MPP^+ administration produced a marked decrease of viability of SK-N-SH

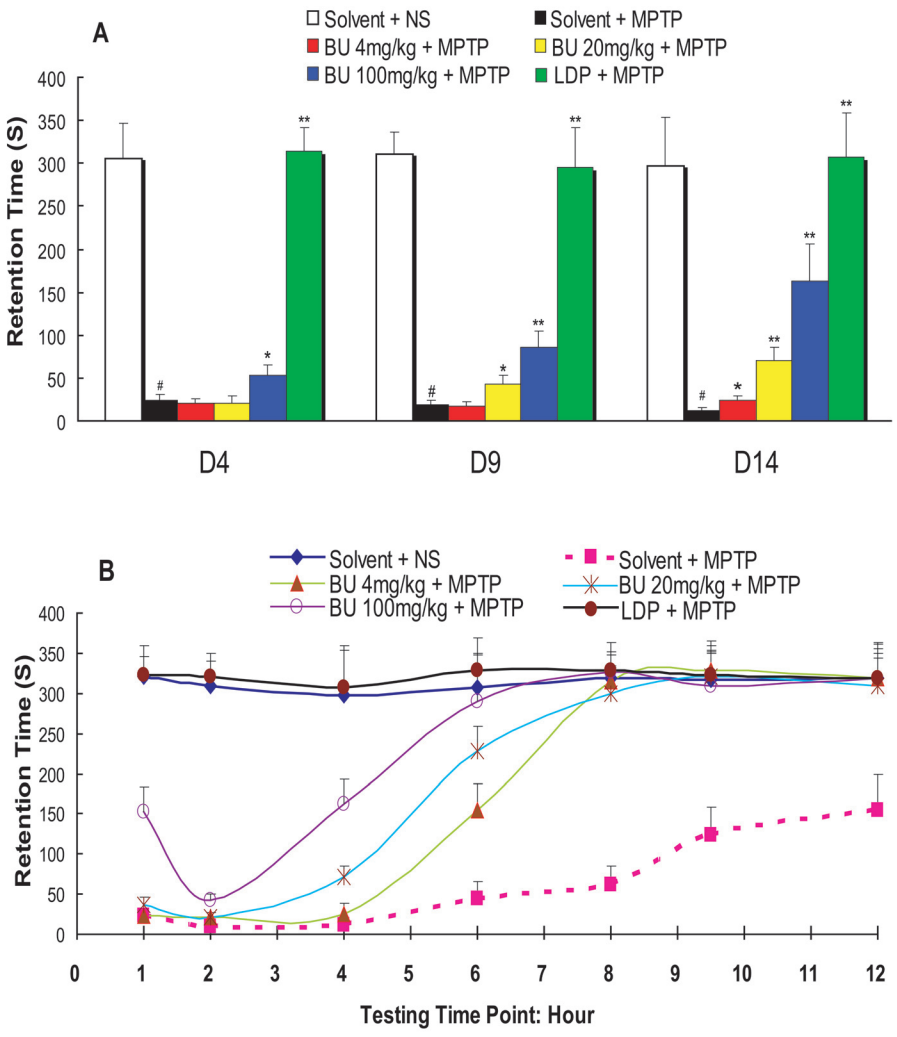

Figure 4. Effect of BU and L-DOPA (LDP) on parkinsonian behavior in MPTP-treated mice. A. Effect of BU on retention time in MPTP mice 4 h post-administration of MPTP, respectively on experimental day 4, 9, and 14 (D4, D9, and D14, respectively). B. Effect of BU on mice retention at differing time points (from 1 h to 12 h) after MPTP injection on the last experimental day. Mice in the normal (solvent + NS) group were administered with 0.9 % normal saline 30 min following vehicle solvent treatment. Model group (solvent + MPTP) was treated with vehicle solvent and MPTP. Treatment groups (4 mg/kg + MPTP, 20 mg/kg + MPTP, 100 mg/kg + MPTP) was treated with BU at a dose of 4, 20, and 100 mg/kg respectively, at 30 min prior to MPTP administration. "LDP + MPTP" represents positive group with 70 mg/kg of LDP and MPTP. $^{\#}P < 0.01$, compared to solvent + NS; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to solvent + MPTP. Values are the mean ± SEM of 8–10 mice (survival rate, 80–100 %).

cell, whereas this cytotoxicity was notably prevented by GBR12,935 (Fig. 2), implying that neuronal injury occurred via DAT uptake. Comparatively, BU also significantly mitigated the MPP^+-induced decrease of cell viability with similar efficacy to GBR12,935. This action was produced under the condition of ruling out the false positive that was possibly due to variation in cell viability produced by the testing of the compound itself, because BU alone did not affect cell viability (Fig. 2). The efficacy profile of BU in SK-N-SH cells, a human neuroblastoma cell line, is consistent with our previous result that was produced in D8 cells [9], providing further evidence to support its DAT inhibitory effect and neuroprotective action. The mechanism for the protective action may be through DAT suppression, which would contribute to counteracting uptake of MPP^+ into cytosomes and prevent mitochondria from subsequent injury [21, 22]. This protective effect was evaluated by MTT assay and verified by CCK-8 test. Assay with WST-8 may escape harassment by a false negative that results from possible enhancement of formazan exocytosis by some testing compounds with unknown activity in MTT test [23] and is generally believed to be more sensitive than MTT test [24], though both MTT and WST-8 are mainly reduced by pyridine nucleotide redox systems [25, 26] and commonly adopted for reflecting mitochondrial activity [24]. The convincing activity suggests that BU may be a promising dopaminergic protective agent for Parkinson's disease.

Similar to the *in vitro* profile of BU for inhibition of monoamine transports as previously reported in transgenic CHO cells [9], the present *ex vivo* assay showed that repeated dose injection of BU produced a decrease in DA/NE uptake in brain synaptosomes from the BU-dosed animals (Fig. 3A and B), most likely reflecting that BU can penetrate the blood-brain barrier and thereby elicited a subsequent plasmalemmal DAT inhibition. This supposition is supported and confirmed by the evidence that there

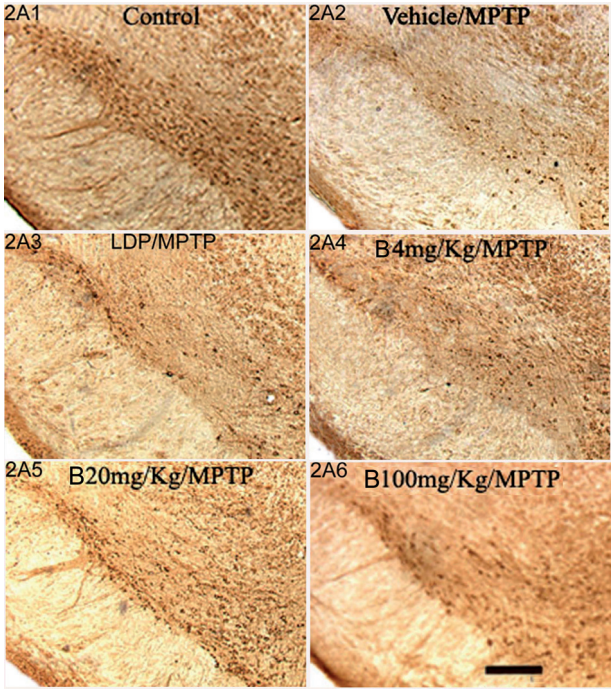

Figure 5. Immunohistochemical study on the effect of BU and LDP on TH-immunoreactive neurons in the substantia nigra in MPTP-treated mice (treatment design seen in Fig. 4). Sections with scale bar of 200 μm (*A*) showed TH-specific immunohistochemical stainings in substantia nigra of mice in the normal (solvent + NS) group (A1, Control), model group (A2, Vehicle/MPTP), positive group (A3, LDP/MPTP), and BU treatment groups (A4, B4 mg/kg / MPTP; A5, B20 mg/kg / MPTP; A6, B100 mg/kg / MPTP; B represents BU).

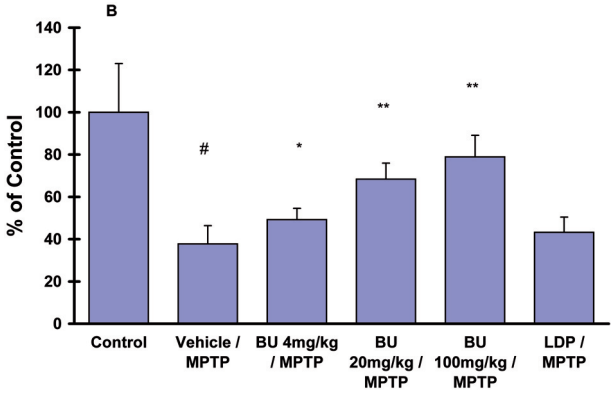

Figure 5. (*continued*) Quantification of TH-immunoreactive neurons in the substantia nigra was shown in (*B*). $^{@}P < 0.01$ compared to control; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to Vehicle/MPTP. Values are mean ± SEM of five mice.

was a striking decrease of WIN35,428 binding on these tested striatum samples in a dose dependent manner (Fig. 3C). Because cocaine can competitively inhibit DAT activity and its isotope-labeled analog WIN35,428 is commonly used to determine DAT quantity based on its binding sites [9, 27], the loss of WIN35,428 binding on administration of BU indicates

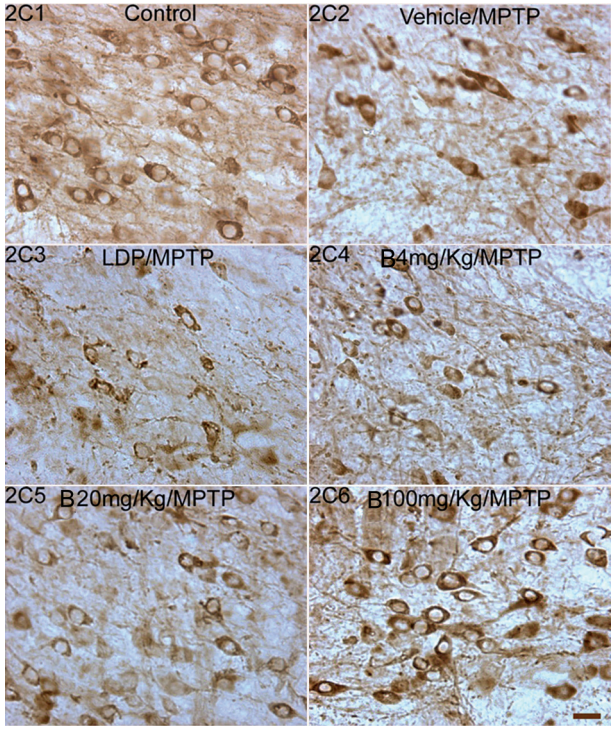

Figure 5. (*continued*) Cell shapes of TH-positive neurons were shown in sections (scale bar = 20 μm) of C1, C2, C3, C4, C5, and C6, representing normal group (Control), model group (Vehicle/MPTP), LDP positive group (LDP/MPTP), 4 mg/kg BU treatment group (B 4 mg/kg/MPTP), 20 mg/kg BU treatment group (B 20 mg/kg/MPTP), and 100 mg/kg BU treatment group (B 100 mg/kg/MPTP), respectively.

that DA-uptake sites of the synaptosomal DAT protein could have been occupied by this competitive DAT blocker BU before they were targeted by [^{3}H]WIN35,428, further confirming the inhibitory efficacy of the IP dosed BU on synaptosomal monoamine transporters. Furthermore, the plasmalemmal DAT inhibition, in rats administered with BU, suggests its potential, promising efficacy for disease treatment in related animal models such as the PD model.

Behavioral investigation in this study demonstrated that BU administration produced a significantly antiparkinsonian-like effect in MPTP mice (Fig. 4A and B). Synaptic dopamine, the level of which is modulated by DAT [28], is essential for regulating motility and locomotion; and MPTP-induced dopamine depletion in the nigrostriatal dopaminergic system contributes to parkinsonism in animals [29]. The older bl/c57 mice intoxicated with parkinsonian toxin MPTP reflect the threshold level causing the abnormal DA transmission and the concomitant motor dysfunction [29]. In this experiment, 13 month old male mice were used, receiving a successive 14 day MPTP administration. The MPTP mice exhibited a series of parkinsonian-like symptoms such as immobility, tremble, and poor performance on rotarod,

Table 3. Data in CD3OD, BB-DEPT-135 NMR spectra

No.	Chemical shift	Chemical shift ®	Hydrogen atom number
16	24.50	23.70	CH3
14	30.53	30.10	CH3
15	30.53	30.10	CH3
9	44.12	43.00	C
10	45.81	44.10	CH
13	71.74	71.00	C
18	112.87	112.70	CH 2
3	116.74	121.80	CH
5	116.74	121.80	CH
11	124.44	123.10	CH
8	128.74	126.80	CH
2	128.74	127.30	CH
6	128.93	127.30	CH
1	131.29	135.80	C
7	135.92	138.00	CH
12	124.67	141.50	CH
17	147.70	145.50	CH
4	158.28	149.90	C

R, reference of Labbe et al (1996) [17].

indicating the validity of the PD model. In contrast, BU treatment markedly improved the abnormality in a time- and dose-dependent manner in these MPTP mice. Our previous report showed that BU markedly reversed locomotion reduction in mice pretreated with catecholamine depleter reserpine [9]. It is suggested that, by a combination of our previous and present findings (*in vitro*, *ex vivo*, and *in vivo*), the activity improvement in MPTP mice by BU in this study could be attributable to activating the lesioned monoaminergic transmission by DAT blockade in nigrostriatal system.

Morphological evidence obtained from this MPTP model (Fig. 5 A, B, and C) further corroborates the antiparkinsonian-like effect of BU. The loss of TH-positive neurons in the SN in MPTP mice was significantly reversed and concomitantly the abnormal cell shapes also were recovered to differing degrees, following repeat BU administration. The morphological effect of BU is consistent with the aforementioned finding that BU can significantly mitigate the MPP^+-induced decrease of SK-N-SH-cell viability, further indicating its neuroprotective action. This *in vivo* neuroprotective profile of BU is possibly due to reducing the depression of mitochondria respiratory chain enzymes through inhibiting the gate action of DAT [30] or up-regulating a-synuclein [22] in dopaminergic subregions of brain. There was no dopaminergic protection as shown in the group treated with L-DOPA, even though L-DOPA (at dosage up to 70 mg/kg) displayed a potent behavioral effect (the rotarod latency of the L-DOPA treated mice was almost similar to that of normal group). Additionally, as mentioned above, administration of BU produced a notable inhibition of NE uptake in synaptosomes from the dosed rats (Fig. 3A). NE is able to activate neurotrophins such as nerve growth factor and brain derived neurotrophic factor [31], and NE itself has antioxidant properties and trophic effects on dopaminergic neurons [32, 33]. Damage to the norepinephrinergic system markedly deteriorated motor activity deficits in MPTP-treated mice [34], NET blockade by nisoxetine confers protection against dopaminergic lesion [35], and NET-knockout produces antiparkinsonism-like effects [35] in this PD model. Thus, NET inhibition could be a partial mechanism of neuroprotection in our MPTP mice with BU. Moreover, principal meroterpene bakuchiols have been proven effective in protecting against oxidative stress in rat liver microsomes and mitochondria [36], suggesting that antioxidation could be another potential mechanism underlying BU's protection against dopaminergic lesion in these old MPTP mice.

A series of studies has indicated an important role of monoamine re-uptake blockers such as brasofensine [37], GBR12,909 [14, 15], and methylphenidate [38] in PD treatment. BU, as a novel monoamine transporter inhibitor, may be effective for PD patients and evaluated as a pharmacotherapy for PD. In fact, empirically, the water extract of fructus psoraleae (rich in BU) has been used clinically to prevent brain aging and treatment of memory deficit, sexual dysfunction, enuresis, frequent urination, and fatigue syndrome in China for hundreds of years; the fructus psoraleae that is commonly used with a chronic, oral dosing of 9–30 g is known to have no toxic or addictive liability as reported by ancient Chinese clinicians [9]. Our previous study also showed that BU had no intrinsic rewarding or aversive effects on mice in conditioned place preference paradigm [9]. Additionally, toxicological experiment revealed a high i. p. LD_{50} value (1101.8 mg/kg) for BU in mice, further corroborating its relative safety in application.

Collectively, BU exhibits dopaminergic neuroprotection in immortalized neuronal cell line and antiparkinsonian-like effects, including improvement of akinesia and prevention of dopaminergic loss in MPTP mice, the mechanism of which may be due to monoamine-transporter suppression. These findings provide preliminary evidence for its potential as a clinically meaningful antiparkinsonian medication, including PD prevention, improvement of PD symptoms, and adjunctive therapy with L-DOPA.

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