BIOCHEMICAL PHARMACOLOGY 75 (2008) 1835–1847

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Bakuchiol analogs inhibit monoamine transporters and regulate monoaminergic functions

Gang Zhao [a,b], Shao-Yun Zang [a], Xiang-Wei Zheng [b], Xiao-Hua Zhang [b], Li-He Guo [a,b,*]

[a] Cell Star Bio-Technologies Co., Limited, 898 Halei Road, Shanghai 201203, People's Republic of China
[b] Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, People's Republic of China

ARTICLE INFO

Article history:
Received 12 November 2007
Accepted 28 January 2008

Keywords:
Bakuchiol analogs
Monoamine transporter
Dopamine transporter
Norepinephrine transporter
Reuptake inhibitor

ABSTRACT

Monoamine transporters play key roles in controlling monoamine levels and modulating monoamine reuptake. The objective of the present study was to identify monoamine transporter inhibitors from herbal sources. We discovered that bakuchiol analogs isolated from *Fructus Psoraleae* inhibited monoamine transporter uptake to differing degrees. The bakuchiol analog, Δ^3,2-hydroxybakuchiol was the most potent and efficacious reuptake blocker and was thus selected as the candidate target. Monoamine transporter inhibition by Δ^3,2-hydroxybakuchiol was more selective for the dopamine transporter (DAT) ($IC_{50} = 0.58 \pm 0.1$ μM) and norepinephrine transporter (NET) ($IC_{50} = 0.69 \pm 0.12$ μM) than for the serotonin transporter (SERT) ($IC_{50} = 312.02 \pm 56.69$ μM). Δ^3,2-Hydroxybakuchiol exhibited greater potency (pEC_{50} for DAT and NET) than bupropion and exhibited similar efficacy (E_{max} for DAT and/or NET) to bupropion and GBR12,935. Pharmacokinetically, Δ^3,2-hydroxybakuchiol competitively inhibited DAT and NET with partial reversibility and occupied cocaine binding sites. Moreover, Δ^3,2-hydroxybakuchiol counteracted 1-methyl-4-phenylpyridinium-induced toxicity in cells expressing DAT with similar efficacy to GBR12,935. *In vivo* studies showed that Δ^3,2-hydroxybakuchiol increased the activity of intact mice and improved the decreased activity of reserpinized mice. In the conditioned place preference test, preference scores in intact mice were unaffected by Δ^3,2-hydroxybakuchiol treatment. Bakuchiol analogs, especially Δ^3,2-hydroxybakuchiol, are monoamine transporter inhibitors involved in regulating dopaminergic and noradrenergic neurotransmission and may have represented potential pharmacotherapies for disorders such as Parkinson's disease, depression, and cocaine addiction.

* *Corresponding author at*: Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, People's Republic of China. Tel.: +86 21 54921392; fax: +86 21 54921391.
E-mail address: lhguo@sibs.ac.cn (L.-H. Guo).

Abbreviations: CHO, Chinese hamster ovary; DAT, dopamine transporter; DPM, disintegrations-per-minute; fr., fraction; GABA, γ-aminobutyric acid; GAT-1, γ-aminobutyric acid transporter; GBR12,935, 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)homopiperazine hydrochloride; HBSS, Hank's balanced salt solution; i.p., intraperitoneal; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NET, norepinephrine transporter; PBS, phosphate-buffered saline; SERT, serotonin transporter; Tr-CHO, transgenic Chinese hamster ovary; WIN35,428, (−)-2β-carbomethoxy-3β-(4-fluorophenyl) tropane.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.01.014

1. Introduction

The classic biogenic amine (or monoamine) neurotransmitters—dopamine (DA), norepinephrine (NE) and serotonin (5HT) control a variety of functions, including locomotion, autonomic function, hormone secretion, and complex behaviors associated with affect, emotion, and reward [1]. In the CNS, functional deficiencies in monoamine signaling has been implicated in the pathophysiology of various mental disorders [2]. A key step that determines the intensity and duration of monoamine signaling at synapses is the reuptake of the transmitter released into nerve terminals through high-affinity plasma membrane monoamine transporters, members of the members SLC6 (solute carrier family 6) family, by electrogenic Na^+ and Cl^- transport-coupled mechanisms [3–6]. Monoamine transporters for DA, NE, and 5HT DAT, NET and SERT respectively are expressed exclusively in monoamine-synthesizing neurons and contribute to monoamine homeostasis. Monoamine transporters can also transport other substrates including amphetamines and neurotoxins. For example, the DAT functions as a gate switch in modulating transporting molecular toxins, such as 1-methyl-4-phenylpyridinium (MPP^+; an active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP] [7]), 6-hydroxydopamine [8], and paraquat [9], into dopaminergic neurons and plays a pivotal role in selective dopaminergic toxicity. Monoamine transporter dysfunction may underlie many disorders associated with neurotransmitter imbalance, including depression, attention deficit hyperactivity disorder (ADHD), schizophrenia, and drug addiction [10]. In particular, the DAT plays a primary role in the reinforcing and behavioral-stimulant effects of psychostimulants in animals and humans [11]. The DAT, NET, and SERT represent established targets for many pharmacological agents that affect brain function, including psychostimulants and antidepressants [12,13]. Our major interest is to develop novel pharmacotherapies targeting monoamine transporters for the treatment of mental and neural disorders, such as drug dependence, depression, and Parkinson's disease. We established transgenic Chinese hamster ovary (Tr-CHO) cell lines expressing neurotransmitter transporters to screen for possible target chemicals from several hundred herbs. Bakuchiol analogs, meroterpenoids isolated from *Fructus Psoraleae*, seeds of *Psoralea corylifolia* L (Leguminosae), were found to be potent monoamine transporter blockers and to have regulation monoaminergic transmission.

2. Materials and methods

2.1. Reagents

Ascorbic acid, pargyline hydrochloride, fluoxetine hydrochloride, 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)-homopiperazine hydrochloride (GBR12,935), desipramine hydrochloride, bupropion hydrochloride, tiagabine hydrochloride, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 1-methyl-4-phenylpyridinium iodide, dopamine hydrochloride, and norepinephrine bitartrate were obtained from Sigma Chemicals (St. Louis, MO, USA). Cocaine hydrochloride was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Reserpine injection was obtained from Fudan Fuhua Pharmaceutical Co., Ltd. (Shanghai, China). Bakuchiol analogs were prepared in our laboratory. [^{3}H]dopamine (8.8 Ci/mmol), [^{3}H]serotonin (111 Ci/mmol), [^{3}H]γ-aminobutyric acid (GABA) (88 Ci/mmol), and [^{3}H]norepinephrine (40.0 Ci/mmol) were purchased from GE Healthcare UK Limited (Buckinghamshire, UK). [^{3}H](−)-2β-carbomethoxy-3β-(4-fluorophenyl) tropane (WIN35,428) (86 Ci/mmol) was obtained from PerkinElmer (Boston, MA, USA). RMPI 1640 medium, fetal bovine serum, and Geniticin (G418) were obtained from Invitrogen (Carlsbad, CA, USA). All other chemicals were of the highest purity and were obtained from Sigma Chemicals. Compounds were prepared just before use in saline (NaCl 0.9%). The hydrochlorides, bitartrate, and iodide were dissolved in deionized water for *in vitro* study or in saline (NaCl 0.9%) for *in vivo* study. Bakuchiol analogs were dissolved in deionized water containing 1% dimethylsulfoxide for *in vitro* study or in refined soybean oil for *in vivo* study. All compound solutions were prepared just before experimentation. The injection volume per gram animal weight used to administer compounds was 0.01 ml.

2.2. Extraction and isolation of free bakuchiol analogs

We previously reported that the inhibitory effects of the petroleum ether extract fraction separated from *Fructus Psoraleae* on monoamine transporters are the most potent among the distinct fractions of *Fructus Psoraleae* extracts [14]. In this study, further isolation of active compounds from the petroleum ether extract of *Fructus* Psoraleae was performed by concomitant bioactivity tracing. The method for uptake assay is shown in Section 2.4 below, and data are shown in Fig. 1. Dry, ripe *Fructus Psoraleae* (4000 g) was purchased from Sichuan Province, China, and powdered and extracted with 95% ethanol three times under reflux. Evaporation of the solvent under reduced pressure provided the ethanolic extract (860.4 g; yield rate: 21.51%). The ethanolic extract was partitioned in a petroleum ether: H_2O (1:1, v/v) mixture. Removal of the solvents *in vacuo* from the petroleum ether-soluble fraction (fr.) yielded 167.8 g of the petroleum ether extract of *Fructus Psoraleae*. Normal-phase silica gel column chromatography [3 kg, *n*-hexane–EtOAc (15:1 → 10:1 → 5:1 → 1:1, v/v) → $CHCl_3$:MeOH:H_2O (6:4:1, v/v/v) → MeOH] of the extract gave nine fractions [fr.1 (16.2 g), fr.2 (17.5 g), fr.3 (26.4 g), fr.4 (17.8 g), fr.5 (17.5 g), fr.6 (20.5 g), fr.7 (16.4 g), fr.8 (17.5 g), fr.9 (16.8 g)]. As shown in Fig. 1A, fr.3 displayed the strongest inhibitory action on DAT or NET among the fractions and was thus used for further isolation. The candidate fr.3 (3.0 g) was separated by reverse-phase silica gel column chromatography [0.15 kg MeOH–H_2O (60:40 → 80:20, v/v) → MeOH] to yield five fractions, of which fr.3-3 was a candidate for suppressive effects (Fig. 1B), and its chemical configuration was identified (fr.3-3, bakuchiol; 1.1 g; yield rate: 0.242%). Meanwhile, fr.6 (20 g) was separated by reverse-phase silica gel column chromatography [300 g, MeOH–H_2O (60:40 → 70:30 → 80:20, v/v) → MeOH] to yield six fractions [fr.6-1 (568 mg), fr.6-2 (2228 mg), fr.6-3 (4020 mg), fr.6-4 (3218 mg), fr.6-5 (4211 mg), fr.6-6(5197 mg)] followed by bioactivity tracing (Fig. 1C). The preferential fr.6-3 (4000 mg) was

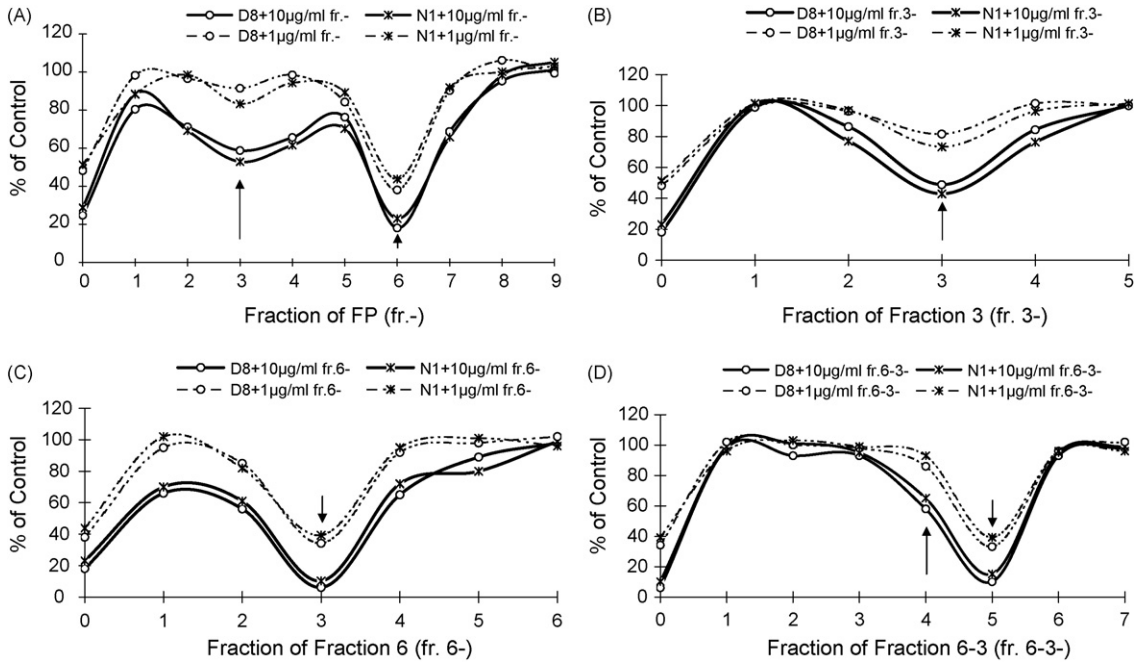

Fig. 1 – Bioactivity tracing of active compounds from petroleum ether extract fraction (FP) of Fructus Psoraleae. Activity was detected by [^{3}H]-DA or [^{3}H]-NE uptake assay in D8 or N1 cells. Each *x*-axis represents fraction number, wherein 0 is the primary fraction prior to further isolation. (A) Assay for activity of FP fractions. Candidate fractions (*arrows*) were chosen for further isolation. (B) Assay for activity of fractions of fr.3 (fr.3-0 to 5). The chemical structure of fr.3-3 was identified as bakuchiol (B1). (C) Assay for activity of fractions of fr.6 (fr.6-0 to 6). (D) Assay for activity of fractions of the candidate fr.6-3 (fr.6-3-0 to 7). The fr.6-3-4 and fr.6-3-5 were structurally confirmed to be Δ^1,3-hydroxybakuchiol (B2) and Δ^3,2-hydroxybakuchiol (BU), respectively. Values are expressed as the means of triplicate cell samples (each S.E.M. is less than 20% of mean) and defined by % of D8/N1 control cells (means of DPM values in (A)–(D): 22364.31/19451.27, 25567.11/18756.75, 24344.32/22157.33, and 26114.31/23561.66, respectively).

separated by high-performance liquid chromatography (Beckman Coulter, Fullerton, CA, USA) [isopropanol–H_2O (40:60, v/v)] to yield seven fractions. As shown in Fig. 1D, two isolates, fr.6-3-4 (Δ^1,3-hydroxybakuchiol; 640.1 mg; yield rate: 0.016%) and fr.6-3-5 (Δ^3,2-hydroxybakuchiol; 1569.8 mg; yield rate: 0.039%), markedly inhibited monoamine transporter activity. The molecular configurations of the final pure samples were elucidated by 1H NMR (400HZ) (Varian, Palo Alto, CA, USA). The purities of bakuchiol, Δ^1,3-hydroxybakuchiol, and Δ^3,2-hydroxybakuchiol were 94.1, 92.2, and 96.54%, respectively, which was analyzed by the normalization method following high-performance liquid chromatography detection. The three isolates can be classified structurally as bakuchiol analogs (Fig. 2).

2.3. Cell culture and DAT transfection

Chinese hamster ovary (CHO) cells expressing the rat DAT (rDAT; GI 310097), rat SERT (rSERT; GI 207086), mouse γ-aminobutyric acid transporter-1 (mGAT-1; GI 37590748), and human NET (hNET; GI 189257) were used in this study as described previously [14,15]. rDAT-pCDNA3 was transfected into CHO cells using electroporation methods. Selection of the transfected cells was conducted in culture by the addition of G418. CHO cells stably expressing DAT then were subcloned by limiting dilution methods. Several subclones were selected using a [^{3}H]-DA uptake assay (procedure shown in Section 2.4 below). 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, S6, or N1 cells, respectively) was obtained. As shown in Table 1, 20–30 times enhancement of neurotransmitter uptake occurred after CHO cells were transfected with the corresponding neurotransmitter transporter. GBR12,935 (selective DAT inhibitor), desipramine (selective NET inhibitor), fluoxetine (inhibitor of SERT), and tiagabine (inhibitor of GAT-1), at concentrations of 10, 100, 100, and 100 μM, respectively, inhibited the enhanced uptake with disintegrations-per-minute (DPM) values similar to background measured in CHO cells, conferring validity and feasibility of the screening model. Replicate variabilities were less than 10%.

2.4. [^{3}H]monoamine uptake assay in vitro

D8 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) and 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. [^{3}H]-DA, ascorbic acid, and pargyline were added to final concentrations of 100 nM, 100 μM, and 100 μM, respectively. Cells then were incubated

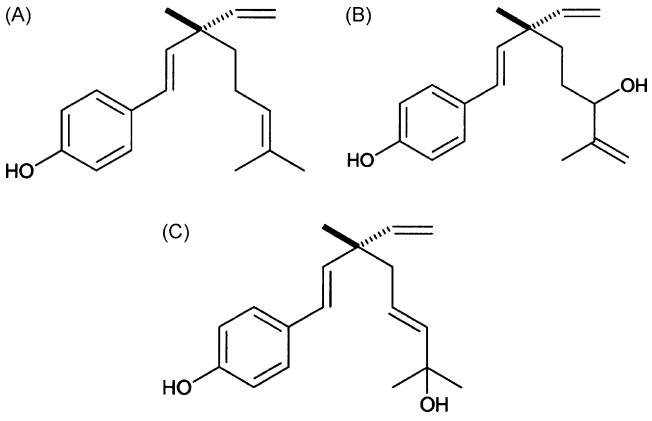

Fig. 2 – Chemical structure of bakuchiol analogs: (A) bakuchiol, (B) Δ^1,3-hydroxybakuchiol, and (C) Δ^3,2-hydroxybakuchiol.

at 37 °C for 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 then were solubilized in 2N NaOH, and an aliquot was measured by liquid scintillation (TRI-CARB2900TR, PerkinElmer, Waltham, MA, USA) to quantify uptake of [^{3}H]dopamine. Specific uptake was defined as the difference between total uptake by the Tr-CHO cells and non-specific accumulation determined in CHO cells. To explore the effects of fractions of the petroleum ether extracts of *Fructus Psoraleae* and bakuchiol analogs on transporters, different concentrations of extract or bakuchiol analog solutions were individually added to Tr-CHO cell cultures at the beginning of the uptake assay. To assay 5HT and NE transport, the procedure was similar to that used for DA uptake, with the exception that 50 nM [^{3}H]-5HT or 25 nM [^{3}H]-NE was used instead of [^{3}H]-DA for S6 cells and N1 cells, respectively. For the GABA uptake assay, 50 nM [^{3}H]GABA was used instead of [^{3}H]-DA and ascorbic acid and pargyline in the assay for G1 cells. The [^{3}H]-labeled neurotransmitter uptake assay in the human neuroblastoma cell line SK-N-SH, known to express endogenous DAT and NET, was performed similarly to that for Tr-CHO cells. GBR12,935, bupropion, fluoxetine, and tiagabine were used as positive controls for [^{3}H]-DA, [^{3}H]-NE, [^{3}H]-5HT, and [^{3}H]GABA uptake, respectively. The concentration–effect curve was established to measure IC_{50} values (concentration required to inhibit specific monoamine uptake by 50%), E_{max} (maximal inhibition rate), and EC_{50} (effective concentration to reach 50% of E_{max}). Inhibition rate = [(DPM value of vehicle-treated Tr-CHO cells − DPM value of drug treated Tr-CHO cells)/(DPM value of vehicle-treated Tr-CHO cells − DPM value of CHO cells) × 100%]. pEC_{50} = −log [concentration of Δ^3,2-hydroxybakuchiol, μM].

The pharmacokinetic inhibition characteristics were evaluated using the Tr-CHO platform. The method of competitive analysis was reported previously by Xu et al. [15]. Reversal analysis was performed in D8 or N1 cells. The cells were pretreated with HBSS or different concentrations of Δ^3,2-hydroxybakuchiol at room temperature for 10 min. The solution then was removed, and the cells were washed with PBS three times. An additional 10 min incubation was conducted after Δ^3,2-hydroxybakuchiol or vehicle was added into vehicle-pretreated or Δ^3,2-hydroxybakuchiol-pretreated plate wells, respectively, in addition to overall concomitant addition of [^{3}H]-DA (or [^{3}H]-NE), ascorbic acid, and pargyline into the wells. Subsequently, DPM values were measured by a liquid scintillation counting analyzer.

2.5. *Uptake of DA and NE by rat brain synaptosomes*

The procedure previously described [16] was used with modifications. Male Sprague–Dawley rats (200–250 g) were sacrificed by decapitation, and the striatum and hippocampus were dissected. Fresh brain tissues were homogenized with a glass homogenizer (with 10–20 strokes) in ice-cold 0.32 M phosphate-buffered sucrose and centrifuged at 1000 × *g* at 4 °C for 10 min. The supernatants (S1) then were centrifuged at 22,000 × *g* at 4 °C for 15 min. 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_2PO4 1 mM, $NaHCO_3$ 27 mM, and glucose 5.4 mM at 37 °C for 5 min. The incubation was continued for another 10 min in the same medium containing 100 μM pargyline, 100 μM ascorbic acid, and 100 nM [^{3}H]-DA or 25 nM [^{3}H]-NE (100 μl final volume). 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. Pellets were resuspended with 1.2 ml ice-cold Li^+-Krebs Ringer Henseleit medium and centrifuged again as described above. The pellets then were decomposed by NaOH (2N, 100 μl), and an aliquot was used for determination of radioactivity. The

Table 1 – Functional confirmation of *in vitro* screening

	CHO + vehicle	Tr-CHO + vehicle	Tr-CHO + antagonist
[^{3}H]dopamine uptake by D8	807.44 ± 81.37*	25485.66 ± 2168.33	881.11 ± 61.59* (GBR12,935)
[^{3}H]norepinephrine uptake by N1	628.45 ± 109.99*	20198.11 ± 1497.65	687.21 ± 98.39* (desipramine)
[^{3}H]serotonin uptake by S6	764.33 ± 89.36*	16509.13 ± 1439.33	669.47 ± 87.28* (fluoxetine)
[^{3}H]GABA uptake by G1	979.97 ± 110.58*	32866.98 ± 2461.19	917.44 ± 78.36* (tiagabine)

Dopamine, norepinephrine, serotonin, and GABA uptake assays were performed using Tr-CHO cells expressing DAT (D8 cells), NET (N1 cells), SERT (S6 cells), and GAT-1 (G1 cells), respectively. The results showed 20–30 times uptake enhancement was induced by transfection of transporters. GBR12,935 (10 μM), desipramine (100 μM), fluoxetine (100 μM), and tiagabine (100 μM) are selective inhibitors of DAT, NET, SERT, and GAT-1, respectively. *$p < 0.001$, compared with control (D8, N1, S6, and G1 cells), respectively, inhibited the enhanced uptake to nearly background levels. Values are mean ± S.E.M. of triplicate cell samples.

specific uptake of DA or NE 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. To study the effect of Δ^3,2-hydroxybakuchiol on DA and NE uptake by synaptosomes, different concentrations of Δ^3,2-hydroxybakuchiol, GBR12,935, and desipramine were added at the beginning of the uptake assay. EC_{50} (effective concentration of 50% of maximal inhibition rate) was determined by concentration–effect curves. Inhibition rate = [(DA uptake produced by vehicle − DA uptake produced by drug)/(specific DA uptake produced by vehicle)] × 100%.

2.6. [^{3}H]WIN35,428 binding assay

D8 cells were assayed for membrane binding of the [^{3}H]-labeled cocaine analog WIN35,428 as described previously [14]. Briefly, D8 cells were incubated with 2 nM [^{3}H]WIN35,428 at 4 °C for 120 min. 100 μM (−)-cocaine was added to parallel incubations to provide estimates of nonspecific binding. Three washes with ice-cold buffer terminated the binding reaction. Analysis of the effect of Δ^3,2-hydroxybakuchiol on binding was performed by the addition of increasing concentrations of Δ^3,2-hydroxybakuchiol into the reaction system.

2.7. Effect of Δ^3,2-hydroxybakuchiol on D8 cell viability after MPP^+ administration

The effect of Δ^3,2-hydroxybakuchiol on cell viability was evaluated by the MTT assay. D8 cells were cultured in RMPI 1640 medium and inoculated in 96 culture wells (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Plate cells were approximately 30,000 per well after 24 h incubation. GBR12,935 (10 μM) and increasing concentrations of Δ^3,2-hydroxybakuchiol were added into the cell wells and incubated for 4 h. MPP^+ was subsequently added into the wells to a final concentration of 33.3 μM with a total volume of 200 μl and incubated for an additional 48 h. MTT reagent (with a final concentration of 0.5 mg/ml) then was added to the system, and the dyeing reaction persisted for 4 h until purple precipitate was visible. The cells were solubilized by 100 μl dimethylsulfoxide, and the optical density (OD) value was recorded at 570 nm in Multiscan MK3 (Nova and Technology Development Co. Ltd., Beijing, China). For assessment of the effect of Δ^3,2-hydroxybakuchiol on cell viability, vehicle solvent was used instead of MPP^+. EC_{50} of Δ^3,2-hydroxybakuchiol or GBR12,935 was defined by concentration of 50% of maximal protection rate. Protection rate = [(OD value of Δ^3,2-hydroxybakuchiol-treated toxic cells − OD value of vehicle-treated toxic cells)/(OD value of control cells − OD value of vehicle-treated toxic cells)] × 100%.

2.8. Animals

Male C57BL/6 mice (weighing 18–22 g, 2 months old) were purchased from the Laboratory Animal Center of the Chinese Academy of Science (Shanghai, China). Mice were housed at room temperature (22 ± 3 °C) with 50–60% humidity under a standard 12-h light/12-h dark cycle (lights on at 07:00 a.m.) with unlimited access to food and water. They were housed for 1 week prior to the experiments. The experimental protocols in the animal studies 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.

2.9. Locomotor activity measurements in intact mice

Locomotor activity was measured in opaque plastic cages (60 cm × 40 cm × 25 cm), with 24 squares (10 cm × 10 cm) drawn by brush pen on the bottom of each cage. The animals were separated randomly into five groups (n = 8) and habituated to the cages for 60 min before administration of 0.9% saline, cocaine (15 mg/kg i.p.), and Δ^3,2-hydroxybakuchiol (4, 20, and 100 mg/kg), respectively. Locomotor activity was recorded for 2 h by a video camera mounted on the ceiling above the center of the apparatus. The camera was interfaced with a computer, and line breaks per 15 min were counted by observers blind to treatment condition.

2.10. Locomotor activity measurements in reserpinized mice

Locomotor activity of reserpinized mice was examined by the DigBehv (Locomotor) System (Jiliang Software Technologies Co., Ltd., Shanghai, China). The open-field apparatus consisted of a large black opaque, sound-attenuated plastic box (0.6 m × 0.6 m × 0.8 m) with a smaller square Plexiglas chamber (50 cm × 50 cm × 80 cm) inside, with six video cameras mounted above the apparatus. The mice were subcutaneously injected with 10 mg/kg reserpine (injection diluted with 0.9% NaCl) and remained in the cage for 24 h to be reserpinized. After grouping, the reserpinized mice were gently transferred into the smaller chamber and were habituated for 1 h. Animals then were injected (i.p.) with vehicle or different doses of Δ^3,2-hydroxybakuchiol (5, 20, or 100 mg/kg) or bupropion (20 mg/kg) followed by a 100-min recording session. All experimental events were controlled and recorded automatically by a computer located in an adjacent room. Spontaneous locomotor activity was analyzed by the Locomotor System software.

2.11. Conditioned place preference

The conditioned place preference paradigm was performed as described previously [17] with slight modifications. The place preference conditioning schedule consisted of three phases: preconditioning, conditioning, and testing. During the preconditioning phase, C57BL/6 mice were placed in the middle of the neutral area and allowed to freely explore the entire apparatus for a 20-min session daily for the first 2 days. Their locations were recorded by the DigBehv (CPP) System (Jiliang Software Technologies Co., Ltd., Shanghai, China) for 15 min on the third day to assess baseline place preferences. During the conditioning phase, mice were treated alternately at 8 a.m. and 2 p.m. for five consecutive days with daily injections (i.p.) of cocaine (20 mg/kg), Δ^3,2-hydroxybakuchiol (10 and 100 mg/kg), or the petroleum ether extract of *Fructus Psoraleae* (100 mg/kg) in the drug-paired compartment and saline in vehicle-paired compartment. Each pairing lasted 20 min. Control animals received saline each day. During the testing phase, locations of the mice were recorded for 15 min at 24 h after the

final conditioning session. Preference score (time spent in the drug-paired compartment vs. total time spent in all compartments, expressed as a percentage of total time) was analyzed by DigBehv (CPP) software.

2.12. Data analysis

Data were analyzed using SPSS v.13.0 (SPSS, Chicago, IL, USA). Differences among groups were assessed using analysis of variance (ANOVA) followed by *post hoc* comparisons. Differences were considered statistically significant with $p < 0.05$. IC_{50} values were analyzed by linear regression ($r^2 > 0.95$). pEC_{50} and E_{max} were analyzed by GraphPad Prism v.4.01 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Effects of bakuchiol analogs on monoamine uptake by Tr-CHO cells

The inhibitory effects of three bakuchiol analogs isolated from the petroleum ether extract of *Fructus Psoraleae* on DAT, NET, and SERT were evaluated by neurotransmitter uptake assay in Tr-CHO cell lines. As shown in Fig. 3, we found differing monoamine uptake inhibition in D8, N1, or S6 cells after a 20-min incubation of bakuchiol, Δ^1,3-hydroxybakuchiol, or Δ^3,2-hydroxybakuchiol (10 μM) ($p < 0.001$ compared with respective control), while no effect on GABA uptake was observed by G1 cells ($p > 0.05$ compared with control cells). For corroboration of these results, increasing concentrations of bakuchiol analogs were added to the screen. Half-logarithmic curves of concentration–effects were obtained, and pEC_{50} and E_{max} were analyzed. pEC_{50} and E_{max} (expressed as inhibition rate) of bakuchiol analogs for monoamine transporters are shown in

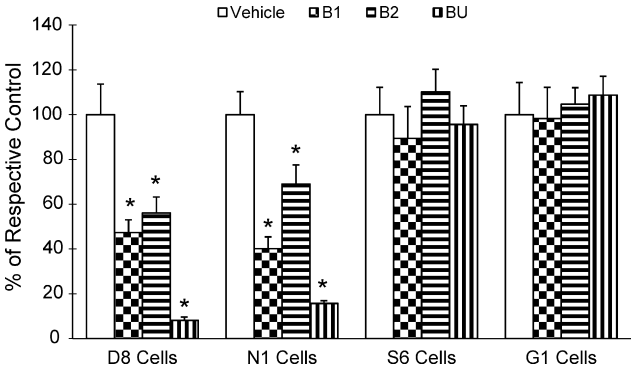

Fig. 3 – Effects of bakuchiol analogs on monoamine transporters. Bakuchiol (B1) and its analogs, Δ^1,3-hydroxybakuchiol (B2) and Δ^3,2-hydroxybakuchiol (BU), at a concentration of 10 μM, inhibited DA, NE and 5-HT uptake by respective Tr-CHO cells (D8, N1, or S6). Values are expressed as mean ± S.E.M. of triplicate cell samples and defined by % of D8, N1, S6, or G1 control cells (DPM value: 24117.11 ± 1457.24, 23427.77 ± 1746.75, 14844.22 ± 1617.33, and 35174.81 ± 2161.37, respectively). * $p < 0.001$, compared with D8, N1, or S6 control cells (one-way ANOVA). pEC_{50} and E_{max} values are shown in Table 4.

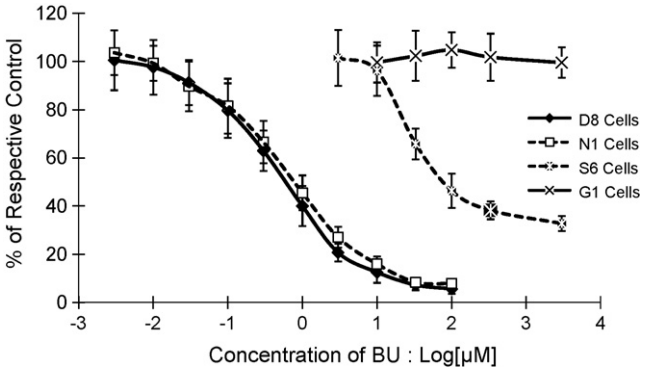

Fig. 4 – Effect of different concentrations of Δ^3,2-hydroxybakuchiol (BU) on isotope-labeled transmitter, DA, NE, 5-HT, or GABA, uptake by respective Tr-CHO cells. Values are defined by % of D8, N1, S6, or G1 control cells (DPM value: 25017.11 ± 2151.24, 20727.77 ± 1916.55, 15584.22 ± 1067.67, and 31184.81 ± 2551.07, respectively). IC_{50} or pEC_{50} and E_{max} values are shown in Tables 2 and 4, respectively.

Table 4. The three isolates exhibited different pEC_{50} and E_{max} values for the monoamine transporters, wherein the two values for DAT and NET were substantially higher than for SERT. Bakuchiol analogs at concentrations up to 3 mM did not affect [^{3}H]GABA uptake by G1 cells (data for bakuchiol and Δ^1,3-hydroxybakuchiol not shown; data for Δ^3,2-hydroxybakuchiol shown in Fig. 4). Of the bakuchiol analogs, Δ^3,2-hydroxybakuchiol exhibited stronger potency and higher efficacy and was thus chosen for further testing. Δ^3,2-Hydroxybakuchiol at concentrations of 0.003-3000 μM, added cumulatively in increments of 0.5 log units, dose-dependently inhibited monoamine uptake (IC_{50}: DAT, 0.58 ± 0.1 μM; NET, 0.69 ± 0.12 μM; SERT, 312.02 ± 56.69 μM) (Table 2, Fig. 4). Compared with bupropion, Δ^3,2-hydroxybakuchiol exhibited stronger inhibitory actions on DAT and NET with slightly higher pEC_{50} values, despite sharing similar E_{max} values. Compared with GBR12,935, Δ^3,2-hydroxybakuchiol exhibited a lower pEC_{50} value for DAT inhibition, but the two had similar E_{max} values. In addition, Δ^3,2-hydroxybakuchiol pEC_{50} and E_{max} values for SERT were less than those of fluoxetine (Table 4).

Similar DA and NE uptake inhibition by Δ^3,2-hydroxybakuchiol was found in human neuroblastoma cell line SK-N-SH (Fig. 5A and B), human pheochromocytoma cell line PC12 (data not shown), and rat brain synaptosomes (Fig. 6A and B). Δ^3,2-Hydroxybakuchiol IC_{50} values in SK-N-SH cells and synaptosomes, and pEC_{50} and E_{max} in synaptosomes, are shown in Tables 2 and 5, respectively.

3.2. Pharmacokinetics of Δ^3,2-hydroxybakuchiol inhibitory effects on DAT and NET

The Michaelis–Menten constant (K_m) and the maximal reuptake rate (V_{max}) of the transporters were determined by incubating Tr-CHO with varying concentrations of substrate. A curve typical of competitive inhibition was shown in

Table 2 – IC_{50} values (μM) of Δ^3,2-hydroxybakuchiol acting on monoamine transporters

	DAT	NET	SERT	GAT-1	n
Trans-CHO Cells	0.58 ± 0.10	0.69 ± 0.12	312.02 ± 56.69	>1000	3
SK-N-SH Cells	0.46 ± 0.13	0.85 ± 0.24	nd	nd	3
Symnaptosomes	1.02 ± 0.24	1.55 ± 0.28	nd	nd	3

nd, not detected. Δ^3,2-Hydroxybakuchiol selectively inhibited DAT and NET. IC_{50} for dopamine and norepinephrine uptake inhibition was significantly less than for serotonin or GABA uptake inhibition. Tr-CHO Cells: representative CHO cells expressing DAT (D8), NET (N1), SERT (S6), or GAT-1 (G1). The methods for isotope-labeled neurotransmitter uptake by Tr-CHO cells, SK-N-SH cells, and synaptosomes are shown in Section 2 (Sections 2.4 and 2.5). Values are mean ± S.E.M. of three different experiments performed in triplicate.

the Δ^3,2-hydroxybakuchiol-D8 cells reaction system (Fig. 7A). Δ^3,2-Hydroxybakuchiol at 0.5 μM and 2 μM had no marked effects on V_{max} of dopamine uptake, though the K_m value dose-dependently increased (Table 3). With regard to the curve characteristics of NE uptake in N1, Δ^3,2-hydroxybakuchiol treatment displayed a curve typical of competitive inhibition, in which Δ^3,2-hydroxybakuchiol had no effect on V_{max} but elicited a dose-dependent increase in K_m (Table 3, Fig. 7B). After the D8 cells pretreated with Δ^3,2-hydroxybakuchiol were washed with PBS three times, about 12–43% of [^{3}H]-DA uptake inhibition was recovered (Fig. 7C). In addition, about 14–35% of [^{3}H]-NE uptake blockade was restored (Fig. 7D). At concentrations of 0.03–333 μM, Δ^3,2-hydroxybakuchiol also dose-dependently reduced [^{3}H]WIN35,428 binding in D8 cells (Fig. 8).

3.3. *Effect of Δ^3,2-hydroxybakuchiol on cell viability and MPP$^+$-induced toxicity in D8 cells*

The MTT test revealed no clear difference in cell viability between D8 cells pretreated with Δ^3,2-hydroxybakuchiol (1–500 μM) and control cells (D8/vehicle) (Fig. 9). In contrast, cell viability decreased to about 25% of control when pretreated with MPP$^+$ (33 μM). However, Δ^3,2-hydroxybakuchiol dose-dependently prevented MPP$^+$-induced cell toxicity. Δ^3,2-Hydroxybakuchiol pEC_{50} and E_{max} values for protection against D8 lesion (expressed as protection rate) were −0.34 ± 0.036 and 82.31 ± 6.78%, respectively. The Δ^3,2-hydroxybakuchiol pEC_{50} value was significantly less than that for GBR12,935 (1.44 ± 0.23), whereas no difference was observed between E_{max} values for Δ^3,2-hydroxybakuchiol and GBR12,935 (85.45 ± 10.17%) (Table 5).

3.4. *Effect of Δ^3,2-hydroxybakuchiol on locomotor activity in intact mice*

Δ^3,2-Hydroxybakuchiol dose-dependently increased locomotor activity (line break score), especially at doses of 20 and 100 mg/kg (4 mg/kg mildly increased activity with no significant difference from the saline group) (Fig. 10). An initial 15–30 min low score of locomotor activity was followed by a long-lasting time-dependent stimulant effect, until about 70 min when activity was highest (line breaks: 260.32 ± 42.78). The pronounced stimulant action of Δ^3,2-hydroxybakuchiol persisted until about 90 min and then gradually declined. In contrast, the locomotor-stimulating effect of cocaine began with a steep curve during the initial 30 min (line breaks: 546.88 ± 113.19) that declined quickly. Compared with cocaine, Δ^3,2-hydroxybakuchiol increased locomotor activity to a lesser extent, with a slower onset and a longer duration of action after systemic administration.

3.5. *Effect of Δ^3,2-hydroxybakuchiol on locomotor behavior of reserpinized mice*

Spontaneous activity in mice was almost completely abolished after reserpine treatment (10 mg/kg) (Fig. 11). Δ^3,2-Hydroxybakuchiol at doses of 5, 20, and 100 mg/kg markedly and dose-dependently increased horizontal movement in reserpinized mice, with a response curve similar to intact mice. Likewise, bupropion at 20 mg/kg stimulated activity, with distance traveled similar to mice treated with the same dose of Δ^3,2-hydroxybakuchiol at different time points.

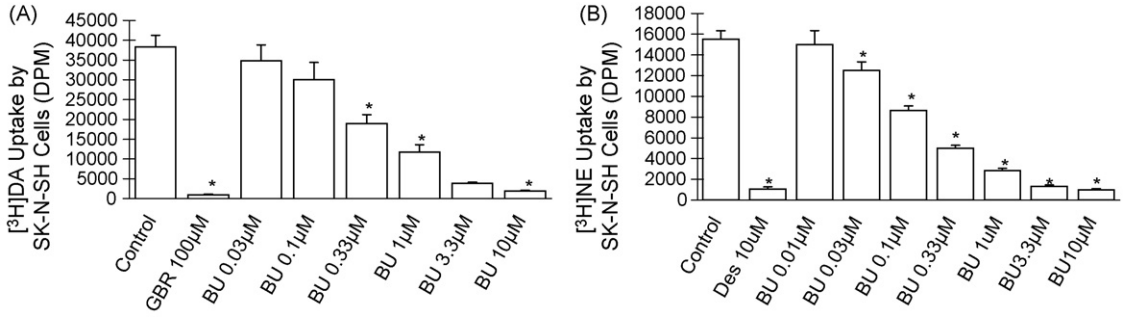

Fig. 5 – Inhibitory effect of different concentrations of Δ^3,2-hydroxybakuchiol (BU) on [^{3}H]-DA (A) and [^{3}H]-NE (B) uptake by SK-N-SH cells. Control cells were treated with the same volume of solvent. GBR12,935 (GBR) or desipramine (Des), positive control and representative unspecific uptake. $^*p < 0.01$, compared with control group. Values are mean ± S.E.M. of five samples.

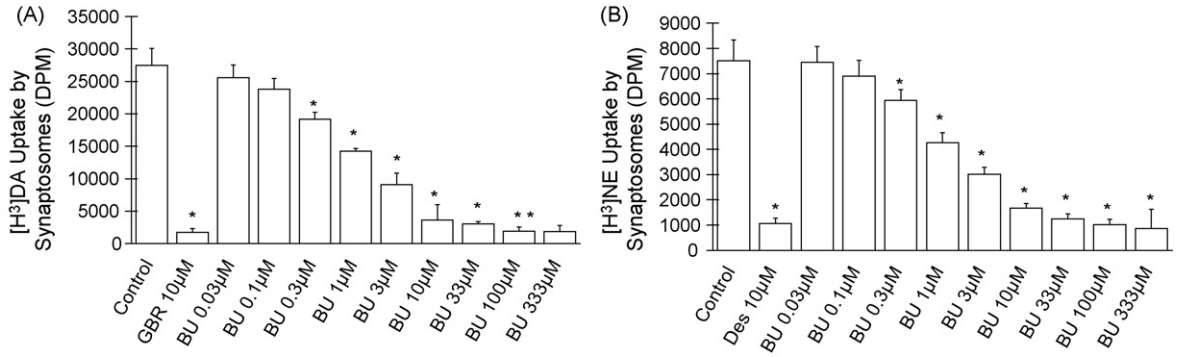

Fig. 6 – Effect of Δ^3,2-hydroxybakuchiol (BU) on [^{3}H]dopamine (A) or [^{3}H]norepinephrine (B) uptake by synaptosomes from rat striatum or hippocampus. Synaptosomes of the control group were treated with the same volume of solvent. GBR12,935 (GBR) or desipramine (Des), as positive control and representative unspecific uptake. $^*p < 0.01$, compared with control group. Values are mean ± S.E.M. of five samples. pEC_{50} and E_{max} values are shown in Table 5.

3.6. *Effect of Δ^3,2-hydroxybakuchiol on the reward system of C57BL/6 mice*

To explore the dependence liability of Δ^3,2-hydroxybakuchiol, preference scores were evaluated using conditioned place preference. The DAT antagonist, cocaine, a drug with high abuse potential, at a dose of 20 mg/kg, produced a substantial rewarding effect in mice. Preference scores of mice treated with 100 mg/kg Δ^3,2-hydroxybakuchiol and the petroleum ether extract of *Fructus Psoraleae* were not significantly different from normal mice (Fig. 12).

4. Discussion

The sensitivity of monoamine transporters to substrates or antagonists was examined in heterogeneous expression systems (HEK-293 and PC12 cell lines over-expressing monoamine transporters) established by other groups [18,19]. Our lab established a drug screening model using a recombinant technique in which monoamine transporters and amino acid transporters are stably expressed in an immortal CHO cell line. The model displayed 20–30 times enhancement of transmitter uptake compared with wildtype CHO cells, conferring extraordinary sensitivity. Conversely, the elevated DPM values were reduced to background levels in the presence of saturating concentrations of uptake inhibitors, demonstrating the validity and feasibility of the screening model (Table 1).

We screened active compounds targeting monoamine transporters from a variety of Chinese herbal extracts by the established Tr-CHO bioactivity tracking system. Bakuchiol analogs were isolated from the petroleum ether extract of *Fructus Psoraleae* (Figs. 1 and 2). Data shown in the uptake assay showed that bakuchiol analogs selectively inhibited monoamine transporters and had no effect on GAT-1 (Figs. 3 and 4, Table 4). Of the bakuchiol analogs, bakuchiol and Δ^1,3-hydroxybakuchiol exhibited moderate potency and efficacy for inhibition of DAT and NET, and mild potency and efficacy for SERT. Compared with bakuchiol and Δ^1,3-hydroxybakuchiol, Δ^3,2-hydroxybakuchiol exerted higher potency and efficacy for DAT and NET, and lower potency and efficacy for SERT (Table 4). Δ^3,2-hydroxybakuchiol, therefore, was chosen for further *in vitro* and *in vivo* studies. Δ^3,2-Hydroxybakuchiol had markedly lower IC_{50} values for DAT and NET compared with SERT (Table 2), revealing DAT and NET selectivity. Bupropion is a clinically accepted dual DAT/NET antagonist and was used as a reference compound to evaluate whether Δ^3,2-hydroxybakuchiol would have distinct activities. Despite a similar E_{max} value, Δ^3,2-hydroxybakuchiol displayed slightly higher potency and affinity for DAT and NET than bupropion (Tables 4 and 5). Although the potency of Δ^3,2-hydroxybakuchiol for DAT was lower than that of GBR12,935, their efficacies were similar (Tables 4 and 5). Efficacy has been considered to be more important than potency in evaluating the effect of a drug [20,21]. The greater efficacy of Δ^3,2-hydroxybakuchiol underscores its pharmacological potential in regulating abnormal monoaminergic transmission. Furthermore, the suppressive effects of Δ^3,2-hydroxybakuchiol on monoamine uptake were shown in three additional auxiliary screening systems that assessed inhibitory activity: SK-N-SH cells (Fig. 5), PC12 cells, and synaptosomes prepared from rat brain (Table 5, Fig. 6).

Pharmacokinetic parameters, including V_{max} and K_m, were calculated from a Lineweaver–Burke plot (Fig. 7A and B) and are summarized in Table 3. Δ^3,2-Hydroxybakuchiol at the concentrations of 0.5 and 2 μM significantly increased K_m values for DA or NE uptake, though had no significant effect on V_{max} values, suggesting that DAT and NET inhibition occurs through a typical competition mechanism. Reversible analyses revealed that inhibition was somewhat recovered after the cells preincubated with Δ^3,2-hydroxybakuchiol were washed with PBS (Fig. 7C and D), suggesting partially reversible competitive inhibition.

Protein DAT represents a primary target for the psychostimulant cocaine. The isotope-labeled cocaine analog WIN35,428 is commonly used to determine DAT quantity based on its binding sites [22]. Δ^3,2-Hydroxybakuchiol dose-dependently reduced WIN35,428 binding (Fig. 8). Δ^3,2-Hydroxybakuchiol may occupy cocaine binding sites, thereby disrupting DAT binding. The present finding is consistent with the aforementioned conclusion of competitive DAT inhibition, since cocaine also is a competitive DAT inhibitor

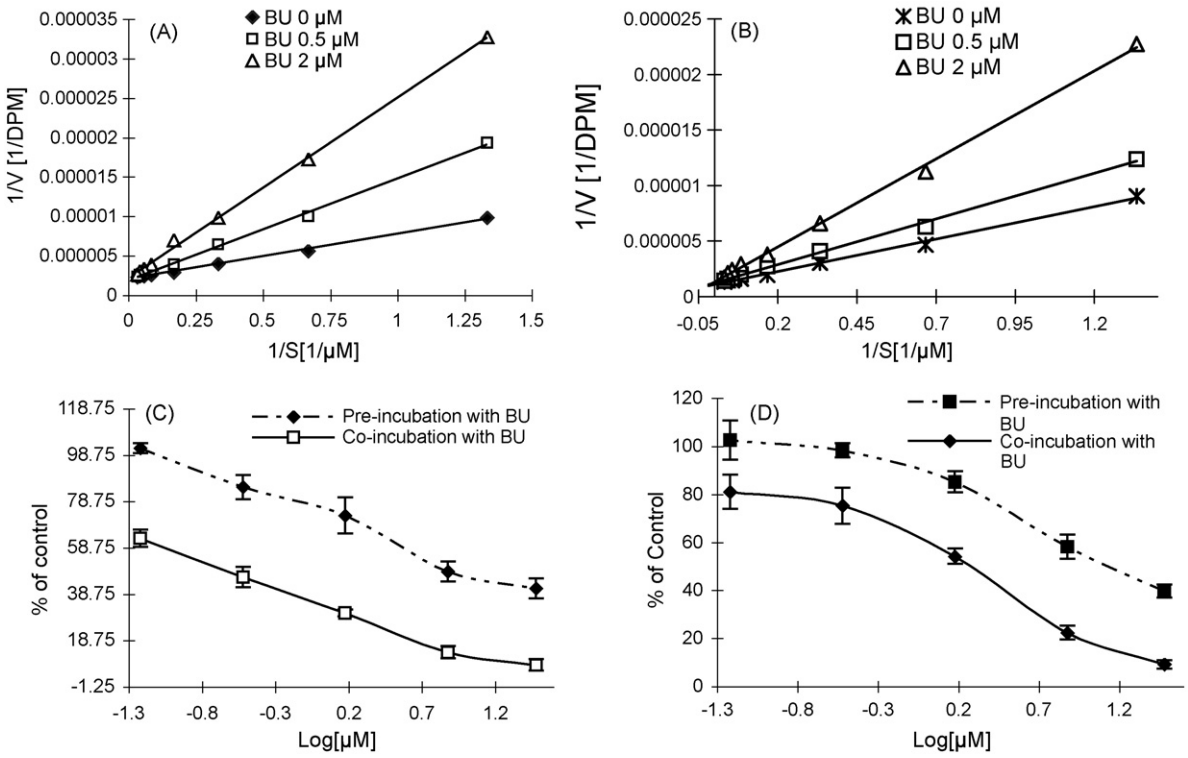

Fig. 7 – Pharmacokinetic mechanisms of Δ³,2-hydroxybakuchiol (BU) in the regulation of monoamine function. Effects of Δ³,2-hydroxybakuchiol on kinetic constants were explored by co-incubation with increasing concentrations of monoamine (hot and cold) and Δ³,2-hydroxybakuchiol (0, 0.5, or 2 μM). Lineweaver–Burke plots demonstrate competitive-type inhibition of DAT and NET. V, velocity (the amount of uptake divided by time). S, concentration of DA or NE. The respective kinetic parameters are summarized in Table 3. Reversible analyses revealed partial reversible inhibition of DAT (C) and NET (D). Values are expressed as % of D8 or N1 control cells (DPM value: 22387.45 ± 1258.74 and 24317.77 ± 1336.44, respectively).

[23]. In addition, disruption of cocaine binding implies that Δ³,2-hydroxybakuchiol has the potential to counteract the reinforcing effects of cocaine.

Furthermore, the DAT has been shown to catalyze the reuptake of MPP^+, 1-benzyl-1,2,3,4-tetrahydroisoquinoline derivatives [24], and β-carboline [25], in addition to regulating synaptic neurotransmitter content. The DAT thus may be considered a molecular gate for neurotoxins. The present results showed a marked decrease in D8 cell viability after MPP^+ administration, implying neuronal injury occurs via DAT. Δ³,2-Hydroxybakuchiol mitigated MPP^+-induced decreased cell viability with similar efficacy to GBR12,935 (Table 5, Fig. 9). The mechanism for the protective effect of Δ³,2-hydroxybakuchiol may be through DAT suppression, which would counteract MPP^+ uptake into cytosomes and prevent mitochondria from subsequent injury [26,27]. The protective effects further support the DAT inhibitory effect of Δ³,2-hydroxybakuchiol. Δ³,2-Hydroxybakuchiol also may be a

Table 3 – Summary of effects of Δ³,2-hydroxybakuchiol on the kinetic constants for monoamine transporters

Δ³,2-Hydroxybakuchiol concentration (μM)	V_{max} (DPM × 10^{-5})		K_m (μM)		n
	DAT	NET	DAT	NET	
0	7.08 ± 2.12	12.23 ± 3.02	3.75 ± 0.93	4.26 ± 1.13	6
0.5	7.08 ± 2.12	11.75 ± 2.21	7.91 ± 1.09*	9.25 ± 1.27*	6
2	8.01 ± 2.05	10.98 ± 1.87	23.79 ± 8.31*#	19.65 ± 3.48*#	6

D8 and N1 cells were incubated at 37 °C for 20 min in 100 μl medium with increasing concentrations of dopamine (cold and ^{3}H-labeled) from 1.5 to 24 μM or norepinephrine (cold and ^{3}H-labeled) from 0.75 to 20 μM, in the presence and absence of Δ³,2-hydroxybakuchiol at concentrations of 0, 0.5, and 2 μM. Means of *n* different experiments were performed in quadruplicate. Results showed a concentration-dependent increase in K_m values following administration of D8 or N1 cells with Δ³,2-hydroxybakuchiol at concentrations of 0.5 and 2 μM. Data were analyzed by Lineweaver–Burke plots. $^*p < 0.01$, compared with control (Δ³,2-hydroxybakuchiol, 0 μM); $^\#p < 0.01$, compared with Δ³,2-hydroxybakuchiol 0.5 μM.

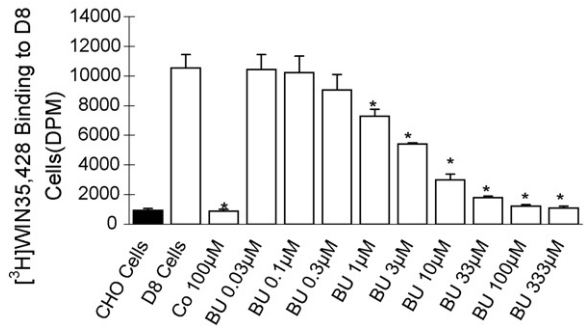

Fig. 8 – Effect of Δ^3,2-hydroxybakuchiol (BU) on (−)-2β-carbomethoxy-3β-(4-fluorophenyl) tropane (WIN35,428) binding to DAT. [^{3}H]WIN35,428 binding in CHO cells or D8 cells applied with 100 μM cocaine (Co) were taken as negative and positive control, respectively. Cocaine also was used to define nonspecific binding. Values are mean ± S.E.M. of quadruple samples. $^*p < 0.001$, compared with D8 cells (first open bar).

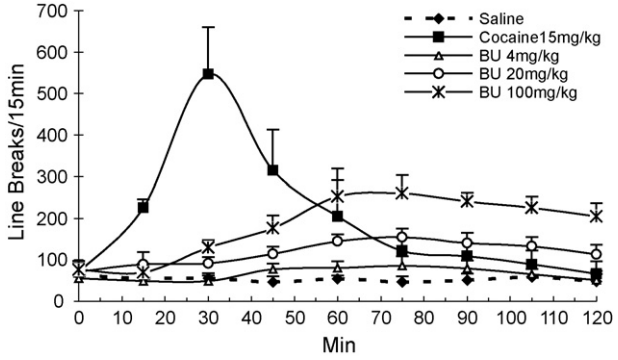

Fig. 10 – Effect of Δ^3,2-hydroxybakuchiol (BU) on locomotor behavior of intact C57/BL mice. Numbers of line breaks are shown on the *y*-axis and represent the horizontal distance traveled. Cocaine served as the positive control. *n* = 8 mice per group.

promising dopaminergic protective agent for Parkinson's disease.

The present behavioral study showed that Δ^3,2-hydroxybakuchiol induced a dose-dependent increase in locomotor activity (Fig. 10). The stimulant action may be attributable to inhibition of DAT and NET and subsequent enhancement of corresponding neurotransmitters in the synapses, further supporting the effects of Δ^3,2-hydroxybakuchiol as a potent

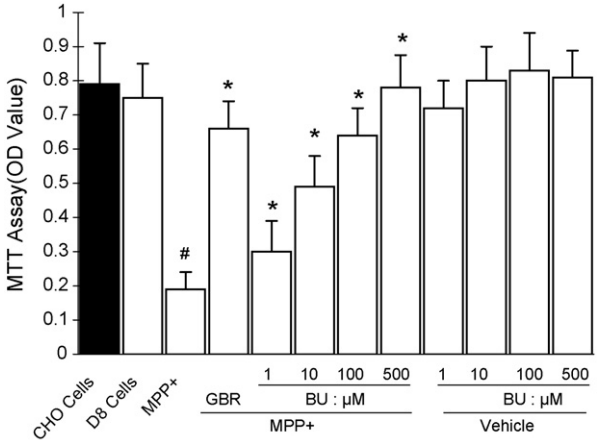

Fig. 9 – Effect of different concentrations of Δ^3,2-hydroxybakuchiol (BU) on cell toxicity induced by MPP$^+$. D8 cells (open bars) were preincubated with vehicle or Δ^3,2-hydroxybakuchiol solvent for 4 h followed by 48 h incubation with 1-methyl-4-phenylpyridinium (MPP$^+$). To assess the effect of Δ^3,2-hydroxybakuchiol on cell viability, vehicle solvent was added to the wells instead of MPP$^+$. GBR12,935 (GBR) at 10 μM served as positive control. $^\#p < 0.01$, compared with D8 control (first open bar). $^*p < 0.01$, compared with MPP$^+$-lesioned cells (second open bar). Values are mean ± S.E.M. of four well samples (one-way ANOVA). pEC_{50} and E_{max} values are shown in Table 5.

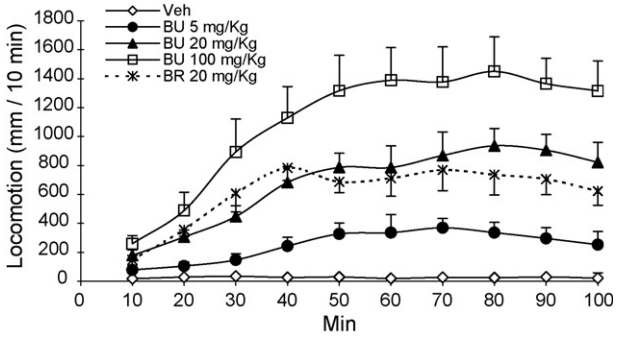

Fig. 11 – Δ^3,2-Hydroxybakuchiol (BU) improved abnormal behavior in reserpine-pretreated mice. Locomotor activity of the reserpinized mice after i.p. injection of vehicle solvent (Veh) or Δ^3,2-hydroxybakuchiol were assayed. Bupropion (BR) served as the positive control. *n* = 8 mice per group. $^*p < 0.05$, $^{**}p < 0.01$ compared with vehicle.

monoamine transporter blocker. However, the mode of stimulation was different than that for cocaine. Low locomotor activity scores were observed for the first 15–30 min after Δ^3,2-hydroxybakuchiol injection, followed by a long-lasting, time-dependent stimulant effect until about 70 min when locomotor activity scores were highest. The stimulant action occurred until about 90 min and then gradually declined. Δ^3,2-Hydroxybakuchiol was found to enhance locomotor activity with a lower peak, slower onset, and longer duration than cocaine. The rapid rise and decline of extracellular dopamine levels may contribute to the reinforcing properties and abuse liability of addictive drugs such as cocaine [28], suggesting that the slower onset and offset of the stimulant effects of Δ^3,2-hydroxybakuchiol make it less reinforcing than cocaine. The pattern of locomotor activation induced by Δ^3,2-hydroxybakuchiol suggests that it might be developed into a therapeutic drug with little addictive potential. In fact, the water extract of *Fructus Psoraleae* has been used clinically in China for hundreds of years, and addictive liability has not been reported for *Fructus Psoraleae* with chronic, oral dosing of 9–30 g. Mice given different doses of Δ^3,2-hydroxybakuchiol and the petroleum

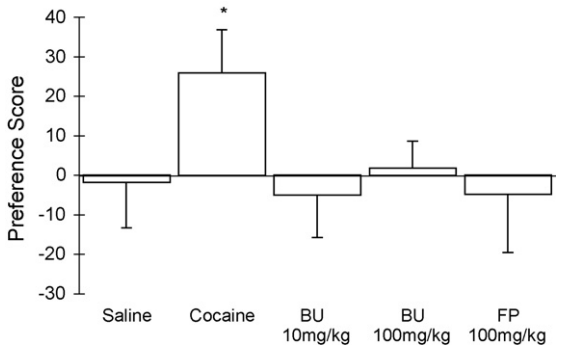

Fig. 12 – Effect of Δ^3,2-hydroxybakuchiol (BU) on conditioned place preference. Neither animal treated with the petroleum ether extract of *Fructus Psoraleae* (100 mg/kg) nor Δ^3,2-hydroxybakuchiol (10 and 100 mg/kg) showed any significant place preference response ($p > 0.05$ vs. saline). Cocaine (20 mg/kg) significantly increased preference score. $^*p < 0.01$, compared with saline. Data are expressed as difference in preference score between the testing phase and the preconditioning phase. Values are mean ± S.E.M. of 9–10 mice.

ether extract of *Fructus Psoraleae* exhibited place preference scores similar to untreated mice (although the high-dose group exhibited a slightly higher score) (Fig. 12), thus demonstrating no intrinsic rewarding or aversive effects.

Administration of reserpine causes DA and NE to remain exposed within the cell and broken down by monoamine oxidase, thereby profoundly reducing DA and NE content [29]. As shown in Fig. 11, reserpine-treated mice displayed no locomotor response to a novel environment. Δ^3,2-Hydroxybakuchiol administration in reserpinized mice markedly stimulated horizontal movement with a time-response curve similar to the locomotion observed in intact mice. A similar locomotor-stimulating effect was seen in response to bupropion. The activity improvement provides additional evidence to confirm the functional blockade of monoamine transporters by Δ^3,2-hydroxybakuchiol and the possible mechanism by which it upregulates deficient dopaminergic and noradrenergic function in mice. Moreover, the observed effect on locomotor activity suggests an anti-parkinsonian-like effect because reserpinized mice are known model of Parkinson's disease [30].

Bakuchiol analogs are structurally classified as meroterpene analogs. The principal meroterpene, bakuchiol has been reported to be effective in protecting against microbial infection [31], oxidative stress [32], liver injury, and cirrhosis [33,34]. The chemical structure of Δ^3,2-hydroxybakuchiol was first identified in 1996 [35] and shown to have inhibitory activity against antigen-induced degranulation [36]. The present study demonstrated that bakuchiol analogs had selective antagonist activity on DAT and NET and subsequent stimulatory action on locomotor behavior related to monoaminergic transmission. The DAT limits the intensity and

Table 4 – Summary of pEC_{50} and E_{max} values for monoamine transporter inhibition

	D8 cells		N1 cells		S6 cells	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}
Δ^3,2-hydroxybakuchiol	0.25 ± 0.031	105.56 ± 11.21	0.13 ± 0.014	99.68 ± 8.32	−2.37 ± 1.34	64.31 ± 6.78
Bakuchiol	−0.83 ± 0.012$^\$$	72.11 ± 6.31*	−0.7 ± 0.091$^\$$	74.31 ± 5.34*	<−3	nd
Δ^1,3-Hydroxybakuchiol	−1.05 ± 0.025$^\$$	64.77 ± 13.14*	−1.15 ± 0.16$^\$$	61.32 ± 8.32$^\$$	<−3	nd
Bupropion	0.071 ± 0.015*	94.28 ± 13.33	−0.083 ± 0.01*	101.21 ± 23.11	nd	nd
GBR12,935	1.74 ± 0.14*	98.24 ± 6.32	nd	nd	nd	nd
Fluoxetine	nd	nd	−1.06 ± 0.21$^\$$	87.58 ± 13.69	0.25 ± 0.04$^\$$	92.33 ± 15.6*

Bakuchiol analogs, bakuchiol, Δ^1,3-hydroxybakuchiol, and Δ^3,2-hydroxybakuchiol inhibited monoamine transporters with differing potencies and efficacies. Δ^3,2-Hydroxybakuchiol achieved higher values for pEC_{50} and E_{max} for DAT and NET than bakuchiol and Δ^1,3-hydroxybakuchiol, higher pEC_{50} values for DAT and NET than bupropion, and lower pEC_{50} values for DAT than GBR12,935. Δ^3,2-Hydroxybakuchiol also exhibited weak potency and slight efficacy for SERT with remarkably less pEC_{50} values and E_{max} values compared with fluoxetine. pEC_{50}, −log (μM). E_{max}, % of control. nd, not detected. $^*p < 0.05$ and $^\$p < 0.01$, compared with Δ^3,2-hydroxybakuchiol-treated cells, respectively. Values are mean ± S.E.M. of three independent experiments.

Table 5 – Summary of pEC_{50} and E_{max} values for uptake inhibition in synaptosomes and for protection against 1-methyl-4-phenylpyridinium (MPP^+) induced D8 cell lesion

	Dopamine uptake by synaptosomes		Norepinephrine uptake by synaptosomes		Protection against D8 lesion	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}
Δ^3,2-Hydroxybakuchiol	0.10 ± 0.016	95.56 ± 8.21	0.066 ± 0.013	95.68 ± 6.32	−0.34 ± 0.036	82.31 ± 6.78
Bupropion	−0.26 ± 0.042*	99.28 ± 13.33	−0.21 ± 0.052*	100.36 ± 11.36	nd	nd
GBR12,935	1.33 ± 0.15 $	98.24 ± 6.32	nd	nd	1.44 ± 0.23$	85.45 ± 10.17

Δ^3,2-Hydroxybakuchiol significantly inhibited dopamine or norepinephrine uptake by synaptosomes with higher pEC_{50} values than bupropion, but lower than GBR12,935, E_{max} value for Δ^3,2-hydroxybakuchiol was similar to bupropion and GBR12,935. Δ^3,2-Hydroxybakuchiol displayed protection against D8 cell toxicity induced by MPP^+ with similar efficacy to GBR12,935. pEC_{50}, −log (μM). E_{max}, % of control for uptake or % of total protection. nd, not detected. $^*p < 0.05$ and $^\$p < 0.01$, compared with Δ^3,2-hydroxybakuchiol-treated cells, respectively. Values are mean ± S.E.M. of three independent experiments.

duration of DA action at pre- and postsynaptic receptors and is essential for maintaining normal dopamine homeostasis in the brain [1]. DAT-like immunoreactivity has been detected mainly in the striatum, substantia nigra, nucleus accumbens, ventral tegmental area, cingulate, and medial prefrontal cortex [37]. DAT regulates dopaminergic transmission involved in locomotion, cognition, emotion, and reward. Deficits in the dopaminergic system contribute to the development of several neurological and psychiatric conditions, such as Parkinson's disease, depression, and attention-deficit hyperactivity disorder [38]. Δ^3,2-hydroxybakuchiol, therefore, may be beneficial for treating these disorders. Δ^3,2-Hydroxybakuchiol also inhibits NET with high potency and efficacy. NET, mainly confined to the hippocampus and cortex, mediates noradrenergic signaling involved in emotion, neuroplasticity [39], memory [40], and neuroprotection [41]. Mechanisms of depression [42] and dementia [43] also are considered to be related to deficient noradrenergic function, in addition to the established mechanism of insufficiency in dopaminergic and serotonergic transmission. Recent reports showed that damage to the noradrenergic system markedly deteriorated motor activity deficits in MPTP-treated mice [44]. In contrast, NET blockade can elevate extracellular dopamine concentrations [45], and NET-knockout mice show attenuated parkinsonism-like effects [46]. Deficiency in the noradrenergic system plays a role in the development of Parkinson's disease, and NET blockers could be an alternative or complementary approach to Parkinson's disease treatment. The results observed in the reserpinized model, together with previous reports, further indicate a possible role of bakuchiol analogs in antiparkinsonism. Therefore, the DAT/NET inhibitor Δ^3,2-hydroxybakuchiol may improve psychiatric disorders such as cocaine dependence, attention-deficit hyperactivity disorder, depression and dementia and protect against parkinsonism or enhance L-DOPA efficacy in Parkinson's disease treatment.

In summary, bakuchiol analogs, especially Δ^3,2-hydroxybakuchiol, inhibit monoamine transporters with specificity for DAT and NET and regulate monoaminergic transmission. Despite greater inhibition potency than bupropion for DAT/NET, Δ^3,2-hydroxybakuchiol possesses generally similar inhibition properties as bupropion. Δ^3,2-hydroxybakuchiol, therefore, may be evaluated as a pharmacotherapy for depression in addition to other related diseases. Similar to bupropion, the potency of Δ^3,2-hydroxybakuchiol is slightly lower than other antidepressants. Future studies will screen other chemicals that have greater activity, and Δ^3,2-hydroxybakuchiol as a lead compound may be structurally modified to obtain new compounds with higher activity. Efficacy evaluations of Δ^3,2-hydroxybakuchiol in animal models will be considered in future studies.

Acknowledgements

We thank Mrs. Sheng Li and Mr. Jie Wang their assistance with cell culture. We also thank Mr. Guo-Wei Qin, Mr. Jian Fei, and Mr. Zhi-Hua Jiang for direction in phytochemistry and pharmacology. Research was supported by a grant from the Shanghai government (05DZ19339) and Chinese Academy of Science.

REFERENCES

[1] Torres GE. The dopamine transporter proteome. J Neurochem 2006;97(suppl. 1):3–10.
[2] Gainetdinov RR, Caron MG. Monoamine transporters: from genes to behavior. Annu Rev Pharmacol Toxicol 2003;43:261–84.
[3] 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.
[4] Nelson N. The family of Na^+/Cl^- neurotransmitter transporters. J Neurochem 1998;71:1785–803.
[5] Miller GW, Gaintedinov RR, Levey AI, Caron MG. Dopamine transporters and neuronal injury. Trends Pharmacol Sci 1999;20:424–9.
[6] Greengard P. The neurobiology of slow synaptic transmission. Science 2001;294:1024–30.
[7] Barc S, Page G, Barrier L, Garreau L, Guilloteau D, Fauconneau B, et al. Relevance of different striatal markers in assessment of the MPP^+-induced dopaminergic nigrostriatal injury in rat. J Neurochem 2002;80:365–74.
[8] Berretta N, Freestone PS, Guatteo E, de Castro D, Geracitano R, Bernardi G, et al. Acute effects of 6-hydroxydopamine on dopaminergic neurons of the rat substantia nigra pars compacta in vitro. Neurotoxicology 2005;26:869–81.
[9] Kuter K, Smialowska M, Wieronska J, Zieba B, Wardas J, Pietraszek M, et al. Toxic influence of subchronic paraquat administration on dopaminergic neurons in rats. Brain Res 2007;1155:196–207.
[10] Jayanthi LD, Ramamoorthy S. Regulation of monoamine transporters: influence of psychostimulants and therapeutic antidepressants. AAP 2005;7:E728–38.
[11] Howell LL, Kimmel HL. Monoamine transporters and psychostimulant addiction. Biochem Pharmacol 2008;75:196–217.
[12] Amara SG, Sonders MS. Neurotransmitter transporters as molecular targets for addictive drugs. Drug Alcohol Depend 1998;51:87–96.
[13] Schroeter S, Apparsundaram S, Wiley RG, Miner LH, Sesack SR, Blakely RD. Immunolocalization of the cocaine- and antidepressant-sensitive l-norepinephrine transporter. J Comp Neurol 2000;420:211–32.
[14] Zhao G, Li S, Qin GW, Fei J, Guo LH. Inhibitive effects of Fructus Psoraleae extract on dopamine transporter and noradrenaline transporter. J Ethnopharmacol 2007;112:498–506.
[15] 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.
[16] 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.
[17] Maldonado R, Saiardi A, Valverde O, Samad TA, Roques BP, Borrelli E. Absence of opiate rewarding effects in mice lacking dopamine D2 receptors. Nature 1997;388:586–9.
[18] Schonn JS, Desnos C, Henry JP, Darchen F. Transmitter uptake and release in PC12 cells overexpressing plasma membrane monoamine transporters. J Neurochem 2003;84:669–77.
[19] Scholze P, Nørregaard L, Singer EA, Freissmuth M, Gether U, Sitte HH. The role of zinc ions in reverse transport mediated by monoamine transporters. J Biol Chem 2002;277:21505–13.

[20] Becker DE. Drug therapy in dental practice: general principles: part 2. Pharmacodynamic considerations. Anesth Prog 2007;54:19–23.
[21] Oates JA. The science of drug therapy. In: Brunton LL, Lazo JS, Parker KL, editors. Goodman and Gilman's the pharmacological basis of therapeutics. 11th ed., New York: McGraw-Hill; 2006. p. 117–36.
[22] Ding J, Shi J, Cui D, Xu L, Duan S, Guo L, et al. Development of peptidic dopamine transporter inhibitors via aromatic modification-mediated conformational restriction. J Med Chem 2006;49:4048–51.
[23] Stuber GD, Roitman MF, Phillips PE, Carelli RM, Wightman RM. Rapid dopamine signaling in the nucleus accumbens during contingent and noncontingent cocaine administration. Neuropsychopharmacology 2005;30: 853–63.
[24] Kawai H, Makino Y, Hirobe M, Ohta S. Novel endogenous 1,2,3,4-tetrahydroisoquinoline derivatives: uptake by dopamine transporter and activity to induce parkinsonism. J Neurochem 1998;70:745–51.
[25] Storch A, Hwang YI, Gearhart DA, Beach JW, Neafsey EJ, Collins MA, et al. Dopamine transporter-mediated cytotoxicity of β-carbolinium derivatives related to Parkinson's disease: relationship to transporter-dependent uptake. J Neurochem 2004;89:685–94.
[26] Cassarino DS, Parks JK, Parker Jr WD, Bennett Jr JP. The parkinsonian neurotoxin MPP^+ opens the mitochondrial permeability transition pore and releases cytochrome *c* in isolated mitochondria via an oxidative mechanism. Acta Biochim Biophys Sin 1999;1453:49–62.
[27] Vila M, Vukosavic S, Jackson-Lewis V, Neystat M, Jakowec M, Przedborski S. α-Synuclein up-regulation in substantia nigra dopaminergic neurons following administration of parkinsonian toxin MPTP. J Neurochem 2000;74:721–9.
[28] Desai RI, Kopajtic TA, Koffarnus M, Newman AH, Katz JL. Identification of a dopamine transporter ligand that blocks the stimulant effects of cocaine. J Neurosci 2005;25:1889–93.
[29] Florin SM, Kuczenski R, Segal DS. Effects of reserpine on extracellular caudate dopamine and hippocampus norepinephrine responses to amphetamine and cocaine: mechanistic and behavioral considerations. J Pharmacol Exp Ther 1995;274:231–41.
[30] Marino MJ, Williams Jr DL, O'Brien JA, Valenti O, McDonald TP, Clements MK, et al. Allosteric modulation of group III metabotropic glutamate receptor 4: a potential approach to Parkinson's disease treatment. Proc Natl Acad Sci USA 2003;100:13668–73.
[31] Newton SM, Lau C, Gurcha SS, Besra GS, Wright CW. The evaluation of forty-three plant species for in vitro antimycobacterial activities: isolation of active constituents from *Psoralea corylifolia* and *Sanguinaria canadensis*. J Ethnopharmacol 2002;79:57–67.
[32] Haraguchi H, Inoue J, Tamura Y, Mizutani K. Antioxidative components of *Psoralea corylifolia* (Leguminosae). Phytother Res 2002;16:539–44.
[33] Park EJ, Zhao YZ, Kim YC, Sohn DH. Protective effect of (*S*)-bakuchiol from *Psoralea corylifolia* on rat liver injury *in vitro* and *in vivo*. Planta Med 2005;71:508–13.
[34] Park EJ, Zhao YZ, Kim YC, Sohn DH. Bakuchiol-induced caspase-3-dependent apoptosis occurs through c-Jun NH_2-terminal kinase-mediated mitochondrial translocation of Bax in rat liver myofibroblasts. Eur J Pharmacol 2007;559:115–23.
[35] Labbe C, Faini F, Coll J, Conolly JD. Bakuchiol derivatives from the leaves of *Psoralea glandulosa*. Phytochemistry 1996;42:1299–303.
[36] Matsuda H, Sugimoto S, Morikawa T, Matsuhira K, Mizuguchi E, Nakamura S, et al. Bioactive constituents from Chinese natural medicines: XX. Inhibitors of antigen-induced degranulation in RBL-2H3 cells from the seeds of *Psoralea corylifolia*. Chem Pharm Bull (Tokyo) 2007;55:106–10.
[37] Torres GE, Gainetdinov RR, Caron MG. Plasma membrane monoamine transporters: structure, regulation and function. Nat Rev Neurosci 2003;4:13–25.
[38] Adinoff B. Neurobiologic processes in drug reward and addiction. Harv Rev Psychiatry 2004;12:305–20.
[39] Morton DB, Bredt DS. Norepinephrine increases cyclic GMP levels in cerebellar cells from neuronal nitric oxide synthase knockout mice. J Neurochem 1998;71:440–3.
[40] Soto-Moyano R, Valladares L, Sierralta W, Perez H, Mondaca M, Fernandez V, et al. Mild prenatal protein malnutrition increases α_{2C}-adrenoceptor density in the cerebral cortex during postnatal life and impairs neocortical long-term potentiation and visuo-spatial performance in rats. J Neurochem 2005;93:1099–109.
[41] Troadec JD, Marien M, Darios F, Hartmann A, Ruberg M, Colpaert F, et al. Noradrenaline provides long-term protection to dopaminergic neurons by reducing oxidative stress. J Neurochem 2001;79:200–10.
[42] Laifenfeld D, Klein E, Ben-Shachar D. Norepinephrine alters the expression of genes involved in neuronal sprouting and differentiation: relevance for major depression and antidepressant mechanisms. J Neurochem 2002;83: 1054–64.
[43] Kalinin S, Gavrilyuk V, Polak PE, Vasser R, Zhao J, Heneka MT, et al. Noradrenaline deficiency in brain increases β-amyloid plaque burden in an animal model of Alzheimer's disease. Neurobiol Aging 2007;28:1206–14.
[44] Archer T, Fredriksson A. Influence of noradrenaline denervation on MPTP-induced deficits in mice. J Neural Transm 2006;113:1119–29.
[45] Carboni E, Silvagni A, Vacca C, Di Chiara G. Cumulative effect of norepinephrine and dopamine carrier blockade on extracellular dopamine increase in the nucleus accumbens shell, bed nucleus of stria terminalis and prefrontal cortex. J Neurochem 2006;96:473–81.
[46] Rommelfanger KS, Weinshenker D, Miller GW. Reduced MPTP toxicity in noradrenaline transporter knockout mice. J Neurochem 2004;91:1116–24.