Neurotrophic and Neuroprotective Effects of Tripchlorolide, an Extract of Chinese Herb *Tripterygium wilfordii* Hook F, on Dopaminergic Neurons

Feng-Qiao Li, Xiao-Xin Cheng,[1] Xi-Bin Liang, Xin-Hong Wang, Bing Xue, Qi-Hua He, Xiao-Min Wang,[2] and Ji-Sheng Han

Neuroscience Research Institute, Peking University, 38 Xueyuan Road, Beijing 100083, People's Republic of China

It has been reported recently that the immunosuppressant FK506 produced neurotrophic and neuroprotective effects on dopaminergic neurons *in vitro* and *in vivo*. We investigated whether tripchlorolide, an immunosuppressive extract of Chinese herb *Tripterygium wilfordii* Hook F, could exert similar neurotrophic and neuroprotective effects similar to those of FK506. It was found that tripchlorolide promoted axonal elongation and protected dopaminergic neurons from a neurotoxic lesion induced by 1-methyl-4-phenylpyridinium ion (MPP$^+$) at concentrations of as low as 10^{-12} to 10^{-8} M. *In situ* hybridization study revealed that tripchlorolide stimulated brain-derived neurotrophic factor (BDNF) mRNA expression. *In vivo* administration of tripchlorolide (1 μg/kg, ip) for 28 days effectively attenuated the rotational behavior challenged by D-amphetamine in the model rats by transection of the medial forebrain bundle. In addition, tripchlorolide treatment (0.5 or 1 μg/kg/day for 28 days) increased the survival of dopaminergic neurons in substantia nigra pars compacta by 50 and 67%, respectively. Moreover, tripchlorolide markedly prevented the decrease in amount of dopamine in the striatum of model rats. Taken together, our data provide the first evidence that tripchlorolide acts as a neuroprotective molecule that rescues MPP$^+$ or axotomy-induced degeneration of dopaminergic neurons, which may imply its therapeutic potential for Parkinson's disease. The underlying mechanism may be relevant to its neurotrophic effect and its efficacy in stimulating the expression of BDNF. © 2002 Elsevier Science (USA)

Key Words: tripchlorolide; *Tripterygium wilfordii* Hook F; immunosuppressant; Chinese herb; neurotrophic; brain-derived neurotrophic factor; Parkinson's disease.

[1] Current address: Department of Pathogeny Biology, Dalian Medical University, Dalian, Liaoning, China.

[2] To whom correspondence should be addressed. Fax: 86-10-8207-2207. E-mail: xmwang@bjmu.edu.cn.

INTRODUCTION

Protection or regeneration of the dopaminergic (DA)[3] system would be of significant therapeutic value for Parkinson's disease (PD). Several growth factors, such as glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), have been identified to be critical for the survival of dopaminergic neurons (7). However, clinical utility has been limited by its inability to reach the brain after systemic administration.

The immunosuppressant FK506 and its analogs have been reported to have neurotrophic activities in a variety of *in vitro* and *in vivo* models (8, 21), suggesting that immunosuppressants might be a resource of neurotrophic chemicals. Extracts of the Chinese herb *Tripterygium wilfordii* Hook F (TWHF) have been found to have anti-inflammatory and immunosuppressive properties equivalent to FK506 and have been used successfully in traditional Chinese medicine for the treatment of rheumatoid arthritis (25). Tripchlorolide (designated as TW397) is one of the active ingredients of TWHF, which is a diterpene triepoxide and structurally analogous to the major active ingredient, triptolide (Fig. 1) but with less gastrointestinal stimuli. Therefore, TW397 is more promising to be developed into oral medication. Both TW397 and triptolide inhibit proliferation of human peripheral blood lymphocytes, Jurkat T-cells, and human bronchial epithelial cells (17) and reduce the expression of interleukin

[3] Abbreviations used: 6-OHDA, 6-hydroxydopamine; AMPH, amphetamine; BDNF, brain-derived neurotrophic factor; DA, dopamine; DIG, digoxigenin; ECD, electrochemical detection; GDNF, glial cell line-derived neurotrophic factor; HPLC, high-performance liquid chromatography; IL, interleukin; IOD, integrated optic density; ISH, *in situ* hybridization; MFB, medial forebrain bundle; MPP$^+$, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; PFA, paraformaldehyde; SNpc, substantia nigra pars compacta; SSC, standard saline citrate; TH, tyrosine hydroxylase; TNF, tumor necrosis factor; TWHF, *Tripterygium wilfordii* Hook F; VTA, ventral tegmental area.
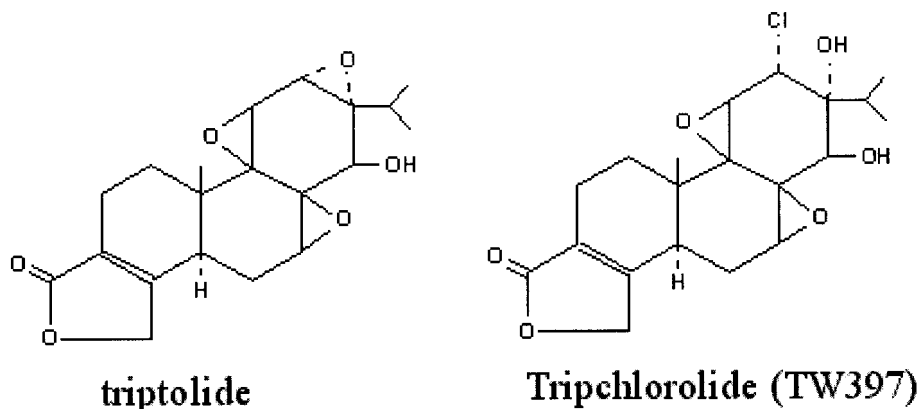
FIG. 1. Structure of tripchlorolide (TW397) and triptolide.

(IL)-1, IL-2, and IL-6 and the secretion of prostaglandin E_2 (PGE$_2$) (25, 30, 31). The mechanisms of triptolide inhibition of cytokine gene expression involve nuclear inhibition of transcriptional activation of NF-κB and the purine-box regulator which operates at the antigen receptor response element (ARRE)/nuclear factor of activated T-cells (NF-AT) at a step after specific DNA binding (17). It has been recently reported that triptolide promotes nerve regeneration after heterografting (4). We thereby investigated whether TW397 would exert a neurotrophic effect in primary cultured mesencephalic neurons of rats. To elucidate the underlying mechanisms, we also studied the influence of TW397 on mRNA expression of BDNF in the same cultured model.

Unilateral transection of the medial forebrain bundle (MFB) is often used to set up an animal model of Parkinson's disease. Axotomy of the MFB produces a precisely defined lesion of dopaminergic nigrostriatal afferents which results in degenerative changes in a subpopulation of dopaminergic cell bodies in the pars compacta of the substantia nigra (SN) with the subsequent degeneration of the striatal dopaminergic nerve terminals (1, 27). Winter *et al.* reported recently that FK506 protected against neuronal death in the substantia nigra pars compacta (SNpc) following transection of the rat MFB (29). Since degeneration of SNpc neurons is the main pathogenesis of Parkinson's disease, protection of SNpc neurons by immunosuppressants might offer a new strategy for retardation of this neurodegenerative disorder. We thereby tested the neuroprotective effect in the same model to ascertain the effectiveness of TW397.

MATERIALS AND METHODS

Source of tripchlorolide. Tripchlorolide (TW397) was generously provided by Professor Jia-Run Zheng (Institute of Dermatology, Chinese Academy of Medical Sciences). The material was in the form of white needle-like crystals, with a melting point of 256–258°C, molecular weight 397, and 98% purity.

Preparation of mesencephalic neuronal cultures. Embryonic mesencephalic neurons were obtained from timed pregnant Sprague-Dawley rats on embryonic day 16.5 (E16.5). Briefly, the ventral mesencephalons were dissected out, mechanically dissociated in D-Hanks' solution (Ca^{2+} and Mg^{2+} free), and then mildly triturated with a fire-polished Pasteur pipet. The dispersed cells were seeded in 24-well plates precoated with poly-D-lysine (12.5 μg/ml) at a density of 5×10^5/well in a nutrient medium (DMEM, Gibco) containing 10% heat-inactivated fetal bovine serum and 2 mM glutamine (Sigma). Cultures were incubated at 37°C in a water-saturated atmosphere of 5% CO_2 and 95% air for 24 h. A serum-free supplemented medium was then provided, containing DMEM/Hams' F12 (1/1, Sigma), 2% N_2 supplement (Gibco), and 2 mM glutamine.

To monitor the neurite outgrowth-promoting effect, TW397 (10^{-13} to 10^{-6} M) was added into the serum-free medium on the second day. Neurons were randomly photographed for 20 fields per well at $200\times$ magnification after treatment with TW397 for 48 h. Lengths of axons (defined as the longest process per neuron) were measured using reversed-phase contrast microscope (DMIRB, Leica) equipped with SPOT-2 digital camera (Diagnostic Instruments Inc., Sterling Heights, MI) and MetaMorph image processing and analysis software (Universal Imaging Corporation, Downingtown, PA). Only processes over $2\times$ cell body sizes were measured. Data obtained from identically treated wells (two per group) were pooled and histograms were constructed.

MPP$^+$ treatment and tyrosine hydroxylase (TH) immunocytochemistry. Cultures were exposed to freshly prepared MPP$^+$ (Research Biochemicals Inc., Natwick, MA) in serum-free medium at day 4 *in vitro* for 24 h. TW397 (10^{-12} to 10^{-8} M) were applied 30 min before

and during MPP$^+$ (10 μM) treatment. For visualization of dopaminergic neurons, cultures were fixed with 4% paraformaldehyde (PFA) and incubated with TH antibody (1:10,000, monoclonal, Sigma, St. Louis, MO) overnight at 4°C. The subsequent procedures were performed as described by the protocol of ABC kit (Sino-American Biotechnology Co., LuoYang City, China). The neurite length of TH-positive neurons was measured as described above.

In situ hybridization (ISH) with BDNF oligodenucleotide probe. A nonradioactive *in situ* hybridization approach was used to evaluate the level of BDNF mRNA expression in the primary mesencephalic neurons. The BDNF oligo probes complementary to 746–795 of the pig BDNF cDNA (10) was synthesized by Sangon Company (Shanghai, China). This oligonucleotide has 90% identity with the rodent sequence and the specificity has been verified (5). It was labeled with a digoxigenin (DIG) oligonucleotide 3'-end labeling kit according to an existing protocol (Boehringer Mannheim, Mannheim, Germany). The efficiency of DIG labeling was quantitated by dot blotting. After treating with various concentrations of TW397 (10^{-12} to 10^{-6} M) for 48 h, cultures were fixed with 4% PFA for further *in situ* hybridization analysis as described previously (10). Briefly, after rinsing in phosphate buffer and pretreatment with proteinase K (1 μg/ml), the slides were transferred into PFA/PBS and were then acetylated. After prehybridization, the sections were covered with 25 μl hybridization mixture (0.5 μg/ml DIG-labeled oligo probe, 20% deioned formamide, 10% dextran sulfate, 500 μg/ml salmon sperm DNA, 2× SSC, and yeast tRNA) and hybridized for 12–16 h at 37°C. Posthybridization washes were undertaken at 37°C in 2×, 1×, and 0.25× SSC with gentle shaking. The procedures of immunodetection and staining were performed as recommended in the detection kit (Boehringer Mannheim). Methodological controls of ISH were performed without oligo probe added to the hybridization buffer.

For evaluating the level of BDNF mRNA expression, slides were observed under the Leica microscope at 200× magnification and analyzed using an image-analysis system (MetaMorph, Universal Imaging Corporation). The value of gray scale was presented as integrated optic density (IOD) units. The IOD data were obtained by randomly measuring 200–400 positive neurons from different fields. Data from two slices of the same group were combined for statistical analysis.

Medial forebrain bundle (MFB) axotomy. Adult male Sprague–Dawley rats weighing 180–200 g were obtained from the Laboratory Animal Center, Peking University, and housed in a standard 12-h on/off light cycle with food and water *ad libitum* in the home cage. Rats were anesthetized with chloral hydrate (400 mg/kg, ip) and positioned in a stereotaxic apparatus (David

Kopf Instruments, Tujunga, CA) with the tooth bar set at −3.3 mm. Lesions of the right MFB were made using a retractable wire knife (Scouten knife, David Kopf Instruments) as described by Brecknell *et al.* (2) and Tseng *et al.* (26). Briefly, the knife was lowered through a drillhole 3.8 mm posterior and 2.4 mm lateral to bregma to a ventral position of 8.0 mm below bregma. The blade was extended by 2.0 mm and the knife slowly moved upward by 2.5 mm and subsequently down again by 2.5 mm. The blade was then retracted and the knife was withdrawn. Thereafter, the skull surface was covered with fibrosponge and the skin was sutured.

Application of TW397. Rats were divided into three groups and randomly treated with TW397 (0.5 or 1 μg/kg) or vehicle. TW397 was freshly prepared and was applied as single daily injection at 8:00 AM. Rats received a single dose of 0.5 or 1 μg/kg injected intraperitoneally once per day from 2 days preaxotomy up to 26 days postaxotomy (totally for 28 days).

Rotational behavior assay. Rats were placed into cylinders attached to rotameter to examine the rotational behavior induced by amphetamine on the second day after the final TW397 injection. They were allowed to rest for 10 min for adapting to the testing environment and then were injected intraperitoneally with 2.5 mg/kg D-amphetamine sulfate (Sigma) dissolved in normal saline. Measurement of rotational activity began 5 min after injection and tested for 45 min under minimal external stimuli. The lights were turned off, and the room was sealed from noise. The rotameter recorded the number of full clockwise and counterclockwise turns the animals made during the testing period. Clockwise turns (ipsilateral to the lesion) were counted as positive turns, whereas counterclockwise turns (contralateral to the lesion) were counted as negative turns. The net number of turns made during the entire 45-min testing period was counted and divided by 45 to calculate the rotational rate.

Tissue collection and processing. For morphologic studies, three rats from each group were randomly selected on the second day after assay of rotational behavior, deeply anesthetized with sodium pentobarbital (70 mg/kg, ip), and then transcardially perfused by 100 ml saline followed with 200 ml 4% paraformaldehyde in phosphate buffer. Brains were dissected and postfixed in the same fixative and cryoprotected by 30% sucrose for 3–5 days. The brains were frozen on powdered dry ice and then blocked for frontal sectioning according to the rat brain atlas of Paxinos and Watson (16a). Frozen sections (40 μm in thickness) were cut with cryostat at −20°C and were used for immunohistochemistry. Other rats were decapitated and the bilateral striatum were dissected quickly and stored in −80°C for HPLC-ECD determination of dopamine content.

Histologic examination. All sections spanning the SN (bregma −4.80 mm to −6.30 mm; 16a) were collected and stained for TH immunohistochemistry. The procedure of TH immunohistochemistry followed those described above.

TH-positive neurons with distinct nuclei were counted in six sections 300 μm apart throughout the entire rostrocaudal extent of the substantia nigra (A9 + A8). All sections were coded and examined blind. The percentage of survival in the SNpc was determined by counting the number of TH-immunoreactive (TH-IR) neurons on the lesioned side (right) relative to the number of TH-IR neurons on the unlesioned side (left).

Neurochemical analysis. For biochemical studies, the contents of DA, dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were determined by high-performance liquid chromatography apparatus with an electrochemical detector (Shimadzu LC-6A HPLC-ECD system). Briefly, tissues were homogenized in 200 mM ice-cold perchloric acid (solution A). The homogenate was placed in an ice bath for 30 min. Subsequently, the sample was centrifuged at $15,000g$ for 20 min at 4°C and the supernatant was transferred to another 1.5-ml Eppendorf tube. One-half volume of solution B containing 20 mM potassium citrate, 300 mM potassium dihydrogen phosphate, and 2 mM EDTA · 2Na was added and mixed thoroughly to deposit perchloric acid. After incubating in ice bath for 30 min, the mixed solution was centrifuged again at $15,000g$, for 20 min at 4°C. The supernatants were then injected directly into the HPLC system for analysis. The mobile phase was 125 mM sodium citrate buffer containing 20% methanol, 0.1 mM EDTA · 2Na, 0.5 mM 1-octanesulfonic acid sodium salt (OSA, Acros) adjusted to pH 4.3. The flow rate was set at 1.2 ml/min.

Statistical analysis. Data were processed by commercially available software Graph Pad Prism 3.0. Results were typically presented as means ± SEM. Statistical significance was assessed using a one-way ANOVA followed by Dunnet's *post hoc* test. Significance was set at $P < 0.05$.

RESULTS

Effect of TW397 on Neurite Outgrowth in Primary Culture of Mesencephalic Neurons

After 3 days *in vitro,* neurons developed long axon-like processes. Treatment with TW397 (10^{-13} to 10^{-6} M) promoted neurite elongation with a bell-shaped dose–response curve (Fig. 2A). The maximal effect was observed at the 10^{-10} M concentration, at which the average of neurite length was increased by 43% compared to the vehicle-treated group. However, the compound was less effective at a higher (10^{-6} M) concentration, at which TW397 might be toxic to neurons. The number of processes per cell was also counted and no
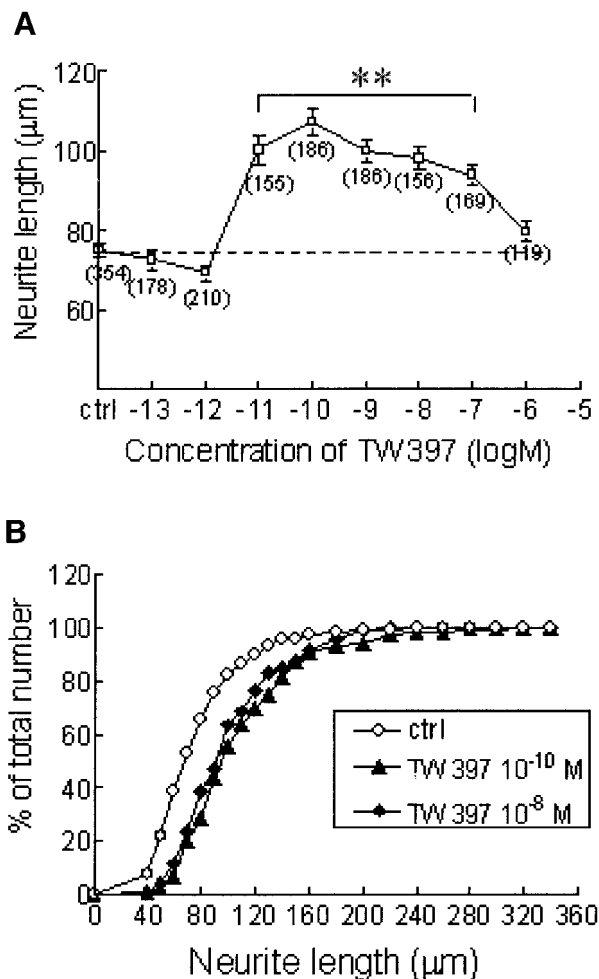

FIG. 2. Effect of TW397 on the neurite outgrowth of primary cultured mesencephalon neurons. (A) Concentration–response curve constructed from the mean axonal lengths of tested groups after treatment for 48 h in serum-free medium. (**$P < 0.01$, ANOVA with post hoc Dunnet's test). (B) Cumulative histogram showing the distribution of axonal lengths demonstrates a shift to the right after treatment with TW397 (0.1 nM), indicating that there are more neurons bearing longer processes.

significant difference was observed between groups. Cumulative histograms were constructed to show the distribution of neurite length (Fig. 2B). Compared to the control group, a significant ($P < 0.01$) shift to the right was observed in the group treated with TW397 at 10^{-10} and 10^{-8} M for 48 h, indicating that there were more neurons bearing longer processes.

Protection against MPP$^+$ Neurotoxicity

Treatment of cultures with 10 μM MPP$^+$ at 4 DIV for 24 h produced clear indications of structural damage to dopaminergic fibers, as was reported previously (16), without significant influence on the number of TH-positive neurons (data not shown). In the control group (treated with MPP$^+$ only), TH-positive neurons were
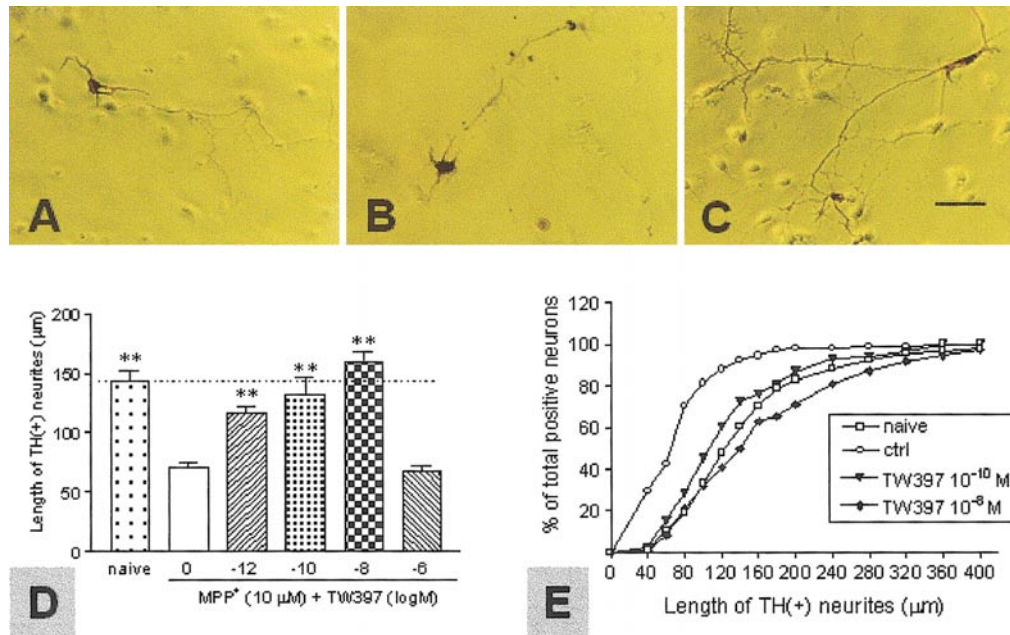
FIG. 3. TW397 protects primary cultured mesencephalic dopaminergic neuron against MPP^+-induced damages. Dopaminergic neurons were visualized by anti-TH immunocytochemistry. (A) Naive group (without MPP^+ and TW397). (B) Control group (treated with MPP^+ 10 μM for 24 h). (C) TW397 (10^{-8} M) + MPP^+ (10 μM). (D) Decrease of axonal length by MPP^+ and its reversal by TW397 at concentration of 10^{-12} to 10^{-8} M. (E) Cumulative histogram showing a shift to the left of the curve by MPP^+ and a reversal of MPP^+ effect by TW397. $**P < 0.01$ compared with control group. Magnification, 200 ×. Bar, 100 μm.

shown to have very short or completely truncated processes (Fig. 3B). This effect of neurotoxicity could be reversed by treatment of TW397 (10^{-12} to 10^{-8} M) with a bell-shaped dose–response curve (Figs. 3C and 3D). The peak effect of TW397 was observed at 10^{-8} M, where the toxicity of MPP^+ was completely reversed (Fig. 3D). The cumulative curves showed that TW397 not only reversed the toxic effect of MPP^+, but also promoted the elongation of the neurite of dopaminergic neurons.

Effect of TW397 on mRNA Expression of BDNF

In the control group, we found a low level of BDNF mRNA expression in the primary cultured mesencephalic neurons. Treatment with TW397 (10^{-12} to 10^{-8} M) for 48 h induced visible up-regulation of BDNF mRNA expression. A typical bell-shaped dose–response curve was observed with the most effective concentration being at 10^{-10} M, during which the average IOD was 14% higher than that of the control group ($P < 0.01$). The effect of up-regulation extinguished at the concentration of 10^{-6} M.

Postsurgical Recovery

Compared with saline-treated controls, TW397 substantially improved the recovery from surgery of Kopf knife lesion. Within 8 h, rats were actively moving around the cage, showing a normal feeding and drinking behavior, without other signs of postsurgery stress.

These rats were able to maintain their body weight during the entire period of TW397 application. In contrast, saline-treated rats were unable to keep their body weight and some of them died of hypoalimentation.

TW397 Administration Improves Functional Recovery after Unilateral MFB Axotomy

MFB axotomy leads to the degeneration of dopaminergic neurons of the SN. Administration of amphetamine, an indirect agonist of DA receptor, could elicit the rotational behavior to the ipsilateral side (lesioned side). In saline-treated controls, animals exhibited higher rotation rate, that is, 5.6 ± 1.1 turns per minute examined at 28 days after unilateral MFB axotomy. However, treatment with TW397 (1 μg/kg) for 28 days significantly reduced the amphetamine-induced number of turns (2.4 ± 0.6 turns per minute, $P < 0.05$) by 56%, compared with the control group (Fig. 5). At the dose of 0.5 μg/kg, TW397 diminished the rotational behavior although no statistical significance between the two groups was found.

Immunohistochemical Analysis of SNpc

A significant sparing of the TH-positive SN neurons was observed in the animals treated with TW397 and the dendritic processes surrounding the TH-positive neurons was largely preserved. In contrast, animals that received saline treatment showed a marked loss of
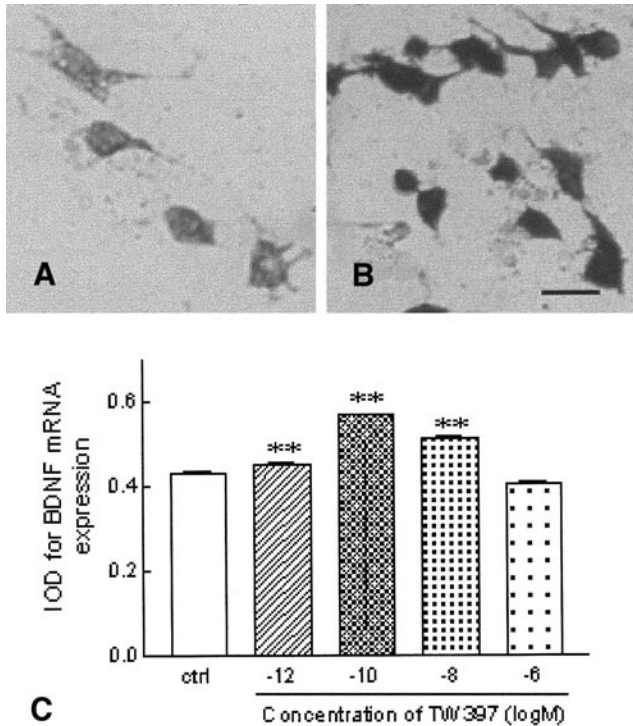
FIG. 4. TW397 increases BDNF mRNA expression in primary mesencephalic cultures as revealed by *in situ* hybridization with oligo probe. Representative photographs show digoxigenin staining after treatment with vehicle (A) or TW397 10^{-10} M (B) for 48 h, respectively. Magnification, 200 ×. Bar, 25 μm. The mean integrated optic density (IOD) representing the levels of BDNF mRNA expression after different treatment is illustrated in (C). Data are presented as means ± SEM. **$P < 0.01$ (ANOVA with post-hoc Dunnet's test).

TH-positive neurons and their dendrites (Fig. 6). TW397 at the dose of 1 μg/kg preserved as many as 71.8% TH-positive cells on the lesioned side, compared with those on nonlesioned side. In contrast, in the control group injected in saline, only 22.3% (ANOVA, $P < 0.05$) TH-positive neurons in the SNpc of the lesioned side were found, compared with those on the nonlesioned side. TW397 at the dose of 0.5 μg/kg was less effective, showing a preservation of 44.6% without statistical significance.

Levels of Dopamine and Its Metabolites in the Striatum

The adequacy of the MFB lesion was ascertained by a decrease of striatal dopamine content. DA, DOPAC, and HVA levels after MFB axotomy were significantly reduced in striatum by 71.7, 65.5, and 42.7%, respectively (vs. naive group). Treatment with TW397 (1 μg/kg, ip) for 28 days significantly attenuated DA depletion in the striatum (Table 1). The DA level of striatum in TW397-treated group was 140% higher than that of control group, although it was still significantly lower than that of the naïve level.

DISCUSSION

According to our knowledge, this is the first study to demonstrate that TW397, an immunosuppressive component of the traditional Chinese herb *T. wilfordii* Hook F, possesses the neurotrophic and neuroprotective actions for dopaminergic neurons *in vitro* as well as a neuroprotective effect *in vivo*.

Immunosuppressive and Neuroprotective Effects of TW397

Results obtained from the present study showed that TW397 promoted axonal elongation and protected DA neurons against MPP$^+$-induced damage at very low concentrations ranging from 10^{-12} to 10^{-8} M. Our findings are consistent with that of FK506 demonstrated by Costantini *et al.* in the same primary culture (3), although the concentration required for TW397 to promote neurite outgrowth is much lower than that of FK506, suggesting that TW397 has stronger neurite-promoting effect than FK506.

A closer look revealed that in contrast to an optimal concentration of 10^{-8} M for neuroprotective effect, TW397 showed an effect of neurite elongation at a concentration of 10^{-10} M. In other words, the neuroprotective effect of TW397 and its neurite-promoting effect seem to be separable. On the other hand, TW397 exerts a maximal neuroprotective action (i.e., protecting against MPP$^+$ neurotoxicity) at the concentration of about 10^{-8} M, which was the concentration exhibiting a potent immunosuppressive property as previously reported (30, 31). These results seem to suggest that the immunosuppressive activity of TW397 might be in
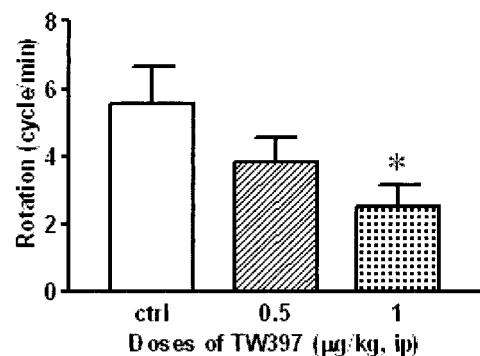
FIG. 5. Effect of TW397 treatment on AMPH-induced rotation behavior of MFB-axotomized rats. Rats were randomly grouped and then pretreated with TW397 (0.5, 1 μg/kg/day, ip) or vehicle 2 days before surgery and subsequently for 26 days after surgery (totally for 28 days). On the 28th day, rats were injected with D-amphetamine (2.5 mg/kg, ip) to challenge rotational behavior. Total turns were counted by computerized rotameter for 45 min and the rotation rate expressed as turns per minute was regarded as the index of degree of lesion. One-way ANOVA with a Dunnet's *post hoc* test for multiple comparisons with a control (vehicle) was used to statistically assess differences among groups. $n = 11$–16. *$P < 0.05$.

Unlesioned side   Lesioned side
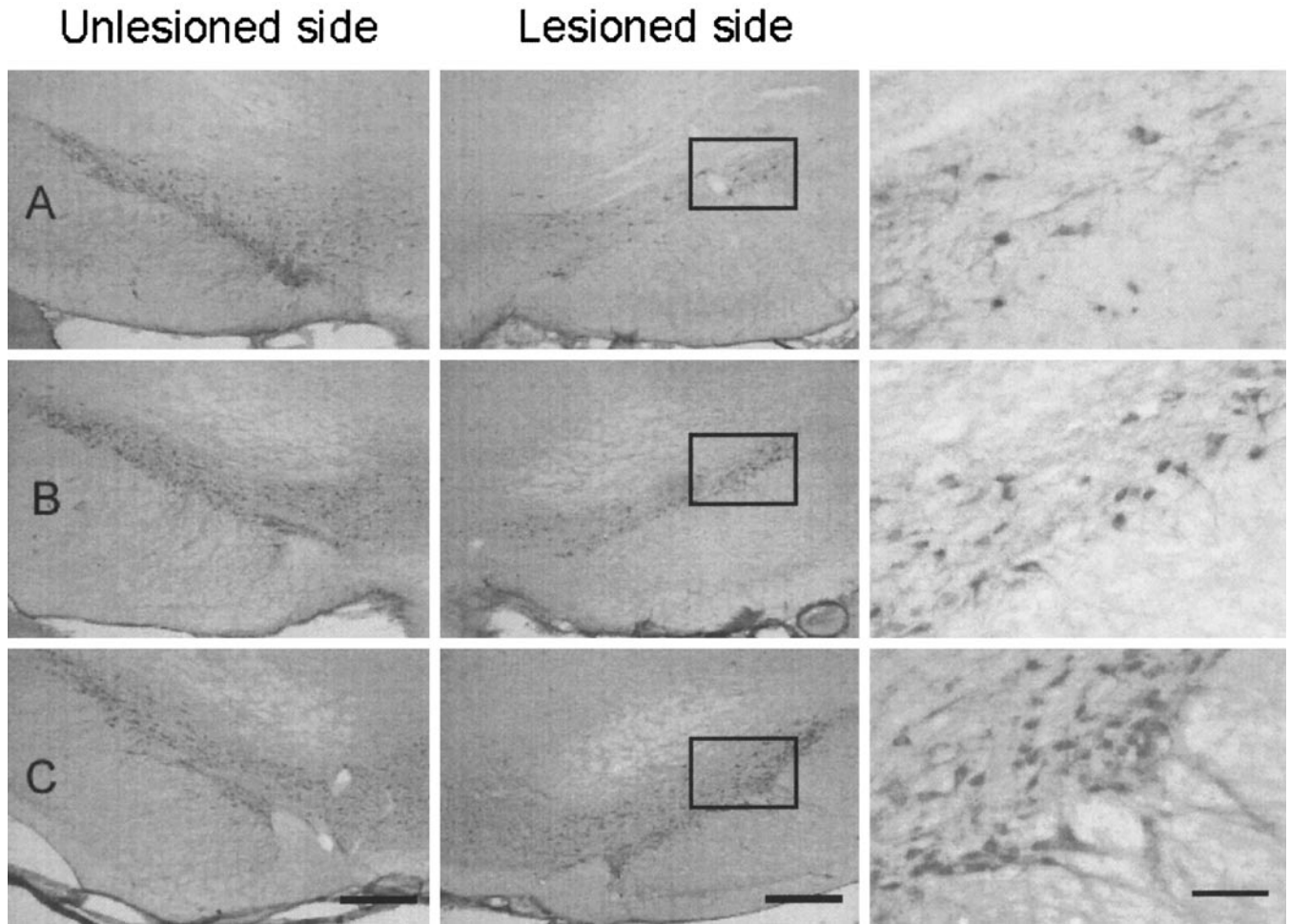
FIG. 6. TH immunohistochemical staining showing the dopaminergic neurons in the VTA and SNpc. The photographs were captured by the imaging analysis system (SPOT-2) at the magnification 50 × (left and the middle; bar, 400 μm) and 200 × (right; bar, 100 μm). Row A, control; row B, TW397 0.5 μg/kg; row C, TW397 1 μg/kg.

some way related with its neuroprotective effect. Interestingly, recent studies suggest that inflammation may also be a mechanism involving the degeneration of nigrostriatal DA neurons. Mogi *et al.* reported marked increase of the contents of proinflammatory, immunoreactive cytokines, including IL-1β, IL-2, IL-6, and tu-

TABLE 1

Amount of DA, HVA, and DOPAC in Striatum of MBF-Lesioned Rats with TW397 Treatment (Mean ± SEM ng/mg Wet Tissue)

Group	DA	HVA	DOPAC
Sham control	5.41 ± 0.69	1.36 ± 0.11	2.60 ± 0.30
Lesioned	1.53 ± 0.42*	0.47 ± 0.06*	1.49 ± 0.24*
TW397 0.5 μg/kg	1.54 ± 0.41	0.71 ± 0.21	1.42 ± 0.57
TW397 1.0 μg/kg	3.68 ± 0.75**	1.02 ± 0.13*	2.16 ± 0.44

* $P < 0.05$ vs. sham control group;   ** $P < 0.05$ vs. lesioned group, $n = 11$–16.

mor necrosis factor (TNF)-α in the striatal dopaminergic regions and the cerebrospinal fluid of PD patients (11, 12). The animal experiments also showed that systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) to mice induced marked elevation of the contents of IL-1β and TNF-α in the striatum (14). Treatment with immunosuppressant FK506 may suppress such an abnormal increase, thereby preventing DA neurons from degeneration (15, 29). In contrast, GPI-1046, the nonimmunosuppressive analog of FK506, failed to protect DA neurons from the toxicity of MPP$^+$ (6). Previous studies have shown that TW397 significantly inhibited the production of IL-1β and IL-6 at the concentration of 5–20 ng/ml (1.2×10^{-8}–5×10^{-8} M) (30, 31). Thus, it is reasonable to speculate that TW397 might exert its neuroprotective effect via attenuating abnormal elevation of levels of cytokines induced by MPP$^+$.

Our preliminary *in vivo* data obtained from the MFB axotomy model provided further evidence for the effectiveness of TW397 in protecting the dopaminergic neurons from degeneration after systemic administration. TW397 not only rescues axotomized SNpc neurons, but also ameliorates the imbalance behavior and preserves the expression of TH, the pacemaker enzyme for DA synthesis. However, the underlying mechanism of its effectiveness is unknown so far. Recent studies suggest the indirect relevance between its immunosuppressive effect and its neuroprotective action. Revuelta *et al.* reported recently that massive activated microglia and astroglia were found throughout the ventral mesencephalon, especially in the most medial part of SN, suggesting that reactive microglia and astroglia might be involved in the degenerating process of dopaminergic neurons (19). In fact, accumulating evidence suggests that activated microglia may take an active role in neuron destruction (24). Moreover, these activated microglia may produce a great deal of proinflammatory cytokines, such as IL-1β, IL-2, IL-6, and TNF-α, which can be detrimental to neurons. Therefore, any compounds that can inhibit astroglial reaction, macrophage/microglial activation, and inflammatory cytokine production in the CNS may be beneficial for the survival of CNS neurons.

FK506 has been reported to inhibit microglial activation and thereby protect against neuronal death after MFB axotomy, while its nonimmunosuppressive analog GPI-1046 did not possess such an effect (29). We speculate that the neuroprotective effect of TW397 observed in this study may be correlated with its immunosuppressive property similar to that of FK506. Further studies are needed to elucidate mechanisms of action between those two factors. Our next study is to investigate the relationship between the survival of DA neurons and microglia activation after MFB axotomy as well as their responses to the TW397 treatment.

Indirect Neurotrophic Effect of TW397

The present study revealed that TW397 augmented the expression of BDNF mRNA at the concentration of 10^{-10} M, a concentration that is optimal for promoting axonal elongation. This is in accordance with the findings that BDNF promoted neurite outgrowth *in vitro* (18, 28). BDNF also promoted the survival of fetal mesencephalic dopaminergic neurons *in vitro* (22) and protected them from MPP$^+$ and 6-OHDA-induced degeneration *in vivo* (20, 23). Therefore, the increase of BDNF mRNA expression induced by TW397 treatment may contribute to its neuroprotective effect.

BDNF is abundant and widely distributed within the brain including the striatum and SN (9), and the levels of BDNF were found to be decreased in the nigrostriatal dopaminergic regions of patients with PD (13) as well as in MPTP-induced parkinsonism mice (14).
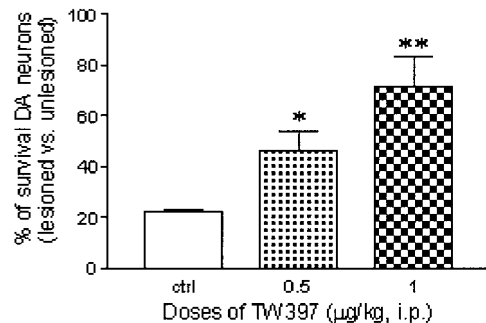
FIG. 7. Survival ratio of TH-positive neurons in SNpc of MFB-axotomized rats after treatment with TW397 (0.5, 1 μg/kg/day for 28 days), versus those treated with vehicle. $n = 3$–5; $*P < 0.05$; $**P < 0.01$ (one-way ANOVA with *post hoc* test).

Based on these findings, the putative potential of TW397 to facilitate the survived neurons and/or glial cells to secrete neurotrophic factors such as BDNF might benefit the regeneration of damaged neurons and/or prevent them from progressive degeneration.

Coherence between in Vitro and in Vivo Studies

The lipophilic character together with its small molecular size (MW 396) makes TW397 a promising candidate for clinical utility in the treatment and/or prevention of PD. Therefore, we used TW397 in a rat model of PD to assess whether systemically administered TW397 will produce a therapeutic effect on the experimentally induced PD syndrome. We demonstrate that intraperitoneal injection of TW397 at an extremely low dose (1 μg/kg) once a day for 4 weeks produces a 56% reduction of the rotational behavior challenged by D-amphetamine (Fig. 5) and an effective rescue of the dopaminergic neuronal death (Fig. 7), as well as a prevention of DA depletion (Table 1) induced by MFB axotomy. Assuming a homogenous distribution of the TW397 in the body, a dose of 1 μg/kg should lead to a maximal concentration of 10^{-6} g/kg, approximately 2.5×10^{-9} M in molar concentration, which is quite close to the effective concentration in the *in vitro* studies (Figs. 2–4).

The key issue for the potential use of TW397 for the treatment of PD is its safety, that is, the dose ratio between therapeutic and toxic effects. The therapeutic effects remained when the dose of TW397 was increased to 1 μg/kg, as assessed from the behavior index of rotational test (Fig. 5) and the morphologic index of survival of DA neurons (Fig. 7). However, neurochemical observation revealed that in terms of the content of DA and its metabolites, HVA and DOPAC, an increase of the dose of TW397 from 1 to 5 μg/kg produced a deterioration of the therapeutic effect (preliminary data, not shown). Thus, results obtained from the *in vitro* study showed a dose–response relationship over a wide range of 3–4 orders of magnitude, that is, 10^{-7}–

10^{-11} M in neurite growth (Fig. 2) and 10^{-8}–10^{-12} M in the size of the neuronal processes (Fig. 3) and BDNF mRNA expression (Fig. 4). Results obtained from *in vivo* studies (Figs. 5 and 7) showed a much narrower safety range. This is an issue deserving a closer look before the results of the present study can be extrapolated to practical use.

CONCLUSION

We report that TW397 at nanomolar concentrations exerts potent neurotrophic effects and protects dopaminergic neurons from degeneration induced by both chemotoxic (e.g., MPP$^+$) and mechanical injuries. *In situ* hybridization study shows that TW397 could stimulate the expression of BDNF mRNA, which might contribute to its neurite-promoting and neuroprotective effects. Its immunosuppressive effect is also absolutely necessary for neuronal protection. We thereby propose that TW397 may serve as a promising leading compound in the development of drugs for Parkinson's disease.

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