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Original Article

Chiral 1,4-oxazepan-3-one Targeting Schwann Cells Exhibits Morphometrically Inhibitory Effects on Wallerian Degeneration

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Abstract : Schwann cell is a unique neuroglial cell and has essential functions for regulating Wallerian degeneration and peripheral nerve regeneration after nerve injury. When Wallerian degeneration is inefficient or irreversible due to non-function or malfunction of Schwann cells, peripheral nerve regeneration doesn't occur and peripheral neurodegenerative diseases are induced, such as diabetic neuropathies. However, effective medicinal therapeutics has not been developed. In this study, we employed a synthesized chiral 1,4-oxazpan-3-one (1,4-DZ) compound to investigate neuroanatomically degenerative characteristics of Schwann cells during Wallerian degeneration. We also used *ex vivo* sciatic nerve explant system to exclude macrophage effects on demyelination, performed immunostaining by using teased nerve fiber samples to observe the outlook of myelin sheath during Wallerian degeneration, and identified its inhibitory effects on the degeneration. Thus, our results suggested that 1,4-DZ could be a prototype to develop a new small drug for peripheral neurodegenerative diseases.

Keywords : Schwann cells, Peripheral nerve degeneration, *Ex vivo* culture system, Morphometric analysis, Diazepam derivatives

INTRODUCTION

Wallerian degeneration (anterograde degeneration) during peripheral nerve degeneration is an essential process in the distal part of the nerve injury site. Inefficient or irreversible Wallerian degeneration loses the regenerative capacity of peripheral nerves and induces peripheral neurodegenerative diseases such as diabetic peripheral neuropathies [1]. During Wallerian degeneration, Schwann cells, as unique neuroglial cells in the peripheral nervous system (PNS), exhibit pivotal characteristics (demyelination, dedifferentiation, and proliferation) and regulate axonal degradation [2]. Malfunctional or non-functional Schwann cells cannot effectively regulate Wallerian degeneration and result in contributing to the induction of neurodegenerative diseases. Thus, regulation of the degenerative characteristics in Schwann cells may be a key factor in managing Wallerian degener-

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ation.

Small molecules, especially synthesized organic compounds, are broadly used as medicine tablets to treat some diseases because the compounds are easy to be taken and mass-produced. Among them, diazepam, as a benzodiazepine family, is very famous and commonly used to treat anxiety, seizures, and insomnia [3]. Because of its significant neuro-effectiveness, its diverse derivatives have been synthesized and developed as neurological drugs for various neurological diseases. 1,4-oxazepanone (1,4-DZ) is one of the derivatives which are known to have various bioactivities, such as anti-bacterial and anti-tumor effects [4,5]. However, in previous studies, diazepam and its derivatives have studied for reducing pain in peripheral neuropathy [6,7], but not for Wallerian degeneration.

Here, we investigated the inhibitory effect of 1,4-DZ on Wallerian degeneration. First, we used an *ex vivo* sciatic nerve culture system as a Wallerian degeneration animal model to exclude the macrophage effect. Second, we used morphometrical analysis for quantifying the drug effects based on microanatomy of peripheral nerves focusing on degenerative characteristics of Schwann cells such as dedifferentiation, demyelination, and proliferation. Further, we also checked morphometrically axonal degradation as an anatomical factor of nerve degeneration to identify the effect of 1,4-DZ.

MATERIALS AND METHODS

1. Animals

Adult male C57BL/6J mice (4-weeks old) were purchased from Orientbio™ (Sungnam, Korea). Animals were housed under a 12 hours light/dark cycle and temperature/humidity-controlled environment with free access to food and water. All experiments were conducted in accordance with the guidance prepared by Korean Academy of Medical Science and approved by Kyung Hee University Committee of Animal Research [KHSASP-21-463]. We made an effort to minimize the number of animals used and their suffering.

2. *Ex vivo* sciatic nerve culture

Sciatic nerve culture was described previously [8]. Briefly, sciatic nerves, including connective tissues, were obtained from the male mice by using a fine iris scissor (Fine Science Tools, Foster City, CA, USA) under a sterile environment. After the connective tissues were removed in the phosphate-buffered saline (PBS), the nerves were transferred into ice-cold PBS and then washed three times with PBS. Each nerve was split by fine iris scissors into 3 to 4 mm size lengths. Then, the explants were transferred into the cell culture medium [DMEM containing 9% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin] and incubated for three days in a humidified chamber at 37°C and 5% CO_2 with or without 1,4-DZ treatment. Three days after incubation, the nerves were washed in PBS and fixed with 4% paraformaldehyde (PFA).

3. Immunohistochemistry

For immunostaining, briefly, after post-fixation with 4% PFA, teasing sections in which nerve fibers were put on slides were incubated in 0.3% triton-X100, blocking solution, containing 10% FBS (Sigma) in PBS for 1 hour and then incubated with primary antibodies [anti-lysosomal-associated membrane protein 1 (LAMP1, 1 : 1000, sc-19992, Santa Cruz Biotechnology, Dallas, TX, USA); anti-myelin basic protein (MBP, 1 : 1000, ab980, Millipore, Bedford, MA, USA); anti-neurofilament H&M (NF, 1 : 1000, MAB1592, Millipore); anti-cyclin D1 (CCND1, 1 : 1000, MA5-14512, Invitrogen, Waltham, MA, USA)] at 4°C overnight. This step was followed by incubation with corresponding secondary antibodies coupled to a fluorescent dye for 2 hours at room temperature. After three times washing with PBS, the sections were counterstained with DAPI (10236276001, Roche, Basel, Switzerland) to visualize cell nuclei.

4. 1,4-DZ synthesis

Synthesis of 1,4-DZ [(*R*)-4-(benzyloxy)-2,2-dimethyl-5-phenacyl-1,4-oxazepan-3-one] (Fig. 1A) was described previously [9]. To a solution of δ-hydroxy-α,β-unsaturated carbonyl (0.10 mmol), α-bromohydroxamate (0.10 mmol), and catalyst (0.01 mmol) in triflurotoluene (1.0 mL) was added Cs_2CO_3 (0.10 mmol). After the reaction, the resulting mixture was filtered through the plug of celite and concentrated in vacuo. Yield 71%, $[\alpha]_D^{22} = -68.8$ ($c = 0.42$, $CHCl_3$); 98% ee. All chemicals were purchased from Acros (Geel, Belgium) and Sigma-Aldrich (St. Louis, MO, USA), and solvents were obtained from

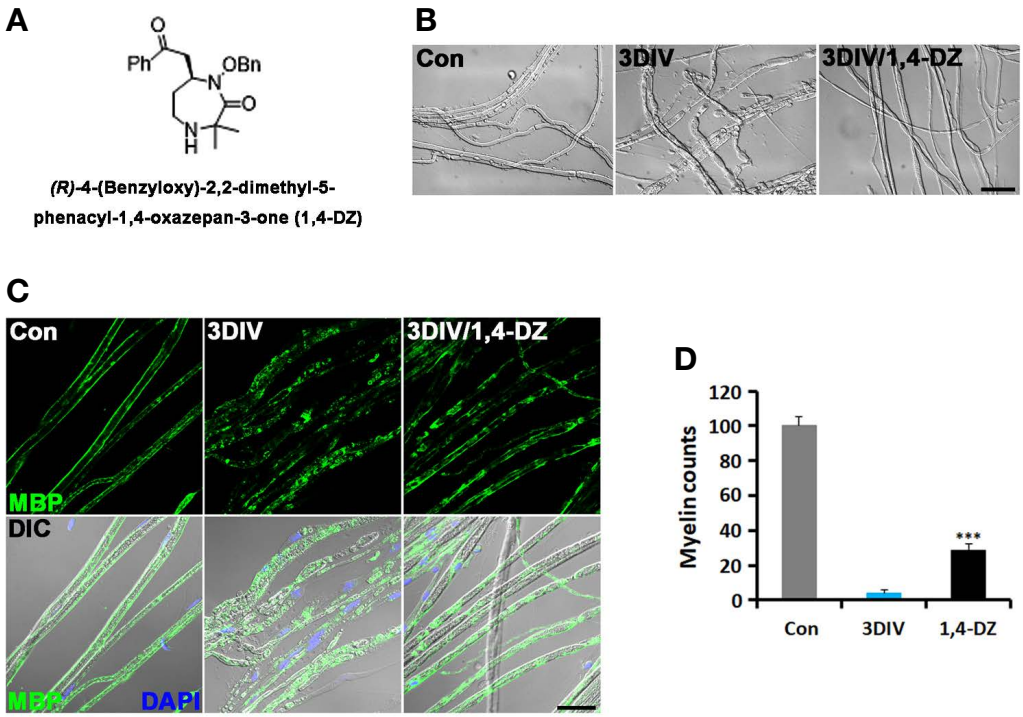

Fig. 1. 1,4-DZ effectively inhibits Schwann cell demyelination during Wallerian degeneration. (A): Structure of 1,4-DZ. 1,4-DZ; (*R*)-4-(Benzyloxy)-2,2-dimethyl-5-phenacyl-1,4-oxazepan-3-one. (B): Ovoid-shaped myelin fragmentation. Sciatic nerve fibers were cultured for 3DIV with or without treatment of 1,4-DZ. Scale bar = 50 μm. (C): Myelin sheath was investigated using an anti-myelin basic protein (MBP, green) antibody counterstained with DAPI. Scale bar, 50 μm. (D) Quantitative analysis of myelin in Schwann cells. Myelin counts were estimated by calculating the 100 μm-long consecutive lines of MBP-marked nerve fibers. $P^{***} < 0.001$.

Daejung (Siheung, Korea).

5. Morphometric indices

Myelin counts were estimated by calculating the 100 μm-long consecutive lines of MBP (a marker for myelin sheath)-marked nerve fibers. NF (a marker for peripheral axon) counts were calculated by counting the 100 μm-long consecutive lines. The intensity of LAMP1 (a marker for dedifferentiation in Schwann cells) expression was calculated in the teased sciatic nerve fibers within $200 \times 200\ \mu m^2$ widths of teasing slides. CCND1 (a marker for cell cycle) counts were proceeded by calculating CCND1-marked signals out of 200 DAPI-positive ones.

6. Statistical analysis

Two-tailed unpaired Student's t-test was used in order to compare the 1,4-DZ treated group with non-treated group through IBM SPSS Statistics Ver. 23 (Armonk, NY, USA). P^{**} (<0.005) and P^{***} (<0.001) values were considered statistically significant. All data are presented as means ± SEM.

RESULTS

1. Inhibitory effects of 1,4-DZ on *ex vivo* demyelination of Schwann cells

Among the degenerative characteristics of Schwann cells, demyelination means myelin fragmentation in Schwann cells [10]. During Wallerian degeneration, peripheral nerve fibers exhibit many ovoid-shaped fragments and the fragmentation disappears at the end of the degeneration. Thus, the quantification of myelin fragments can represent the degree of Wallerian degeneration and be used to evaluate the drug effect on the degeneration [11]. First, we investigated the effect of 1,4-DZ (100 μM) on myelin fragmentation. Since in the concentration of 1 and 10 μM, 1,4-DZ did not show the inhibitory effect (data not shown), further evaluation was carried out by using 100 μM of 1,4-DZ. At 3 days

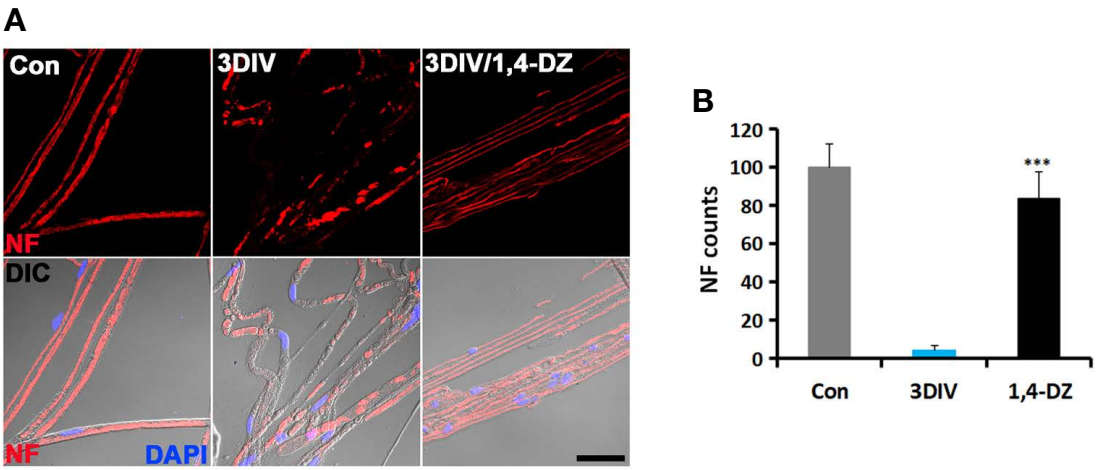

Fig. 2. 1,4-DZ effectively inhibits axonal degradation during Wallerian degeneration. (A): Sciatic nerve fibers were immunostained. Neurofilament (NF, red) and 4′,6-Diamidine-2′-phenylindole (DAPI, blue) were used as markers for axons and nucleus, respectively. Scale bar = 50 μm. (B): Quantitative analysis of axons in Schwann cells. NF counts were calculated by counting the 100 μm-long consecutive lines. $P^{***} < 0.001$.

in vitro (3DIV) culture, teased nerve fibers exhibited several ovoid-like fragments compared with control (non-injured or non-incubated) (Fig. 1B). Whereas, 1,4-DZ-treated nerve fibers showed no ovoid fragments compared with the fiber at 3DIV (Fig. 1B).

Next, to morphometrically analyze the degree of demyelination, we immunostained myelin sheath using anti-MBP antibody as a marker of myelin protein. Control exhibited double-consecutive lines around nerve fibers, whereas the fibers at 3DIV showed irregular signals and round/ovoid-shaped structures (Fig. 1C). However, 1,4-DZ-treated fibers showed the similar phenotype of the control, including intact double-line structures (Fig. 1C). Fig. 1D indicated the quantification of myelin fragmentation during Wallerian degeneration. Thus, our results demonstrated that 1,4-DZ significantly inhibits the demyelination of Schwann cells during Wallerian degeneration.

2. Inhibitory effects of 1,4-DZ on *ex vivo* axonal degradation by Schwann cells

Axonal degradation is a degenerative phenotype of axons during Wallerian degeneration. However, the degradation is induced indirectly by the mechanical crush of Schwann cells [10]. Thus, we investigated the axonal degradation to identify the inhibitory effect of 1,4-DZ on Wallerian degeneration. For immunostaining a biomarker of axons, we used anti-NF antibody and confirmed that the control showed intact line signals (Fig. 2A). Compared to the control, nerve fibers at 3DIV exhibited broken axonal signals and decreased intensity of the signals, whereas 1,4-DZ-treated fibers showed intact single-line signals similar to the control (Fig. 2A). Quantitative analysis also showed the inhibitory effect of 1,4-DZ on axonal degradation. Thus, these results indicated that 1,4-DZ significantly suppresses axonal degradation during Wallerian degeneration.

3. Inhibitory effects of 1,4-DZ on *ex vivo* dedifferentiation of Schwann cells

Intact peripheral nerves are wrapped by a myelin sheath, which is made by differentiated/myelinating Schwann cells. However, during Wallerian degeneration, Schwann cells return to their developing/dedifferentiated form [12]. The dedifferentiated Schwann cells express unusual proteins compared to the myelinating Schwann cells. LAMP1 is one of the degenerative proteins in Schwann cells during Wallerian degeneration [13]. To investigate the effect of 1,4-DZ on the Schwann cell dedifferentiation, we immunostained the teased nerve fibers with anti-LAMP1 antibody. In control nerve fibers, LAMP1 was not expressed, whereas the fibers at 3DIV exhibited increased LAMP1 signals in the cytoplasm of Schwann cells (Fig. 3A). However, 1,4-DZ-treated fibers showed reduced LAMP1-positive signals compared to those at 3DIV (Fig. 3A). Quantitative LAMP1 intensity also showed the decrease of its signal af-

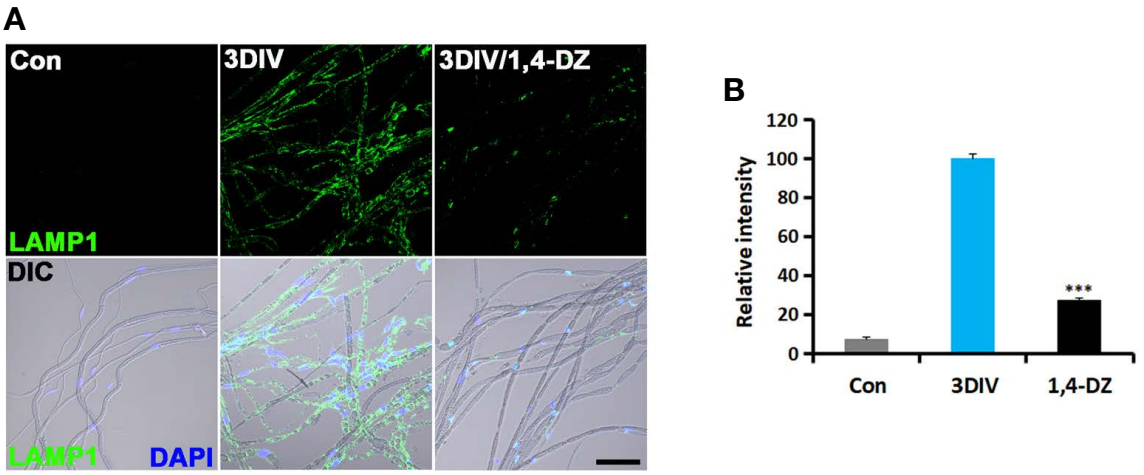

Fig. 3. 1,4-DZ effectively inhibits the dedifferentiation of Schwann cells during Wallerian degeneration. (A): Immunostaining with LAMP1 (green) co-stained with DAPI (blue) in *ex vivo* sciatic nerve fibers was performed. Scale bar = 50 μm. (B): Quantitative analysis of LAMP1 expression in Schwann cells. The intensity of LAMP1 expression was calculated in the teased sciatic nerve fibers within 200 × 200 μm^2 widths of teasing slides. $P^{***} < 0.001$.

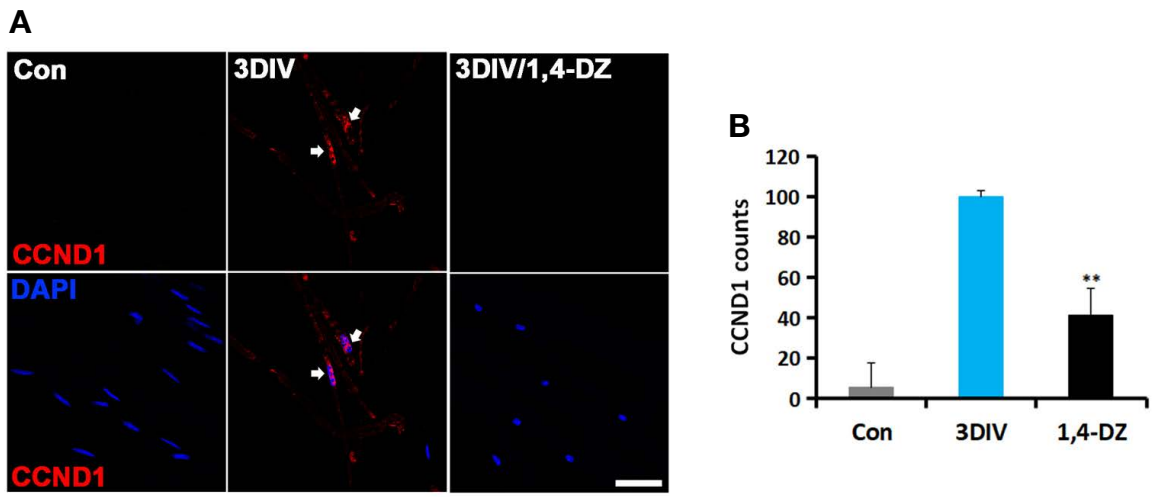

Fig. 4. 1,4-DZ effectively inhibits the proliferation of Schwann cells during Wallerian degeneration. (A): CCND1 (red) and DAPI (blue) were immunostained in *ex vivo* sciatic nerve fibers. The arrows indicated CCND1/DAPI double-positive signals. Scale bar = 50 μm. (B): Quantitative analysis of CCND1 expression in Schwann cells. CCND1 counts were proceeded by calculating CCND1-marked signals out of 2.

ter 1,4-DZ treatment. Thus, these results indicated that 1,4-DZ effectively inhibits the dedifferentiation of Schwann cells during Wallerian degeneration.

4. Inhibitory effects of 1,4-DZ on *ex vivo* proliferation of Schwann cells

During Wallerian degeneration, Schwann cells obtain the proliferative capacity and prepare the nerve regeneration. CCND1 is important for Schwann cell proliferation during its development and after peripheral nerve injury [14]. To investigate the effect of 1,4-DZ on Schwann cell proliferation, we employed CCND1 as a marker of cell proliferation and immunostained the teased nerve fibers with anti-CCND1 antibody. In the immunostaining, intact nerve fibers showed no CCND1-positive signals, whereas the fibers at 3DIV expressed the CCND1 signals in the nuclei of Schwann cells (Fig. 4A). However, CCND1-positive

signals were not expressed in the nucleus of 1,4-DZ-treated Schwann cells compared to those at 3DIV (Fig. 4A). Counting CCND1/DAPI double-positive signals also showed the inhibitory effect of 1,4-DZ on Schwann cell proliferation. Thus, these results indicated that 1,4-DZ significantly inhibits Schwann cell proliferation during Wallerian degeneration.

DISCUSSION

After peripheral nerve injury, differentiated Schwann cells transform into developing/dedifferentiated cells and start to regulate Wallerian degeneration. During the degeneration, dedifferentiated Schwann cells exhibit several degenerative characteristics such as demyelination, proliferation, and migration, and the characteristics influence the morphological phenotypes of Wallerian degeneration and peripheral nerve regeneration. Thus, Schwann cells are essential for regulating Wallerian degeneration. However, besides mechanical injury, systemic injury such as genetic or hyperglycemic conditions can cause irreversible non-function or malfunction of Schwann cells [1], and subsequently, irreversible nerve degeneration occurs, and ultimately, peripheral nerves lose their regenerative capacity. The whole process is involved in the pathophysiology of peripheral neurodegenerative diseases such as diabetic neuropathy. Because the nerves cannot regenerate, the therapeutic strategy is not to boost their regenerative capacity but to delay or stop their degenerative process. Until now, several treatments have been known, but effective one is not present clinically.

In previous studies, diazepam derivatives showed several pharmacological activities. For example, the derivatives have inhibitory effects on acute ischemia by vascular occlusion, antibacterial/antifungal effects, antitumor activity, and analgesic effects [4,5,15-17]. Among the derivatives, 1,4-DZ was already known as an inhibitor of *in vitro* proliferation in Schwann cell lines [9]. Its anti-proliferative effect may affect to delay or stop *ex vivo* Wallerian degeneration. In Fig. 4, we demonstrated that 1,4-DZ inhibited CCND1 (a marker of the cell cycle) expression during Wallerian degeneration, and the inhibitory effects may affect Wallerian degeneration via inhibition of Schwann cells proliferation similar to those effects on tumors shown in previous studies [4,5].

On the other hand, the anti-proliferative effects may come from the inhibitory effects of Schwann cell dedifferentiation. Dedifferentiated Schwann cells express unusual proteins compared to intact Schwann cells, and the proteins are associated with cell proliferation and migration [18]. A main feature of dedifferentiated Schwann cells is non-myelinated, and their lysosomes engulf and digest myelin debris during the nerve degeneration. A diazepam derivative is known as a potent protein kinase C (PKC) modulator [19], and lysosome expression is regulated by PKC [20]. Thus, 1,4-DZ could interact directly or indirectly with PKC and affect PKC-dependently lysosomal activity in Schwann cells. Schwann cell proliferation also could be suppressed subsequently by its inhibited dedifferentiation. To clear this hypothesis, further studies are needed near future.

An *ex vivo* sciatic nerve culture system may be the best way to evaluate the drug efficacy of peripheral neurological drugs targeting Schwann cells. In *in vivo* animal model, macrophage recruitment in the injury sites can interrupt the action of Schwann cells on removing myelin debris because both Schwann cells and macrophages are involved in myelin clearance [21]. Thus, unique drug effects on Schwann cells alone, besides those of macrophages during Wallerian degeneration, can be just assessed by an *ex vivo* system.

In conclusion, delaying or stopping Wallerian degeneration is actually the most possible therapeutic strategy for irreversible peripheral neurodegenerative diseases, and the discovery of synthetic small compounds for these diseases is the easiest present approach. For the development, we used 1,4-DZ as a diazepam derivative and *ex vivo* sciatic nerve degeneration model. Using morphometrical analysis, we evaluated the drug efficacy of 1,4-DZ and found that 1,4-DZ significantly inhibited the degenerative characteristics of Schwann cells during Wallerian degeneration, such as Schwann cell dedifferentiation, proliferation, and demyelination. Thus, it could appeal for 1,4-DZ to be used in the treatment of irreversible peripheral neurodegenerative diseases.

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