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# Flavopiridol protects against oxidative stress in Schwann cells and maintains their myelin structure during peripheral nerve degeneration: pharmaco-neuroanatomical approaches

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## ABSTRACT

Flavopiridol (Flavo) is a synthetic flavonoid analog and a broad cyclin-dependent kinase (CDK) inhibitor based on an extract of *Dysoxylum binectariferum* that effectively decreases the activity of CDK1, CDK2, CDK6, CDK7, and CDK9 in living cells. This property has allowed Flavo to be tested in clinical trials for acute myeloid leukemia and for the suppression of cancer cell proliferation. The proliferation of Schwann cells, unique glia in the peripheral nervous system, is the main morphological phenotype during peripheral nerve degeneration. We determined that Flavo induced Schwann cell proliferation during peripheral nerve degeneration. Flavo also protected other phenotypes, such as demyelination, axonal degeneration, transcriptional regulation, and Schwann cell trans-dedifferentiation. One of the causes of peripheral nerve degeneration is oxidative stress. A variety of cellular events in oxidatively damaged Schwann cells induce the onset of neurodegeneration, resulting in irreversible neurodegenerative conditions. In Schwann cells, hydrogen sulfide ($H_2S$) increases the protective response to oxidative stress and several signaling pathways involved downstream of $H_2S$. Flavo significantly inhibited the $H_2S$/CDK pathway in Schwann cells during peripheral nerve degeneration. Thus, our study suggests that Flavo is an effective inhibitor for peripheral neurodegeneration and could be a candidate to develop a therapeutic drug for peripheral neurodegenerative diseases.

Keywords: Schwann cells, flavopiridol, oxidative stress, peripheral nerve degeneration, hydrogen sulfide



## INTRODUCTION

Schwann cells are unique glia in the peripheral nervous system (PNS) that function to maintain the myelin sheath around peripheral axons, which causes saltatory conduction. Peripheral nerve

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degeneration occurs in the PNS under abnormal conditions, such as mechanical or toxic injury, and Schwann cells play roles both degenerating and repairing injured Schwann cells functionally and morphologically.[1] After completing nerve degeneration, Schwann cells promote regeneration of axons to the destination and remyelinate axons to recover saltatory conduction. Therefore, Schwann cells have an all-round capability to solve whatever occurs in the PNS. However, aberrant peripheral nerve degeneration under oxidative stress conditions, such as diabetes mellitus, induces irreversible nerve degeneration and ultimately causes peripheral neurodegenerative diseases, such as peripheral diabetic neuropathy.[2] No pathogenetically targeted treatment has been identified.

Oxidative stress is one of the main causes of peripheral neurodegenerative diseases.[2] Previous studies have shown the harmful effect of oxidative stress on peripheral nerve degeneration, targeting Schwann cells in particular. Schwann cells increase the expression of heme oxygenase 1 (HO1) after nerve injury, which degrades heme into biliverdin, carbon monoxide, and ferrous iron.[3] Ferrous iron functions as a pro-oxidant and induces free radicals, leading to oxidative stress, which affects peripheral nerve degeneration. During peripheral nerve degeneration, nitric oxide (NO) is upregulated by nitric oxide synthase 1 (NOS1) and NO induces cytotoxic reactive nitrogen species on Schwann cells, which promote peripheral nerve degeneration.[4] Schwann cells exhibit protective events against oxidative stress as well as cytotoxic events. Hydrogen sulfide ($H_2S$) is an antioxidant that increases to decrease oxidative damage in injured Schwann cells.[5-7] Cystathionine $\gamma$-lyase (CSE) is an enzyme that regulates $H_2S$ expression in Schwann cells. Controlling $H_2S$ protects against oxidative stress during peripheral nerve degeneration.[5-7] Therefore, therapeutic methods targeting $H_2S$-related signaling pathways in Schwann cells could be an effective mode against oxidative-stress-induced peripheral neurodegenerative diseases.

Herein, we conducted drug screening to identify compounds that effectively inhibit peripheral nerve degeneration and involve $H_2S$-related signaling pathways in Schwann cells using an ex vivo drug screening system. Among the drugs, flavopiridol (Flavo) is a synthetic analog of the natural product rohitukine, which is extracted from *Dysoxylum binectariferum* and is a cyclin-dependent kinase (CDK) inhibitor that decreases cyclin D1 (CCND1).[8,9] Our study determined that Flavo had the best inhibitory effect on ex vivo phenotypes of peripheral nerve degeneration, including demyelination, axonal degradation, Schwann cell trans-dedifferentiation, Schwann cell proliferation, and transcriptional regulation. Thus, our results suggest that Flavo could serve as a preventive treatment for peripheral neurodegenerative diseases via the $H_2S$/CDK pathway that stops or delays the speed of neurodegeneration.

## MATERIALS AND METHODS

*Reagents*

Flavo (#F3055), trans-4-[[6-(ethylamino)-2-[[1-(phenylmethyl)-1H-indol-5-yl]amino]-4-pyrimidinyl]amino]-cyclohexanol (CINK4, #SML1352) and trigonelline (Tri, #T5509) were purchased from Millipore Sigma (St. Louise, MO, USA). Fascaplysin (Fas, #341251) was purchased from Calbiochem (San Diego, CA, USA). Primary antibodies against myelin basic protein (MBP; 1:1000, #ab980), neurotrophin receptor p75 (p75 NTR; 1:1000, sc-271708) and lysosomal-associated membrane protein 1 (LAMP1; 1:1000, sc-19992) were purchased from Santa Cruz Biotechnology (Santa Cruz, Ca, USA). Primary antibodies against CSE (1:1000, 12217-1-AP) were purchased from Proteintech Group Inc (Rosemont, IL, USA). Primary antibodies against CCND1 (1:1000, #PA5-119058) and secondary antibodies included Alexa fluor-488 donkey anti-mouse (1:20,000, #A32766), alexa fluor-488 donkey anti-rabbit (1:20,000, #A32790), alexa fluor-594 donkey anti-mouse (1:20,000, #A10037), and alexa fluor-594 donkey anti-rabbit (1:20,000, #A10042)

were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents were purchased from Millipore Sigma (St. Louis, MO, USA).

*Animals and ex vivo protocol*

Healthy male C57BL/6 mice (the age of 5 weeks) were obtained from the Samtako (Osan, Korea). Animals were housed at 23 ± 1 °C under a 12/12 hours light/dark cycle and had free access to food and water. All of the animal care and experimental procedures conformed to the guidelines of animal experimentation established by The Korean Academy of Medical Science. All procedures were reviewed and approved by Kung Hee University Committee on Animal Research (protocol number #KHSASP-21-463). For ex vivo experiments, the sciatic nerve explant culture was performed using mice according to a previously described method.[3] Briefly, sciatic nerves were carefully dissected from 5-week-old male C57BL/6 mice using fine iris scissors (Fine Science Tools, Foster City, CA, USA) under sterile surgical conditions. After dissection, surrounding connective tissues were meticulously removed in phosphate-buffered saline (PBS) under a stereomicroscope. Each nerve was subsequently sectioned into 3–4 segments, approximately 2–3 mm in length, using the same fine scissors. These nerve fragments were then transferred into Dulbecco's Modified Eagle Medium (DMEM; #SH30243.01, HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Explants were cultured with or without test compounds for three days in a humidified incubator maintained at 37 °C and 5% $CO_2$. For experiments requiring single nerve fiber analysis, each nerve segment was gently teased using fine-tipped forceps (Dumont #5) on a gelatin-coated glass slide coated with a thin layer of PBS. Then, we removed connective sheathes from the nerve and were carefully teased apart from nerve fascicles on the slides. Individual nerve fibers were separated longitudinally by slowly pulling along the nerve axis to avoid mechanical damage. Care was taken to maintain the integrity of axon–Schwann cell units during the teasing process. This approach allowed for high-resolution visualization of Schwann cell morphology, myelin structure, and molecular marker expression at the single-fiber level.[6]

*Cell culture and treatment*

RT4-D6P2T cell line was purchased from the American Type Culture Collection (ATCC). Cells were maintained in DMEM (Gibco, Grand Island, NY, USA), supplemented with 10% heat-inactivated bovine serum (FBS, Gibco, Grand Island, NY, USA) and 1% Penicillin-Streptomycin Solution (Gibco, Grand Island, NY, USA) at 37 °C with 5% $CO_2$. For western blot analysis with anti-NOS1 antibody, cells were cultured in DMEM containing 1 μM of Flavo for 1 day.

*Immunohistochemistry*

Slides with teased fibers were fixed in 4% paraformaldehyde (4% PFA) solution, frozen by dry ice, and sectioned at 6 μm thickness. The slides blocked with 10% bovine serum albumin (BSA) at room temperature (RT) for 1 hour and primary antibodies were applied overnight at 4 °C in PBS containing 2% BSA. The slides were then washed in PBS followed by incubation with secondary antibodies labeled with Alexa Fluor 488- and 594 at RT for 2 hours. All images were obtained by a laser confocal microscope (LSM700, Carl Zeiss, Oberkochen, Germany) and six random fields in each slide were captured to assess the quantitative difference.

*Western blot*

The extracted proteins were lysed in 2% sodium dodecyl sulfate (SDS) containing 1% protease inhibitor cocktail (Roche Molecular Biochemicals, Nutley, NJ, USA) and analyzed on 10% SDS- polyacrylamide gel electrophoresis (PAGE) followed by Coomassie Brilliant Blue

staining for confirming quality of the proteins. The proteins were electrophoretically separated by SDS-PAGE and transferred onto polyvinylidine difluoride membranes (Amersham Biosciences, Buckinghamshire, UK). After protein transfer, the membranes were treated with the 5% non-fat milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST) at 4 °C for 12 hours followed by incubation with horseradishp (HRP)-conjugated secondary antibodies.

*Morphometric indices*

To evaluate morphologically the degree of peripheral nerve degeneration, we used strip, ovoid, myelin and neurofilament (NF) indices. Strip index is the number of white or black transverse stripes within 500 μm of sciatic nerve explants under a stereoscope. Ovoid index is the number of myelin ovoids within 200 μm of one teased nerve fiber under a differential interference contrast (DIC)-filtered microscope. Myelin index is the number of the nerve fibers which have intact myelin stains with longer than 50 μm among 100 teased nerve fibers under a microscopic field. NF index is the number of 100 μm-long NF-marked intact stains of axons among 100 nerve fibers.

*Statistical analysis*

Data were shown as mean ± standard error of the mean (SEM). The statistical significance of differences was assessed using two-tailed Student's *t* test using SPSS 21.0 software (IBM, Armonk, NY, USA). All the experiments were conducted at least four times and $p < 0.05$ was considered to indicate significant differences.

## RESULTS

*Flavo exerts a concentration-dependent inhibitory effect on peripheral nerve degeneration*

The CSE enzyme is a key regulator that produces $H_2S$ in Schwann cells, and its downstream molecules are involved in various cellular events.[5-7] CDK is a protein related to progression through regulating cell cycle, binding a regulatory protein called a cyclin.[8,9] To investigate the effect of $H_2S$/CDK on peripheral neurodegeneration, we used Flavo.[9-13] We used CINK4 (a CDK4 inhibitor) and Fas (a CDK4/6 inhibitor, Fas) as positive controls, and Tri, an inhibitor of nuclear factor erythroid 2–related factor 2 (NRF2), as a negative control. In our previous study, Tri inhibited the $H_2S$/NRF2 pathway, in which NRF2 is a basic leucine zipper transcription factor regulating antioxidant gene expression and whose activity is modulated by $H_2S$, but failed to suppress peripheral nerve degeneration.[14,15] (Fig. 1A).

During peripheral nerve degeneration, the continuous, uneven ridge-like structure representing myelinated nerves on the sciatic nerve surface disappeared on day 3 of in vitro culture (3DIV) (Fig. 1A). With this gross property, we performed a pharmacoactivity screening test using an ex vivo explant culture system and various concentrations of the compounds.[6] Tri, which is associated with the $H_2S$/NRF2 signaling pathways, did not inhibit the disappearance of the continuous structure in the sciatic nerve explants at 3DIV (Fig. 1A). Among the compounds associated with the $H_2S$/CDK signaling pathway, CINK4, Fas and Flavo inhibited the disappearance of the continuous structure on sciatic nerve explants at 3DIV (Fig. 1A and B). CINK4 significantly suppressed nerve fiber fragmentation, consistent with previous studies,[15] however, its effect was confirmed to be weaker than that of Flavo and Fas (Fig. 1B). Fas and Flavo showed pharmacoactivity at 10 and ≥ 100 μM concentrations, respectively. Here, we used 10 μM of Fas and 100 μM of Flavo for further evaluation since the pharmacological effects of Fas on Schwann cells have been previously reported.[15]

Additionally, the transverse stripes (bands of Fontana) in sciatic nerves is also a morphologically effective phenotype of peripheral nerve degeneration.[3] Flavo inhibited the disappearance of the transverse stripes in sciatic nerve explants at 3DIV (Fig. 1C). We used *N*-ethylmaleimide (NEM) as a positive control compound, as it effectively inhibits all Schwann cell phenotypes during peripheral nerve degeneration.[6] Excessive CSE activity leads to dysregulated $H_2S$ signaling, increased oxidative stress, and mitochondrial dysfunction, which promote abnormal Schwann cell dedifferentiation and myelin breakdown. Inhibition of CSE using NEM reduces oxidative damage, preserves mitochondrial integrity, and stabilizes axon–Schwann cell interactions, thereby suppressing degeneration-associated Schwann cell responses and attenuating peripheral nerve degeneration.[5,6]

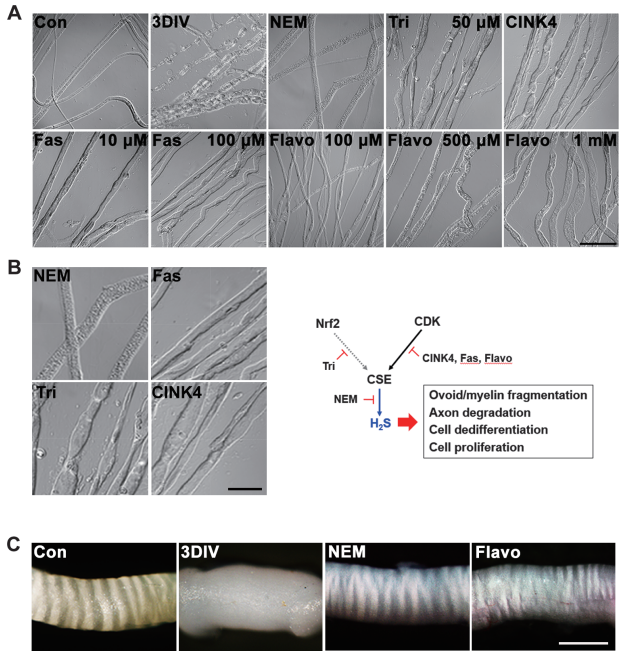


**Fig. 1** Fas inhibits protects ovoid formations and the disappearance of transverse stripes during ex vivo peripheral nerve degeneration

**Fig. 1A:** Sciatic nerve explants were cultured for 3DIV in absence or presence of the drugs under a differential interference contrast (DIC)-filtered microscope. NEM, CINK4 and Fas were as positive control drugs. Tri was a negative control drug. Scale bar = 100 μm.

**Fig. 1B:** High-magnification images of the positive and negative controls. Fas was applied at a concentration of 100 μM. Scale bar = 50 μm.

**Fig. 1C:** Dose-dependent treatment for showing transverse stripe patterns in sciatic nerve explants. Scale bar = 600 μm.

3DIV: 3 days in vitro
CINK4:trans-4-[[6-(ethylamino)-2-[[1-(phenylmethyl)-1H-indol-5-yl]amino]-4-pyrimidinyl]amino]-cyclohexanol
Con: control
CDK: cyclin-dependent kinase
CSE: cystathionine γ-lyase
Fas: fascapysin
Flavo: flavopiridol
$H_2S$: hydrogen sulfide
NEM: *N*-ethylmaleimide
Nrf2: nuclear factor erythroid 2–related factor 2
Tri: trigonelline

*Flavol effectively inhibits maintains the myelin structure and axonal degradation*

The key phenotypes of peripheral nerve degeneration are demyelination and axonal degradation discovered by English neurophysiologist Augustis Volney Waller in the 19th century. To investigate whether Flavo inhibits these two morphological phenotypes of peripheral nerve degeneration, we performed immunostaining with antibodies for MBP (a myelin sheath marker) and NF (an axonal marker) using an ex vivo explant culture. MBP staining was observed as consecutive lines in control nerve fibers (sciatic nerve with no injury), while the nerve fibers displayed cleaved lines and irregular margins of the MBP immunostain at 3DIV (Fig. 2A), indicating demyelinated Schwann cells during peripheral nerve degeneration. However, the nerve fibers treated with Flavo at 3 DIV showed the similar pattern of MBP immunostaining as the control, such as consecutive lines and regular margins, maintained during ex vivo culture. To estimate quantitatively the ex vivo morphological pharmacoactivity of Flavo, we used morphometric indices, ovoid (Fig. 2B) and myelin (Fig. 2C), which indicate demyelination.[6]

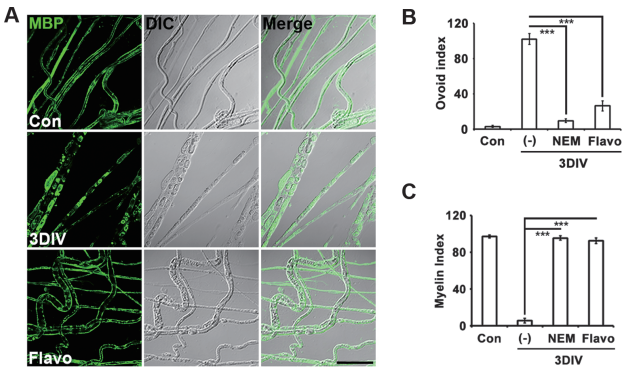


**Fig. 2** Flavo inhibits demyelination during ex vivo peripheral nerve degeneration

**Fig. 2A:** Confocal images show immunolabeling for myelin basic protein (MBP, a myelin marker, green). Scale bar = 100 μm.

**Fig. 2B, C:** Ovoid (B) and myelin (C) indices show quantitative degree of ex vivo peripheral nerve degeneration (n = 4 mice). $^{***}p < 0.0001$. NEM was a positive drug.

3DIV: 3 days in vitro
Con: control
DIC: differential interference contrast
Flavo: flavopiridol
MBP: myelin basic protein
NEM: *N*-ethylmaleimide

After nerve injury, axons degrade directly by a self-destructive pathway or indirectly by mechanical destruction of Schwann cells.[1] NF immunostaining of the control revealed consecutive lines in the longitudinal middle parts of the nerve fibers (Fig. 3A). In our experiment, NF immunostaining showed consecutive lines (red color) in control nerve fibers while the nerve fibers revealed an increase of broken consecutive lines at 3DIV. Flavo-treated nerve fibers did not have broken lines and maintained the continuity of the staining. Next, to estimate quantitatively axonal degradation of Flavo, we used NF indices (Fig. 3B). Flavo revealed similar pharmacoactivity to that of NEM for the ovoid index, and Flavo had the similar pharmacoactivity as NEM for the myelin and NF indices (Figs. 2 and 3). Thus, these results indicate that Flavo effectively inhibits demyelination and axonal degradation and, consequently, maintains myelin structure during peripheral nerve degeneration.

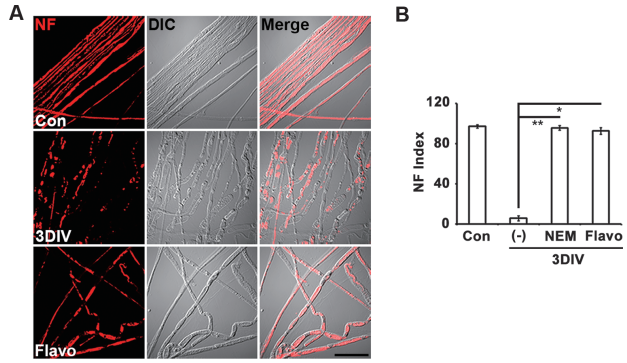


**Fig. 3** Flavo inhibits axonal degradation during ex vivo peripheral nerve degeneration
**Fig. 3A:** Confocal images show immunolabeling for NF (an axon marker, red). Scale bar = 100 μm.
**Fig. 3B:** NF index shows quantitative degree of ex vivo peripheral nerve degeneration (n = 4 mice). $^{*}p < 0.05$ and $^{**}p < 0.001$. NEM was a positive drug.
3DIV: 3 days in vitro
Con: control
DIC: differential interference contrast
Flavo: flavopiridol
NEM: *N*-ethylmaleimide
NF: neurofilament

*Flavo effectively inhibits trans-dedifferentiation and proliferation of Schwann cells*

Schwann cells have similar requirements during nerve degeneration to immature Schwann cells during nerve development, which is called Schwann cell trans-dedifferentiation, and is a peculiar property of the peripheral neurodegenerative process. To confirm whether Flavo regulates Schwann cell trans-dedifferentiation during nerve degeneration, we investigated the expression

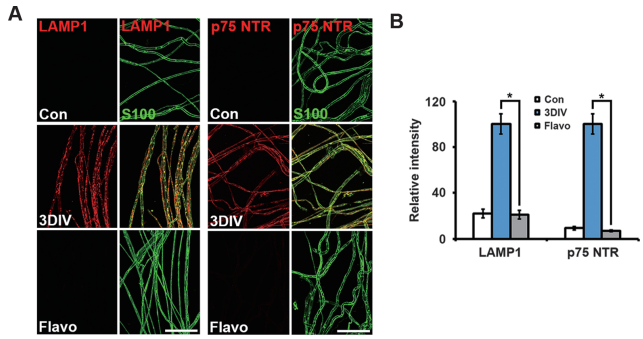


**Fig. 4** Flavo inhibits Schwann cell trans-dedifferentiation during ex vivo peripheral nerve degeneration
**Fig. 4A:** Confocal images show immunolabeling for LAMP1 (left panel, red) and p75 NTR (right panel, red). S100 was used as a Schwann cell marker (green). Scale bar = 100 μm.
**Fig. 4B:** Quantitative data shows the expression patterns of LAMP1 and p75 NTR in the absence or presence of Flavo (n = 3 mice). $^{*}p < 0.05$.
3DIV: 3 days in vitro
Con: control
Flavo: flavopiridol
LAMP1: lysosomal-associated membrane protein 1
p75 NTR: neurotrophin receptor p75

pattern of several trans-dedifferentiation markers in Schwann cells, including LAMP1 and p75 NTR.[3,6] Immunostaining with the LAMP1 and p75 NTR antibodies showed that LAMP1 and p75 NTR upregulation was effectively inhibited by Flavo in ex vivo explants at 3DIV (Fig. 4). Thus, these results indicate that Flavo is an effective inhibitor that protects against the transformation of normal Schwann cells into trans-dedifferentiated Schwann cells during peripheral nerve degeneration.

Another main phenotype of Schwann cells during peripheral nerve degeneration is proliferation. To identify the effect of Flavo on Schwann cell proliferation during nerve degeneration, we immunostained ex vivo sciatic nerve fibers with the anti-Ki67 antibody as a marker of cell proliferation. Ki67 was not immunostained in the control fibers, but degenerated nerve fibers showed 4′,6-diamidino-2-phenylindole (DAPI)- or SRY-box transcription factor 10 (Sox10)-double-positive Ki67 signals at 3DIV, whereas Flavo-treated nerve fibers did not express Ki67 at 3DIV (Fig. 5A). CCND1 is another cell proliferation marker and an effector that represents the pharmacoactivity of Flavo.[9-13] CCND1 was upregulated at 3DIV in untreated explants and it was also suppressed by Flavo at 3DIV (Fig. 5B). Thus, these results indicate that Flavo is an effective inhibitor that suppresses Schwann cell proliferation during peripheral nerve degeneration.

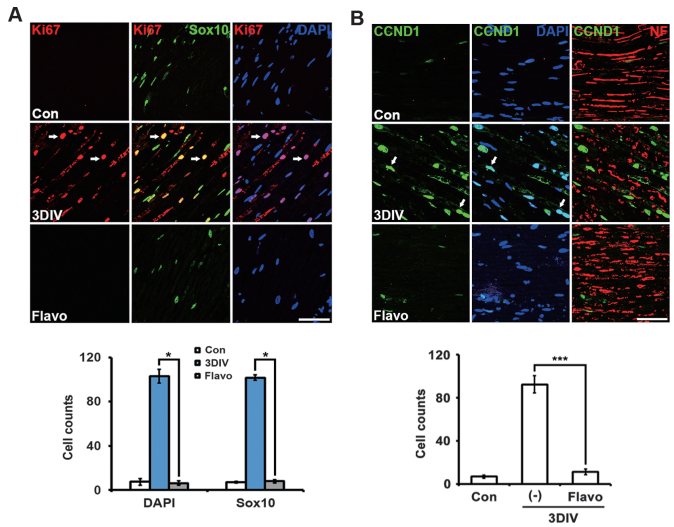


**Fig. 5** Flavo inhibits Schwann cell proliferation during ex vivo peripheral nerve degeneration

**Fig. 5A:** Confocal images (upper panel) show immunolabeling for Ki67 (red). DAPI and Sox10 are used for nucleus markers (green). Scale bar = 100 μm. The number of cells (lower panel) was counted with Ki67 immunolabeling out of 200 DAPI or Sox10-(+) nuclei in teased sciatic nerve fibers as compared with that of the control (n = 4 mice). In y-axis, count 1 = 1 Ki67/DAPI or Sox10 double-positive Schwann cell. $^{*}p < 0.05$.

**Fig. 5B:** Confocal images (upper panel) show immunolabeling for CCND1 (green). DAPI and S100 were used for a nucleus (blue) and axon (red) marker, respectively. Scale bar = 100 μm. The number of cells (lower panel) was counted with CCND1 immunolabeling out of 200 DAPI-(+) nuclei in teased sciatic nerve fibers as compared with that of the control (n = 4 mice). In y-axis, count 1 = 1 CCND1/DAPI double-positive Schwann cell. $^{***}p < 0.0001$.

3DIV: 3 days in vitro
CCND1: cyclin D1
Con: control
DAPI: 4′,6-diamidino-2-phenylindole
Flavo: flavopiridol
Sox10: SRY-box transcription factor 10

*Flavo regulates downstream of $H_2S$ and inhibits oxidative damage in Schwann cells*

Because Flavo is an inhibitor of CDKs, which is downstream of $H_2S$.[9-11] Flavo should not inhibit upstream of CDKs, particularly CSE as a specific enzyme that produces $H_2S$ in Schwann cells.[6] To confirm whether Flavo specifically regulates downstream of $H_2S$ during peripheral nerve degeneration, we performed immunostaining with an anti-CSE antibody using an ex vivo model. NEM is a specific CSE inhibitor[6] (Fig. 1) and NEM effectively inhibited CSE expression at 3DIV compared with untreated nerve fibers, while Flavo did not affect the decrease of CSE expression in degenerating Schwann cells (Fig. 6A and B). Thus, we determined that Flavo is an inhibitor of downstream of $H_2S$ in Schwann cells during peripheral nerve degeneration.

Next, to confirm whether Flavo has a protective effect against oxidative stress in Schwann cells, we performed a western blot analysis using RT4-D6P2T cells (rat Schwann cell-line). NOS1 is a specific enzyme used to produce NO and the main resource for free radical damage in Schwann cells, not NOS2 or NOS3 during peripheral nerve degeneration.[3] After treatment with 1 μM of Flavo, RT4-D6P2T cells decreased the expression of NOS1 compared with untreated control RT4-D6P2T cells (Fig. 6C and D). Therefore, our results indicate that Flavo effectively protects against oxidative stress in Schwann cells during peripheral nerve degeneration by inhibiting downstream of $H_2S$.

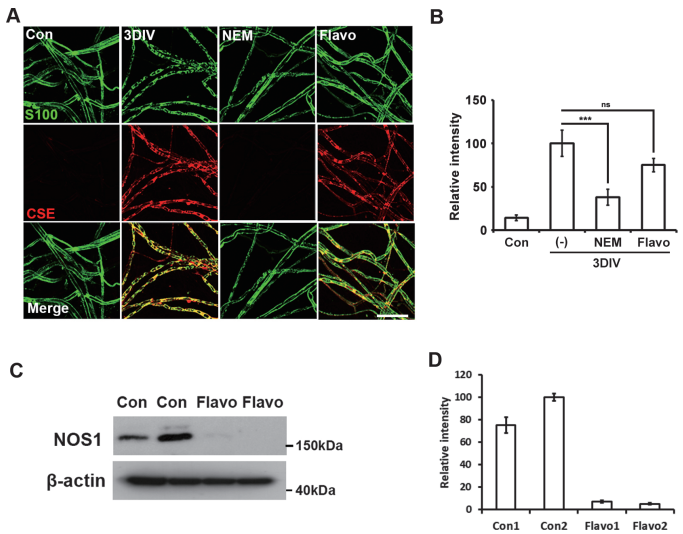


**Fig. 6** Flavo inhibits oxidative stress in in vitro and ex vivo dedifferentiated Schwann cells

**Fig. 6A:** Confocal images show immunolabeling for CSE (red) and S100 (a marker of Schwann cells, green). Scale bar = 100 μm.

**Fig. 6B:** Quantitative data shows the expression patterns of CSE in the absence or presence of NEM and Flavo (n = 3 mice). $^{***}p < 0.0001$.

**Fig. 6C:** Protein lysates (30 μg) from RT4-D6P2T in the absence or presence of Flavo were analyzed by Western blotting (n = 4 experiments).

**Fig. 6D:** Quantitative data shows the relative intensities of nitric oxide synthase 1 (NOS1) in the absence or presence of Flavo (n = 3 experiments).

3DIV: 3 days in vitro
Con: control
CSE: cystathionine γ-lyase
Flavo: flavopiridol
NEM: *N*-ethylmaleimide

## DISCUSSION

Peripheral neurodegenerative diseases, such as diabetic neuropathy, are a worldwide problem, and effective treatment has not been identified. One of the causes of peripheral neurodegenerative diseases is oxidative damage to Schwann cells. A variety of oxidative resources, such as reactive oxygen species (ROS), reactive nitrogen species and free iron, are involved in the onset of peripheral nerve degeneration, and long-term exposure to oxidative stress induces irreversible peripheral neurodegeneration.[2,15] Thus, effective regulation against oxidative stress in Schwann cells may be a reasonable therapeutic target for peripheral neurodegenerative diseases.

*Flavo directly targets oxidative stress in Schwann cells during peripheral nerve degeneration*

Flavo is a synthetic flavone structurally related to an analog of rohitukine from *D. binesctariferum*.[8] Flavones are a class of flavonoids backboned by 2-phenylchromen-4-one (2-phenyl-1-benzopyran-4-one) and have antioxidant properties, as well as anti-inflammatory, antitumor, anti-allergic, neuroprotective, cardioprotective and antimicrobial effects in living cells.[2,16] Flavo was initially called Alvocidib and was developed to clinically treat acute myeloid leukemia.[17] Clinical trials have been performed to develop treatments for various cancers as well as leukemia.[18]

Although Flavo is a CDK inhibitor, it could have antioxidant properties because it is a synthetic flavone. In previous studies, flavones suppress the activity of central free radical-producing enzymes, such as nicotinamide adenine dinucleotide phosphate oxidase or NOS,[16] and have been suggested to be a possible therapeutic antioxidant to treat diabetic neuropathy.[2] Our results also revealed that Flavo inhibited NOS1 expression in dedifferentiated Schwann cells, in which NOS1 is a specific NOS in Schwann cells[3]; NOS2 and NOS3 are not expressed in Schwann cells under any conditions (Fig. 6C and D). Thus, Flavo reduced oxidative stress in Schwann cells as an antioxidant targeting NOS1 during peripheral nerve degeneration and this effect may protect against the neurodegenerative process and maintain the myelin structure of injured peripheral nerves (Fig. 2).

*Flavo canonically inhibits the main Schwann cell phenotypes during peripheral nerve degeneration*

The canonical function of Flavo is as a CDK inhibitor and it targets positive transcription elongation factor b (P-TEFb),[19] which is why Flavo is used as a cancer drug, particularly for leukemia.[16-18] Schwann cells have similar properties as cancer cells. Schwann cells re-acquire the ability to proliferate during nerve degeneration, which is revealed during their development. Proliferation is dependent on cyclin protein dynamics and regulating cyclins in Schwann cells protects against nerve degeneration.[20] Because oxidative stress induces peripheral nerve degeneration, Flavo not only canonically regulated the neurodegenerative process but also non-canonically functioned as an antioxidant. In our study, Flavo effectively suppressed Schwann cell proliferation according to a Ki67 immunostaining analysis (Fig. 5A). We also discovered that Flavo inhibited CCND1 in Schwann cells during peripheral nerve degeneration, in which CCND1 is a protein related to progression through the G1 phase of the cell cycle (Fig. 5B). Because CCND1 interacts with CDK9 under cell proliferative conditions, Flavo may also regulate the action of CCND1 by inhibiting CDK9/P-TEPb in Schwann cells during peripheral nerve degeneration.[21] Therefore, oxidative-stress-induced peripheral neurodegeneration could activate Schwann cell proliferation through the CDK9/CCND1 pathway and Flavo may be a reasonable compound targeting the CDK9/CCND1 pathway for Schwann cell proliferation, leading to delayed or terminated peripheral neurodegeneration.

In conclusion, we determined that Flavo had an inhibitory effect on peripheral nerve degeneration. Representative phenotypes, such as demyelination, axonal degradation, transcriptional

dysregulation, trans-dedifferentiation, and proliferation of Schwann cells, were effectively inhibited by Flavo in in vitro and ex vivo peripheral nerve degeneration models. Flavo may be involved in the $H_2S$/CDK signaling pathway and indirectly or directly regulate oxidative stress in Schwann cells during peripheral nerve degeneration. Because Flavo has already been tested in clinical trials for several diseases, such as acute myeloid leukemia, it is worth attempting a clinical trial for peripheral neurodegenerative diseases. Thus, we hope that our study will help develop a novel therapeutic strategy for peripheral neurodegenerative diseases.

## AUTHOR CONTRIBUTIONS

H-JC and JSC contributed equally to this study. Conceptualization, data curation, formal analysis, H-JC, JSC, NYJ, and JJ; funding acquisition, H-JC, NYJ, and JJ; investigation, methodology, project administration, resources, supervision, validation, visualization, writing-original draft and writing-review and editing, NYJ, H-JC and JJ; software, JSC and WWM. All authors have read and agreed to the published version of the manuscript.

## DISCLOSURES

The authors declare that they have no conflicts of interest in the studies described.

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