Duloxetine attenuates paclitaxel-induced peripheral nerve injury by inhibiting p53-related pathways

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ASCO, American Society of Clinical Oncology; CIPN, Chemotherapy-induced peripheral neuropathy; DRG, Dorsal root ganglia; PIPN, Paclitaxel-induced peripheral neuropathy; PTX, paclitaxel; PARP, poly ADP-ribose polymerase; p53, tumor suppressor gene; pft-α, Pifithrin-α.

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Abstract

Paclitaxel (PTX) is an antineoplastic drug extracted from the *Taxus* species, and peripheral neuropathy is a common side effect. Paclitaxel-induced peripheral neuropathy (PIPN) seriously affects patient quality of life. Currently, the mechanism of PIPN is still unknown, and few treatments are recognized clinically. Duloxetine is recommend as the only potential treatment for chemotherapy-induced peripheral neuropathy (CIPN) by the American Society of Clinical Oncology (ASCO). However, this guidance lacks a theoretical basis and experimental evidence. Our study suggested that duloxetine could improve PIPN and provide neuroprotection. We explored the potential mechanisms of duloxetine on PIPN. As a result, duloxetine acts by inhibiting PARP cleavage and p53 activation and regulating the Bcl2 family to reverse PTX-induced oxidative stress and apoptosis. Taken together, the present study shows that using duloxetine to attenuate PTX-induced peripheral nerve injury and peripheral pain may provide new clinical therapeutic targets for CIPN.
Significance Statement

This study reported duloxetine significantly alleviates neuropathic pain induced by Paclitaxel, and related to PARP, p53 and the Bcl2 family. Our findings thus not only provide an important guidance to support duloxetine to become the first standard chemotherapy-induced peripheral neuropathy (CIPN) drug, but also will find potential new targets and positive control for new CIPN drug development.
Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is an oft-reported dose-limiting side effect of neurotoxic chemotherapeutic agents (for example, paclitaxel (PTX), oxaliplatin and vincristine) (Cata et al., 2006). PTX stabilizes microtubules and is commonly used in the treatment of breast, ovarian and cervical cancer (Hagiwara and Sunada, 2004). However, patients treated with PTX often suffer from paraesthesia, allodynia (Pain caused by stimulus that produce no pain in general) and hyperalgesia (increased pain from stimulus which cause pain in general), such as tingling or numbness in the hands or feet, pain provoked by normal behaviors of daily life, and et al.; this paclitaxel-induced peripheral neuropathy (PIPN) is a serious problem for most patients and reduces quality of life (Dougherty et al., 2004; Jensen TS and Finnerup NB, 2014). Unfortunately, there are few effective medications for this peripheral neuropathic pain (Wolf et al., 2008; Gihyun L and Sun K, 2016; Brandolini et al., 2019). Therefore, most of the drugs currently used to modulate neuropathic pain are painkillers, including opioids, cannabinoid receptor modulators and sodium channel blockers (Jensen TS and Finnerup NB, 2014).

Apoptosis is a process of polygene regulation. Many proteins are involved in PTX-induced apoptosis, such as p53 and PARP, caspase-3, caspase-9 and the Bcl2 family (Yin et al., 2012; Xu et al., 2015; McNeish et al., 2016). The tumour suppressor gene p53, a major transcriptional regulator, is upregulated after cellular stress signalling, including DNA damage, hypoxia, and chemotherapy. The accumulation of p53 regulates many cellular processes, including apoptosis and
autophagy. Cisplatin inhibits activity of PIK3CA promoter and weakens survival pathways of PI3K / AKT cell through activating p53 in sensitive cells (Thakur and Ray, 2016). Furthermore, studies have shown that PTX remarkably upregulated the expression of PARP-1, Bax and some of the caspase family such as caspase-3 and caspase-9 and downregulated the expression of Bcl-2 in K562 cancer cells, which indicates that PTX regulates the intrinsic apoptotic signalling pathway to induce cell apoptosis (Wang et al., 2017). P53 and its downstream pathways play a key role in preventing tumour formation (Neitemeier et al., 2014). P53 dysfunction leads to neuronal damage, and p53 can mediate cell death by inducing Bax (Bcl2 family proteins) and the BH3-only proteins Bid, PUMA, and NOXA (Culmsee and Landshamer, 2006; Vousden and Prives, 2009; Culmsee and Mattson, 2005). Moreover, knockout or inhibition of p53 has a beneficial effect on nerve damage (Xiang et al., 1996; Culmsee et al., 2003; Plesnila et al., 2007). Poly-ADP-ribose polymerase-1 (PARP-1) is a protease with multiple physiological functions involved in DNA damage repair. Reserches shew that oxidative stress produced by chemotherapy or high glucose-induced nerve damage causes excessive activation of PARP1, and ATP and nicotinamide adenine dinucleotide (NAD\(^+\)) are depleted in cells, which ultimately lead to cell apoptosis (Haince et al., 2005; Langelier et al., 2008; Kuzhandaivel et al., 2010; Wang et al., 2016).

At present, the main treatments for PIPN have many side effects; the mechanisms of actions of these drugs are still unclear, and clinical application has not been recognized. Duloxetine, an antidepressant drug that inhibits serotonin and
norepinephrine uptake, has been approved by the United States FDA for a wide range of chronic neuropathic pain, including diabetic peripheral neuropathic pain and fibromyalgia (Yang et al., 2012; Miyazaki et al., 2012; Takenaka et al., 2013; Finnerup et al., 2015). Duloxetine was also recommended by American Society of Clinical Oncology (ASCO) as the only clinical drug for the treatment of CIPN. However, studies of duloxetine have mainly focused on clinical applications. Early case reports and small-scale open clinical studies suggest that duloxetine is effective for CIPN, but relevant research is still limited, and there is no basis for the theoretical system. There are fundamental problems that have not yet been answered. The mechanism of action of duloxetine when used to treat CIPN is an important issue.

Therefore, we aimed to clarify the effects of duloxetine on PTX-induced peripheral neuropathic pain in vivo and in vitro and to determine whether the p53 apoptotic signalling pathway induced by PTX is associated with the preventive impact of duloxetine on PIPN.
Materials and Methods

In vitro studies

Drugs

Paclitaxel (PTX, Sigma, United States) was dissolved in dimethyl sulfoxide (DMSO, Solarbio, China) to a concentration of 300 μM. Duloxetine (D, Shanghai Yuanye, China) was dissolved in DMSO to a concentration of 10 mM. All drugs were then diluted in the appropriate medium to a specified working standard concentration. Pifithrin-α (pft-α, p53 inhibitor, Nanjing Jiancheng Bioengineering Institute, China) was dissolved in DMSO to a concentration of 74 mM.

Cell Culture and drug treatments

Dorsal root ganglia (DRG) were dissected from Sprague-Dawley rats within 1-3 days of birth and immediately incubated with collagenase I solution (3 mg/mL, Worthington Biochemical Corporation, United Kingdom) at 37°C for 50 min. The suspension was centrifuged at 1500 rpm for 2 minutes and then resuspended, and the cells were seeded in plates at a density of $10^5$ cells/mL in Neurobasal medium (Gibco, United States) added in 1% penicillin/streptomycin (Gibco, United States), 10% foetal bovine serum (FBS, Gibco, United States), 0.5 mM glutamine, 1× B-27 supplement (Gibco, United States), 0.2% glucose and 10 μg/mL human glial-derived neurotrophic factor (GDNF, Peprotech, United States) in a 37°C, 5% CO₂ incubator for 24 hours. All culture plates were previously coated with poly-L-lysine (3 μg/mL, Sigma, United States). Then, the cells were exposed to culture medium containing 2% FBS with PTX (300 nM) for the model group and duloxetine (300 nM) for the treatment group.
(Meng et al., 2019). The untreated cells were cultured in complete medium without any drugs.

**Cell viability**

Cell viability was detected by CCK-8 assay (Yeasen, China), which is based on the ability of cells to convert WST-8 substrate into yellow formazan. Briefly, after treatment with PTX (300 nM) and/or varying concentrations of pft-α for 24 h, CCK-8 solution at 10 μL/well was added to cells in 96-well plates and incubated for 4 h at 37 °C. Then, the colour intensity reflecting cell growth was examined at 450 nm using a Multimode Plate Reader (Tecan, Switzerland).

**Cell apoptosis**

Cell apoptosis were analysed by flow cytometric analysis. Briefly, after drug treatment, the cells were washed and collected, according to the directions of the Annexin V-FITC/PI Apoptosis Detection Kit (KeyGen, China). The cells were resuspended in 500 μL of Binding Buffer, and 5 μL of Annexin V-FITC and propidium iodide were added and incubated for 15 min in the dark. The proportion of apoptotic cells was determined and analysed by flow cytometry (Accuri C6, BD, United States).

**Antitumour activity**

The cancer cell line PA-1 (ovarian cancer) was cultured to assess the effects of duloxetine and pft-α on PTX-induced cell death using the CCK-8 assay. PA-1 cells were incubated in high-glucose DMEM (HyClone, United States) containing 10% FBS (Gibco, United States) and 1% penicillin-streptomycin. After PTX treatment (300
nM) and different concentrations of duloxetine and pft-α, CCK-8 solution (10 μL) was added to each well. After 4 h, absorbance was measured at 450 nm.

**In vivo studies**

**Animals**

All procedures involving animals were conducted in accordance with the ethical guidelines established by the International Association for the Study of Pain (Eum et al., 2013) and the protocols were approved by the Animal Committee of Nanjing University of Chinese Medicine (Approval number, ACU171001). In the study, we used C57BL/6 mice (18-22 g; Nanjing QingLongShan, China). Food and water were freely available. The animals were housed in a room with a normal 12-h light-dark cycle. All animals were habituated in the room for a week before experiments; all experiments were evaluated in a blinded manner.

**Drugs**

paclitaxel (Shanghai Jinhe, China) was dissolved with Cremophor EL and ethanol at a ratio of 1:1 at concentration of 6 mg/mL and then diluted to 2 mg/mL with 0.9% sterile saline. Duloxetine (D, Shanghai Yuanye, China) was dissolved in 0.9% sterile saline at a concentration of 3 mg/mL and then diluted to 2 mg/mL and 1 mg/mL.

**PTX-induced neuropathic pain model**

The mice were randomly divided into the following 6 groups with 10 mice per group: the control group (Control); the paclitaxel model group (PTX); the duloxetine
treatment group with high, medium and low concentrations of duloxetine [P+D (H), P+D (M), and P+D (L)]; and the duloxetine alone treatment group (D). The mice were treated with PTX intraperitoneally (i.p.) at a dose of 20 mg/kg every other day (days 1, 3, 5, 7; cumulative dose, 80 mg/kg) to model PTX-induced peripheral pain (Melli et al., 2006; Krukowski et al., 2015). Control mice were injected with vehicle only. There were three duloxetine treatment groups with different doses of duloxetine: 10 mg/kg [P+D (L)], 20 mg/kg [P+D (M)] and 30 mg/kg [P+D (H)] (Toyama et al., 2017; Shidahara et al., 2016). Duloxetine was injected i.p. daily; in the treatment groups, duloxetine was administered 1 hour before an injection of PTX.

Detection of intracellular oxidative stress

MitoTracker Red CM-H₂XRos (Yeasen, China) was employed to detect the levels of intracellular oxidative stress. When the treatment was completed, cells were washed three times with PBS and then loaded with MitoTracker Red CM-H₂XRos (500 nm) in fresh Neurobasal medium for 45 min at 37°C. After dyeing, the cells were washed three times with PBS and fixed with pre-warmed 4% paraformaldehyde solution for 20 min. After washing with PBS, the cells were stained with Hoechst for 20 min. The fluorescence was then quantified, and the images were collected using fluorescence microscopy (IX71, Olympus, Japan).

Behaviour studies

Behaviour was evaluated after drug injection weekly (days 0, 7, 14, 21, and 28)
and conducted at room temperature. The body weights of the mice were recorded weekly. The investigator was blinded to the treatment groups (n=10).

**Measurement of mechanical hyperalgesia**

To measure mechanical hyperalgesia, we used the Dynamic Plantar Aesthesiometer (DPA, Ugo Basile, Italy). The mice were placed in a plastic chamber to acclimate for half an hour prior to the test. Force was transferred to the hind paw at a frequency of 1 g/s. To minimize damage, the cut-off force was set at 10 g. The nociceptive threshold was determined when paw withdrawal occurred, which was assessed on each hind paw for three trials in total per mouse.

**Measurement of thermal nociception**

The thermal withdrawal thresholds were assessed using a plantar test (37370, Ugo Basile Plantar Test Apparatus, Italy). Mice were allowed to acclimate to the glass floor for 30 minutes before the test. An infrared source was located at the centre of the hind paw of the mice, and heat could transfer rapidly. The time of withdrawal was recorded when the heat source was switched off. To avoid injury, a cut-off period of 20 s was maintained. The thermal withdrawal threshold was determined by the average of three treatments per mouse.

**Measurement of cold allodynia**

The mouse was placed in a fixing apparatus with only the tail exposed. The tail of the mouse was immersed in a water bath maintained at 4°C until tail withdrawal. To avoid damage to the tail, the cut-off time was set at 20 s. The behaviour test was repeated three times for each mouse with a time interval of at least 15 minutes.
between two measurements.

**Western blot analysis**

On the last day at the end of week 4, the DRG were removed from the mice for Western blot analysis. The DRG were homogenized in RIPA buffer and PMSF (a protease inhibitor) at a ratio of 100:1. Then, homogenates were centrifuged at 12,000 x g for 15 min at 4°C. Using a BCA protein assay kit, the protein supernatants were collected and measured. Proteins (20 μg) were separated by 10% SDS polyacrylamide gels and transferred onto PVDF membranes in transfer buffer for 60 min. The membranes were soaked in Tris-buffered saline and tween-20 (TBST) containing 5% nonfat milk for 60 min at room temperature and incubated with rabbit polyclonal antibodies against Bax (1:2000; Abcam, MA, USA), Bcl2 (1:2000; Abcam), and PARP1 (1:2000; Abcam) and mouse monoclonal antibodies against β-actin (1:8000; Cell Signaling Technology) and p53 (1:2000; Cell Signaling Technology) overnight at 4°C. The membranes were then washed in TBST three times and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000; Cell Signaling Technology) or horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000; Cell Signaling Technology) for 60 min at room temperature. The membranes were washed in TBST three times again, and the bands were detected by enhanced chemiluminescence. The grey values were quantified using ImageJ software.

**Immunohistochemistry**
On the last day at the end of week 4, the L4 and L5 DRG were removed from mice for immunohistochemical analysis. The DRG were fixed in 4% paraformaldehyde overnight and dehydrated with 30% sucrose solution. The DRG were cut in 10-μm thickness and mounted on glass slides. After treating with 0.5% TritonX-100 in PBS for 20 min and washing with PBS three times quickly, the sections were blocked in 5% normal goat serum and 0.5% Tween-20 in PBS for 1 h at room temperature. The sections were washed with PBS three times again and incubated in primary antibodies containing p53 (1:1000; Cell Signaling Technology) overnight at 4°C. After washing with PBS three times, the sections were incubated in FITC-conjugated secondary antibodies (1:100) for 1 h in the dark at room temperature. Then, the sections were washed three times and stained with DAPI. The immunostained DRG were viewed under an inverted fluorescence microscope.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from DRGs using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA was used to synthesize cDNA with ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO CO. LTD. Life Science Department, Japan). Real-time PCR was conducted with an Applied Biosystems 7500 RealTime PCR System (Life Technologies, USA) using TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China). GAPDH was used as an internal reference, and primers were synthesized by Shanghai Sangon Biotech. The primer sequences were as follows: GAPDH, (forward) 5’-GGT TGT CTC CTG CGA CTT CA-3’, (reverse)
5’-TGG TCC AGG GTT TCT TAC TCC-3’; p53, (forward) 5’-CGC CGA CCT ATC CTT ACC AT-3’, (reverse) 5’-TCT TCT GTA CGG TCT CTC-3’; and PARP1, (forward) 5’-CAG TGC CAG TCA GCT CAA GG-3’; (reverse) 5’-TGT TCC ATC CAC CTC GTC AC-3’. In all cases, the effectiveness of amplification was determined by the presence of a single peak in the melting temperature analysis and linear amplification throughout the PCR cycles. The $2^\Delta \Delta C_T$ relative quantification method was used to calculate the relative mRNA impression of the target genes.

**RNA Interference**

The transfection of p53 siRNA into DRG cells was performed according to standard protocols. Cells were cultured in plates with Neurobasal medium for 24 h. The next day, the cells were incubated with 20 μM p53 siRNA and transfection reagent in serum-free Neurobasal medium for 4-6 h, and then, Neurobasal medium containing 2% FBS was added to each well for 24 h. Then, the cells were cultured with PTX and/or duloxetine. Furthermore, the cells were also transfected with a fluorescein-labelled control siRNA to detect the transfection efficiency.

**Statistical analysis**

All experimental data were analysed using GraphPad Prism 5. The differences between groups were analysed by two-way ANOVA followed by Bonferroni post-tests. $P < 0.05$ was considered significant.
Results

Duloxetine Prevented PTX-Induced Pain Hypersensitivity in the Mice

The mice were injected with duloxetine daily, and pain behaviour was assessed on the first day of every week. At baseline, there was no significant difference in status between the six groups of mice. Pain behaviour tests showed that, compared with the vehicle-controlled mice, the PTX-treatment group showed significant decreases in the paw withdrawal latency from the second week to the 4th week in the mechanical hyperalgesia test and from the first week to the 4th week in the thermal nociception test (Fig. 1A and B). In the PTX-treated group, the paw withdrawal threshold rapidly decreased from 6.02±0.65 g before PTX to 4.25±0.69 g at week 2 (P <0.001 vs. vehicle control, n=8-10 in each group) and persisted until week 4 in the mechanical hyperalgesia test. Similarly, in the model group, the paw withdrawal latency time was significantly decreased from 14.60±1.28 s to 12.75±1.04 s at week 1 (P <0.001 vs. vehicle control, n=8-10) and to 8.80±1.66 s at week 4 in thermal nociception tests. On the other hand, the paw withdrawal threshold was increased in the mice treated with duloxetine before PTX compared with the PTX-treated group for mechanical hypersensitivity (Fig. 1A), and the mice pretreated with duloxetine, whether at the high, middle or low dose, all had higher reaction latency times compared with the model group for heat sensitivity (Fig. 1B). In contrast, the mice treated with PTX, with or without duloxetine, showed no difference in the tail withdrawal latency times compared with vehicle-treated group in the cold allodynia...
test (Fig. 1C), which indicated that PTX did not cause cold hypersensitivity in the mice.

**Duloxetine Did Not Change PTX-Induced Body Weight**

To examine whether duloxetine affected body weight in the model of PTX-induced peripheral pain, body weight was assessed in mice with or without duloxetine after PTX treatment. The assessment revealed that, compared with the vehicle control mice, there was no significant effect on body weight after PTX treatment, and the administration of duloxetine did not induce any alteration or enhancement on the physiological body weight compared with the model group (Fig. 1D). Body weights were measured weekly starting from the first injection of duloxetine through the end of the experiment.

**Duloxetine Had No Effect on the Antitumour Activity of PA-1 cells to PTX**

Then, we measured PA-1 cell viability to evaluate the impact of duloxetine on the antitumour activity of PTX. The cells were exposed to different concentrations of PTX and co-treated with different doses of duloxetine with the best concentration of PTX. As shown in Fig. 2A, each concentration of PTX had a strong ability to kill cancer cells. At or above 1 nM concentration, PTX caused more than 50% of PA-1 cells death; cell viability was only 30% at 300 nM, and co-treatment with different concentrations of duloxetine with 300 nM PTX did not decrease the ability of PTX to
Duloxetine Suppressed PTX-Induced Oxidative Stress in DRG Neurons

MitoTracker Red CM-H2XRos, a reduced red fluorescent dye, does not fluoresce itself but is oxidized to fluoresce when it enters cells; thereby, it can be used to detect oxidative stress levels in DRG cells. Hoechst is a nuclear marker that stains the nucleus blue. Compared with the control group, the fluorescence intensity of CM-H2XRos was significantly enhanced after PTX treatment, and the fluorescence intensity was weakened after the intervention of duloxetine, as shown in Fig. 2C. Quantitative histograms showed a significant difference between the model group and the control group, and there was a significant difference between the duloxetine-administered group and the model group. The fluorescence intensity of the duloxetine-alone group was not significantly different from that of the blank control group. It can be noted that PTX causes oxidative stress in DRG cells, leading to peripheral nerve injury, and duloxetine can significantly reduce the level of oxidative stress induced by PTX.

Duloxetine Alleviated PTX-Induced p53 and PARP1 Levels

To investigate the effects of cell apoptotic signalling pathway in PIPN, we examined protein levels in DRG tissues and the sciatic nerve. Western blotting suggested that the expression of p53 and cleaved-PARP1 was increased after PTX
treatment in DRG tissues (Fig. 3A) and the sciatic nerve (Fig. 3B). In addition, at a
dose of 30 mg/kg, 20 mg/kg or 10 mg/kg, duloxetine decreased PTX-induced p53 and
cleaved-PARP1 expression in DRG tissues (Fig. 3A) and the sciatic nerve (Fig. 3B).
The changes of expression were similar to that in primary rat DRG neurons
(Supplementary Fig. S1). We next investigated p53 expression in the DRG in vivo and
in vitro using immunostaining (Fig. 4C, Supplementary Fig. S2). The results showed
that duloxetine attenuated PTX-induced immunoreactivity compared with the vehicle
control group, and the p53 protein expression levels were the same as noted with
Western blotting. The immunoreactive DRGs co-localized with DAPI. To further
characterize p53 and PARP1 in neuropathic DRG tissues and the sciatic nerve, the
mRNA expression levels of p53 and PARP1 were determined by real-time PCR.
Compared with the control group, the data revealed that p53 and PARP1 mRNA
expression was increased in PTX-treated mice. In addition, duloxetine attenuated the
PTX-induced increases in p53 and PARP1 mRNA expression levels in the DRG
tissues (Fig. 3D) and sciatic nerve (Fig. 3E). The changes of mRNA expression were
similar to that in primary rat DRG neurons (Supplementary Fig. S3).

Duloxetine Blocked PTX-Induced Bcl2 Family in Neuropathic Mouse
DRG Tissues and the Sciatic Nerve

The Bcl2 family of proteins plays a central role in the apoptosis process. The
Bcl2 family includes Bcl2, Bax, Bad, and Bcl-xL (Zheng et al., 2016; Rahman et al.,
2015). Bax and Bad proteins promote apoptosis, while Bcl2 and Bcl-xL proteins
inhibit apoptosis. In fact, the ratio of Bax to Bcl2 determines the sensitivity to cell death (Amin et al., 2014). Therefore, we employed western blotting to determine whether the Bcl-2 family was involved in cell apoptosis caused by PTX. PTX treatment increased Bax expression and markedly decreased the expression of the pro-apoptotic protein Bcl2 in DRG tissues and the sciatic nerve, as shown in Fig. 4A and B. However, pretreatment with duloxetine effectively balanced this change; these data reveal that Bcl2 family is involved in PTX-induced neuropathic pain, and duloxetine can attenuate their upregulation in DRGs. Then, we examined gene levels in the DRG and the sciatic nerve. The results suggested that duloxetine markedly regulated the mRNA expression of Bax/Bcl2 in the DRG and the sciatic nerve (Fig. 4C, D), indicating that duloxetine has remarkable neuroprotective effects through apoptosis resistance.

Effect of p53 Inhibitor on Neurotoxicity and Cell Apoptosis Induced by PTX in Primary DRG Neurons

One of the most significant functions of p53 is inducing cell apoptosis. We have demonstrated that PIPN can be reduced by inhibiting p53. Pifithrin-a (pft-a), one of the most commonly used inhibitors of p53, has been reported to have efficacy in stroke models, seizures, and PD models (Duan et al., 2002; Culmsee et al., 2003; Engel et al., 2010). Hence, we used the CCK-8 assay to evaluate pft-a-induced neuroprotection. As shown in Fig. 5A, 300 nM PTX caused approximately 40% neurotoxicity in DRG cells, and varying concentrations of pft-a (4.63-37 μM)
protected against PTX-induced neurotoxicity. Moreover, 18.5 μM pft-a could better alleviate the neurotoxicity caused by PTX. To examine whether pft-a had effect on the antitumour activity of PTX, we identified that 300 nM PTX killed at least 50% of PA-1 cancer cells, achieving a half-lethal dose, while pretreatment of pft-a with various concentrations enhanced the capacity of PTX to kill PA-1 cancer cells (Fig. 5B). To determine whether inhibiting p53 could protect peripheral nerves by decreasing PTX-induced cell apoptosis, a flow cytometric analysis experiment was performed. We discovered that PTX treatment in DRG cells caused apoptosis after 24 h, and the proportion of apoptotic cells increased significantly (30.57%); however, apoptosis decreased markedly after treatment of 18.5 μM pft-a (21.41%), indicating that pft-α affects PTX-induced apoptosis, as show in Fig. 5C.

Duloxetine Attenuated PTX-Induced Cell Apoptosis via Inhibiting p53

Apoptosis plays a significant role in maintaining various cellular functions (Sharafi and Rahimi, 2012; Hassan et al., 2014). P53 is a cell cycle checkpoint protein which is in response to DNA damage via mediating cell cycle stagnation or apoptosis, thereby contributing to the maintenance of genetic stability (Renaud et al., 2014; Guan et al., 2008). Because of the importance of p53 in the cell cycle, we investigated the effects of p53 siRNA on cell viability and Bcl2 family expression. As expected, when siRNA was used to reduce the level of p53, p53 siRNA strongly attenuated the PTX-induced decrease in cell viability, and cells were resistant to PTX toxicity (Fig.
6A, B). On the side, duloxetine provided the same neuroprotection. This result indicated that lowering p53 levels may be the main mechanism by which duloxetine participates in neuroprotection. On the other hand, Western blotting showed that p53 siRNA treatment decreased cell apoptosis by decreasing the expression of Bax/Bcl2 (Fig. 6C). Activation of p53 during DNA damage has been reported to activate downstream signalling to regulate apoptosis in cells, like the Bcl2 family. Taken together, the above experimental results obviously demonstrated that duloxetine suppressed PTX-induced cytotoxicity and apoptosis by inhibiting p53 and the downstream Bcl2 family.
Discussion

CIPN is the main dose-limiting toxicity caused by various chemotherapeutic agents. PTX is a chemotherapeutic drug, and it has been widely used in various solid tumours. However, peripheral neuropathic pain caused by PTX leads to loss of body function, a significant decline in quality of life, and even a delay in treatment. The peripheral neurotoxicity caused by PTX is rarely disabling, but it often lasts for several months to years after stopping the drug, causing inconvenience and impacting the patient’s quality of life (Kiya et al., 2011). PTX-induced peripheral neurotoxicity usually occurs within 24-72 hours of administration, which is dose-accumulating with an incidence of 59%-78% (Wilkes, 2007).

With the proposal of the mechanism of PIPN, some prevention and treatment methods have emerged accordingly. Although there are many clinical trials on the treatment of PIPN, there is no unified standard in clinical treatment. To date, there have been 42 randomized controlled clinical trials of drug prophylaxis against CIPN, but none of these trials has provided any convincing evidence, and the specific mechanism is unclear. The ASCO guidelines note that duloxetine is the only drug available to treat CIPN. There is a lack of high-quality, consistent data to support the use of drugs other than duloxetine. Moreover, duloxetine is considered to be related to the regulation of neuropathic pain.

Based on a previous report, we used DRG neurons herein extracted from primary rats (Meng et al., 2019). Our results showed that PTX caused an increase in mitochondrial oxidative stress levels in the DRG. The intervention of duloxetine
inhibits peroxidation and protects cells from nerve damage, partially alleviating CIPN. Many experimental and basic research studies conducted by scholars on PIPN have found that PIPN is in connection with structural damage of mitochondria and peripheral nerves in the DRG (Zheng et al., 2011). Damage to intracellular mitochondria causes oxidative stress, inflammation, and pro-apoptotic signalling (Mecocci et al., 1994). Oxidative stress is an important and common way to prevent or reverse CIPN. When cells have weak antioxidant defence ability, they are particularly susceptible to oxidative damage induced by chemotherapy (Low et al., 1997). Duggett N A et al. found that during the early development of neuropathic pain caused by PTX, ROS levels increased in tissue, producing oxidative stress (Duggett et al., 2016).

Neuropathic pain is evoked by damage or disturbances that affect the somatosensory system, involving the activation of the pain and analgesia pathway (Lisi et al., 2015; Jensen et al., 2011). Duloxetine is an important drug. The behavioural evaluation of mice indicated that duloxetine can effectively improve PTX-induced thermal pain and mechanical allodynia in mice for the treatment of abnormal reactions to to innocuous stimuli (hyperpathic pain) or noxious stimuli (hyperlgesia) (Bellingham and Peng, 2010). In our experiments, we had access to a mouse PIPN model with reference to the modelling method (Mellie et al., 2006; Zhu et al., 2013; Krukowski et al., 2015). The results showed that PTX injected intraperitoneally every other day caused hyperalgesia and thermal hyperalgesia in mice, but not sensitivity to cold pain stimulation. PTX also had little effect on body
weight. It was found that duloxetine can effectively improve PTX-induced thermal and mechanical allodynia in mice. It has been reported in previous studies that chemotherapy-induced neuropathic pain is related to enhancive oxidative stress (Di et al., 2012). Inhibition of ROS accumulation is a significant mechanism to prevent nerve cell damage. Our study suggested that duloxetine inhibited oxidative stress, and pretreatment of duloxetine prevented pain hypersensitivity, indicating that duloxetine improves neuropathic pain through reducing oxidative stress.

Apoptosis is a cascade of activating cell death after stimulation by varieties of death-inducing signaling complex and plays a significant role in the maintenance of multiplex cellular functions. P53 and PARP1 are involved in cellular regulation as apoptosis-related proteins. The present researches showed that the nuclear translocation of p53 is increased when tumour cells are treated with microtubule-targeted drugs, including PTX (Carney et al., 2012; Giannakakou et al., 2000). It has been reported that Bcl2 was down-regulated, while p53 and Bax were up-regulated, when cells were treated with PTX, suggesting that apoptosis initiates through a p53-dependent pathway (Thomas et al., 2015). PARP is a DNA-sensitive enzyme which can identify single-stranded DNA strand breaks and initiate repair through the BER pathway. Besides, it has been shown that activation of PARP is able to evoke a variety of apoptotic signalling events (Schreiber et al., 2006). Our experimental results proved that PTX-induced apoptosis was connected with p53 and an imbalance in the expression of the Bcl-2 family members, nevertheless pretreatment with duloxetine significantly inhibited the upregulation of p53 and Bax.
and the downregulation of Bcl2. In addition, cleaved-PARP can lead to apoptosis. The quantitative expression of PARP1 in protein and gene levels further confirmed the function of PARP1 in PTX-induced apoptosis. However, as indicated by PARP cleavage, pretreatment with duloxetine markedly blocked the process of apoptosis. Additionally, pft-α provided a neuroprotective effect, which revealed that inhibiting p53 can reduce PTX-induced apoptosis and achieve neuroprotection. This finding also provides a practical basis for the use of pft-α to improve CIPN. Moreover, our further studies revealed that p53 siRNA had a neuroprotective effect on cell neurons and downregulated the expression levels of Bax and Bcl2, thereby partially inhibiting cytotoxicity and cell apoptosis induced by PTX. These above results demonstrated that duloxetine has the possibility to improve PIPN through inhibiting cell apoptosis mediated by p53 through its downstream signals, the Bcl2 family and PARP1.

In summary, our study suggested that duloxetine exerts an anti-hyperalgesic effect on mechanical and heat hyperalgesia in mice and alleviates PTX-induced cytotoxicity and apoptosis by suppressing oxidative stress and activating p53 and the Bcl2 family and PARP1. Therefore, duloxetine has potential as a new therapeutic agent targeting p53-related apoptotic pathways to ameliorate PTX-induced peripheral neuropathic pain and nerve injury and decrease adverse reaction of antitumour drugs.
Authorship Contribution

Participated in research design: Y Lu, P Zhang, Q Zhang, C Yang, and J Zhu.

Conducted experiments: Y Lu, P Zhang, Q Zhang, Y Qian, J Suo, and X Tao.

Contributed new reagents or analytic tools: Q Zhang, and J Zhu.

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Footnotes

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Figure legends

Figure 1. Duloxetine alleviates PTX-induced hypersensitivity in C57BL/6 mice without affecting their body weight.

(A) Paw withdrawal threshold was significantly decreased at week 2 and maintained for more than 3 weeks in mice treated with PTX. Duloxetine at doses of 10, 20 and 30 mg/kg all attenuated PTX-induced mechanical hyperalgesia. (B) Paw withdrawal threshold was significantly decreased at week 1 and maintained for more than 4 weeks in mice treated with PTX. Duloxetine at doses of 10, 20 and 30 mg/kg all attenuated PTX-induced thermal nociception. (C) PTX did not have a significant effect on cold tail withdrawal latency. Similarly, no effect was observed with duloxetine on mice treated with or without PTX. (D) The effect of duloxetine on body weight in mice treated with or without PTX. No significant difference between the model group and the duloxetine-treated group in mice. (** P < 0.01, *** P < 0.001 vs. Control; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. PTX, n=8-10).

Figure 2. Duloxetine has no effect on the antitumour activity of PTX in DRG neurons, but it can alleviate the oxidative stress induced by PTX.

(A) When PA-1 cancer cells were grown for 24 h, PTX showed toxicity and reduced their cell viability by 40-80%. (B) Various concentrations of duloxetine (10 μM – 300 nM) together with PTX (300 nM) showed no significant changes in cell viability compared with treatment with PTX alone. (C) Primary rat DRG neurons were grown for 24 h and then treated with PTX (300 nM) and/or duloxetine for another 24 h.
Cells were then subjected to MitoTracker Red CM-H2XRos staining. The fluorescence intensity was observed using fluorescence microscopy (200×). (** vs. Control, P < 0.001; ### vs. PTX, P < 0.001, α =0.05, n=6).

Figure 3. Effect of duloxetine on p53 and PARP1 in DRG tissues and sciatic nerve.
(A) Duloxetine significantly decreased the expression of p53 and actived-PARP1 protein in the DRG of PTX-treated mice. (B) Duloxetine significantly decreased the expression of p53 and acitved-PARP1 protein in the sciatic nerve of PTX-treated mice. (C) The DRGs were double-stained with DAPI and p53. The fluorescence intensity was observed using fluorescence microscopy (400×). (D) Duloxetine significantly decreased the mRNA expression of p53 and PARP1 in the DRG of PTX-treated mice. (E) Duloxetine significantly decreased the mRNA expression of p53 and PARP1 in the sciatic nerve of PTX-treated mice. (* P < 0.05, ** P < 0.01, *** P < 0.001 vs. Control; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. PTX, n=5).

Figure 4. Effect of duloxetine on Bax and Bcl2 in DRG tissues and the sciatic nerve.
(A) Duloxetine significantly decreased the expression of Bax/Bcl2 protein in the DRG of PTX-treated mice. (B) Duloxetine significantly decreased the expression of Bax/Bcl2 protein in the sciatic nerve of PTX-treated mice. (C) Duloxetine significantly decreased the mRNA expression of Bax/Bcl2 in the DRG of PTX-treated mice. (D) Duloxetine significantly decreased the mRNA expression of
Bax/Bcl2 in the sciatic nerve of PTX-treated mice. (** P < 0.01, ### P < 0.001 vs. Control; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. PTX, n=5).

Figure 5. Effect of pft-a on neurotoxicity and cell apoptosis caused by PTX in primary DRG neurons.

(A) Primary rat DRG neurons were grown for 24 h and then treated with PTX (300 nM) and/or various concentrations of pft-a for another 24 h. (B) When PA-1 cancer cells were grown for 24 h, various concentrations of pft-a together with PTX (300 nM) showed slight changes in cell viability compared with treatments with PTX alone. (C) After DRG cells were cultured for 24 h, they were then exposed to PTX (300 nM) with or without pft-a (18.5 μM) for another 24 h. Cells were double-stained with annexin V-FITC/PI. (** P < 0.001 vs. Control; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. PTX, n=6).

Figure 6. Effect of duloxetine on PTX-induced cell apoptosis via p53.

(A) After treatment with p53 siRNA, protein expression was examined via Western blotting methods. The expression of p53 in the Blank group was the same as that in the NC siRNA group, while p53 expression was significantly decreased in p53 siRNA group. (** vs. Blank, P < 0.001, α=0.05, n=4). (B) After treatment with p53 siRNA, cell viability was assessed (** vs. Control, P < 0.001; # vs. PTX, P < 0.05, ### vs. PTX, P < 0.001, α=0.05, n=4). (C) Treatment with p53 siRNA and duloxetine
markedly balanced the protein expression of Bax/Bcl2 in PTX-treated DRG cells.

(*** vs. PTX, P < 0.001, α=0.05, n=4).
Figure 1.
Figure 2.

A: Paclitaxel toxicity in PA-1

B: PA-1

C: Hoechst

CM-H2Ros

Merge

Cell viability

Control  PTX  P+D  D
Figure 3.
Figure 4.

A

B

C

D

Figure 4.
Figure 5.
Figure 6.