Peptides 104 (2018) 24–34

Contents lists available at ScienceDirect

Peptides

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

A synthetic snake-venom-based tripeptide (Glu-Val-Trp) protects PC12 cells from MPP^+ toxicity by activating the NGF-signaling pathway

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ARTICLE INFO

Keywords:
Snake-venom peptides
Neuroprotection
Neurotrophin receptors trkA (tropomyosin receptor kinase A)
MPP^+ (1-methyl-4-phenylpyridinium)
PC12 cells

ABSTRACT

Venom small peptides that target neurotrophin receptors might be beneficial in neurodegeneration, including Parkinsońs disease (PD). Their small size, ease of synthesis, structural stability and target selectivity make them important tools to overcome the limitations of endogenous neurotrophins as therapeutic agents. Additionally, they might be optimized to improve resistance to enzymatic degradation, bioavailability, potency and, mainly, lipophilicity, important to cross the blood brain barrier (BBB). Here, we evaluated the neuroprotective effects and mechanisms of the synthetic snake-venom-based peptide p-BTX-I (Glu-Val-Trp) in PC12 cells treated with MPP^+ (1-methyl-4-phenylpyridinium), a dopaminergic neurotoxin that induces Parkinsonism *in vivo*. The peptide p-BTX-I induced neuritogenesis, which was reduced by (i) k252a, antagonist of the NGF-selective receptor, trkA (tropomyosin receptor kinase A); (ii) LY294002, inhibitor of the PI3 K/AKT pathway and (iii) U0126, inhibitor of the MAPK-ERK pathway. Besides that, p-BTX-I also increased the expression of GAP-43 and synapsin, which are molecular markers of axonal growth and synaptic communication. In addition, the peptide increased the viability and differentiation of cells exposed to MPP^+, known to inhibit neuritogenesis. Altogether, our findings suggest that the synthetic peptide p-BTX-I protects PC12 cells from MPP^+ toxicity by a mechanism that mimics the neurotrophic action of NGF. Therefore, the molecular structure of p-BTX-I might be relevant in the development of drugs aimed at restoring the axonal connectivity in neurodegenerative processes.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease in elderly, preceded only by Alzheimeŕs disease [1]. It is a progressive chronic neurodegenerative disease, characterized by loss of brain function with resulting motor deficits, tremors, and postural instability [2,3]. Despite extensive research, the cause of neuronal loss in various neurodegenerative diseases is not entirely understood [4]. Recent evidence shows that axonal degeneration and loss of connectivity in the nigrostriatal dopaminergic pathway are early and predominant events in PD [5,6]. Therefore, neuroprotective and/or neurorestorative interventions might counteract the dopaminergic degeneration that starts at axons during the early asymptomatic stages of PD, and might also restore de connectivity already lost by the time of symptoms onset. Neurotrophins and molecules with neurotrophic activity might be able to restore the axonal connectivity, minimizing the symptoms and slowing the progression of the disease. The therapeutic application of natural neurotrophins, such as NGF and BDNF (brain-derived neurotrophic factor), is limited by poor oral bioavailability, uncertain pharmacokinetics and inability to cross the blood brain barrier (BBB). Besides that, their large-scale production is difficult and expensive. Small organic molecules that mimic the neurotrophic action of neurotrophins can be modified to easily cross the BBB and therefore, reach the brain parenchyma after oral administration [7,8]. Therefore, the development of small molecules with favorable pharmacokinetics and that target specific neurotrophin receptors, such as tropomyosin-related kinase (trk) receptors, is of great interest in the treatment of neurodegenerative diseases [7,9–11]. In this scenario, snake venom peptides might be useful since they play important roles in signaling processes and can be obtained in large amounts by chemical

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https://doi.org/10.1016/j.peptides.2018.04.012
Received 24 January 2018; Received in revised form 12 April 2018; Accepted 16 April 2018
Available online 20 April 2018
0196-9781/

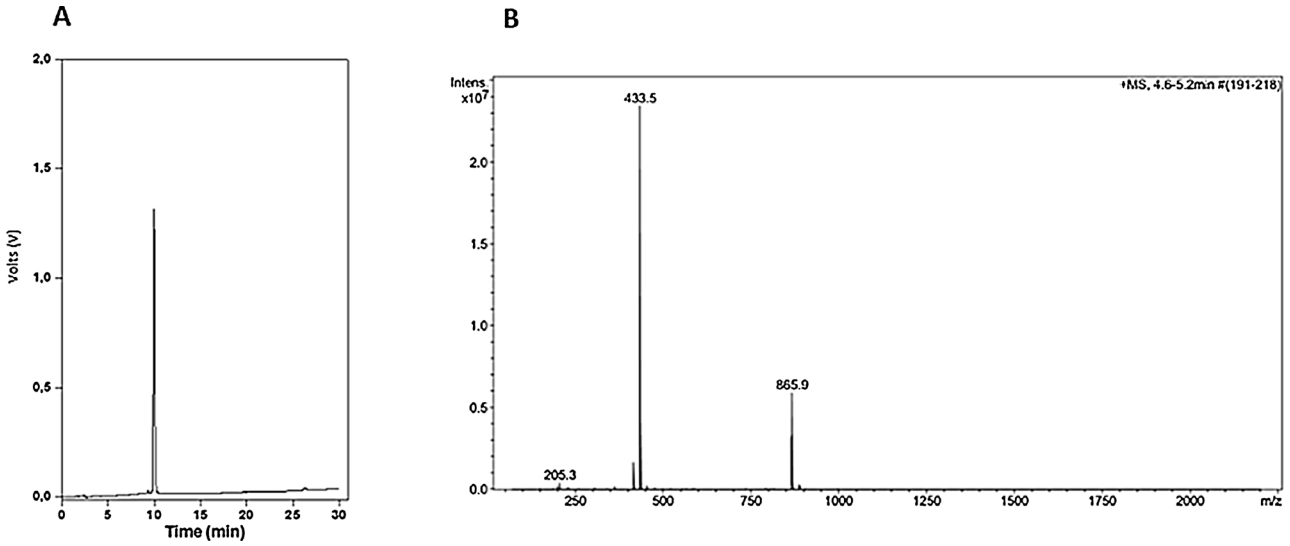


Fig. 1. (A) HPLC profile and (B) electrospray ionization mass spectrometry of the synthetic peptide p-BTX-I.

synthesis. Snake venoms are cocktails of proteins and peptides with diverse biological activities [12]. These biomolecules act disturbing fundamental processes in the envenomed victim, leading to morbidity and even to death. However, they might also trigger signaling pathways that lead to beneficial effects in specific diseases. Over the years, significant effort has been made to identify new molecules from snake venoms and convert them into therapeutic tools [13–18]. Since the development of Captopril, the first drug derived from a peptide of snake venom, many other toxins have been described as biologically active and potential models for the development of new drugs [12,19].

Our research group has been working with snake venoms in order to identify small peptides that might have beneficial effects on cellular models related to Parkinsońs disease and, therefore, be used as models for drug design. We have previously reported the neuroprotective and neurotrophic effects of a tripeptide from *Bothrops atrox* venom in PC12 cells exposed to the dopaminergic neurotoxin MPP+, known to cause Parkinsonism *in vivo* [20]. We have now synthesized a tripeptide (p-BTX-I) based on that natural peptide in order to (i) evaluate its neurotrophic and neuroprotective potential in the same cellular model, and (ii) to investigate the involvement of the NGF-signaling pathway in the mechanism of action of this synthetic peptide.

2. Materials and methods

2.1. Reagents

MPP^+ (1-Methyl-4-phenylpyridinium iodide) (D048), LY-294.002 hydrochloride (L9908), MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (M2128), K252a (K2015), U0126 monoethanolate, HEPES (H3375), Sodium Bicarbonate (S5761), Dulbecco's Modified Eagle Medium (DMEM) (D5648), Ham's F12K medium, Trypsin (59427C), NGF (Nerve Growth Factor from *Vipera lebetina* venom − N8133), Collagen Type-IV (C5533) and bovine serum were purchased from Sigma-Aldrich (MO, USA). Equine serum and antibiotic mix (PSN, 5 mg/mL penicillin, 5 mg/mL streptomycin and 10 mg/mL neomycin) were purchased from GIBCO® (Life Technologies Corporation, USA). All reagents were of analytical grade.

2.2. Synthesis of the peptide p-BTX-I

The synthetic peptide was based on the natural tripeptide formed by Glu-Val-Trp, which was isolated from *Bothrops atrox* venom as we have previously described [20]. Solid phase peptide synthesis (SPPS) was performed manually based on a method previously described [17], using Fmoc/tBu (9-fluorenylmethyloxycarbonyl) protocols on the Fmoc-Trp(Boc)-Wang resin to obtain the carboxylic C-terminus. The side chain protecting group tBut (t-Butyl) were used for Glu. Ninhydrin test was used to assess coupling and deprotection [18]. The α-amino group deprotection steps were performed in 20% methyl-piperidine/dimethylformamide (DMF) for 1 and 20 min. Cleavage from the resin and removal of the side chain protecting groups were performed with a solution of 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane (TIS) for 2 h. After synthesis, the peptide was cleaved and precipitation was performed using cold diethyl ether. However, due to its size or hydrophobicity, the peptide was not precipitated. Then, the cleavage solution containing the peptide was evaporated in a rotary evaporator. Crude peptides were purified by semi-preparative HPLC (Shimatzu system, Japan) using a reverse-phase C18 column. The peptide p-BTX-I was obtained with a high level of purity (> 95%).

2.3. Electrospray mass spectrometry

In order to identify the peptide, diluted samples were introduced by a syringe pump (2.5–10 μL/min) into amazon ion trap mass spectrometer (Bruker Daltonics) using electrospray positive ionization (ESI). Liquid chromatograph with mass spectrometer (LC–MS) system was also used in order to identify the correspondent peptide peak after rotary evaporation.

2.4. Cell culture

The PC12 cell line from rat pheochromocytoma was obtained from the American Type Culture Collection (Manassas, VA, USA). PC12 cells were cultured and maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% equine serum, 5% fetal bovine serum and 1% antibiotic mixture (PSN, GIBCO™). PC12 cells were cultured in 75 cm^2 tissue culture flasks at 37 °C under a humidified atmosphere containing 5% CO_2 and 95% air. The growth medium was renewed every 2–3 days. For harvesting, trypsin was used to remove adherent cells, and equal volume of growth medium was used to stop the enzymatic reaction of trypsin.

2.5. Determination of neurite outgrowth

For the differentiation assay, PC-12 cells were plated in collagen IV-coated 24- well plates (2×10^5 cells/well). After 24 h for adhesion, the culture medium was replaced by the differentiation medium (GIBCO F–12 K) Nutrient Mixture Kaighn's Modification supplemented with 1%

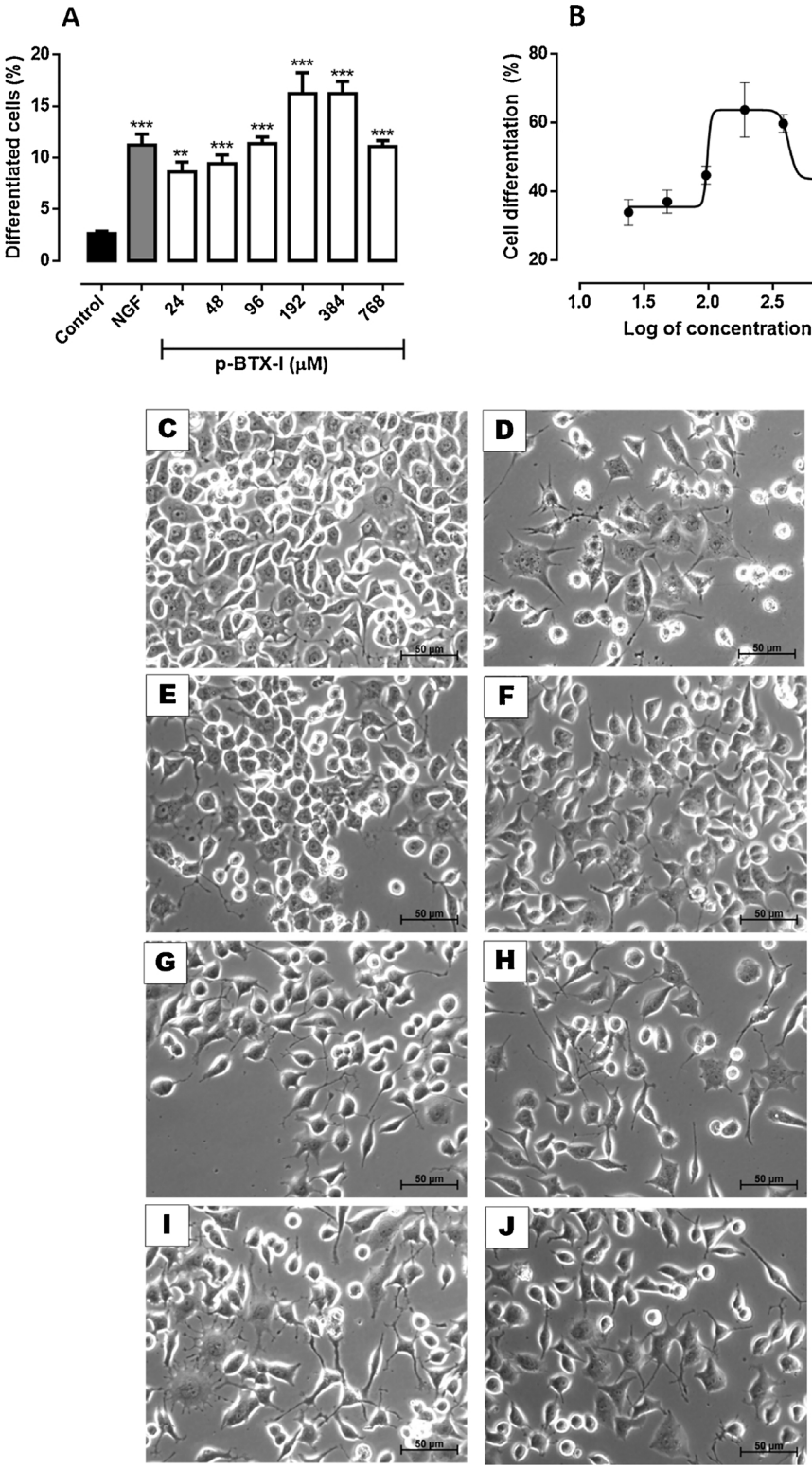


Fig. 2. Effects of p-BTX-I on the differentiation of PC12 cells.

Cells were incubated for 72 h with/without NGF (100 ng/mL) or p-BTX-I (24–768 μM). (A) Bar graph represents the mean ± SEM of 3 different experiments performed in triplicate, (B) Concentration-response curve showing the bell-shaped profile of the effect of different concentrations of p-BTX-I on neurite outgrowth, and (C–I) Inverted Phase-contrast photomicrographs of (C) Control (untreated), (D) NGF (100 ng/mL), (E) p-BTX-I (24 μM), (F) p-BTX-I (48 μM), (G) p-BTX-I (96 μM), (H) p-BTX-I (192 μM), (I) p-BTX-I (384 μM) and (J) p-BTX-I (768 μM). Cells with at least one neurite with a length equal to/greater than the cell body were counted and expressed as a percentage of total cells in the field (n = 3). *Significantly different from controls, *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$).

equine serum and 1% antibiotic mixture (PSN, GIBCO™). Then, cells were incubated at 37 °C for 72 h with one of the following additions: p-BTX-I (24–768 μM), NGF (100 ng/mL), NGF (100 ng/mL) + MPP^+ (100 μM) or *p*-BTX-I (192 μM) + NGF (100 ng/mL) + MPP^+ (100 μM). Untreated cells were used as negative controls. Neurite growth was assessed by inverted-phase contrast microscopy (Carl Zeiss Axio Observer A1 Inverted Microscope, magnification of 400 x). Phase contrast photomicrographs of four fields per well were taken after 24, 48 and 72 h of treatment. The percentage of cells with neurites and the length of neurites were determined on digitalized images by using the open source software Image J [21]. Only the cells with at least one neurite with a length equal to or greater than the diameter of the cell body were considered differentiated cells [22].

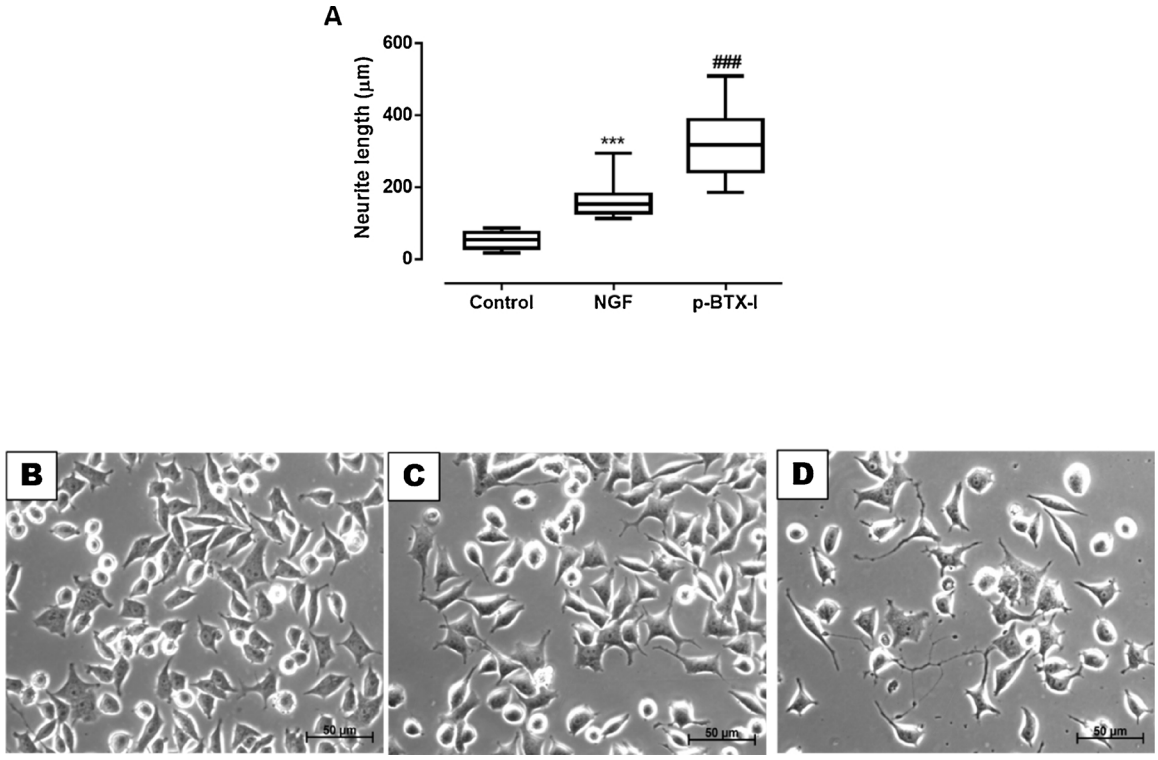


Fig. 3. Effects of p-BTX-I on the length of neurites. (A) Bar graph; (B-D) Photomicrographs of (B) untreated control, (C) NGF and (D) p-BTX-I groups. Additions: NGF, 100 ng/mL; and p-BTX-I (192 µM). Results were expressed as mean ± SEM of 3 different experiments (n = 3) performed in triplicate. ***Significantly different from control ($p < 0.001$). $^{###}$Significantly different from NGF ($p < 0.001$).

2.6. Cell viability

Cells (2×10^4 cells/well, 200 µL final volume) were plated in 96-well plates. After 24 h of incubation, cells were treated with the peptide alone (192 µM), peptide (192 µM) plus MPP^+ (1 mM) or MPP^+ alone (1 mM). After 72 h, cells were treated with 20 µL of MTT solution (5 mg/mL) and incubated again for 3 h at 37 °C. Then, plates were centrifuged at 1000 rpm for 5 min, the supernatant was removed and DMSO (200 µL) was added in each well to lyse cells and solubilize the formazan crystals. Untreated cells were used as negative controls and cells treated with Triton X-100 as positive controls. Plates were mixed (37 °C, 5 min) and the absorbance was determined at 570 nm in a microplate reader (Multiskan FC, Thermo Scientific). This procedure was based on Mosmann [23].

2.7. Inhibition of NGF-signaling pathway

Cells were pretreated for 1 h with one of the following inhibitors: K252a (100 nM), LY294002 (30 µM) or U0126 (10 µM). Then, cells were incubated with or without the additions (192 µM peptide or NGF 100 ng/mL) for 72 h. The determination of neurite outgrowth was performed as described above (Section 2.3) and analyzed as previously described [22].

2.8. Western blot analysis

PC12 cells (2.0×10^5 cells/well) were plated in 24-well plates coated with poly-L-lysine (Sigma-Aldrich®, St. Louis, MO, USA). After 72 h of incubation with the treatments, cells were detached with scraper, transferred to the microcentrifuge tubes and centrifuged (1000 rpm, 5 min, 4 °C). The supernatant was discarded and the cell pellet was suspended in 40 µL Tris-Triton lysis buffer (10 mM Tris, pH 7,4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, Triton X-100 1%, 10% glycerol, 0,1% SDS, 0,5% deoxycholate, 1:200 Protease Inhibitor Cocktail and 1% Phosphatase Inhibitor Cocktail). After 10 min, the cell lysate was centrifuged at 12,000 rpm for 10 min at 4 °C and the supernatant was separated and stored in freezer (−80 °C) until the assay. A 10 µL aliquot of cell lysate was assayed for protein determination by the Bradford method. Briefly, the Protein Assay Dye Reagent (Bio-Rad) was used according to the manufactureŕs instructions. Lysates and dye reagent were diluted with water (1:5) and a calibration curve of BSA (40, 100, 200 and 400 mg/mL) was used. The absorbance (595 nm) was determined in a microplate reader (Multiskan FC, Thermo Scientific) and the concentrations of protein were calculated based on the calibration curve response and multiplied by the dilution factor (x5). Prior to SDS-PAGE, samples were added to an equal volume of Laemmli sample buffer (65.8 mM Tris, pH 6.8, 26.3% glycerol, 2.1% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol) and heated at 98 °C for 5 min. Aliquots of 20 µL containing 10 µg of total protein were applied to 10% polyacrylamide gel (10 wells) and separated by SDS-PAGE (1 h, 160 V, Tris/glycine/SDS buffer). Then, proteins were transferred to nitrocellulose membranes (1 h, 0.37 A, Tris/glycine buffer). Prior to reaction with antibodies, membranes were blocked (30 min, RT, 300 rpm) with 5% non-fat milk or 5% BSA, both prepared in Tween 20/TBS buffer (TTBS). The membranes were incubated with the primary antibodies: anti-GAP-43 (1:1250) or anti-synapsin I (1:1000) overnight at 4 °C, 300 rpm. Then, the membranes were washed with TTBS and incubated (1 h, RT, 300 rpm) with the secondary antibody conjugated with horseradish peroxidase (anti-mouse or anti-rabbit IgG − HRP. 1:20,000). The membranes were washed with TTBS and TBS and treated with 3 mL of chemiluminescence enhancer detection reagent (ECL 1:1); images were captured by using ChemiDoc system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Quantitation was performed on the images based on the optical densitometry (OD) of the bands by using the open source software Image J software [21]. Finally, the membranes were stripped (2% SDS, 62.5 mM Tris pH 6.8 and 100 mM mercaptoethanol) and reprobed for loading control with anti-β-actin (1:3000). OD values of GAP − 43 and synapsin bands were divided by

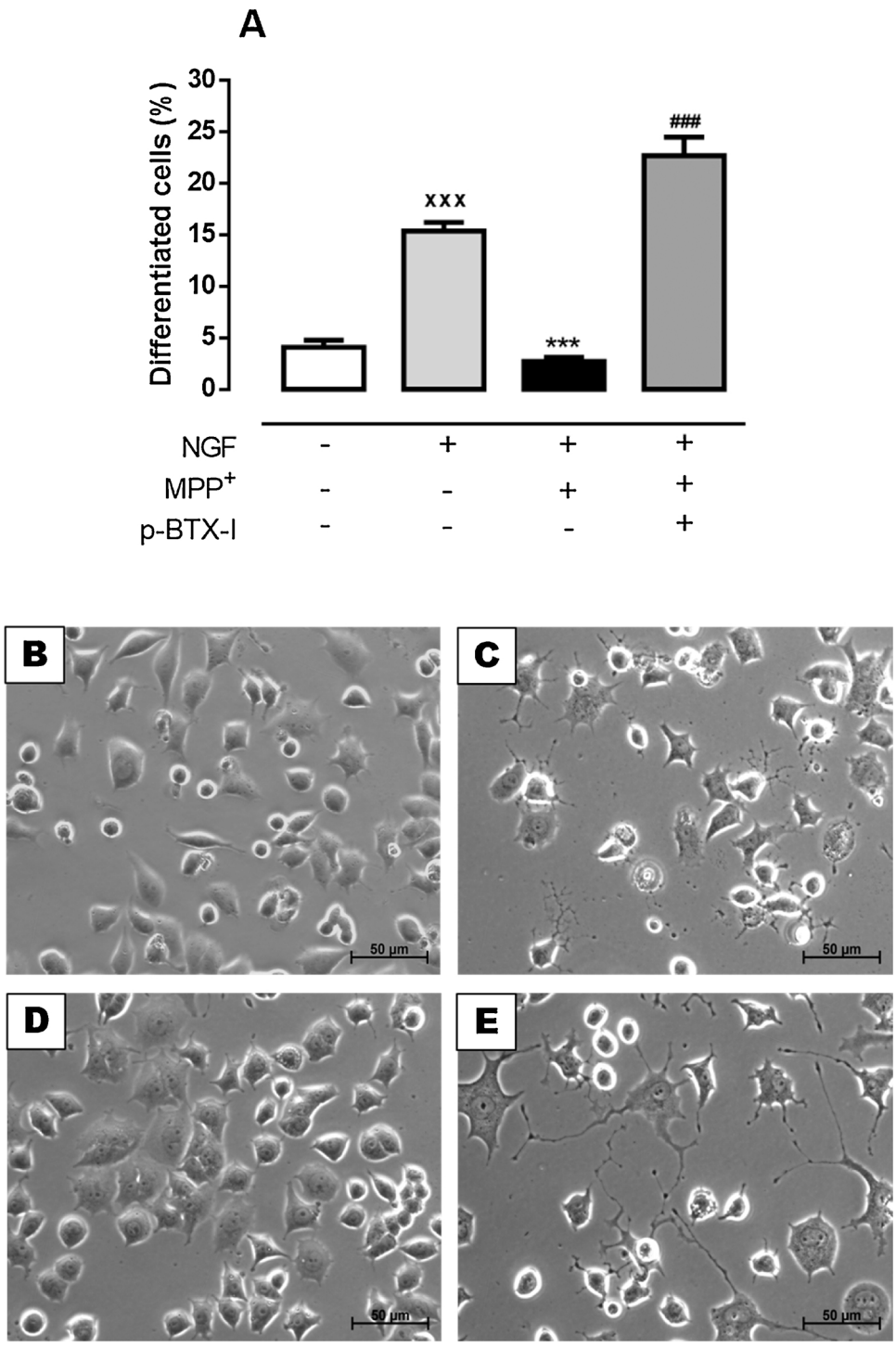


Fig. 4. Effects of p-BTX-I on the differentiation of PC12 cells treated with MPP^+.
(A) Bar graph; (B-E) Photomicrographs of (B) Control (no treatment), (C) NGF, (D) NGF + MPP^+, (E) NGF + MPP^+ + p-BTX-I. Cells with at least one neurite with a length equal to or greater than the cell body were considered as differentiated and expressed as a percentage of the total cells in the field. Additions: NGF, 100 ng/mL; MPP^+, 100 µM and p-BTX-I (192 µM). Results were expressed as mean ± SEM of 3 different experiments (n = 3) performed in triplicate. ^{xxx}Significantly different from control ($p < 0.001$). ***Significantly different from NGF ($p < 0.001$). $^{###}$ Significantly different from NGF + MPP^+ ($p < 0.001$).

the OD values of β – actin for normalization of the results [24].

2.9. Statistical analysis

Data are presented as means ± standard error of means (SEM). Differences among treatments were evaluated by using One-way ANOVA followed by Tukeýs multiple comparisons test (GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com"). Values of $p < 0.05$ were considered significant. Experiments were repeated three times using cells from different cultures (n = 3). The experiment of each day was performed in triplicate.

3. Results

3.1. Analysis of p-BTX-I

The HPLC chromatogram shows the peptide homogeneity, i.e., only one peak with retention time of 9.9 min (Fig. 1A). The molecular weight (432.5 g/mol) obtained in ESI mass spectrometry is on line with the theoretical value (Fig. 1B).

3.2. p-BTX-I induced cellular differentiation in PC12 cells

The percentage of differentiated cells in the NGF-treated group was

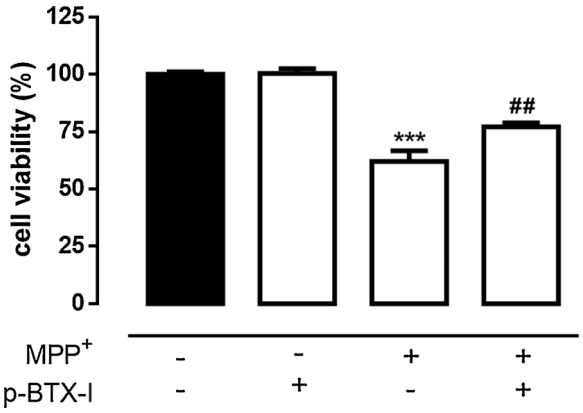


Fig. 5. Effects of p-BTX-I on MPP^+-induced cell death.
Cells were incubated for 72 h with/without p-BTX-I (192 μM) or MPP^+ (1 mM). Results were expressed as mean ± SEM of 3 different experiments (n = 3) performed in triplicate ***Significantly different from control ($p < 0.001$). ## Significantly different from MPP^+ ($p < 0.01$).

significantly higher than in the group of untreated cells. The effect of different concentrations (24; 48; 96; 192; 384 and 768 μM) of p-BTX-I induced a bell-shaped concentration-response curve of differentiation in PC-12 cells, in which the intensity of response increased from 24 up to 192 μM (maximum intensity at 192 μM) and decreased at higher concentrations. Although less intense, the differentiation at higher concentrations was still significantly higher as compared to controls (Fig. 2A and B). The most effective concentration (192 μM) was selected for the subsequent assays. Phase-contrast photomicrographs are presented in Fig. 2C–J.

3.3. p-BTX-I increased the length of neurites

The average length of the neurites induced by p-BTX-I was twice as much higher than the average length of the neurites induced by NGF (Fig. 3A–D).

3.4. p-BTX-I inhibited neurite loss in MPP^+-treated PC12 cells

The peptide p-BTX-I protected PC12 cells against the neurite loss induced by MPP^+ (100 μM, IC50 for neuritogenesis). The IC50 of MPP^+ (100 μM) for neuritogenesis was previously determined by our research group [25]. The percentage of differentiation in the group treated with MPP^+ was significantly lower as compared to the positive control (NGF); whereas in the group treated with peptide plus MPP^+, the differentiation was significantly higher than in the group treated with MPP^+ alone (Fig. 4A). Phase-contrast photomicrographs of controls, NGF, MPP + and p-BTX-I plus MPP + are presented in Fig. 4B–D and E, respectively.

3.5. p-BTX-I increased the viability of MPP^+- treated PC12 cells

The peptide alone did not affect the viability of PC12 cells as compared to controls. MPP^+ (1 mM) significantly decreased the viability of cells as compared to controls; whereas p-BTX-I significantly increased the viability of cells treated with MPP^+(1 mM) as compared to MPP^+ alone (1 mM). The concentration of MPP + used corresponds to the value of IC50 for cell viability, which we have previously determined [25]. Untreated cells were used as controls (set as 100%) and all the other values were normalized based on controls (Fig. 5).

3.6. K-252a (antagonist of trkA receptor), LY294002 (inhibitor of PI3K/AKT pathway) and U0126 (inhibitor of MAPK/ERK pathway) inhibited the neurite outgrowth induced by p-BTX-I

The percentage of differentiated cells was analyzed after incubation for 24, 48 and 72 h. No significant differences were observed between controls (untreated cells) and the groups treated with k252a (Fig. 6A–I), LY294002 (Fig. 7A–I) or U0126 (Fig. 8A–I) after the three periods of incubation. The percentage of differentiated cells increased in the groups treated with p-BTX-I or NGF as compared to controls, after incubation for 24, 48 and 72 h. The effects of NGF and p-BTX-I were inhibited by k252a (Fig. 6A–I), by LY294002 (Fig. 7A–I) and by U0126 (Fig. 8A–I). K252a inhibited the neuritogenesis induced by NGF or p-BTX-1 already after 24 h, whereas the inhibitor U0126 inhibited both effects only from 48 h of incubation. On the other hand, LY294002 inhibited the neuritogenesis induced by p-BTX-I earlier (24 h) than it inhibited the effect of NGF (48 h).

3.7. p-BTX-I increased the expression of neurite-associated proteins and protected against the decrease induced by MPP^+

The peptide p-BTX-I increased the expression of both proteins GAP-43 and synapsin in PC12 cells (not exposed to NGF) when compared to untreated cells. MPP^+ reduced the expression of GAP-43 and synapsin in PC12 cells exposed to NGF; these effects were abolished by p-BTX-I. NGF was used as positive control and it increased the expression of both proteins as compared to untreated cells (Fig. 9A–C).

4. Discussion

Parkinson's disease (PD) is a chronic progressive neurodegenerative disease characterized by loss of dopaminergic neurons in the *substantia nigra* [26]. However, recent evidence suggests that before the death of the dopaminergic neurons, axons are damaged and the synaptic communication in the nigrostriatal system is lost [5,6]. Therefore, therapeutic interventions that improve axonal growth and connectivity might have beneficial effects on the restoration of lost functions, which justifies the search for new molecules with low molecular mass and neurotrophic potential. Animal venoms are important sources of peptides with this profile [8]. We have previously reported the neurotrophic and neuroprotective potential of a tripeptide (PV) isolated from *Bothrops atrox venom*, whose amino acid sequence was Glutamic acid -Valine-Tryptophan [20]. In that study, we showed that PV protected PC12 cells against the apoptotic death induced by MPP^+ and increased the percentage of differentiation even in cells treated with MPP^+, a neurotoxin which inhibits neuritogenesis [20].

We have now synthesized a molecule (p-BTX-I) using the natural peptide PV as a model and assessed its neuroprotective/neurotrophic potential in PC12 cells treated with MPP^+, which is the active metabolite of the putative PD-inducer, MPTP. The PC12-cell-based neuronal model has been widely used in neurobiology to assess the neurotrophic potential or the toxicity of various compounds that target neurite outgrowth [27–30]. After exposure to NGF, PC12 cells undergo physiological and morphological changes that result in differentiation into sympathetic neurons [31]. The advantage of using this neuronal cell model to study neuritogenesis is that, contrarily to neurons, PC12 cells survive in the absence of NGF and the differentiation induced by NGF is reversible, which allows the use of untreated undifferentiated cells as controls [32].

First, we demonstrated that the synthetic peptide induces neuritogenesis in the neuronal model, in the absence of NGF. The response was concentration-dependent with a bell-shaped profile, i.e., the higher the concentration, the higher the differentiation up to 192 μM; above this concentration, the percentage of differentiation decreased, but it was still higher as compared to untreated controls. Other studies reported a bell-shaped profile for the effect of NGF on neurite outgrowth

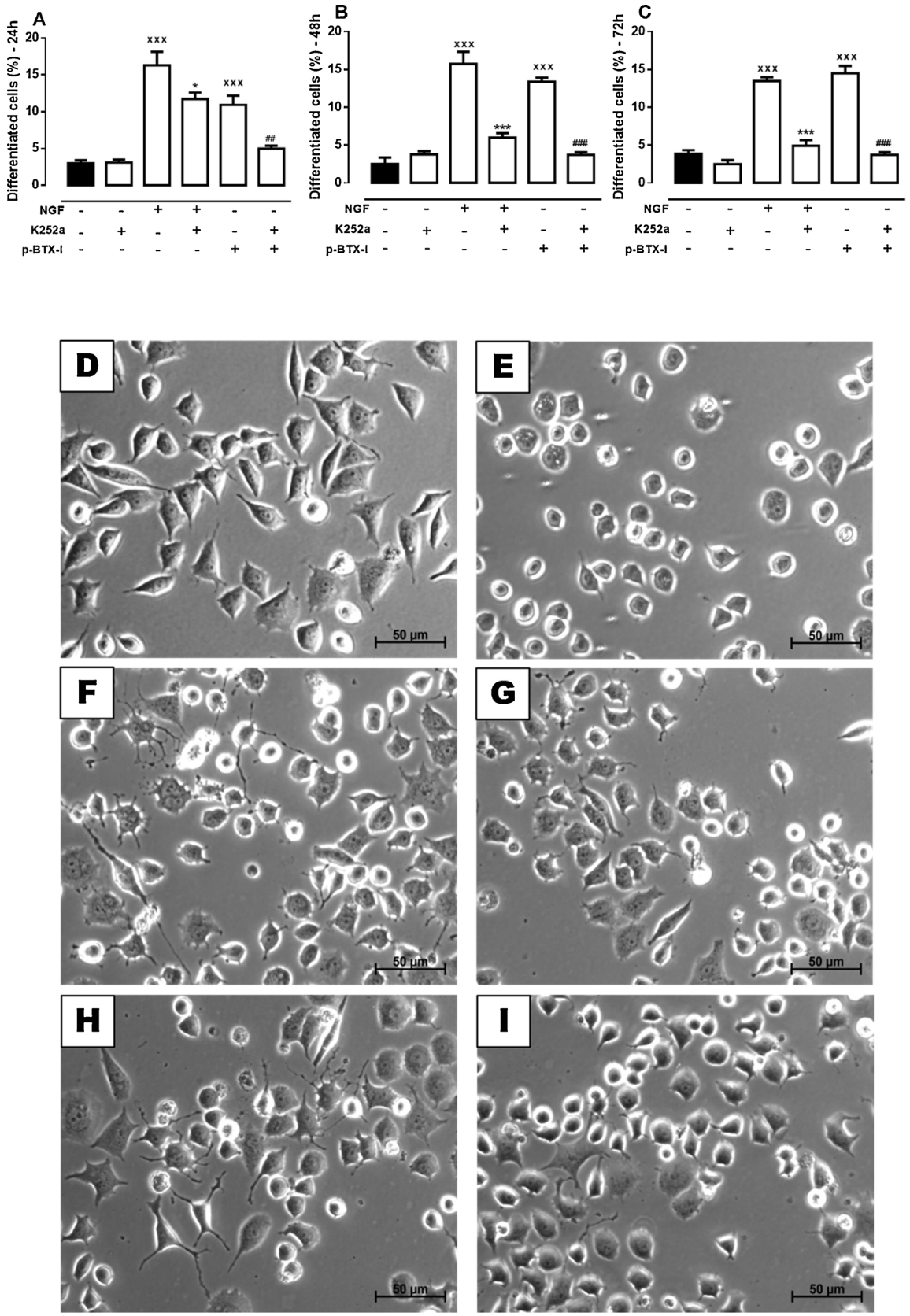


Fig. 6. Effect of K252a (trkA inhibitor) on the differentiation of PC12 cells treated with p-BTX-I. Cells were pretreated with K252a (100 nM) and incubated for 1 h prior to the addition of NGF (100 ng/mL) or p-BTX-I (192 µM). (A, B and C) Bar Graphs after incubation for 24 h, 48 h and 72 h, respectively. Cells with at least one neurite with the length equal to or greater than the cell body were considered as differentiated and expressed as a percentage of total cells in the field. The results are expressed as mean ± SEM of 3 different experiments performed in triplicate (n = 3). ^{xxx} Significantly different from untreated controls ($p < 0.01$) * Significantly different from NGF ($p < 0.05$); *** Significantly different from NGF ($p < 0.01$); $^{###}$ Significantly different from p-BTX-I ($p < 0.01$). (D- I) Inverted Phase-contrast photomicrographs of (D) controls, (E) K252a, (F) NGF, (G) NGF+ K252a, (H) p-BTX-I and (I) p-BTX-I + K252a, after 72 h of treatment.

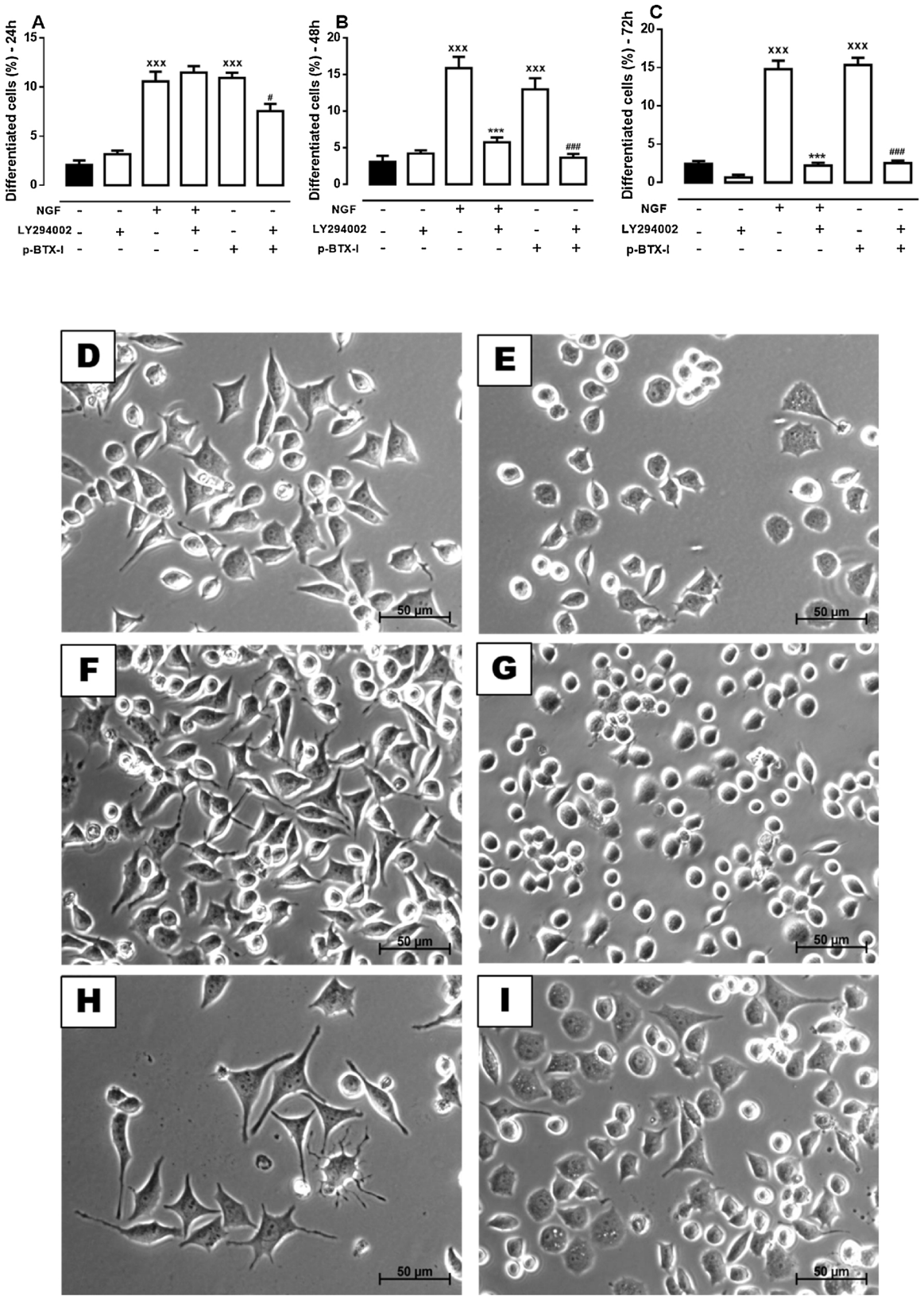
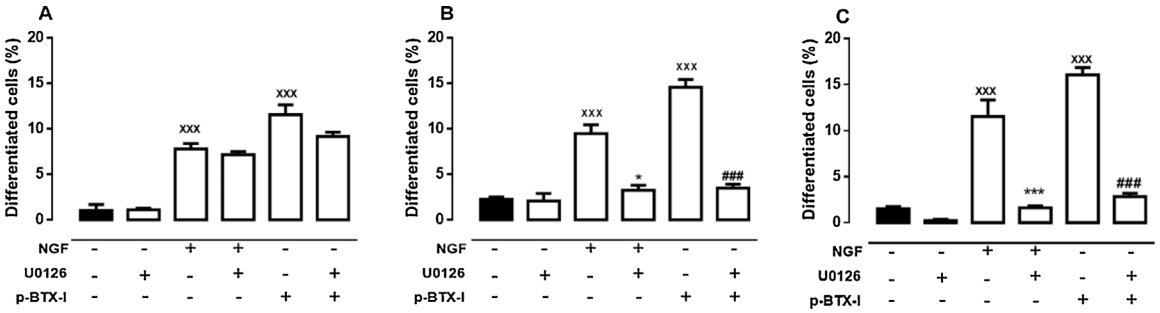


Fig. 7. Effect of LY294002 (PI3 K/AKT pathway inhibitor) on the differentiation of PC12 cells treated with p-BTX-I. Cells were pretreated with LY294002 (30 nM) and incubated for 1 h prior to the addition of NGF (100 ng/mL) or p-BTX-I (192 µM). (A, B and C) Bar Graphs after incubation for 24 h, 48 h and 72 h, respectively. Cells with at least one neurite with the length equal to or greater than the cell body were considered as differentiated and expressed as a percentage of total cells in the field. The results are expressed as mean ± SEM of 3 different experiments performed in triplicate (n = 3). ^{xxx} Significantly different from untreated controls ($p < 0.01$) * Significantly different from NGF ($p < 0.05$); *** Significantly different from NGF ($p < 0.01$); $^{\#\#\#}$ Significantly different from p-BTX-I ($p < 0.01$). (D–I) Inverted Phase-contrast photomicrographs of (D) controls, (E) LY294002, (F) NGF, (G) NGF + LY294002, (H) p-BTX-I and (I) p-BTX-I + LY294002, after 72 h of treatment.



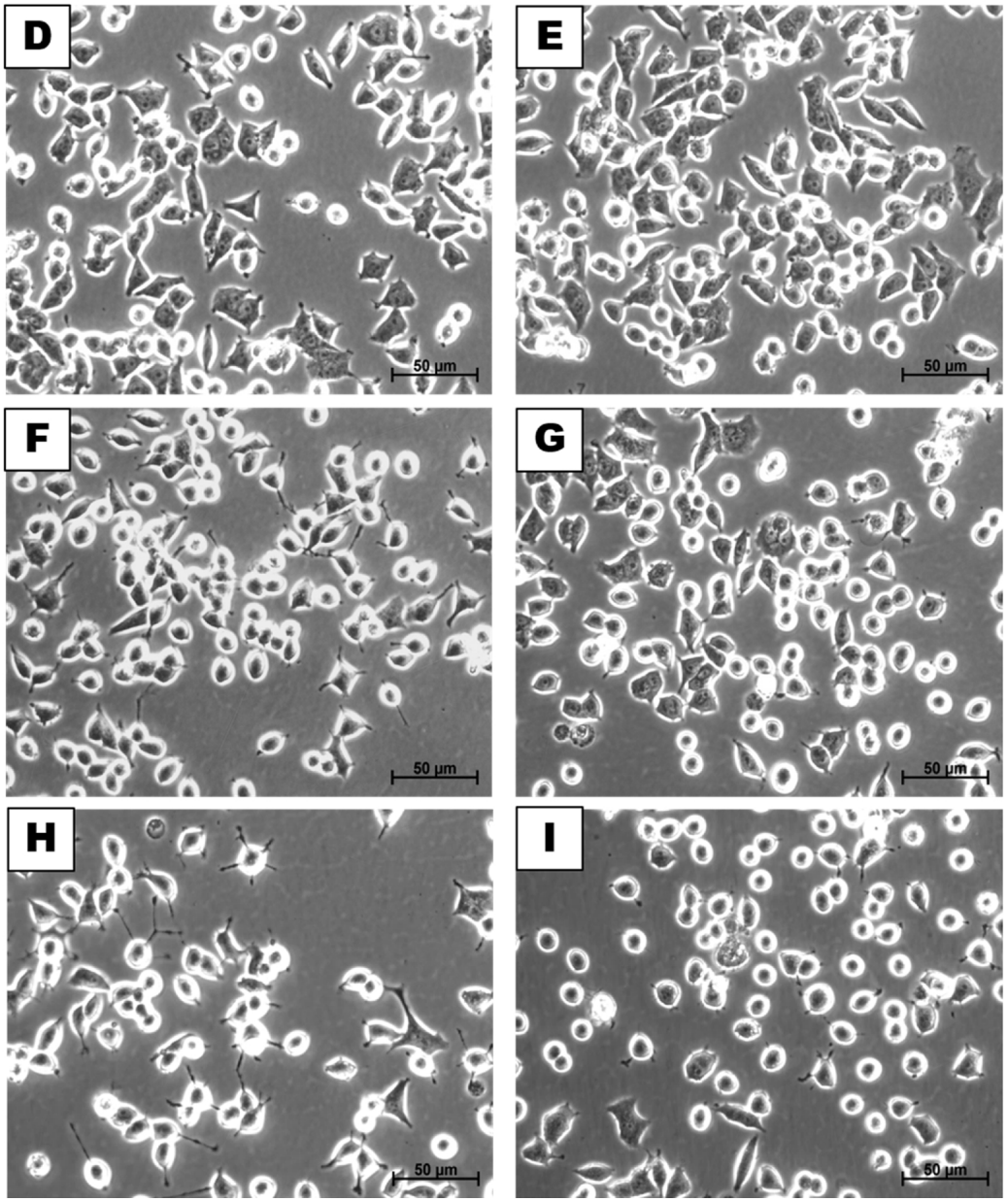


Fig. 8. Effect of U0126 (MAPK-ERK pathway inhibitor) on the differentiation of PC12 cells treated with p-BTX-I. Cells were pretreated with U0126 (10 µM) and incubated for 1 h prior to the addition of NGF (100 ng/mL) or p-BTX-I (192 µM). (A, B and C) Bar Graphs after incubation for 24 h, 48 h and 72 h, respectively. Cells with at least one neurite with the length equal to or greater than the cell body were considered as differentiated and expressed as a percentage of total cells in the field. The results are expressed as mean ± SEM of 3 different experiments performed in triplicate (n = 3). ^{xxx} Significantly different from untreated controls ($p < 0.01$); * Significantly different from NGF ($p < 0.05$); *** Significantly different from NGF ($p < 0.01$); $^{###}$ Significantly different from p-BTX-I ($p < 0.01$). (D–I) Inverted Phase-contrast photomicrographs of (D) controls, (E) LY294002, (F) NGF, (G) NGF + U0126, (H) p-BTX-I and (I) p-BTX-I + U0126, after 72 h of treatment.

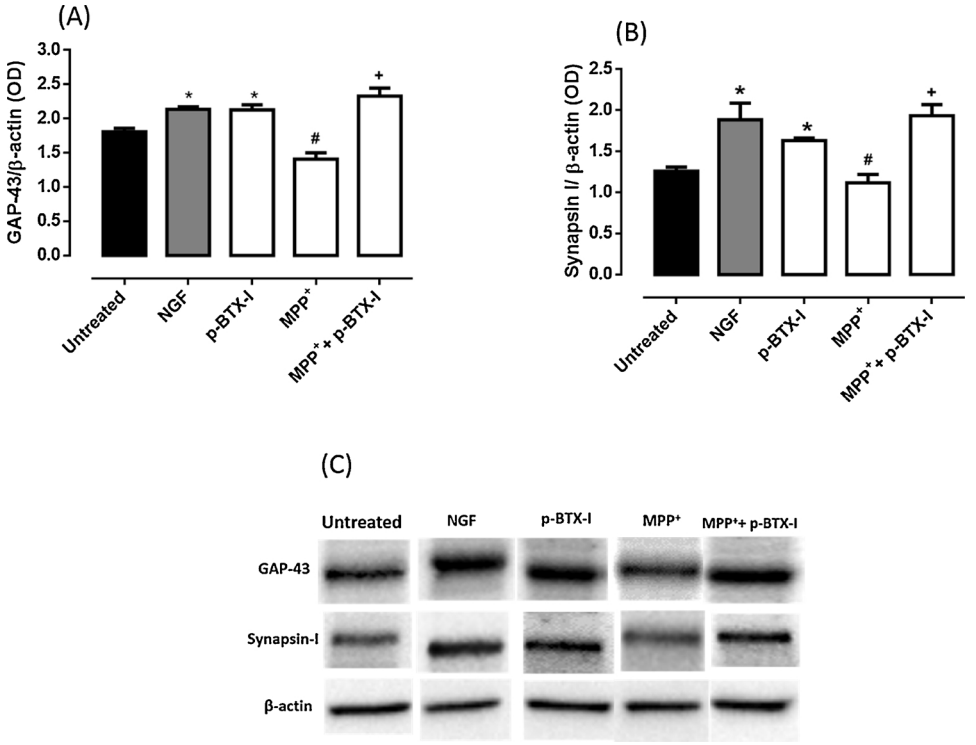


Fig. 9. Effects of p-BTX-I on the expression of axonal proteins in PC12 cells treated with MPP +. (A) Western Blot bands; (B and C) Bar graphs of β-actin-normalized optical densities (OD) of GAP-43 and Synapsin I. Additions: 100 ng/mL NGF, 192 μM p-BTX-I and/or 100 μM MPP^+. Bars indicate means ± SEM of three independent experiments performed in triplicates. *Significantly different from untreated cells ($p < 0.05$); #Significantly different from NGF ($p < 0.05$).

in (i) sensory neurons from de dorsal root ganglion (DRG) [33], and (ii) PC12 cells [34]. According to Calabrese [32] the bell-shaped profile of neurite outgrowth induction is consistent with an hormetic response, which would apply to endogenous neurotrophins such as NGF and exogenous neurotrophic agents, both during normal neuronal development and neuroregeneration. Interestingly, the neurites induced by p-BTX-I were twice as much longer than those induced by NGF. Besides inducing neurite outgrowth in untreated PC12 cells, p-BTX-I (192 μM) also protected MPP^+- treated PC12 cells from the inhibition of neurite outgrowth induced by MPP^+. It has been demonstrated that MPP^+ causes degeneration of neurites in dopaminergic neurons, before the occurrence of cell body loss [35]; we confirmed these findings in PC12 cells. The IC50 of MPP+ for cell viability is 1 mM, which completely blocks neurite outgrowth; however the IC50 for inhibition of neuritogenesis is ten times lower, i.e., 100 μM MPP^+ [25,36]. Therefore, we investigated if the concentration of p-BTX-I that protects PC12 cells from the inhibition of neurites would be able to protect against the cell death induced by the cytotoxic concentration of MPP^+ (1 mM). Our findings show that 192 μM p-BTX-I is not cytotoxic *per se* and protects MPP^+- treated cells from decreased viability, as demonstrated by the MTT assay. In this assay, cell viability is assessed based on the mitochondrial activity, which in turn is measured based on the conversion of MTT to formazan crystals in viable mitochondria [37,38]. Damage to mitochondria might trigger the apoptotic pathway, through the activation of caspase-9 [39]. Accordingly, in our previous study, the natural peptide PV decreased the activity of caspase-9 and caspase-3 that had been increased by MPP^+. Caspase-9 is an initiator caspase that is triggered by the mitochondrial (intrinsic) apoptotic pathway and caspase-3 is the final executioner of apoptosis at the nuclear level [39]. The trophic signaling plays a role in the prevention of apoptosis in neuronal cells. The activation of trkA receptors by NGF activates the anti-apoptotic protein Bcl-2 that blocks cytochrome c release from mitochondria and, consequently, the downstream apoptotic cascade of caspases [40,41]. Therefore, we investigated the hypothesis that p-BTX-I would also act by activating trkA receptors. Neurotrophic effects are mediated by tropomyosin receptor kinase (trk). Each neurotrophin binds to a specific trk receptor; NGF binds selectively to trkA receptors, which are naturally expressed by PC12 cells [42]. Signaling of trk involves the activation of the MAPK-ERK and the PI3K-AKT pathways, which promote neuronal survival and differentiation [43]. Here, we demonstrated the activation of trkA (k252a, antagonist) as well as the involvement of PI3K-AKT (LY294002, inhibitor) and MAPK-ERK (U0126, inhibitor) pathways in the mechanism of action of p-BTX-I, by selectively inhibiting the activation of each of these pathways. Additionally, we also showed the beneficial effect of p-BTX-I on the expression of GAP-43 and synapsin, which are molecular markers axonal plasticity and synaptic communication, respectively. Both proteins are targets of the neurotrophic action of NGF [22]. Here we demonstrated that besides inducing the expression of these proteins in PC12 cells (non-exposed to NGF), p-BTX-I also increases their expression in PC12 cells treated with MPP^+, which decreases both. These findings support the induction of neuritogenesis by p-BTX-I by a mechanism that involves the same neurotrophic pathway of NGF.

5. Conclusion

The synthetic peptide p-BTX-I induces neuritogenesis in PC12 cells, even in the absence of NGF; however the effect of p-BTX-I is mediated by the same receptor (trkA) and pathways (PI3K-AKT and MAPK-ERK) triggered by NGF. In addition, the peptide increases the differentiation and viability of cells exposed to MPP^+. Altogether, our findings suggest that the synthetic peptide p-BTX-I protects PC12 cells from MPP^+ toxicity by a mechanism that mimics the neurotrophic action of NGF. Therefore, this low-molecular-mass peptide might be relevant in the development of drugs aimed at restoring the axonal connectivity in neurodegenerative processes.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors thank FAPESP (Fundação de Amparo à Pesquisa do

Estado de São Paulo, grant numbers: 2015/4808-2, 2011/23236-4 and 2013/07600-3), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for the financial support.

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