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Analgesic and preventive effects of donepezil in animal models of chemotherapy-induced peripheral neuropathy: involvement of spinal muscarinic acetylcholine M2 receptors

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Abstract

Background: donepezil, a cholinesterase inhibitor approved in Alzheimer's disease, has demonstrated analgesic and preventive effects in animal models of oxaliplatin-induced neuropathy. To improve the clinical interest of donepezil for the management and prevention of chemotherapy-induced peripheral neuropathy (CIPN), a broader validation is required in different animal models of CIPN. Methods: using rat models of CIPN (bortezomib, paclitaxel, and vincristine), the analgesic and preventive efficacies of donepezil were evaluated on tactile, cold and heat hypersensitivities. The involvement of muscarinic M2 acetylcholine receptors (m2AChRs) in analgesic effects was investigated at the spinal level. The absence of interference of donepezil with the cytotoxic effect of chemotherapy has been controlled in cancer cell lines. Results: the analgesic efficacy of donepezil was demonstrated for all CIPN models, mainly on tactile hypersensitivity (maximal efficacy at 60 min, $p < 0.05$ vs. vehicle group). This effect was suppressed by an intrathecal injection of methoctramine (m2AChR antagonist). Regarding preventive effects, donepezil limited tactile hypersensitivity induced by paclitaxel, but not for other CIPN models. Donepezil did not modify the viability of cancer cells or the efficacy of anticancer drugs. Conclusions: donepezil had a broad analgesic effect on animal models of CIPN and this effect involved spinal m2AChRs. This work validates the repositioning of donepezil in the management of CIPN.

Keywords

chemotherapy-induced peripheral neuropathy; donepezil; rat; paclitaxel; bortezomib; vincristine; muscarinic M2 acetylcholine receptors

1. Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is a common adverse effect of neurotoxic anticancer drugs (cisplatin, oxaliplatin, paclitaxel, docetaxel, vincristine, bortezomib, and thalidomide) [1]. CIPN is a distal and symmetric polyneuropathy that includes paresthesia (tingling, numbness), and dysesthesia (thermal and tactile allodynia, and neuropathic pain). CIPN prevalence is close to 50% but it can vary considerably depending on the anticancer drugs and doses administered [2–4]. CIPN remains a problematic and long lasting adverse effect associated with a decline of quality of life [5,6], and because no preventive treatment can be recommended and only duloxetine has a moderate recommendation in the management of CIPN (American Society of Clinical Oncology - ASCO and the European Society for Medical Oncology - ESMO) can be recommended [7,8]. Consequently, oncologists must decrease or stop neurotoxic anticancer regimen to limit CIPN severity [9], which may in turn have a negative oncological impact on disease control and progression free survival [10]. Finally, it is expected that CIPN will affect an increasing number of patients and cancer survivors in the coming years. Indeed, by 2035, the number of new cancer cases among older persons is expected to double [11], with the most prevalent cancers being prostate, breast, lung and colorectal. whose management includes neurotoxic anticancer-based chemotherapy [1,11].

Donepezil, a cholinesterase inhibitor approved for Alzheimer disease, has shown promising results in animal models of oxaliplatin-related CIPN. Donepezil was able to prevent and treat tactile allodynia induced by oxaliplatin [12,13]. Besides CIPN, donepezil also produced anti-allodynic effects in rodent models of diabetic neuropathic pain and traumatic neuropathic pain (spinal nerve ligation) [14,15]. More precisely, for oxaliplatin-related CIPN, the analgesic effect of donepezil involved the cholinergic transmission in the posterior insular cortex through the activation of M2 muscarinic acetylcholine receptors (m2AChRs) [12], since the co-administration of methoctramine (m2AChR antagonist) in the posterior insular cortex suppressed the anti-allodynic effect of donepezil [12]. Interestingly, m2AChRs are also expressed at the spinal level and may be involved in nociceptive processes [16]. In the same way, other works suggested that donepezil could exert its analgesic effect through mAChR at the spinal level [15].

Although donepezil could be a new encouraging candidate for the prevention and management of CIPN, many unknowns remain regarding its mechanism of action and its preclinical efficacy and safety in other models of CIPN. It is now accepted that CIPN is not a unique adverse drug reaction with a unique feature, and that disease-causing mechanisms and pathophysiology are heterogeneous among neurotoxic anticancer drugs [17]. In order to complete preliminary works on oxaliplatin-related CIPN [12], the aim of this study was to assess donepezil for the prevention and management of CIPN related to other widely used anticancer drugs, (1) the analgesic efficacy of donepezil on animal models of CIPN (bortezomib, paclitaxel and vincristine), (2) the preventive efficacy of donepezil in these models of CIPN, and (3) the safety of donepezil in cancer cell lines. In the first part, the involvement of spinal m2AChRs will be specifically explored from the mechanistic standpoint.

2. Materials and Methods

2.1. Animals

Experiments were performed with 5-week-old male Sprague-Dawley rats (Janvier Labs, France). Animals were housed (4 per cage) in the animal facility, with water and food ad libitum, and kept in conditions of 12:12 hour light/dark cycle (non-reversed) and 50% hygrometry. Animals were acclimated to the animal facilities for 5 days before the beginning of the experiments. All the experiments were conducted in accordance with the ARRIVE guideline [18]. The present study received ethical approval from the Animal Care and Use Committee of Auvergne (C2EA-02) and from the French Ministry of Higher Education, Research and Innovation (Ministère de l'Enseignement supérieur, de la Recherche et de l'Innovation), with the following agreement numbers N°APAFIS#3058, APAFIS#12877 and APAFIS#15722. The number of experimental animals was kept to a minimum.

2.2. Animal models of CIPN

Paclitaxel (Leancare, UK) was injected four times (2 mg/kg, intraperitoneal – i.p.) on days 0, 2, 4 and 7. For each injection, paclitaxel was diluted to 1 mg/mL in 0.9% NaCl from a 6 mg/mL stock solution (solvent: cremophor®EL (C5135, Sigma-Aldrich) / ethanol, 1/1) [19].

Control animals received the same injection volume of vehicle: cremophor®EL / ethanol (1/1) diluted 1/6 in 0.9% NaCl.

Bortezomib (Leancare, UK) was injected five times (0.2 mg/kg, i.p.) on days 0, 1, 3, 4 and 7. For each injection, bortezomib was diluted to 0.1 mg/mL in 0.9% NaCl (vehicle) from a 1 mg/mL stock solution (solvent: 5% DMSO (276855, Sigma-Aldrich)) (adapted from Yamamoto *et al.* [20]). Control animals received the same injection volume of vehicle: 5% DMSO diluted 1/10 in 0.9% NaCl.

Vincristine (Leancare, UK) was injected five times (0.15 mg/kg, intravenous – i.v.) on days 0, 2, 4, 7 and 9. For each injection, vincristine was diluted to 0.15 mg/mL in 0.9% NaCl (vehicle) from a 1 mg/mL stock solution (solvent: 0.9% NaCl) (adapted from Authier *et al.* [21]). Control animals received the same injection volume of vehicle: 0.9% NaCl.

2.3. Drugs

For oral gavage (*per os* – p.o.), donepezil pills (Aricept® 10 mg, Pfizer) were crushed to powder with a mortar and pestle, and then dissolved in 0.5% carboxymethylcellulose (C5678, Sigma-Aldrich) in water at a concentration of 5 mg/mL. Donepezil was administered at a dose of 5 mg/kg. This donepezil dose was effective in a rat model of oxaliplatin-related CIPN without altering locomotor activity of animals [12].

For drinking water, donepezil (Leancare, UK) was solubilized in tap water at a concentration of 100 mg/L (concentration and dose adapted from Okazaki *et al.* [22]). Donepezil solution was provided to rats through feeding bottles and changed every day (except on week-end). Donepezil solution was added to the drinking water 3 days before starting the CIPN induction (first injection of anticancer drugs) and continued until the end of experiment (Figure 1).

Duloxetine (Leancare, UK) was solubilized in tap water at 10 mg/mL and administered p.o. at a dose of 30 mg/kg [23].

Methoctramine (M105, Sigma Aldrich) was diluted in 0.9% NaCl at a concentration of 1 mg/mL, and was administered by intrathecal route 10 µg/10 µL, under 2% isoflurane anesthesia [24].

2.4. Behavioral assessments

2.4.1. Tactile sensitivity

Tactile nociceptive thresholds were assessed using an electronic von Frey test (Bioseb, France) [25]. Rats were placed individually in plastic compartments on an elevated wire floor and left for habituation for 15 min before each experiment. The von Frey apparatus, consisting of a plastic tip fitted in a hand-held force transducer, was applied perpendicularly to the animal's right hind paw from below and the force applied was gradually increased until paw withdrawal. The maximum force applied (expressed in grams) to induce paw withdrawal was recorded automatically. The two measurements not differing by more than 10 grams were averaged and assigned as the nociceptive threshold [25]. Three days before CIPN induction, each rat was habituated to be in the plastic compartments to avoid any fear or stress during the experiment.

2.4.2. Cold and heat sensitivities

Cold (10°C) and heat (46°C) nociceptive thresholds were assessed using the paw immersion test [26]. The right hind paw up to the ankle was immersed in a thermostatically controlled water bath ($10 \pm 0.5^\circ\text{C}$ or $46 \pm 0.5^\circ\text{C}$, respectively) until shaking or withdrawal was observed. The two measurements not differing by more than 2 seconds were averaged and assigned as the nociceptive threshold. A cut-off time of 15 seconds was applied. Three days before CIPN induction, each rat was habituated to handling by the experimenter to avoid any fear or stress during the experiment.

2.4.3. Locomotor activity

Locomotor activity was measured with the open field test. Rats were individually placed in the center of a squared arena (70 x 70 cm), surrounded by wall (45 cm) of opaque plastic without any prior habituation and left alone for 5 min. Light intensity at the center of the arena was set to 30 lux. A camera was mounted directly above the field to record the animal's behavior. Each recording was analyzed with a specific software (EthoVision®, France). The total distance moved and the mean speed of animals were measured. The arena was cleaned after each assay.

2.5. Biomonitoring of donepezil exposition

2.5.1. Sample preparation

The animals' blood was collected from cardiac puncture under deep 4% isoflurane anesthesia. Thereafter, animals were euthanized by cervical dislocation. The blood was collected in lithium heparin vials and centrifuged (4100 rpm, 20°C, 10 min) to extract plasma. Plasma samples were stored at -80°C until analysis.

Stock solution of donepezil hydrochloride (Leancare, UK) was prepared at 1 g/L in HCl 0.1N aqueous solution. Clomipramine D3 (internal standard, IS) was prepared at 0.1 g/L in methanol. All standard solutions were stored at 4°C. Working solutions of donepezil were prepared at 5 mg/L, 500 µg/L and 50 µg/L in methanol and IS was at 50 mg/L in water.

The extraction procedure was based on the previously validated method of Roche *et al.* [27]. Seven points of calibration curves were constructed in the concentration ranges of 1 – 400 µg/L and different quality controls performed (1, 25, 150 and 400 µg/L). Before on-line purification, 10 µL of IS was added to 200 µL of sample solutions (standards, controls and samples) and protein precipitation was carried out with 200 µL of acetonitrile and 40 µL of methanol. The samples were vortexed vigorously for 10 min at room temperature and centrifuged for 10 min at 14,000 rpm at 4°C. The supernatant was then transferred into a vial.

2.5.2. Liquid chromatography and mass spectrometry

A volume of 20 µL of standard, control or sample preparation was injected into liquid chromatography systems (transcend TLX 1, Thermo Fisher, San Jose, USA). On-line chromatography purification was carried out using three Turboflow® purification columns: a Cyclone, (0.5 x 50 mm, Thermo Fisher). The chromatographic column is a Betasil® phenyl/hexyl 50 x 3 mm with a particle size of 3 µm. The analytical column is thermostated at +30°C. A gradient system with the mobile phase consisting of solvent A (0.1%; v/v; formic acid in water) and solvent B (1%; v/v; formic acid in acetonitrile) ensures a flow rate of 300 µL/min. The chromatographic run lasts 12.5 min. During the first 3 min, the sample is sent to waste through the divert valve. Acquisition by the mass detector is performed over the following 12.5 min.

The mass spectrometry analysis was performed using a Thermo Exactive benchtop Orbitrap® instrument, driven by Xcalibur® software (version 2.2 SP1). We used a heated electrospray ionization source (HESI II). The desolvation temperature was set at 350°C, the curtain gas rate at 40 AU (arbitrary units), auxiliary gas flow at 8 AU, the ionization voltage at 3 kV and

the capillary temperature at 250°C. Acquisition was performed in positive “fullscan” mode over a wide range of masses (m/z): 200–800 amu at a resolution of 50,000 (FWHM) and a range of masses (m/z) 60-800 amu at a resolution of 25,000 (FWHM) for ion quantification and ion confirmation (HCD fragmentation at 30 eV) respectively. Mass extracts were taken at 10 ppm for donepezil ($m/z = 380.22202$ for quantification and $m/z = 91.05432$ for confirmation) and for IS ($m/z = 318.18000$). The concentrations of donepezil were determined by their area ratios to that of the IS using a weight quadratic fit. The lower limit of quantification (LLOQ) of donepezil was 1 $\mu\text{g/L}$ and the upper limit of quantification (ULOQ) was 400 $\mu\text{g/L}$ in plasma.

2.6. Western blot analysis of m2AChR expression in the spinal cord

The dorsal part of the lumbar spinal cord was removed from animals after deep 4% isoflurane anesthesia and decapitation. Tissue samples were immediately immersed in liquid nitrogen and stored at -80°C until analysis.

Tissue samples were homogenized in RIPA (radioimmunoprecipitation assay) buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS 0.5% deoxycholic acid) supplemented with complete protease inhibitor mixture (Roche Diagnostics). Homogenates were cleared by centrifugation at 14,000 rpm for 15 min at 4°C and supernatants were collected and stored at -80°C until use. Total protein concentrations were determined using the BCA Protein Assay kit (Pierce) according to the manufacturer’s recommendations.

For Western immunoblotting, Laemlli loading buffer was added to the samples containing equal amounts of total proteins (30 μg) and heated at 95°C for 5 min. Samples were then separated by SDS-PAGE using mini protean 4-20% ready precast acrylamide gels (Bio-Rad, St Louis, USA) and transferred to nitrocellulose membranes using Trans-Blot Turbo apparatus (Bio-Rad, St Louis, USA).

All membrane incubations were performed on a rotating plate. Nitrocellulose membranes were blocked in TBS-T (50 mM Tris-HCl and 6 mM NaCl containing 0.1% Tween 20) supplemented with 5% nonfat dry milk for 1 h at room temperature. The membranes were then incubated overnight at 4°C with the primary antibodies directed against m2AChRs (1:1500, ab109226, Abcam) and beta-actin (1:5000, Sigma-Aldrich) diluted in 5% nonfat milk in TBS-T. After several washes with TBS-T, the membranes were probed with appropriate HRP-conjugated secondary antibodies (Thermo-Fisher) diluted at 1:10,000 in 5% nonfat dry

milk in TBS-T for 1 h at room temperature. After several washes with TBS-T, Western blots were developed using the Clarity Western ECL kit (Bio-Rad, St Louis, USA). The immunoreactive bands were quantified by densitometric analysis using Bio-Rad imaging software (Image Lab, Chemidoc). The M2 band (i.e., 52 kDa) of each sample was normalized to the corresponding β -actin band.

2.6. Cell culture and viability

To assess donepezil safety on anticancer drug effects and the viability of cancer cells, several combinations of cancer cell lines, anticancer drugs and donepezil were assessed. MDA-MB-231 (human breast cancer cell line) was cultured with paclitaxel, HL60 (human acute promyelocytic leukemia cell line) with vincristine, and LP1 (human myeloma cell line, 30,000 cells/well) with bortezomib. All cell lines were obtained from ATCC. Cells were cultured in complete medium (supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin) and maintained at 37°C in the presence of 5% CO₂. Bortezomib, paclitaxel, and vincristine were obtained as pharmaceutical formulations and solubilized according to the providers' recommendations.

Cancer cells were seeded in 96-well plates (MDA-MB-231: 3,000 cells/well, HL60: 20,000 cells/well, and LP1: 30,000 cells/well) with different concentrations of donepezil (10, 30, and 100 μ M) and cultivated overnight to allow adhesion. The next day, anticancer drugs (bortezomib, paclitaxel, and vincristine) were added into the wells at a range of concentrations (0.1, 0.3, 1, 3, 10, 30, and 100 μ M) and cells were incubated for 72 h at 37°C, 5% CO₂. MTT was then added in each well and incubated for an additional 4 h. The supernatant was discarded and a solution composed of isopropanol/H₂O/HCl (90/9/1, v/v/v) was added. The optical density was determined at 540 nm using Multiskan microplate spectrophotometer (Thermo Fisher). Each experiment was repeated three times (n = 3).

2.7. Study methodology

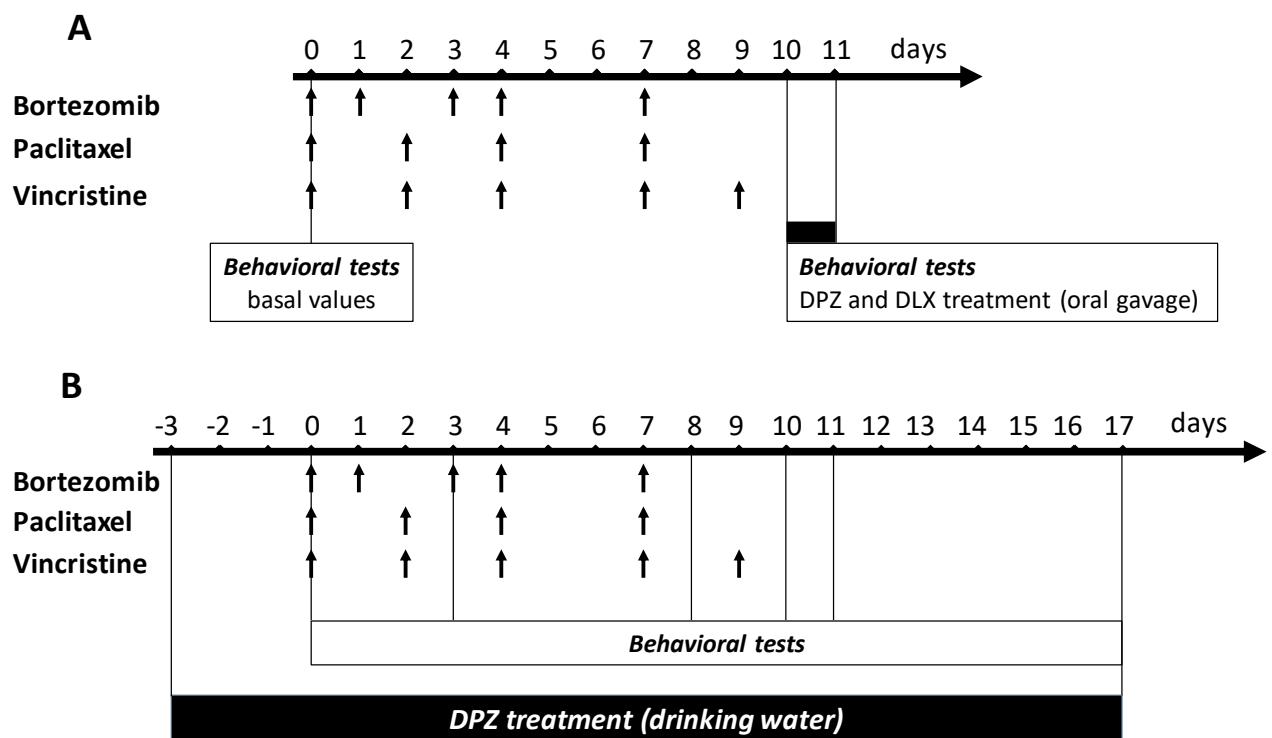
To assess the analgesic effect of donepezil on CIPN-related nociceptive disorders, animal models of CIPN (bortezomib, paclitaxel and vincristine) were developed as described above (control n = 12 and CIPN n = 12 animals). Basal values of tactile sensitivity (electronic von Frey test) and thermal sensitivities (cold and heat paw immersion tests) were assessed at

day 0 before the first injection of anticancer drugs (Figure 1). At the end of anticancer drug injections (day 10), tactile and thermal sensitivities were assessed again. The analgesic effect of donepezil (5 mg/kg, p.o.) was assessed between days 10 and 11, and in comparison to duloxetine (30 mg/kg, p.o.). Among animal models of CIPN, only animals with a 10% decrease of nociceptive thresholds between day 0 and day 10 were included for the assessment of the analgesic effects of donepezil. Thereafter, animals with a decrease of nociceptive thresholds were split into three equilibrated groups of animals to receive a vehicle (named by the anticancer drugs that animals received, BTZ for bortezomib, PTX for paclitaxel, and VCT for vincristine), donepezil (DPZ) and duloxetine (DLX) (Figure 1). Control animals (CTRL) which received a vehicle of anticancer drugs received the donepezil vehicle (0.5% carboxymethylcellulose, p.o.).

To assess the preventive effect of donepezil (100 mg/L, drinking water) on CIPN-related nociceptive disorders, animal models of CIPN (bortezomib, paclitaxel and vincristine) were developed as described above (vehicle group - CTRL n = 12, anticancer drugs (BTZ, PTX or VCT) n = 12, and anticancer drugs + donepezil (BTZ+DPZ, PTX+DPZ, or VCT+DPZ) n = 12). Basal values of tactile sensitivity were assessed at day 0 before the first injection of anticancer drugs. Thereafter, tactile thresholds were assessed on days 3, 8, 11, and 17 (Figure 1).

Figure 1: Chronology of anticancer drug administrations, behavioral tests, and assessment of donepezil effect *in vivo* (A. Analgesic effects and B. Preventive effects)

Black arrows indicate anticancer drug injections (bortezomib - BTZ: 0.2 mg/kg, i.p., paclitaxel - PTX: 2 mg/kg, i.p. and vincristine - VCT: 0.15 mg/kg, i.v.). A. The behavioral tests were the electronic von Frey test (tactile sensitivity), and the paw immersion test (cold sensitivity: 10°C and heat sensitivity: 46°C). Assessment of donepezil (DPZ, 5 mg/kg, p.o.) and duloxetine (DLX, 30 mg/kg, p.o.) was performed between days 10 and 11. The animal groups were as follows: control (vehicle, CTRL), anticancer drug (BTZ, PTX, and VCT), anticancer drug + DPZ, and anticancer drug + DLX. B. The behavioral test was the electronic von Frey test (tactile sensitivity). DPZ was provided through drinking water (100 mg/L). The animal groups were as follows: control (vehicle, CTRL), anticancer drug (BTZ, PTX, and VCT), and anticancer drug + DPZ.



2.8. Statistical analysis

All the quantitative variables are expressed by mean \pm standard deviation (sd). The comparisons of non-repeated quantitative variables (open field test and western blot analysis of m2AChR) were performed using the Mann-Whitney test. The comparisons of repeated quantitative variables (electronic von Frey test, cold and heat immersion tests,

weight of animals, and drinking quantity) were performed using a repeated-measure ANOVA test and followed by a *post hoc* Tukey-Kramer test to compare differences between groups. For multiple comparisons with no repetition of the variable assessed (pharmacological inhibition of m2AChR with methoctramine intrathecal injection, results of open field test, and plasma concentration of donepezil after chronic exposure), a Kruskal-Wallis non-parametric test was performed followed by a *post hoc* Dunn test. For all these analyses, two-tailed tests were run and differences considered statistically significant for a p-value < 0.05. Statistical analyses were performed using XLSTAT (v15.05, Addinsoft).

3. Results

3.1. Analgesic effects of donepezil in animal models of CIPN

All the CIPN models (bortezomib, paclitaxel and vincristine) developed tactile and cold hypersensitivity at day 11, with a decrease of thresholds in comparison to basal value at day 0 ($p < 0.05$) (Figure 2). The bortezomib and paclitaxel models developed heat hypersensitivity ($p < 0.05$) while the vincristine model did not (Figure 2).

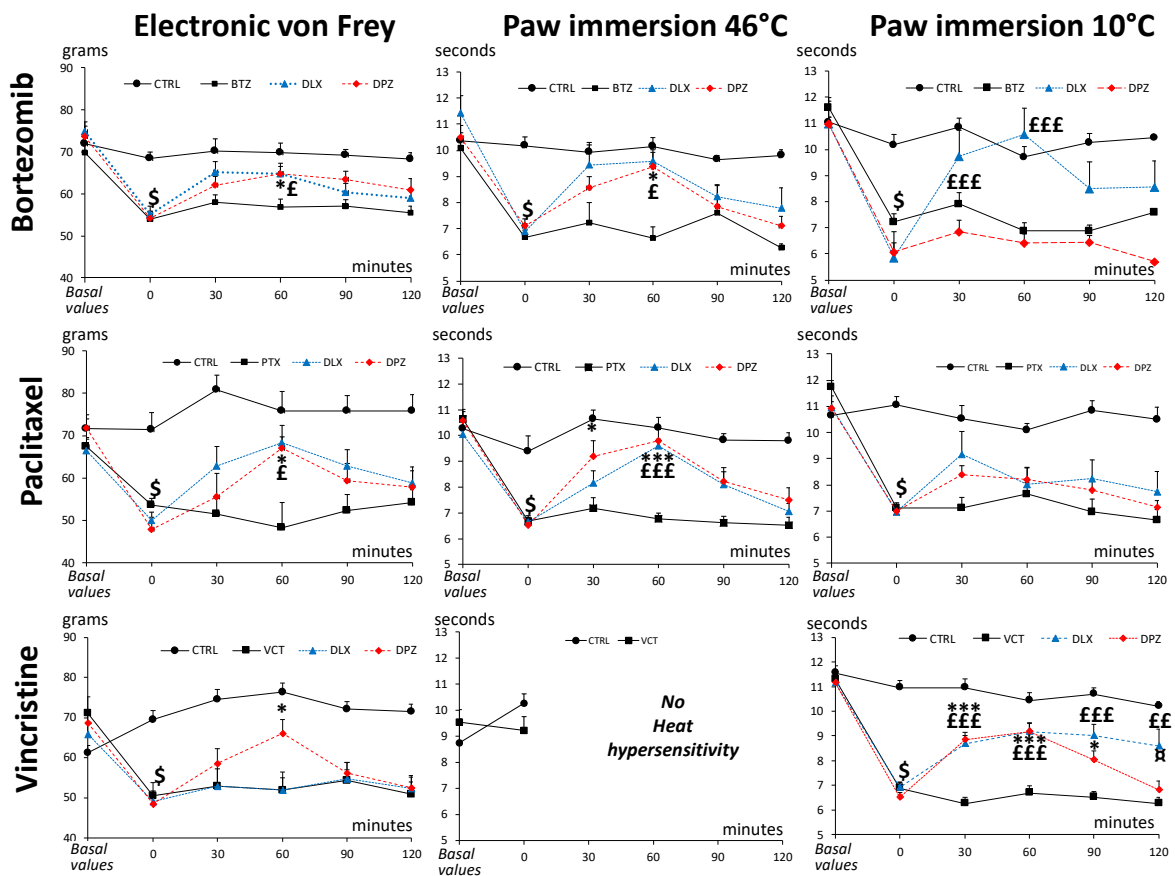
For tactile hypersensitivity induced by bortezomib and paclitaxel, both donepezil (5 mg/kg) and duloxetine (30 mg/kg) improved the tactile thresholds compared to bortezomib and paclitaxel without donepezil or duloxetine at 60 min ($p < 0.05$, for each). For the vincristine model, donepezil improved the tactile thresholds at 60 min ($p < 0.05$), while duloxetine not (Figure 2).

For heat hypersensitivity induced by bortezomib and paclitaxel, both donepezil and duloxetine improved the thermal thresholds at 60 min in comparison to bortezomib and paclitaxel without donepezil or duloxetine ($p < 0.05$ and $p < 0.001$, respectively) (Figure 2).

For the cold hypersensitivity induced by bortezomib, only duloxetine improved the thermal thresholds at 30 and 60 min in comparison to bortezomib without duloxetine or donepezil ($p < 0.05$ and $p < 0.001$, respectively). Neither donepezil nor duloxetine improved the cold thresholds of the paclitaxel model. Both donepezil and duloxetine improved the thermal thresholds starting at 30 min until 90 min (donepezil) and 120 min (duloxetine) for the vincristine model ($p < 0.05$ to $p < 0.001$, both) (Figure 2).

Figure 2: Analgesic effects of donepezil on tactile, heat, and cold sensitivities in animal models of CIPN

Behavioral assessments were performed before (basal values) and at day 11 (time: 0) after the beginning of the anticancer drug injections (bortezomib - BTZ, paclitaxel - PTX, vincristine - VCT). Control animals (CTRL) received vehicle injections. At time 0, donepezil (DPZ, 5 mg/kg, red), duloxetine (DLX, 30 mg/kg, blue) or vehicle (black) were administered to animals treated by anticancer drugs or vehicle of anticancer drugs. The results are presented by the mean \pm error standard of the mean (n = 7-12 per group). Treatment groups: CTRL: vehicle + vehicle; BTZ: bortezomib + vehicle; PTX: paclitaxel + vehicle; VCT: vincristine + vehicle; DPZ: bortezomib or paclitaxel or vincristine + donepezil; DLX: bortezomib or paclitaxel or vincristine + duloxetine. Statistical comparisons: \$ p < 0.05 T0 vs. basal values; * p < 0.05 DPZ vs. BTZ or PTX or VCT; £ p < 0.05 DLX vs. BTZ or PTX or VCT; ££ p < 0.01 DLX vs. BTZ or PTX or VCT; *** p < 0.001 DPZ vs. BTZ or PTX or VCT; £££ p < 0.001 DLX vs. BTZ or PTX or VCT; ¤ p < 0.05 DLX vs. DPZ



Donepezil concentrations in the plasma of naïve animals were assessed from 30 min to 90 min after oral gavage of 5 mg/kg. The mean value of donepezil concentrations was 46.3 ± 12.8 ng/mL ($n = 18$) and concentrations were not different between the sampling timings ($p = 0.423$) (Table 1).

Table 1: Assessment of donepezil concentrations in plasma after a single oral gavage (5 mg/kg) or through drinking water (100 mg/L).

Donepezil concentrations in plasma (ng/mL) were assessed at 30, 60 and 90 min after a single oral gavage (5 mg/kg), and at day 17 after the first anticancer drug injection following a chronic treatment through drinking water (100 mg/L). The results are presented by the mean \pm standard deviation, and number of animals.

Donepezil Route and dose	Timing Animal groups	Donepezil concentration (ng/mL)
Single oral gavage 5 mg/kg	30 min ($n = 6$)	49.7 ± 13.7
	60 min ($n = 6$)	39.8 ± 3.7
	90 min ($n = 6$)	49.3 ± 16.6
Drinking water 100 mg/L	Day 17	
	Bortezomib ($n = 12$)	29.0 ± 15.5
	Paclitaxel ($n = 16$)	20.0 ± 9.3
	Vincristine ($n = 12$)	26.3 ± 12.6

The impact of donepezil and duloxetine on locomotor activity was assessed in naïve animals in comparison to vehicle thanks to the open field test (Table 2). Donepezil (5 mg/kg, p.o.) did not significantly change the total distance moved and velocity in comparison to vehicle. However, duloxetine (30 mg/kg, p.o.) induced a slight but significant decrease of the total distance moved and velocity ($p < 0.05$, both) (Table 2).

Table 2: Assessment of donepezil and duloxetine effects on locomotor activity of animals.

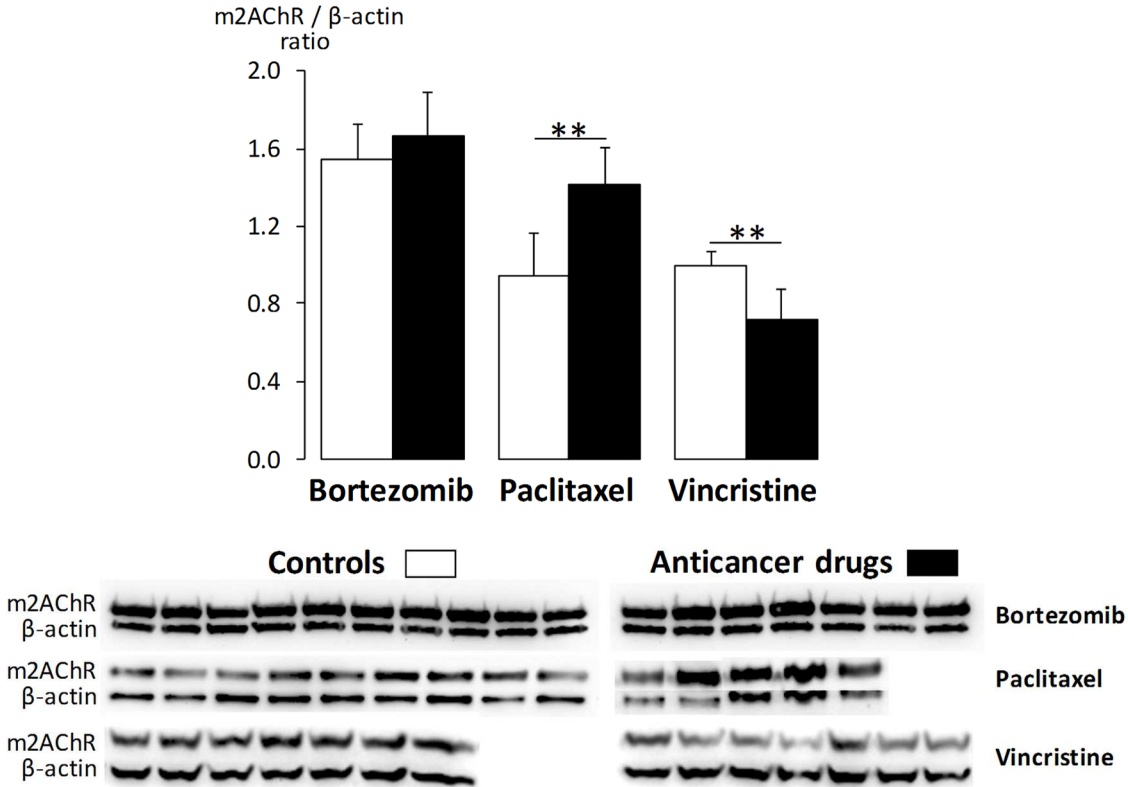
The locomotor activity of rats was assessed with the open field test, measuring the total distance moved (cm) and the velocity (cm/s) of animals for 5 min. Donepezil (5 mg/kg, p.o.) and duloxetine (30 mg/kg, p.o.) effects were assessed 60 min after oral gavage of naïve animals, and in comparison to a vehicle group. Donepezil effects during a chronic exposition (100 mg/L in drinking water) were assessed in paclitaxel treated animals 10 days after the beginning of paclitaxel injections, in comparison to paclitaxel treated animals and control animals who received water. The results are presented by the mean \pm standard deviation.* $p < 0.05$ vs. vehicle.

Treatments	Total distance moved (cm)	Velocity (cm/s)
Donepezil, 5 mg/kg (n = 6)	3132.9 \pm 528.2	10.4 \pm 1.8
Vehicle (n = 6)	3560.5 \pm 255.2	11.9 \pm 0.9
Duloxetine, 30 mg/kg (n = 11)	3109.6 \pm 442.1*	10.4 \pm 1.5*
Vehicle (n = 10)	3490.7 \pm 233.7	11.6 \pm 0.8
Paclitaxel / donepezil, 100 mg/L (n = 9)	3119.5 \pm 1374.0	11.4 \pm 2.6
Paclitaxel / water (n = 9)	3542.3 \pm 1395.1	12.7 \pm 2.3
Control / water (n = 8)	3865.9 \pm 517.0	12.9 \pm 1.7

Protein expressions of m2AChRs were assessed at the spinal level in all animal models of CIPN at day 11 after the beginning of anticancer drug injections (Figure 3). Between control and anticancer drug treated animals, m2AChRs expression was unchanged for bortezomib, increased for paclitaxel ($p < 0.01$) and decreased for vincristine ($p < 0.01$) (Figure 3).

Figure 3: Protein expression of m2AChRs in the lumbar spinal cord.

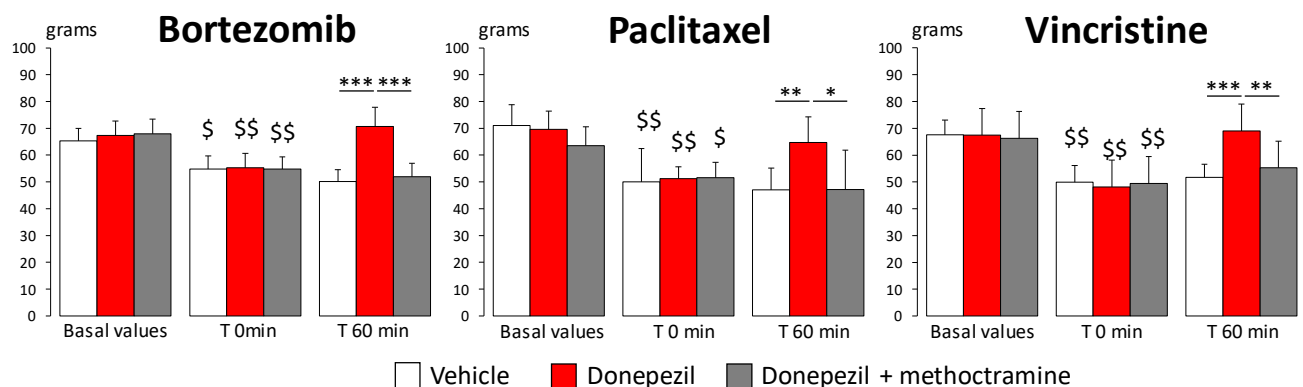
At day 11 after the beginning of anticancer drug injections (bortezomib, paclitaxel and vincristine), m2AChRs were assessed by western blot in the dorsal part of the lumbar spinal cord in animals treated by anticancer drugs in comparison to control animals (vehicle). The results are presented by the mean \pm standard deviation of the ratio of m2AChRs and β -actin expression (n = 7-12 per group). Statistical comparisons: ** p < 0.01



In animal models of CIPN, intrathecal injection of the m2AChRs antagonist, methoctramine (10 µg / 10 µL), 45 min after donepezil administration (5 mg/kg, p.o.) decreased tactile thresholds in comparison to donepezil alone (bortezomib $p < 0.001$, paclitaxel $p < 0.05$ and vincristine $p < 0.01$) (Figure 4).

Figure 4: Involvement of spinal m2AChRs in the analgesic effect of donepezil.

Tactile allodynia was monitored before (basal values) and at days 10 and 11 (pooled) after anticancer drug injections (bortezomib, paclitaxel or vincristine) (T 0 min). Donepezil (5 mg/kg, p.o.) was administered at T 0 min and methoctramine (10 µg / 10 µL, intrathecal injection) at T 45 min. Thereafter, tactile sensitivity was assessed at T 60 min. Animals of the vehicle group received vehicle of donepezil (p.o.) and methoctramine (intrathecal). The donepezil group received an injection of methoctramine vehicle (intrathecal). The results are presented by the mean \pm sd (bortezomib, $n = 9-10$ per group; paclitaxel $n = 7-10$ per group; vincristine, $n = 10$ per group). Statistical comparisons: \$ $p < 0.05$ and \$\$ $p < 0.01$ vs. basal values; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.



3.2. Preventive effect of donepezil in animal models of CIPN

Animals with the bortezomib and paclitaxel-associated CIPN developed tactile hypersensitivity (decrease of tactile thresholds in comparison to control animals, $p < 0.05$) from day 3 until the end of the experiment (Figure 5). Animals with the vincristine-associated CIPN developed tactile hypersensitivity from day 8 until the end of the experiment ($p < 0.05$) (Figure 5).

Chronic treatment with donepezil (100 mg/L drinking water), starting 3 days before the first injections of anticancer drugs, improved tactile hypersensitivity only for the paclitaxel-

related CIPN model, in comparison to paclitaxel-treated animals (water only) on days 7 and 17 ($p < 0.01$ and $p < 0.05$, respectively) (Figure 5). No effect of donepezil was recorded on tactile hypersensitivity induced by bortezomib or vincristine (Figure 5).

Measured at day 10 in paclitaxel-treated animals, chronic exposure to donepezil (100 mg/L drinking water) did not significantly change the locomotor activity in comparison to control and paclitaxel-treated animals receiving water (without donepezil) (Table 2).

Donepezil (100 mg/L drinking water) did not modify weight gain of animal models of bortezomib and vincristine-related CIPN (Figure 6). But a significant decrease of weight gain was recorded for the model of paclitaxel-related CIPN in comparison to control animals (vehicle) (day 4 $p < 0.05$ and day 7 $p < 0.01$) (Figure 6). Importantly, animals treated by bortezomib or vincristine had a significant decrease of weight gain associated with the repetition of anticancer drug injections, which was not the case for paclitaxel-treated animals (Figure 6). Donepezil in drinking water (100 mg/L) did not significantly change the quantities of daily water consumption in comparison to animal receiving only water (Figure 6).

At day 17 after the first anticancer drug injection, the mean plasma concentration of donepezil (all models) was 24.6 ± 12.7 ng/mL and not different between animal models of CIPN ($p = 0.16$) (Table 1).

Figure 5: Preventive effects of donepezil on tactile sensitivity in animal models of CIPN.

Behavioral assessments were performed before (basal values) and at days 3, 7, 11 and 17 after the beginning of the anticancer drug injections (bortezomib - BTZ, paclitaxel - PTX, vincristine - VCT). Control animals (CTRL) received vehicle injections. Donepezil (DPZ, 100 mg/L, red) or water (black) was provided to rats through drinking bottles 3 days before the first injection of anticancer drugs. The results are presented by mean ± standard deviation (n = 12 per group). Group treatments: CTRL: vehicle + vehicle; BTZ: bortezomib + vehicle; PTX: paclitaxel + vehicle; VCT: vincristine + vehicle; DPZ: bortezomib or paclitaxel or vincristine + donepezil. Statistical comparisons: * p < 0.05 vs. CTRL; ** p < 0.01 vs. CTRL; *** p < 0.001 vs. CTRL; § p < 0.05 DPZ vs. BTZ, PTX, VCT; §§ p < 0.01 DPZ vs. BTZ, PTX, VCT.

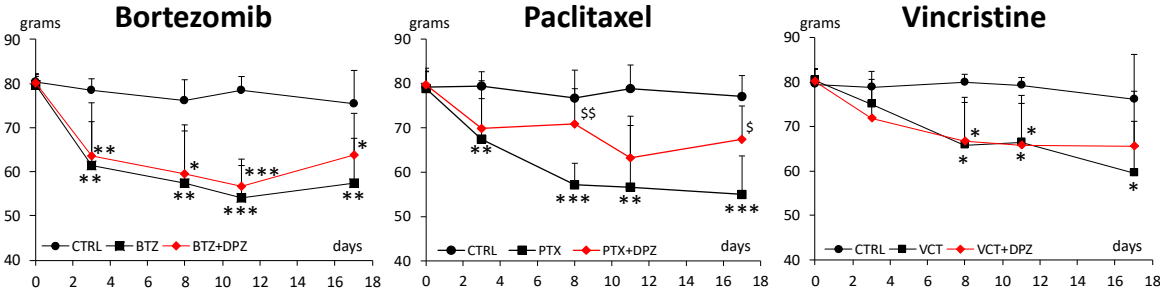
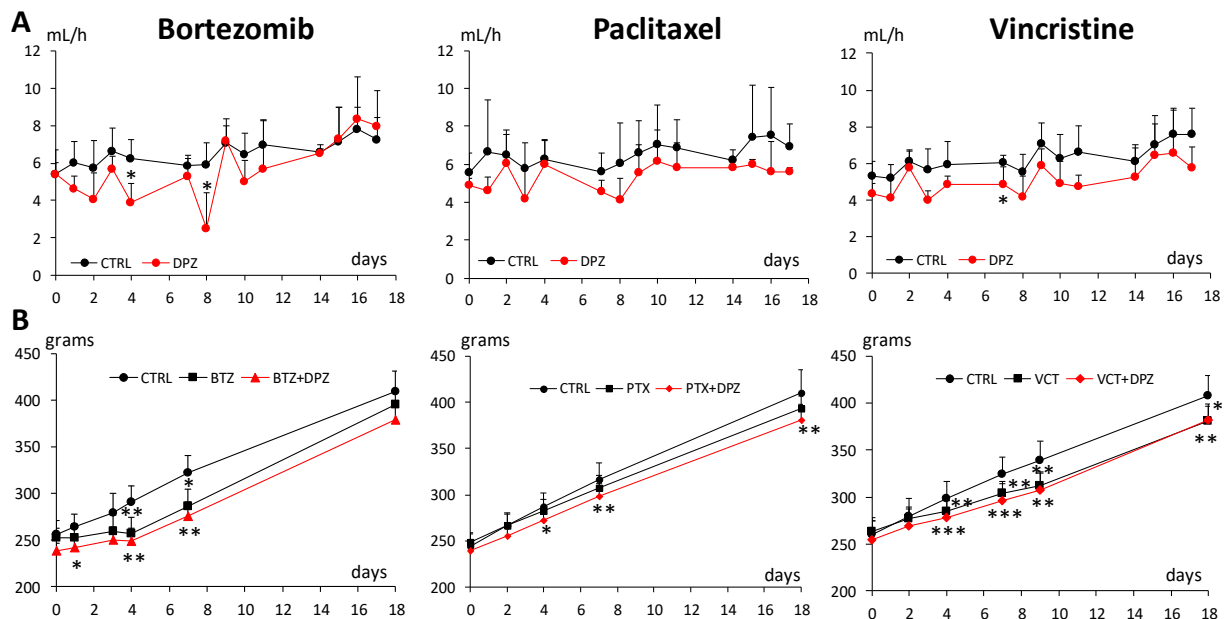


Figure 6: Effects of donepezil on drinking volumes and weight gains of animal models of CIPN.

Donepezil (DPZ, 100 mg/L, red) or water (black) was provided to rats through drinking bottles 3 days before the first injection of anticancer drugs (bortezomib, paclitaxel, or vincristine). A. Drinking volumes were assessed daily, except at the end of the week. Measured volumes were normalized to the time between two measures and expressed in mL/h. The results are presented by the mean \pm standard deviation of drinking volume of animal cages (water n = 6 and donepezil n = 3). Statistical comparisons: * p < 0.05 vs. water. B. The weight of animals was measured each day of anticancer drug injection and at the end of the study (day 17). The results are presented by the mean \pm standard deviation (n = 12 per group). Group treatments: CTRL: vehicle + vehicle; BTZ: bortezomib + vehicle; PTX: paclitaxel + vehicle; VCT: vincristine + vehicle; DPZ: bortezomib or paclitaxel or vincristine + donepezil. Statistical comparisons: * p < 0.05 vs. CTRL; ** p < 0.01 vs. CTRL; *** p < 0.001 vs. CTRL

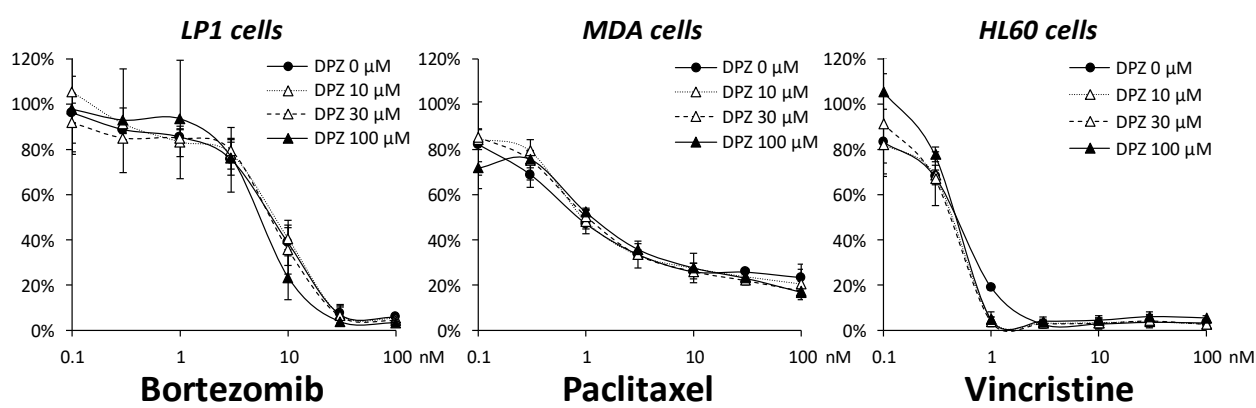


3.3 Assessment of donepezil interaction on cancer cell viability and anticancer drug efficacy

No effect of donepezil (10, 30 and 100 μ M) was observed on the viability of cancer cell lines (LP1, MDA and HL60). Similarly, no effect was observed with the different anticancer drugs tested (bortezomib, paclitaxel and vincristine: 0.1, 0.3, 1, 3, 10, 30 and 100 μ M) (Figure 7).

Figure 7: Effects of donepezil on the viability of cancer cell lines and interaction with anticancer drugs.

Donepezil (DPZ) effect was assessed at 10, 30 and 100 μM . Bortezomib, paclitaxel and vincristine effects were assessed at 0.1, 0.3, 1, 3, 10, 30 and 100 μM , in LP1, MDA and HL60 cancer cell lines, after 72 h of incubation, respectively. The results are expressed with the mean \pm sd of cell viability (percentage in comparison to vehicle of anticancer drugs and donepezil) according to the concentration of anticancer drugs (x-axis expressed by log₁₀) (n = 3).



4. Discussion

First, as demonstrated in oxaliplatin-induced neuropathic pain [12], donepezil (5 mg/kg, p.o.) showed analgesic effects in several animal models of CIPN: on tactile hypersensitivity (induced by bortezomib, paclitaxel and vincristine), on heat hypersensitivity (induced by bortezomib and paclitaxel), and on cold hypersensitivity (induced by vincristine). Donepezil was not effective on cold hypersensitivity induced by bortezomib or paclitaxel. Interestingly, duloxetine (drug recommended by ASCO and ESMO [7,8]) had nearly the same analgesic profile as donepezil to the exception of tactile hypersensitivity induced by vincristine (no analgesic effect) and heat hypersensitivity induced by bortezomib (analgesic effect). Recently, Nakamura *et al.* (2021) demonstrated the analgesic effect of duloxetine (30 mg/kg, p.o.) in a rat model of bortezomib-related CIPN assessed with the von Frey hair test, but not in paclitaxel-related CIPN [28]. To our knowledge, no report exists on the analgesic effects of duloxetine in a rat model of vincristine-related CIPN. Only one study presented a preventive

effect of repeated i.p. injections of duloxetine (5-20 mg/kg) in a mouse model of vincristine-related CIPN assessed with the von Frey hair test [29].

Donepezil (5 mg/kg, p.o.) had no significant impact on the locomotor activity of animals, as previously demonstrated [12]. Although duloxetine (30 mg/kg, p.o.) decreased the locomotor activity of animals, this effect did not bias the results of the behavioral experiments, since duloxetine (and also donepezil) was not effective in all animal models of CIPN and all nociceptive modalities.

The analgesic effects of donepezil on tactile hypersensitivity were mediated through the activation of spinal m2AChRs, since the intrathecal administration of methoctramine (m2AChR antagonist) suppressed the analgesic effect of donepezil, whatever the animal model of CIPN. However, donepezil analgesic effects seemed independent of the expression level of m2AChR at the spinal level (bortezomib: unchanged expression, paclitaxel: increased expression, and vincristine: decreased expression). Previously, Ferrier *et al.* showed increased expression of m2AChRs (gene and protein) in the posterior insular cortex of oxaliplatin treated animals, and made the relation with the activity of donepezil [12]. In diabetic rats, mechanical allodynia is also associated with an upregulation of m2AChRs in the spinal dorsal horn and an increased antinociceptive effect of intrathecal muscarinic agonist administration [30].

From a mechanistic perspective, in the spinal dorsal horn, m2AChRs are expressed in terminals of primary afferents and glutamatergic interneurons, and contribute to a decrease of glutamate release and to analgesic effect [16,31,32]. Donepezil is also an adenosine monophosphate protein kinase (AMPK) activator and blocks the phosphorylation of several kinases involved in nociceptive processes (protein kinase B AKT, mitogen-activated protein kinase MAPK and mammalian target of rapamycin complex 1 mTORC1) [14]. This analgesic effect could also involve spinal nicotinic and GABAergic receptors, but it is still unclear whether this effect of donepezil is directly due to an increase of spinal acetylcholine release [33].

Donepezil (100 mg/L drinking water) showed a moderate preventive effect on paclitaxel-induced tactile hypersensitivity, but not on hypersensitivities induced by bortezomib and vincristine. It has already been reported that donepezil (5 mg/kg, p.o., twice a day and every day, or 1 mg/kg, p.o., once a day and 5 times/week) was able to prevent oxaliplatin-induced tactile hypersensitivity [12,13]. Interestingly, this preventive effect of donepezil on

paclitaxel-associated CIPN could be related to the increase of m2AChRs expression in the spinal cord of animal treated by paclitaxel (as mentioned above). Indeed a similar effect was reported for oxaliplatin-associated CIPN for which m2AChRs expression was increased in the posterior insular cortex of neuropathic animals [12], contrary to bortezomib and vincristine-related CIPN for which no increase of m2AChRs expression was reported in the spinal cord. Moreover, donepezil was able to promote neurite outgrowth in PC12 cells (rat pheochromocytoma cell line characterized by neuronal differentiation in the presence of forskolin) treated by oxaliplatin, but not by paclitaxel or bortezomib [13]. For oxaliplatin-related CIPN, donepezil may have a neuroprotective effect by limiting the degeneration of myelinated fibers in the sciatic nerves of rats and restore superoxide dismutase activity [13]. This neuroprotective effect of donepezil may also be attributed to $\alpha 7$ nicotinic AChR, which resulted in the internalization of NMDA receptors, and the attenuation of glutamate excitotoxicity and calcium influx [34]. Likewise, the activation of m2AChRs and m4AChRs can inhibit the activity of voltage-gated calcium channels in primary sensory neurons [35].

Donepezil did not modify viability of cancer cell lines and pharmacological sensitivity to anticancer drugs (multiple myeloma and bortezomib; breast cancer and paclitaxel; leukemia and vincristine). This has also been reported for oxaliplatin sensitivity in C26-murine colon adenocarcinoma, HCT116-human colon carcinoma, MKN45-human gastric carcinoma and MIA PaCa2-human pancreatic carcinoma [13]. The latter results emphasize the feasibility and safety of using donepezil in cancer patients and survivors.

In the present study, the plasma concentrations of donepezil after a 5 mg/kg oral administration (39.8 ± 3.7 ng/mL) were similar to previous reports in 8 week-old male Fischer rats (39.0 ± 6.0 ng/mL one hour after a 5 mg/kg oral administration, and 15.7 ± 1.4 ng/mL after a 2.5 mg/kg dose) [36]. Importantly, 2.5 and 5 mg/kg oral doses of donepezil significantly decreased cholinesterase activity in the animal brain [36], and a 5 mg/kg oral dose of donepezil increased the concentrations of acetylcholine in the brain [12]. For the chronic treatment, donepezil was administered through drinking water instead of oral gavage. According to the study results, mean water consumption was about 6 mL/h for a cage of four animals (36 mL/day/rat), and consequently, the daily dose of donepezil was about 3.6 mg. Considering a mean weight of animals of 300 g, animals received a daily dose of donepezil of 12 mg/kg/day. Donepezil has a short half-life (57.06 ± 10.10 min [37]) and should have been administered several times a day to achieve a good daily exposure. But

oral gavage was not used to avoid esophageal traumatism, stress and mortality of animals [38]. Administration of analgesic through drinking water has already been done in the context of CIPN with good results [39].

Finally and importantly, analgesic activity of donepezil is probably not restricted to m2AChRs. While inhibiting acetylcholine catabolism, donepezil increases acetylcholine concentrations in nervous tissues [12]. Consequently, acetylcholine may activate other receptors such as muscarinic ones (mAChRs) and nicotinic ones (nAChRs), known to be involved in antinociceptive processes (agonists of m2AChRs, m4AChRs, α 4 β 2nAChRs, α 6 β 4nAChRs, and α 7nAChR) and pronociceptive ones (agonists of α 9nAChRs) [40–42]. So it could be interesting to explore specific agonists of m2AChRs [40], which could also limit autonomic nervous system-related adverse effects [42].

One of the strengths of the work presented is the implementation of different animal models of CIPN to validate donepezil efficacy, thereby increasing the robustness of the results obtained. Because our results clearly showed that all these CIPN models are not equal regarding nociceptive disorders (e.g., no heat hypersensitivity for vincristine-related CIPN), and pharmacological sensitivity (e.g., donepezil efficacy on tactile hypersensitivity induced by vincristine and not duloxetine, and conversely, duloxetine efficacy on cold hypersensitivity induced by bortezomib, and not donepezil). Although CIPN is a common adverse effect of neurotoxic anticancer drugs, these anticancer drugs all have different neurotoxic mechanisms [1]. Platinum-based anticancer drugs reach the neurons of the peripheral nervous system (PNS) and induce nuclear and mitochondrial DNA damage, oxidative stress and ion channel disturbances [1]. Besides microtubule depolymerization disturbances, taxanes induce oxidative stress, damaging neuronal and non-neuronal cells in the PNS, macrophage activation in the dorsal root ganglia (DRG) and peripheral nerves, and microglial activation in the spinal cord [1]. Vinca alkaloids block microtubule polymerization, inducing an increase of microtubule depolymerization, which stops the mitotic cycle and initiates cell apoptosis [1]. The pathogenetic hallmark of bortezomib-induced peripheral neuropathy consists of morphological alterations in the spinal cord, DRG and peripheral nerves, with specific functional alterations in A delta and C sensory nerve fibers [43]. In addition, proteasome inhibition increased α -tubulin polymerization, mitochondrial and endoplasmic reticulum damage, and dysregulation of neurotrophins through the inhibition of nuclear factor kappa B activation that may also contribute to pathogenesis [1].

Clinical studies suggest an analgesic and neuroprotective effect of donepezil. Regarding its analgesic effect, Boyle *et al.* showed in healthy volunteers that donepezil associated with gabapentin improved pain thresholds (sural nerve stimulation), with a better effect than gabapentin alone [44]. Similarly, two other studies in patients with neuropathic pain demonstrated that donepezil increases the analgesic effect of gabapentin [45,46]. Finally, a case report presented an analgesic effect of donepezil in a patient with Alzheimer's disease suffering from pain [47]. Donepezil use in the management of CIPN belongs to the strategy of drug repositioning, aimed at establishing the availability of new treatments for patients [48]. Therefore, donepezil could be rapidly repositioned in the management of CIPN since its safety has been acknowledged in Alzheimer patients [49].

5. Conclusions

The repurposing of donepezil in the management of CIPN could be a real opportunity for cancer patients and survivors suffering from CIPN. The analgesic effects of donepezil was validated across different animal models of CIPN, and associated with its oncological safety. Clinical trials can be now be launched to validate the proof of concept. The preventive effect of donepezil could be more restricted to a specific type of CIPN, such as induced by paclitaxel and oxaliplatin, but further studies would be necessary to precisely define these effects.

CIPN remains an unsolved problem for oncologists, cancer patients and survivors, making it increasingly necessary to seek therapeutic innovations.

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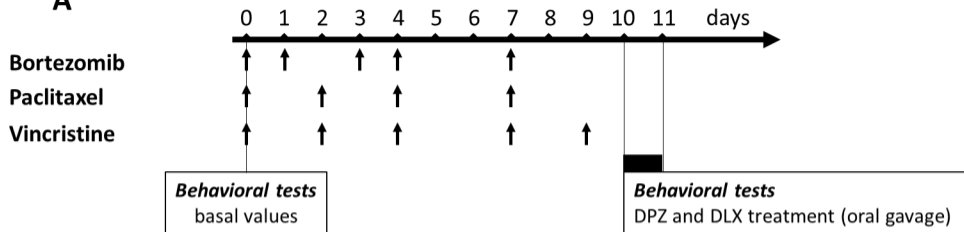
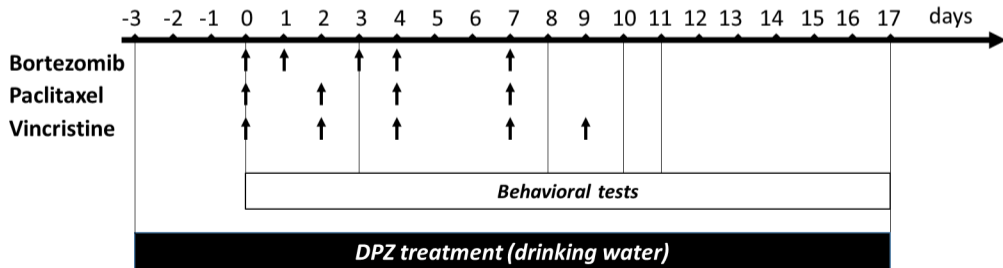
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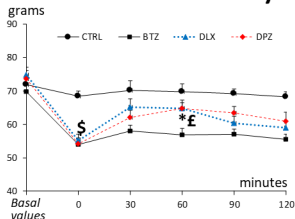
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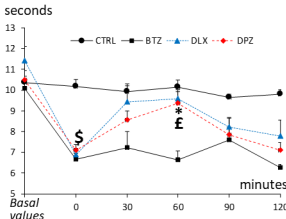
A**B**

Electronic von Frey

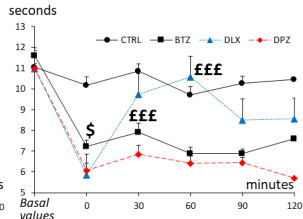
Bortezomib



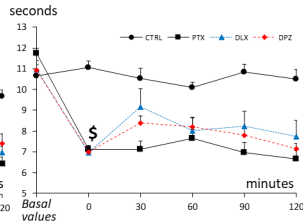
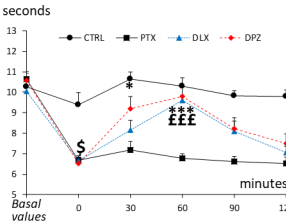
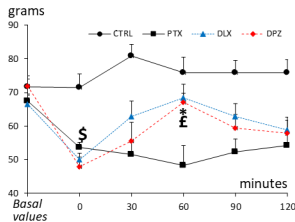
Paw immersion 46°C



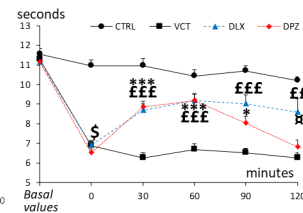
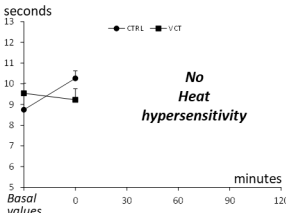
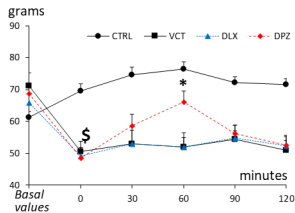
Paw immersion 10°C



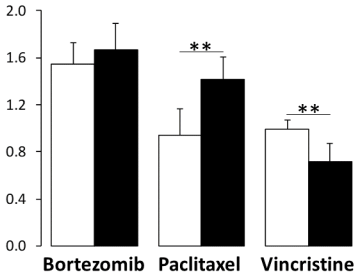
Paclitaxel



Vincristine

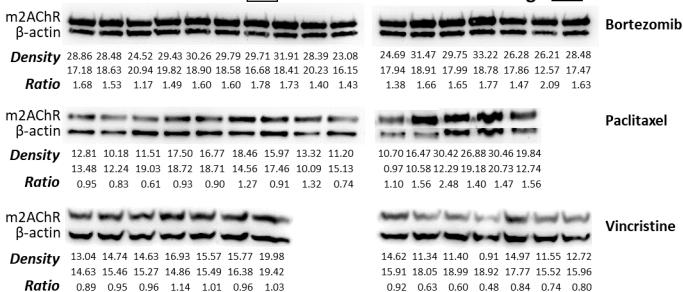


m2AChR / β -actin
ratio

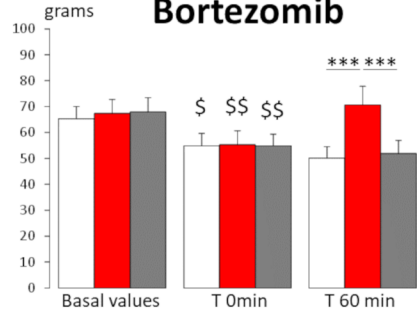


Controls

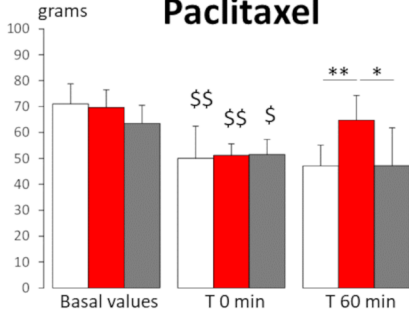
Anticancer drugs



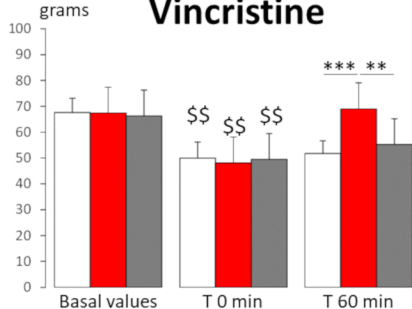
Bortezomib



Paclitaxel

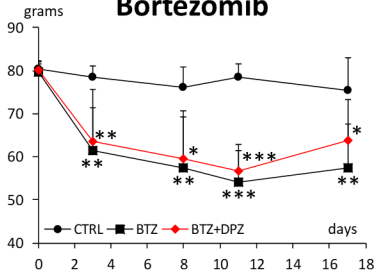


Vincristine

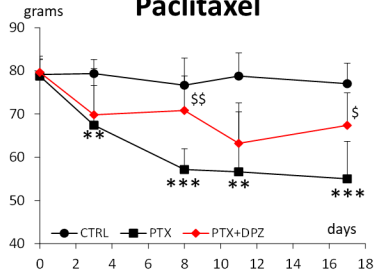


□ Vehicle ■ Donepezil ■ Donepezil + methoctramine

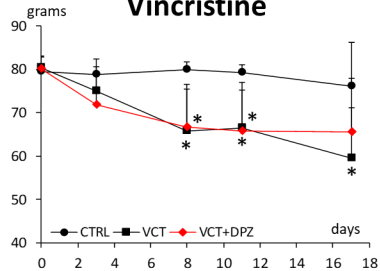
Bortezomib

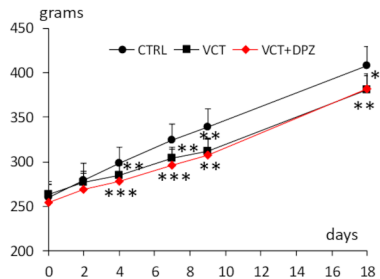
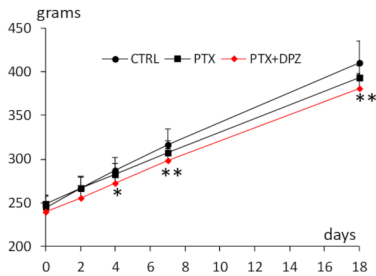
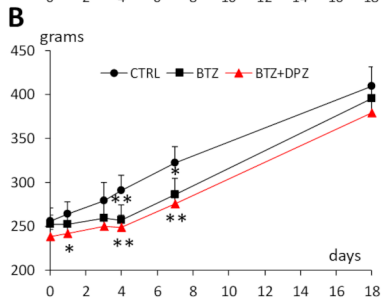
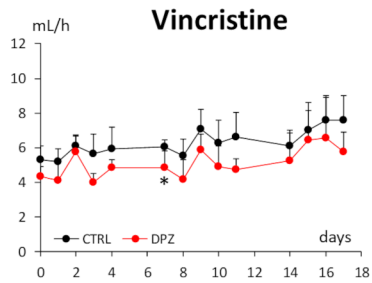
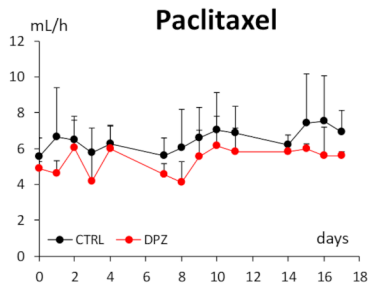
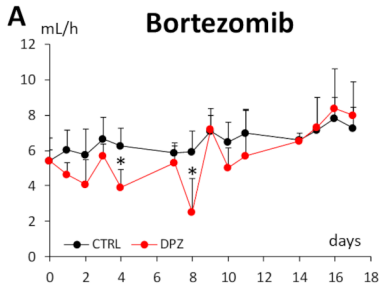


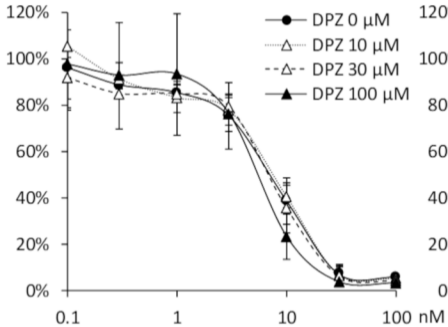
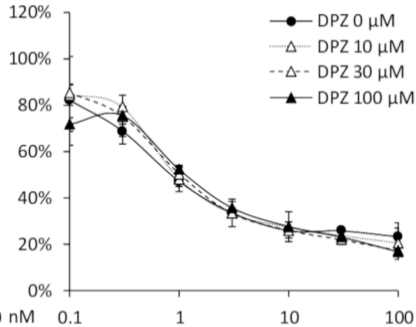
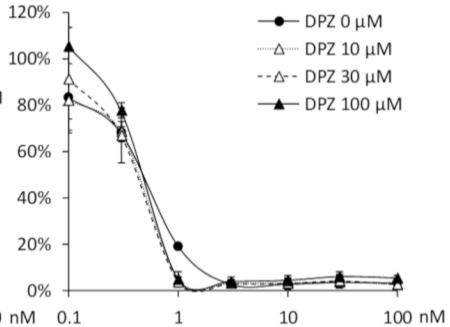
Paclitaxel



Vincristine





LP1 cells**Bortezomib****MDA cells****Paclitaxel****HL60 cells****Vincristine**