Characterization of and Protection from Neurotoxicity Induced by Oxaliplatin, Bortezomib and Epothilone-B

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Abstract. *Aim: To characterize neurotoxicity induced by oxaliplatin, bortezomib, and epothilone-B as well as protection against their neurotoxicity using an in vitro model. Materials and Methods: Neurotoxicity was evaluated using the neurite outgrowth method in PC12 rat pheochromocytoma cells differentiated towards a mature neuronal phenotype, while neuroprotection was explored by simultaneous exposure to 0.5 mM amifostine. The potential markers of neuronal differentiation, cyclin-B2 (Ccnb2) and baculoviral inhibitor of apoptosis repeat-containing 5 (Birc5), were evaluated by quantitative reverse transcription polymerase chain reaction (RT-PCR). Results: Bortezomib, epothilone-B, and oxaliplatin reduced neurite length to 68%, 78% and 66%, respectively ($p<0.05$). The percentage of neurite-forming-cells (discriminating neurotoxicity from general cytotoxicity) decreased from 70% (control) to 55% (bortezomib), 46% (epothilone-B), and 51% (oxaliplatin). Amifostine was neuroprotective against oxaliplatin-induced neurotoxicity, increasing both neurite length and neurite-forming-cells. Quantitative-RT-PCR showed a 2.7-fold decrease in Ccnb2 expression in differentiated PC12 vs. undifferentiated cells. Conclusion: Oxaliplatin, bortezomib, and epothilone-B are neurotoxic in the PC12 model. Amifostine has a neuroprotective effect only against oxaliplatin-induced neurotoxicity, suggesting that these compounds have different mechanisms of neurotoxicity.*

Chemotherapy-induced peripheral neurotoxicity (CIPN) represents a clinically relevant problem in anticancer therapy. CIPN mainly presents with sensory symptoms, and is usually dose-dependent. It typically develops after a cumulative dose, even if acute toxic neuropathy is more common with some drugs (*e.g.* oxaliplatin and taxanes) (1). This side-effect might be dose-limiting or even lead to treatment withdrawal. However, even when neurotoxicity is not dose-limiting, it is an important side-effect that can irreversibly impair the patients' quality of life by causing chronic discomfort (2-5). Not only established anticancer drugs, such as platinum compounds and taxanes, but also novel compounds, such as epothilones and bortezomib, have been reported to be neurotoxic, although the mechanisms of toxicity are likely to be different (6-8).

Bortezomib is a proteasome inhibitor registered for the treatment of multiple myeloma and mantle cell myeloma (9, 10), and is also tested against other diseases, usually in combination with platinated compounds (11). Peripheral neuropathy is among the most frequently-observed toxicities necessitating dose reductions or treatment discontinuation (11). At least one-third of patients under treatment have evidence of clinical sensory peripheral neuropathy (11-13). Although, it is not clear how and where bortezomib affects the peripheral nervous system, treatment withdrawal can result in clinical improvement of the induced neurotoxicity. As a result, further studies are essential in order to define the mechanism of this toxic effect, both alone and in combination with platinated compounds.

Epothilone-B is another new chemotherapeutic agent causing peripheral neurotoxicity due to its interaction with microtubule polymerisation inducing arrest in the G_2/M

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transition (14), similarly to taxanes. Nevertheless, epothilones and taxanes differ in terms of resistance conferred by specific point-mutations in the gene encoding β-tubulin (15-17). Epothilones also induce dose-limiting peripheral neurotoxicity (18). Despite the fact that sensory peripheral neurotoxicity is regarded as a main toxic effect on the nervous system, motor or (rarely) autonomic neuropathy has occasionally been reported. Although no pathological data are available on epothilone-treated patients, dose-dependent axonal damage was demonstrated in animal models, in which recovery was obtained within few weeks after treatment withdrawal (19).

Oxaliplatin is a platinum-based compound with high efficacy in colorectal cancer, when administered in combination with 5-fluorouracil and folinic acid. Oxaliplatin exerts its cytotoxic effects through the formation of DNA adducts, with subsequent impairment of DNA replication and transcription, and causes severe and disabling sensory peripheral neurotoxicity due to accumulation of the drug in the dorsal root ganglia (DRG), where sensory neurons are located (20, 21).

The use of neuroprotective agents may help reduce neurotoxicity caused by chemotherapeutic agents, thus allowing for intensification of chemotherapy. One potential strategy in neuroprotection is the use of detoxicants such as amifostine. Amifostine is a phosphorylated amino-thiol pro-drug, which needs to be de-phosphorylated in order to exert its protective effect, which is selective in normal cells. Amifostine has been postulated to be selectively protective against normal tissue without reducing its antitumor activity (22-24). Amifostine is able to provide protection from the toxic effect of cisplatin in peripheral nerves (25-27).

Few pre-clinical models have been used to study the neurotoxic effects of novel chemotherapeutic drugs and neuroprotective agents (6, 28). Hence, the aims of the present study were: i) to characterize the extent of neurotoxicity induced by bortezomib, epothilone-B and oxaliplatin using the rat PC12 pheochromocytoma cell line to investigate the neurotoxic effects of different drugs; ii) to test the potential role of amifostine in the prevention of chemotherapy-induced neurotoxicity. In this model, neurotoxicity was assessed by standard quantitative morphological methods, including the counting of cells exhibiting neurites and the measurement of neurite length (28). Moreover, we evaluated the potential role of cyclin-B2 (*Ccnb2*) and baculoviral inhibitor of apoptosis repeat-containing 5 (*Birc5*) as surrogate biomarkers of neuronal differentiation using quantitative RT-PCR.

Materials and Methods

Drugs and chemicals. Bortezomib was a gift from Millenium Pharmaceuticals, Inc. (Johnson & Johnson, Cambridge, MA, USA), while oxaliplatin was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and epothilone-B from Calbiochem (Calbiochem, Darmstadt, Germany). Amifostine (S-2-(3-aminopropylamino)-ethylphosphorothioic acid, WR2721, Ethyol1) was obtained from USB Pharma (Nijmegen, the Netherlands). Epothilone-B and bortezomib were dissolved in dimethyl sulfoxide (DMSO), whereas oxaliplatin and amifostine were dissolved in sterile water and phosphate buffered saline (PBS), respectively. The drugs were diluted in culture medium before use. RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were from Gibco (Gaithersburg, MD, USA). All other chemicals were from Sigma.

Cell culture. PC12 rat pheocromocytoma cells (29), were cultured in RPMI (containing 2 mM L-glutamine) supplemented with 10% heat-inactivated FBS, 1 mM sodium pyruvate, insulin-transferrin-sodium selenite medium supplement, penicillin (50 IU/ml) and streptomycin (50 μg/ml). Cells were maintained as monolayer cultures in 75 cm^2 culture flasks (Costar, Cambridge, MA, USA), at 37˚C in 5% CO_2 and 95% air, and harvested with trypsin-EDTA when they were in exponential growth.

Cytotoxicity studies. The growth inhibition caused by bortezomib, epothilone-B and oxaliplatin on PC12 cells was evaluated with the sulforhodamine-B (SRB) assay (30, 31). For this purpose, cells were plated at a density of 20,000 cells/well in 96-well flat-bottom plates (Costar). Twenty-four hours later (day-0) cells were treated using different concentration ranges (0.0001-0.5 μM bortezomib, 0.0001-0.5 μM epothilone-B, and 0.05-100 μM oxaliplatin). The drug exposure time was 72 h (day 3). The IC_{50} and the IC_{80} were the drug concentrations at which cell-growth was inhibited to 50% and 80%, respectively, based on the difference of absorbance values on day 0 and day 3 of drug exposure, as calculated by the sigmoid inhibition model (GraphPad PRISM version 4.0; Intuitive Software for Science, San Diego, CA, USA).

Neurotoxicity and PC12-neurite outgrowth assay. The neurotoxic effects of bortezomib, epothiolone-B and oxaliplatin were investigated using the nerve growth factor (NGF)-induced neurite outgrowth assay, as previously described (29, 32). Briefly, undifferentiated PC12 cells were pre-treated for five days by adding NGF 2.5S (NGF from mouse source; Promega, Madison, WI, USA) to a final concentration of 50 ng/ml, trypsinized, washed using PBS, and plated in 60-mm culture dishes (Costar) at a density of 25,000 cells/dish. Optimal adherence and neurite formation required precoating the plastic culture well using 0.5 mg/ml water solution of polylysine-hydrobromide (Sigma) followed by washing with sterile water. After 48 hours of culture with 10 ng/ml NGF, differentiated cells were exposed to bortezomib, epothilone-B or oxaliplatin at IC_{50} concentrations (0.95 nM bortezomib, 0.75 nM epothilone-B, 0.07 μM oxaliplatin). No drug was added to control dishes.

After 72 h of culture, the interference of cytostatic compounds on neurite outgrowth was evaluated by measuring the neurite length with an automatic image analyser (Quantimet, Leica, Cambridge, UK) on at least 200 randomly selected cells, and the percentage of differentiated cells per culture was calculated (33). For this purpose, cells were considered differentiated when the lengths of their neurite were longer than one-fold the cell body length (34). The morphometric determinations were always performed by the same examiner who was blinded to the treatment of the cell culture under observation. To discriminate specific neurotoxicity from general cytotoxicity, the analysis was performed on the basis of the fraction

of differentiated cells instead of the absolute number of neurite-forming cells. The results, therefore, are given as the percentage of differentiated cells.

In order to evaluate the neuroprotective effect of amifostine, co-incubation experiments were carried out as follows. Differentiated PC12 cells were incubated with 0.5 mM amifostine and 2.7 nM bortezomib, 1.8 nM epothilone-B or 0.2 μM oxaliplatin. After 72 hours of culture cells were scored for neurites.

Quantitative-RT-PCR. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA was dissolved in RNase free-water, and measured at 260 nm. RNA (1 μg) was reverse-transcribed at 37˚C for one hour in 100-μl reaction volume containing 0.8 mM dNTPs, 200 U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT), 40 U of RNase inhibitor, and 0.05 μg/ml of random primers. The cDNA was amplified by quantitative-PCR with the Applied Biosystems 7500HT sequence detection system (Applied Biosystems, Foster City, CA, USA). PCR reactions were performed in triplicate using 5 μl of cDNA, 12.5 μl of TaqMan Universal PCR Master Mix, 2.5 μl of probe and 2.5 μl of forward and reverse primers in a final volume of 25 μl. Samples were amplified using the following thermal profile: an initial incubation at 50˚C for 5 min, to prevent the re-amplification of carryover-PCR products by AmpErase uracil-N-glycosylase (UNG), followed by incubation at 95˚C for 10 min, to suppress AmpErase UNG activity and denature the DNA, 40 cycles of denaturation at 95˚C for 15 sec followed by annealing and extension at 60˚ for 1 min. Forward and reverse primers and probes for rat *Ccnb2* (Rn01530826_g1) and rat glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*, Rn99999916_s1) were obtained from TaqMan® Gene Expression Assays (Applied Biosystems), while forward and reverse primers and probes for rat *Birc5* were obtained as custom TaqMan® Gene Expression Assays (Applied Biosystems), using the File Builder version 2.0 software, on the basis of GenBank database.

Amplification data were normalized to the housekeeping gene *Gapdh*. Quantification of gene expression was performed using standard curves obtained with dilutions of rat cDNA from a mix of PC-12, BCLO, Bara-C and CC531 rat cell lines, and with dilutions of cDNA obtained from quantitative-PCR Human Reference Total RNA (Stratagene, La Jolla, CA, USA). PCR efficiencies were between 96.4 and 100%.

Statistical evaluation. All experiments were performed in triplicate and repeated at least three times. Data are expressed as mean values±SD and were analysed by ANOVA followed by the Tukey's multiple comparison. Differences were considered significant when $p<0.05$.

Results

Cytotoxicity experiments. A concentration-dependent inhibition of cell growth was observed for PC12 cells. PC12 Cells were equally sensitive to bortezomib and epothilone-B (IC_{50} values of 0.9 and 0.7 nM, respectively). Oxaliplatin had a higher IC_{50} (70 nM). The IC_{50} of the single drugs was chosen to evaluate the neurotoxic effects, while the IC_{80} was used to evaluate the neuroprotective role of amifostine.

In vitro neurotoxicity and protective role of amifostine. The standard neurotoxicity assay measuring NGF-dependent neurite outgrowth from the PC12 pheocromocytoma cell line was used to predict neurotoxicity after 72 h incubation with bortezomib, epothilone-B, and oxaliplatin. Drug treatment of differentiated PC12 cells resulted in a statistically significant decrease of neurite length with respect to control cells. Bortezomib, epothilone-B, and oxaliplatin significantly reduced the neurite length to 68±5, 78±3, and 66±4%, respectively, compared to controls (Figure 1A). The percentages of cells expressing neurites, which were used to discriminate neurotoxicity from general cytotoxicity, also decreased significantly from ±3% in the control to 55±2% in bortezomib–, 49±3% in epothilone-B–, and 51±3% in oxaliplatin-treated cells (Figure 1B).

To investigate whether amifostine was able to prevent neurotoxicity induced by bortezomib, epothilone-B, and oxaliplatin in PC12 cells, the neurite length was also measured in PC12 cells exposed to amifostine for 72 h together with the single drugs, at their IC_{80}. Amifostine partially reversed oxaliplatin-dependent neurotoxicity by reducing the shortening of neurite length from 59±3% (oxaliplatin alone) to 74±3% (oxaliplatin with amifostine). The percentage of neurite-forming cells increased from 42±5% to 59±3% for the combination. On the contrary, amifostine did not protect against the decrease of neurite length nor the percentage of neurite-forming cells induced by bortezomib and epothilone-B treatments (Figure 2A and B).

Modulation of Ccnb2 and Birc5 mRNA by bortezomib, epothilone-B, and oxaliplatin. Quantification of neurite outgrowth is the standard method to investigate neurotoxicity *in vitro*, but it is impractical on a large scale. For this reason, we examined changes in *Ccnb2* and *Birc5* mRNA expression in PC12 cells as potential biomarkers of neuronal differentiation. After differentiation towards a neuronal phenotype, PC12 cells showed a 1.6-fold decrease in *Ccnb2* expression compared to the undifferentiated control cells (Figure 3). *Birc5* mRNA expression did not change (data not shown). The most neurotoxic drug in the neurite outgrowth assay, oxaliplatin, led to a 2.0-fold decrease in *Ccnb2* expression at its IC_{50} (Figure 4), while bortezomib and epothilone-B induced a slighter modulation of *Ccnb2*.

Discussion

In the present study, the neurotoxic effects of bortezomib, epothilone-B and oxaliplatin were successfully evaluated in PC12 rat pheocromocytoma cells after neuronal differentiation. In agreement with clinical data, neurotoxicity induced by bortezomib, epothilone-B and oxaliplatin has been shown in our model, by reducing both neurite length and the percentage of neurite-forming cells. We also demonstrated the potential role of the expression of *Ccnb2* mRNA as biomarkers of neuronal differentiation in PC12 rat cells. Moreover, amifostine protected against oxaliplatin-

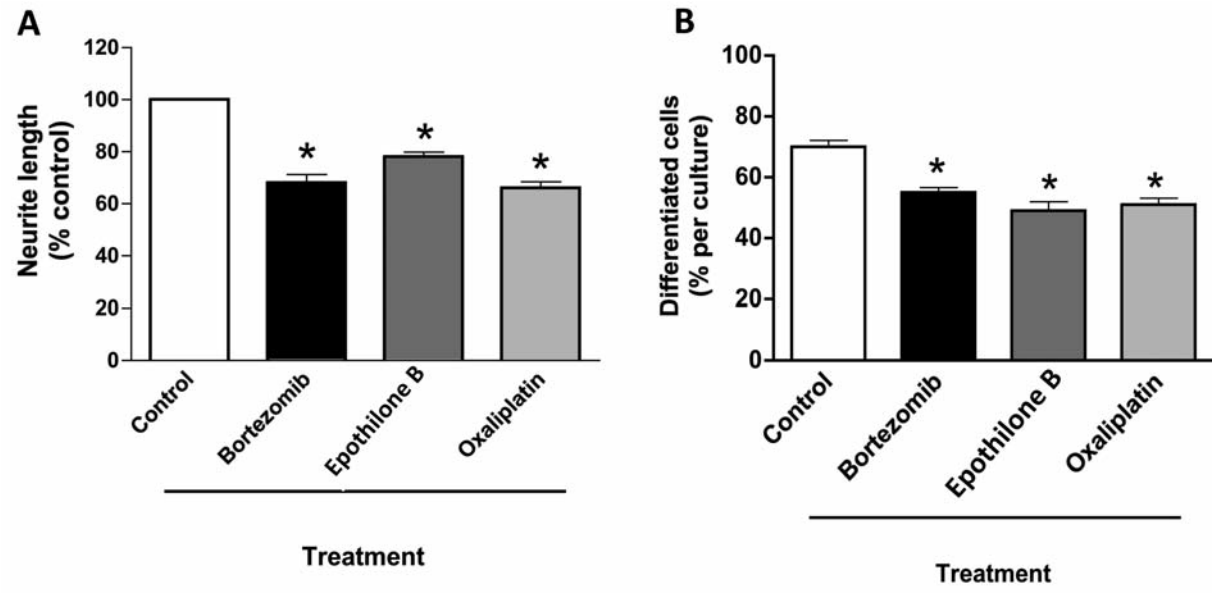

Figure 1. *Effect of bortezomib, epothilone-B and oxaliplatin on neurite length and cell differentiation in PC12 cells. Nerve growth factor (NGF)-differentiated PC12 cells were exposed to half-maximal inhibitory concentrations (IC_{50s}) of bortezomib, epothilone-B and oxaliplatin for 72 h. The neurite length values (a) and the percentage of differentiated cells per culture (b) were calculated as the percentage of values obtained in control cells. Columns, mean values obtained from three independent experiments; bars, SD. *Significantly different from control cells (p<0.001).*

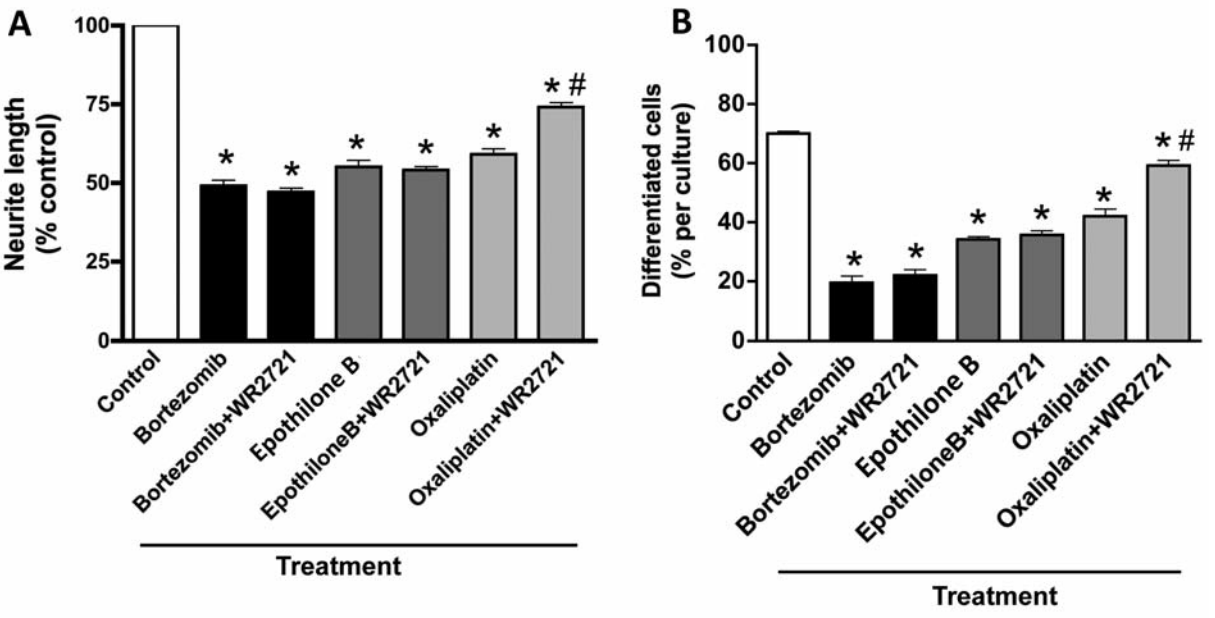

Figure 2. *Effect of amifostine (WR2721) on bortezomib, epothilone-B and oxaliplatin PC12 neurotoxicity in in vitro. Nerve growth factor (NGF)-differentiated PC12 cells were exposed to 80% maximal-inhibitory concentrations (IC_{80s}) of bortezomib, epothilone-B and oxaliplatin alone or in combination with amifostine (0.5 mM) for 72 h. The neurite length values (a) and the percentage of differentiated cells (b) were calculated as the percentage of values obtained in control cells. Columns, mean values obtained from three independent experiments; bars, SD. Significantly different from *control cells (p<0.001), #oxaliplatin-treated cells (p<0.01).*

induced neuropathy with no adverse effect on treatment efficacy, but not against bortezomib and epothilone-B, suggesting a different mechanism of neurotoxicity.

Bortezomib-induced neurotoxicity was mainly characterized in rat models by axonopathy of unmyelinated fibres in nerves and pathological alteration in DRG satellite cells, while DRG

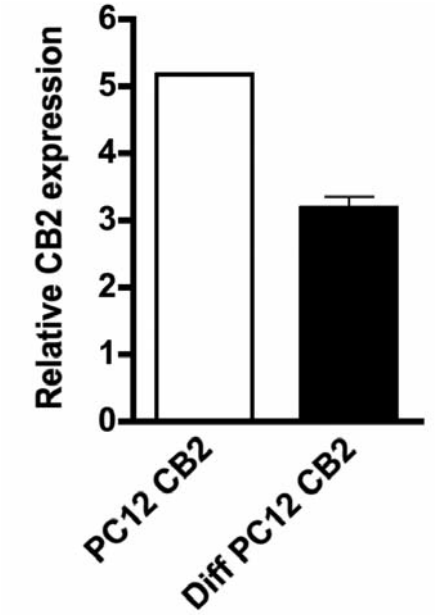

Figure 3. *Effect of differentiation on cyclin B2 (CB2) level in PC12 cells. Modulation of CB2 expression in PC12 cells, as determined by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). Columns, mean values obtained from three independent experiments calculated in comparison with standard curves and with respect to the respective expression values of the housekeeping gene Gapdh; bars, SE.*

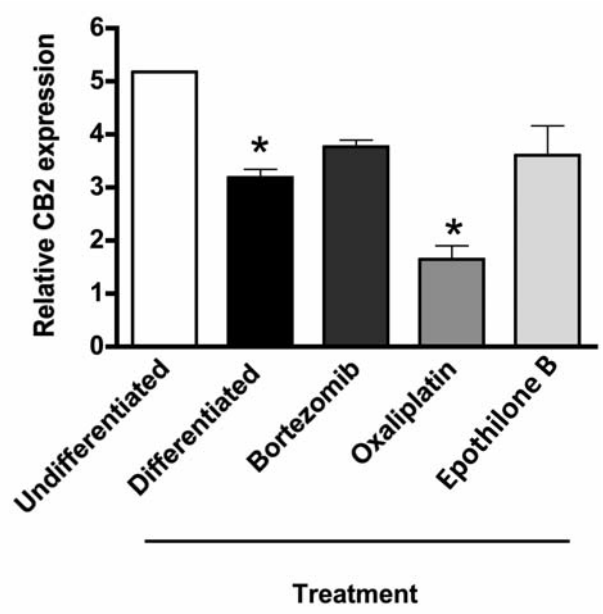

Figure 4. *Effects of bortezomib, epothilone-B and oxaliplatin on cyclin-B2 levels in PC12 cells. Modulation of cyclin-B2 (CB2) expression as determined by real-time reverse transcription polymerase chain reaction (RT-PCR). CB2 expression was studied in nerve growth factor (NGF)-differentiated PC12 cells treated for 72 h with bortezomib, epothilone-B and oxaliplatin at their half-maximal inhibitory concentrations (IC_{50s}) with respect to undifferentiated and NGF-differentiated PC12 cells. Columns, mean values obtained from three independent experiments calculated in comparison with standard curves and with respect to the respective expression values of the housekeeping gene Gadph; bars, SE. Significantly different from *undifferentiated control cells ($p<0.05$);*

neuron degeneration was observed only in mice (28, 35). Epothilone-B effects have not been studied extensively in pre-clinical settings. It is assumed that epothilone-B reduces the nerve conduction velocity (NCV), although no correlation has been found between the concentrations of the drug in tissues with NCV changes (35). Based on two studies, sensory neuropathy induced by ixabepilone (epothilone-B analog) varied from 3 to 59% (37, 38). Oxaliplatin-induced neurotoxicity is characterized by cumulative sensory neurotoxicity due to increased neuronal excitability by alteration of the voltage-gated sodium channels through chelation of calcium by the oxaliplatin metabolite (39). Further characterization of this induced neurotoxicity is crucial in order to find the most effective neuroprotective treatment and minimize the incidence and consequences of such a serious side-effect. Some agents such as vitamin E, carbamazepine, calcium and magnesium infusion, reduced glutathione, N-acetylcysteine and amifostine are considered as neuroprotective treatments, but they have not yet proven to be completely effective (1).

In the present work, we evaluated the potential neuroprotective effect of amifostine on bortezomib, epothilone-B, and oxaliplatin. Oxaliplatin-induced neurotoxicity was partially protected, by restoring neurite length and neurite-forming cells between 35-50% of the neurotoxicity induced by oxaliplatin alone. Importantly, in a clinical trial performed by Lu and colleagues, amifostine reduced the peripheral neurotoxicity due to oxaliplatin by about 60%, resulting in about 20% increase in the number of patients who received multiple chemotherapy cycles (40). The results obtained in our experimental setting thus confirm the reliability of the morphological method used in this study to test the potential neuroprotective activity of different drugs.

In order to evaluate potential biomarkers for neurite outgrowth, we also measured the mRNA expression of *Ccnb2* and *Birc5*. Decreased levels of *Ccnb2* and *Birc5* have been correlated with neurite outgrowth induced by NGF in SH-SY5Y HN cells (41). Accordingly, our studies showed a significant reduction in Ccnb2 expression in NGF differentiated PC12 cells with respect to the control. Conversely, *Birc5* mRNA level was not down-regulated following differentiation. *Ccnb2* is an important regulator of the cell cycle and it associates with cyclin-dependent kinase 1 as its positive regulatory subunit. The decrease in *Ccnb2* expression observed in PC12 cells following NGF differentiation is in accordance with previous data obtained

in the same cellular model. These data suggested that the decrease in *Ccnb2* expression after treatment with agents inducing neuronal differentiation could lead to cyclin-dependent kinase 1 inactivation with a consequent increase in neurite outgrowth (42, 43). The observed effect of oxaliplatin is probably due to reduction of cyclin-dependent kinase 1 activation induced by the antineoplastic drug with a consequent decrease in *Ccnb2* expression, as observed in HCT116 colorectal cancer cells treated with oxaliplatin (44).

In conclusion, the experimental methods used in the present *in vitro* study, including a PCR analysis for *Ccnb2* as a surrogate biomarker of neuronal differentiation, showed similar neurotoxic effects, especially for oxaliplatin. Moreover, we demonstrated that amifostine partially protects against oxaliplatin-induced neurotoxicity. These results prompt future pre-clinical and translational studies to test neurotoxicity of novel platinated compounds, as well as new neuroprotective agents, before starting the treatment.

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