Valproate Protective Effects on Cisplatin-induced Peripheral Neuropathy: An *In Vitro* and *In Vivo* Study

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Abstract. *Background: Antineoplastic drugs, such as cisplatin (CDDP), induce disabling peripheral neuropathies, representing a hindrance to effective cancer treatments. The exact pathogenesis of CDDP-induced neuropathy is not yet understood, and the dysregulation of gene expression has been proposed. Valproate (VPA) is an antiepileptic drug recently discovered to remodel gene expression, with hypothetically putative neuroprotective effects. Materials and Methods: VPA was tested in both, in vitro and in vivo models of CDDP-neurotoxicity. Results: VPA administered in combination with CDDP promoted dorsal root ganglia (DRG) neurons survival. Moreover, this treatment induced in Wistar rats an improvement of body weight, sensory nerve conduction velocity, and DRG morphometric analysis. In contrast, VPA was not able to rescue CDDP pre-treated rats. Conclusion: When used in combination with CDDP, VPA displays a protective action against neuropathy, in our models, suggesting possible future clinical applications.*

Antineoplastic drugs such as those belonging to the platinum or taxane family are severely neurotoxic, inducing the onset of disabling peripheral neuropathies mainly affecting deep and vibratory sensations (1, 2). Unfortunately, these side-effects represent a serious hindrance to an effective treatment of the underlying cancer. The pathogenesis of antineoplastic drug-induced peripheral neuropathies is still poorly understood, although recent reports have led to the formulation of the hypothesis of a putative alteration in the regulation of the gene expression rate at the level of the peripheral nerve cells (3-5).

Acetyl-L-carnitine (ALC) has been recently reported to prevent, and also reverse, peripheral neuropathy in taxane and cisplatin animal models, without interfering with the antitumoral activity of the antineoplastic drugs (6). ALC is a member of the family of carnitines, a naturally occurring compound that has an essential role in intermediary metabolism (7, 8). Recently, some clinical studies on patients with neuropathies of different origin such as chronic diabetes, antiretroviral toxic neuropathy or paclitaxel- and cisplatin-induced peripheral neuropathies have seemed to confirm the neuroprotective action of ALC suggested by the animal model experimental data (9-11). In fact, several results from different experimental paradigms have suggested that ALC treatment promotes peripheral nerve regeneration and has neuroprotective activity (11-14). These effects can be attributed to the ALC metabolic role and it has also been demonstrated *in vitro* that ALC plays a role in histone acetylation and consequently in facilitating gene expression (6, 15). Notably, histone acetylation represents a major mechanism of regulation of the continuous and dynamic transition of the chromatin conformation, resulting in DNA topology relaxation and increased accessibility of transcription factors, which in turn determines an enhancement of the rate of production of specific genes (16). Histone acetylation is strictly regulated by two enzymes with opposite functions, histone acetyl transferase (HAT) and histone deacetylase (HDAC). In recent years, numerous compounds able to inhibit the action of HDAC have been discovered, such as valproic acid (VPA), a well-known and well-tolerated drug which is already extensively used in neuropsychiatry (17, 18), and for which pharmacokinetic and pharmacodynamic data are available.

VPA is a major antiepileptic drug, with demonstrated effectiveness and reasonably low toxicity profile, and it is used to ameliorate the symptoms of painful neuropathies (19, 20). Among the possible mechanisms of action of VPA, a direct effect on the epigenome had been documented (18, 21), with very interesting putative clinical applications besides its use as an antiepileptic agent. In fact, the hyperacetylation of histones has been regarded as a promising anticancer drug strategy, and it is now well-

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established that histone deacetylase inhibitors (HDACi) display the ability to affect several cellular processes which are dysregulated in neoplastic cells (22-25).

Based on this background, and considering its additional ability to act as antineoplastic agent (26, 27), the neuroprotective efficacy of VPA was determined. In fact, with respect to other nowadays available neuroprotective agents, VPA might offer the advantage of targeting not only the peripheral neuropathy, but also the underlying neoplastic disorder, possibly acting synergistically with the main anticancer treatment.

Materials and Methods

Drugs. For the *in vitro* studies, the cisplatin (cis-diammine-dichloroplatinum II, CDDP, Sigma-Aldrich, St. Louis, MO, USA) concentrated solution of 1 mg/ml was made fresh every time in sterile saline solution and diluted to a final concentration of 10 to 5 μg/ml (primary and organotipic cultures, respectively). The antineoplastic drug concentrations used in these experiments were chosen based on the available literature data (28, 29) and preliminary experiments.

The sodium valproate (VPA, Sigma-Aldrich) concentrated solution (1 mg/ml) was made fresh every time in sterile distilled water. The final concentration of this drug was chosen based on the outcome of the preliminary toxicity curves (data not shown), according to the experimental model (see below). The range of concentrations tested in these preliminary experiments was chosen considering the HDAC inhibition potency (IC_{50}: 0.4 mM) of this drug (30).

Rat dorsal root ganglia (DRG) primary cultures. The DRG from Sprague-Dawley rat embryos of embryonic day 15 (E15) were removed and trypsin dissociated. The DRG cells were then cultured for 5 days in collagenated Petri dishes with AN_2 medium (MEM, plus 15% bovine calf serum, 50 μg/ml ascorbic acid, 1.4 mM L-glutamine, 0.6% glucose) supplemented with 5 ng/ml nerve growth factor (NGF) and 10^{-5} M of fluorodeoxyuridine (Fudr) in order to remove satellite cells. The DRG neurons were then incubated with AN_2 medium with 5 ng/ml of NGF for one day before the various treatments. Following drug incubation, the neurons in the same area of each culture dish were photographed at different time-points (0, 24 and 48 hours) and then counted using Image J 1.37v software (National Institutes of Health, Rockville Pike, Bethesda, MD, USA). The neuronal survival rate was calculated as the ratio between the number of neurons at 48 hours, and the number at time-point 0. Each treatment group included three dishes. The VPA was used at a final concentration of 5 and 10 mM.

Rat DRG organotipic cultures. The DRG from Sprague-Dawley rat embryos (E15) were removed and carefully placed in collagenated 35x10 mm Petri dishes with 0.5 ml of AN_2 medium supplemented with 5 ng/ml of NGF for 2 hours, to allow ganglia adhesion to the dish. The different compounds were added to obtain the final concentration in a volume of 1-ml per dish of AN_2 medium supplemented with 5 ng/ml of NGF and 10^{-5} M Fudr in order to inhibit the proliferation of satellite cells. The ganglia of each culture dish were photographed after 24 and 48 hours. The length of the longest neurite of each ganglion was measured using Image J 1.37v software (National Institutes of Health, USA). Each treatment group included four dishes with four ganglia per dish. The VPA was used at a final concentration of 1 and 5 mM.

In vivo experiments. To induce neuropathy 200-g female Wistar rats were used. CDDP was administered by *i.p.* injection (1 ml in saline), at a concentration of 2 mg/kg, twice a week, as previously described (2, 31). The dose of VPA, 300 mg/kg/day orally administered (1.5 ml in distilled water), was selected according to literature data (32-35). Vehicle groups were treated with saline solution.

In Study 1 the drugs were co-administered for four weeks and in Study 2 CDDP neuropathy induction (four weeks) was followed by CDDP withdrawal and VPA treatment (two weeks) and then a follow-up period of two weeks without any drug administration. For each protocol a control group was included. Eight animals were used per group and body weight was assessed twice a week. At the end of the experiments the animals were sacrificed by CO_2 inhalation followed by cervical dislocation.

Sensory nerve conduction velocity (SNCV). The sensory nerve conduction velocity (SNCV) was determined for each animal at the level of the tail, as previously described (36-37), at the end of each treatment period. The antidromic SNCV in the tail nerve was assessed by placing recording ring electrodes distally in the tail, while the stimulating ring electrodes were placed 5 cm and 10 cm proximally of the recording point. The latencies of the potentials recorded at the two sites after nerve stimulation were determined (peak-to-peak) and nerve conduction velocity was calculated accordingly (see 38). All the neurophysiological determinations were performed under standard conditions in a temperature-controlled room (22±2˚C).

Sampling and histological processing of tissues. The DRG samples were obtained from three rats for each group. A few samples were frozen in liquid nitrogen for Western blotting and the rest of the samples were fixed by immersion in 4% paraformaldehyde/2% glutaraldehyde for at least two hours at room temperature (RT) for morphometry studies.

Western blotting. The frozen ganglia were homogenized in Tripure Isolation Reagent (Roche Applied Science, Indianapolis, IN, USA), and the proteins purified according to the manufacturer's instructions. The extracts were then separated overnight by 15% SDS-PAGE and blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Following blocking with 3% non-fat dry milk in 10 mM PBS, pH 7.4/0.1% TWEEN® 20 (Sigma-Aldrich), the membrane blots were incubated overnight at 4˚C with an anti-acetyl-histone H3 (1:5, 000, Upstate Biotechnology, Billerica, MA, USA). After incubation (1 h/RT) with a peroxidase-conjugated goat anti-rabbit secondary Ab (1:1, 000, Chemicon Int., Temecula, CA, USA), the immunoreactive bands were detected using the LiteAblot® chemiluminescent substrate (Euroclone S.p.A., Lugano Italy). For the comparative measurement of the amount of protein applied to each gel, the intensity of actin immunoreactivity was determined in the same blots by incubating with an anti-actin Ab (1:1, 000, 1 h/RT,

Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by an HRP-conjugated donkey anti-mouse secondary Ab (1:500, 1 h/RT, Santa Cruz Biotechnology). Quantification was performed by the Kodak 1D image analysis software Gel Logic (Kodak Scientific Imaging System, New Haven, CT, USA), and the obtained values of acetylated histone H3-like immunoreactivity (~17 kDa) normalized to those of actin-like immunoreactivity (~42.5 kDa).

DRG morphometric analysis. The aldehyde fixed DRG were washed in 0.12 M phosphate buffer solution, postfixed in 1% OsO_4 and, after alcohol dehydration, gradually embedded in epoxy resin. Thin sections were cut with an Reichert-Jung Ultracut E (Leica, Vienna, Austria). Color images of 3-5 ganglia sections (1 μm thick) were captured by a CoolScope microscope (Nikon Instruments S.p.A., Firenze, Italy). The areas of soma, nucleus and nucleolus of at least 200 neurons for each sample, displaying clearly differentiable cell compartments, were measured using the Image J 1.37v software (National Institutes of Health, USA) under blind conditions.

Statistical analysis. All the results were expressed as mean±SEM. One-way ANOVA, followed by the Student-Newman-Keuls Test, were used to assess the significance of the differences between groups. Due to the fact that the neuronal population of the rat DRG follows a bimodal distribution, morphometric data analysis was performed by non-parametric tests. The criteria of significance ($p<0.05$ or 0.01) are indicated in the figure legends.

Results

The survival of rat DRG primary cultures. At the highest concentration used (10 mM), VPA alone did not exert any effect on rat DRG primary cultures (mortality in both vehicle- and VPA-treated cells ~13%, see Figure 1). The CDDP treatment induced a significant reduction of cell survival (~50%, $p<0.002$ *vs.* vehicle and 10 mM VPA). VPA administered in combination with CDDP was effective in protecting from the neurotoxic action of the antineoplastic drug, promoting cell survival at both concentrations used ($p<0.002$ *vs.* CDDP; Figure 1); moreover, a slight, albeit significant, dose-dependent effect for VPA was evident (10 mM VPA/CDDP *vs.* 5 mM VPA/CDDP, $p=0.015$).

The neurite growth of rat DRG organotipic cultures. CDDP induced a reduction of neurite growth in the cultured rat DRG explants, which was more evident after 48 hours of treatment (~45%, $p<0.001$ *vs.* vehicle/48-h, see Figure 2). The VPA treatment (tested alone at the dose of 5 mM), reduced the growth of the neurites in our model to a similar extent to CDDP, displaying a significant dose-dependent synergistic effect when used in combination with the antineoplastic drug (~70% VPA 1 mM/CDDP *vs.* vehicle/48-h, $p<0.001$; ~100% VPA 5 mM/CDDP *vs.* vehicle/48-h, $p<0.001$; Figure 2).

Table I. *Morphometric results obtained from DRG of control and treated rats.*

	Cell area	Nuclear area	Nucleolar area
Control group	897.3±23	132.7±2.7	8.3±0.2
CDDP	798.7±22*	124.3±2.4*	7.1±0.2*
VPA	986.3±22	139.3±2.5	8.6±0.2
CDDP/VPA	909.4±24	134.5±2.6	8.1±0.2

Morphometric analysis of DRG neurons of rats treated with CDDP (2 mg/kg), VPA (300 mg/kg), and the combination of the two drugs for four weeks (Study 1). Values (mean±SEM) are expressed in μm^2. Kruskal-Wallis $p<0.001$ for cell and nucleolar areas, and $p<0.002$ for nuclear areas, followed by Student-Newman-Keuls *post hoc* test, *$p<0.03$ vs. all the other groups; n=3.

In vivo experiments.

Study 1 – VPA and CDDP co-administration. After two weeks of CDDP systemic administration the treated animals already displayed a reduction of body weight ($p<0.01$; Figure 3), which became more evident at the end of the four weeks of treatment ($p<0.001$). An increase of body weight with respect to baseline was shown for the vehicle- and VPA-treated groups, and, although less evident, also for the group treated with the combination of CDDP and VPA.

At the end of the four weeks of treatment, the SNCV assessed at the level of the tail was reduced by ~40% in the CDDP-treated animals with respect to the other groups, while VPA alone did not affect this parameter (Figure 4). The animals treated with the combination of the two drugs displayed higher values of the tail SNCV than the CDDP-treated rats (~75% of vehicle-treated animals, $p<0.05$; Figure 4).

In the CDDP-treated animals, the morphometric analysis of the DRG explants documented a decrease of both cell and nucleolus areas ($p<0.03$; Table I), with a less important reduction of the nuclear area as well, but no modifications were shown in the VPA-treated rats. The animals treated with the combination of the two drugs demonstrated complete protection regarding the CDDP-affected parameters, in line with the neurophysiological data ($p<0.03$ *vs.* CDDP; Table I). Moreover, the rats treated with VPA, alone or in combination, displayed an appoximately three-fold increase of histone H3 acetylation in the DRG samples with respect to the control and CDDP groups (data not shown).

Study 2 – VPA effect on CDDP-induced neuropathy. After four weeks of CDDP treatment the animals displayed both body weight and SNCV reduction, similar to that shown in the previous experiment. In the next four weeks the CDDP-treated rats partially recovered. The sequential VPA treatment for two weeks did not increase the rate of recovery of the SNCV and body weight even after the two weeks of follow-up (data not shown).

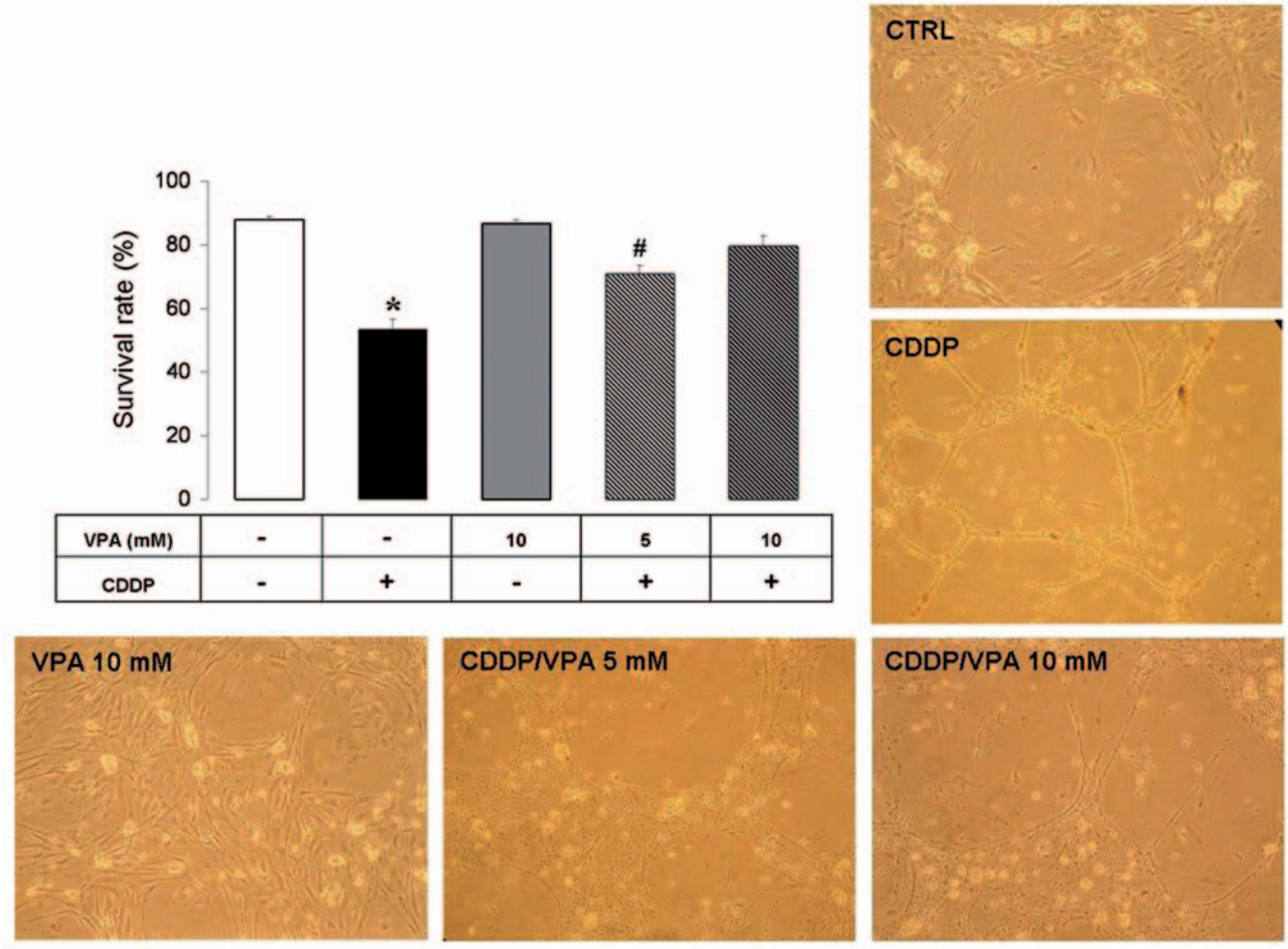

Figure 1. *Effects of CDDP (5 μg/ml) and VPA (5 and 10 mM) combined treatments on neuronal survival of rat dorsal root ganglia primary cultures after 48 hours. ANOVA $p<0.001$, followed by Student Newman-Keuls post hoc test *$p<0.002$ vs. the rest of the treatment groups; #$p=0.015$ vs. vehicle and VPA 10 mM; $n=3$. The photographs show representative examples (48 hours).*

Discussion

Using previously validated models of cisplatin-related neurotoxicity, our data indicated, in both *in vitro* and *in vivo*, that the co-administration of VPA might have an actual neuroprotective effect against the adverse toxic effects of CDDP treatment. For example, VPA co-administration restored to control levels, the survival rate of the DRG neurons in culture. In contrast, in the whole DRG cultures an apparently opposite effect was found. In fact, not only DRG neurite growth was inhibited both by CDDP and VPA administration as reported in other studies (28, 39), but there was a greater extent of inhibition with the combination treatment, suggesting a synergistic effect. This "toxic" effect has already been observed with the same type of neurons in culture by Williams and colleagues (40) who reported an increased growth cone size and decrease of collateral branching following treatment with 1 to 3 mM VPA for 48 hours. These authors suggested that inositol depletion was the mechanism involved, since the effect was abolished by the addition of inositol. However, this result might also possibly be due, at least in part, to the fact that VPA is a non-specific HDAC inhibitor and probably exerts its action on the non-nuclear isoforms of HDAC, such as HDAC6, isoform that acetylates tubulin promoting the stability of a certain population of microtubules (41-43). Hence, the assessment of neurite growth on whole DRG cultures, which has been used to study different neurotoxic drugs and neuroprotectants (28, 44), may not be an adequate experimental model for testing HDAC inhibitors as therapeutic molecules.

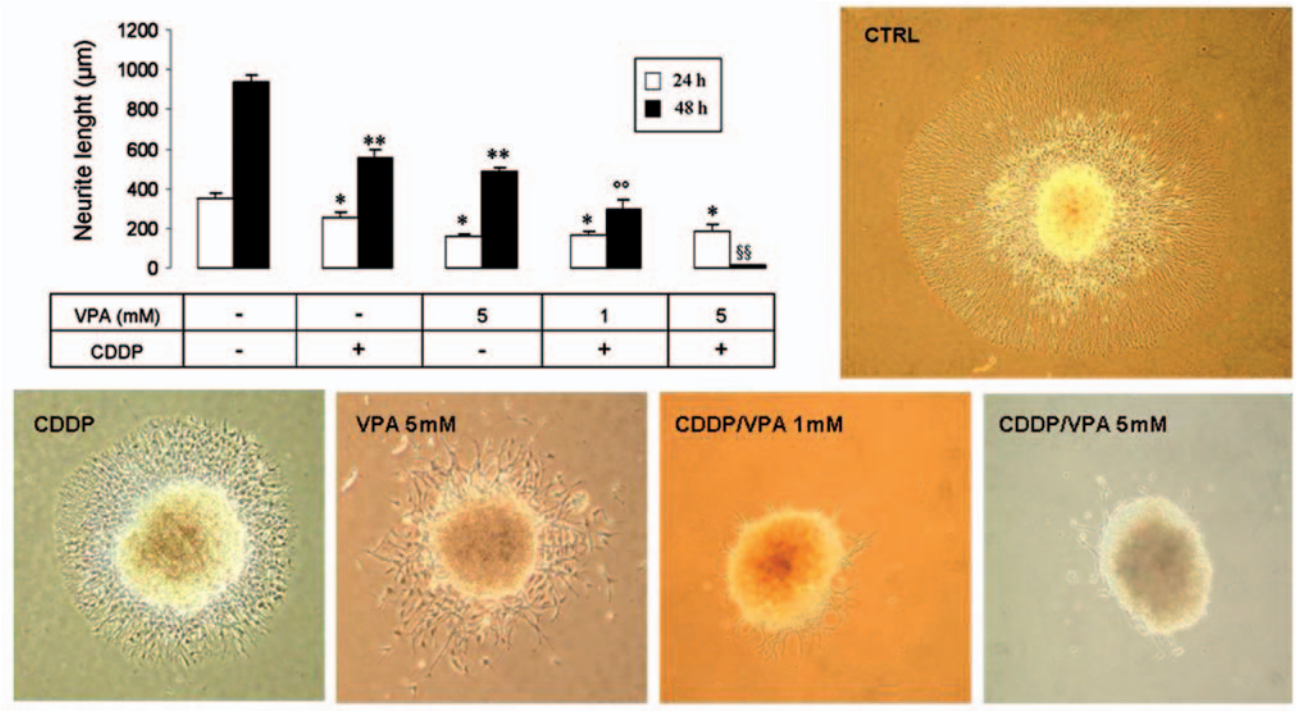

Figure 2. *Effects of CDDP (10 µg/ml) and VPA (1 and 5 mM) combined treatments on the growth of the neurites of DRG in whole explants cultures after 24 and 48 hours. ANOVA p<0.001, followed by Student Newman-Keuls post hoc test, *p<0.02 and **p<0.001 vs. vehicle; °°p<0.001 vs. vehicle, VPA 5 mM, and CDDP; §§p<0.001 vs. the rest of the groups; n=4. The photographs show representative examples (48 hours).*

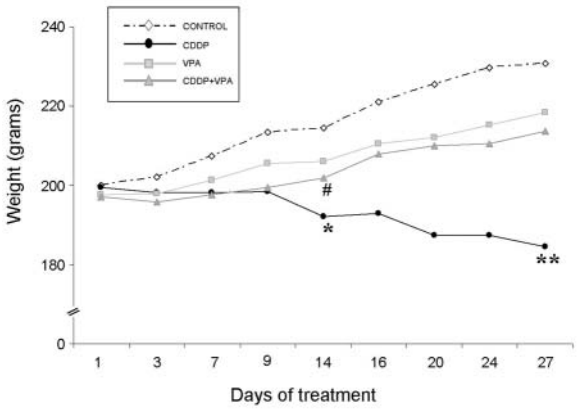

Figure 3. *Effects of CDDP (2 mg/kg) and VPA (300 mg/kg) combination treatment on the weight of the rats throughout the experiment (Study 1). Statistical analysis was performed at days 14 and 27. ANOVA p<0.001, followed by Student-Newman-Keuls post hoc test, *p<0.01, and **p<0.001 vs. all the other groups; #p<0.05 vs. control; n=8.*

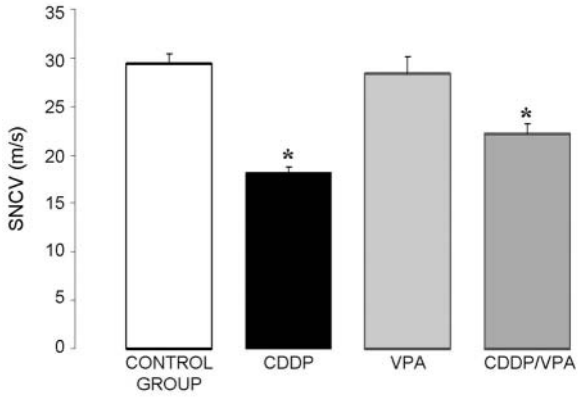

Figure 4. *Effects of CDDP (2 mg/kg) and VPA (300 mg/kg) combination treatment on the sensory nerve conduction velocity (SNCV) at the level of the tail, at the end of the four weeks of treatment (Study 1). ANOVA p<0.001 followed by Student-Newman-Keuls post hoc test, *p<0.05 vs. all the other groups; n=8.*

In the *in vivo* experiment the animals which received the co-treatment of CDDP+VPA (Study 1) had a better outcome in the neurotoxicity tests than those treated with CDDP alone. In fact, the SNCV of the combination group at the end of the co-administration experiment showed values significantly greater than the CDDP group, although not as high as the normal controls or the VPA-treated rats. Even though the neuroprotective action was not complete, the effect of VPA was quite similar to previous experiments performed using other compounds such as nerve growth

factor, ALC and erythropoietin in the same CDDP model (6, 45-47). Interestingly, only for the CDDP group there was a decrease in body weight already at 14 days and the weight of the animals treated with VPA alone or in combination with CDDP tended to increase throughout the whole experiment, although not as markedly as for the control group. Although the effect on CDDP-induced weight loss was not responsible for the neuroprotective action, as already discussed in previous studies (48), it was remarkable that VPA was also able to reduce the general toxicity of CDDP. Therefore, VPA, at this dosage, might partially protect the sensory peripheral nerves from the CDDP toxic effects. VPA did not display a similar action on previously established CDDP-induced peripheral neuropathy (Study 2). In fact, in the animals treated with CDDP for four weeks, which were then treated with VPA for another two weeks, no weight or SNCV rescue was observed. This might have been due to a real ineffectiveness of VPA, or to the schedule of administration and the possibility that better results could be obtained using a longer period of administration or a different dose of VPA cannot be ruled out.

Since VPA reprograms gene expression through histone hyperacetylation and chromatin relaxation, many target genes may be implicated in the observed neuroprotective action of the drug. Among the possible target genes, it has been reported that VPA up-regulates reelin expression through histone acetylation (18). Interestingly, reelin is an extracellular matrix protein present in neonatal peripheral nerves is down-regulated in adults, but induced again when nerve injury occurs (49). Thus, it is possible, for example, that VPA through increasing reelin expression levels contributes to the repair mechanism of the injured nerve. Another putative target gene that could be up-regulated by the effect of VPA is the nerve growth factor (NGF) gene (50), whose protein has neuroprotective effects (47, 51); interestingly, NGF circulating levels are markedly reduced in neuropathic cancer patients (52). However, it is possible that VPA exerts the neuroprotective action observed in our experiments through a mechanism different from HDAC inhibition, and thus, further studies are necessary for clarification. The concurrent increase of DRG histone acetylation following *in vivo* VPA administration suggests, nevertheless, a possible role for epigenetic mechanisms in mediating the observed neuroprotection.

In conclusion, in our opinion, VPA displays a promising neuroprotective action, that might be worth of further investigation, since it is coupled with a very interesting synergistic antineoplastic effect. Hence, VPA, and, possibly, related compounds, might be useful in treating malignancies, in combination with CDDP, reducing the risk of developing problematic side-effects, such as peripheral neuropathies.

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