

Protective Effect of Erythropoietin and Its Carbamylated Derivative in Experimental Cisplatin Peripheral Neurotoxicity

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Abstract Purpose: Antineoplastic drugs, such as cisplatin (CDDP), are severely neurotoxic, causing disabling peripheral neuropathies with clinical signs known as chemotherapy-induced peripheral neurotoxicity. Cotreatment with neuroprotective agents and CDDP has been proposed for preventing or reversing the neuropathy. Erythropoietin given systemically has a wide range of neuroprotective actions in animal models of central and peripheral nervous system damage. However, the erythropoietic action is a potential cause of side effects if erythropoietin is used for neuroprotection. We have successfully identified derivatives of erythropoietin, including carbamylated erythropoietin, which do not raise the hematocrit but retain the neuroprotective action exerted by erythropoietin.

Experimental Design: We have developed previously an experimental chemotherapy-induced peripheral neurotoxicity that closely resembles CDDP neurotoxicity in humans. The present study compared the effects of erythropoietin and carbamylated erythropoietin (50 µg/kg/d thrice weekly) on CDDP (2 mg/kg/d i.p. twice weekly for 4 weeks) neurotoxicity *in vivo*.

Results: CDDP given to Wistar rats significantly lowered their growth rate ($P < 0.05$), with slower sensory nerve conduction velocity ($P < 0.001$) and reduced intraepidermal nerve fibers density ($P < 0.001$ versus controls). Coadministration of CDDP and erythropoietin or carbamylated erythropoietin partially but significantly prevented the sensory nerve conduction velocity reduction. Both molecules preserved intraepidermal nerve fiber density, thus confirming their neuroprotective effect at the pathologic level. The protective effects were not associated with any difference in platinum concentration in dorsal root ganglia, sciatic nerve, or kidney specimens.

Conclusions: These results widen the spectrum of possible use of erythropoietin and carbamylated erythropoietin as neuroprotectant drugs, strongly supporting their effectiveness.

Chemotherapy-induced peripheral neurotoxicity (CIPN) is a major clinical problem because it is a dose-limiting side effect of important and effective antineoplastic drugs (1). The incidence, severity, and clinical symptoms and signs of CIPN depend on the drug given and its schedules. Severe neuropathy can occur in 3% to 7% of treated cases with single agents but can increase to 38% with combined regimens (2, 3). Moreover, even when CIPN is not dose-limiting, it may severely affect the

quality of life of cancer patients and cause chronic discomfort. Therefore, effective prevention and/or treatment of CIPN would be a major advance for cancer patients.

Cisplatin (CDDP) and the other platinum-derived drugs are among the most effective antineoplastic agents, but they are severely neurotoxic. The clinical features of CDDP neurotoxicity in humans are mainly ataxia, pain, and sensory impairment secondary to accumulation of CDDP in the dorsal root ganglia (DRG) and subsequent damage, resulting in secondary nerve fiber axonopathy.

We have developed an experimental model of peripheral neurotoxicity induced by CDDP (4–9) that closely resembles CDDP neurotoxicity in humans. It involves the reversible primary involvement of DRG neurons, with secondary sensory axonal neuropathy and sparing of the motoneurons. This model has already been used to investigate the mechanisms of neurotoxic action of CDDP (4, 5, 7, 9) and to test the effect of putative neuroprotective agents (6, 8–13). Among these agents, cotreatment with neurotrophic agents has been proposed as a means of preventing or reversing CIPN (14).

Among the several substances with trophic action on central neurons and peripheral nerves, erythropoietin has a

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Received 10/5/05; revised 1/16/06; accepted 1/26/06.

Grant support: Fondazione Banca Del Monte di Lombardia (G. Cavaletti).

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doi:10.1158/1078-0432.CCR-05-2177

wide range of neuroprotective effects *in vivo*. Erythropoietin receptors are expressed both in nerve axons and Schwann cells and in DRG neurons and are overexpressed after nerve injury (15–17), presenting a target for pharmacotherapy. In primary neuronal cultures or neuronal cell lines, recombinant human erythropoietin protects from apoptosis (18, 19). *In vivo*, erythropoietin protects neurons from cerebral ischemia and traumatic injury (20) and reduces the severity of experimental autoimmune encephalomyelitis, spinal cord injury, and sciatic nerve compression (21). We recently found that erythropoietin both protects against and lowers the severity of experimental diabetic neuropathy (22). Erythropoietin is also protective against peripheral neuropathy induced by CDDP in rats, evaluated by electrophysiology (compound muscle action potential) and histologic examination of the number of nerve fibers (23).

One major issue in the use of erythropoietin is its erythrodifferentiating action that could be cause of several side effects, including vascular perfusion defects (24). In mice, the increase in hematocrit induced by erythropoietin causes vasoconstriction and cardiac dysfunction due to nitric oxide depletion and endothelin activation (25, 26). In animals and humans, erythropoietin can lead to hypertension (27, 28) or thrombosis (29).

Recently, we have identified derivatives of erythropoietin, including carbamylated erythropoietin (30) and asialo-erythropoietin (21), which are nonerythropoietic but retain neuroprotective action in different animal models, including cerebral ischemia, spinal cord injury, and diabetic neuropathy.

In this study, we investigated the potential role of erythropoietin and carbamylated erythropoietin for neuroprotection against CIPN induced by CDDP.

Because carbamylated erythropoietin does not increase the hematocrit and does not bind the hemopoietic, homodimeric erythropoietin receptor, the present model should enable us to dissociate a peripheral neuroprotective action of erythropoietin from a hemopoietic effect and determine whether neuroprotection can be achieved without the risk associated with the use of erythropoietin in nonanemic patients.

Materials and Methods

Animal husbandry. All the procedures involving animals and their care were conducted in conformity with the institutional guidelines in compliance with national (Law by Decree No. 116, February 18, 1992, *Gazzetta Ufficiale della Repubblica Italiana*, Suppl. 40) and international (European Economic Community Council Directive 86-609, December 12, 1987, in *Official Journal of Law*, p. 358; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996) laws and policies. The protocols for the investigation were reviewed and approved by the Animal Care and Use Committees of the Istituto di Ricerche Farmacologiche "Mario Negri" (Milan) and the Faculty of Medicine, University of Milano "Bicocca".

A total of 72 female Wistar rats (200–220 g at the beginning of the experiment; Harlan Italia, Correzzana, Italy) were used for the study. They were housed in a limited access animal facility with room temperature and relative humidity $22 \pm 2^\circ\text{C}$ and $55 \pm 10\%$ and unrestricted access to food and water. Artificial lighting provided a 24-hour cycle of 12-hour light/12-hour dark (light 7 a.m.–7 p.m.).

Drugs. CDDP (Platamine) was purchased from Pharmacia Italia (Milan, Italy). Erythropoietin was obtained from Dragon Pharmaceuticals (Vancouver, British Columbia, Canada). Carbamylated erythro-

poietin was prepared as described (30) and was kindly provided by H. Lundbeck (A/S, Valby, Copenhagen, Denmark).

In vivo studies. Two separate experiments were done. In a first experiment, we studied the effects of erythropoietin on CDDP-induced neuropathy. Thirty-two rats were randomly divided into four groups (8 per group): CDDP, CDDP plus erythropoietin, erythropoietin, and untreated controls. CDDP was dissolved in sterile saline and rats were injected with CDDP 2 mg/kg i.p. twice weekly for 8 times using a volume of 4 mL/kg (31, 32). The CDDP plus erythropoietin group was treated with the same dose of CDDP plus erythropoietin (50 $\mu\text{g}/\text{kg}$ i.p. thrice weekly). The erythropoietin group received only the drug as above. In a second experiment, we studied the effect of carbamylated erythropoietin (50 $\mu\text{g}/\text{kg}$ i.p. thrice weekly) on CDDP-induced neuropathy. Forty rats were randomly divided into five groups (8 per group): CDDP, CDDP plus erythropoietin, CDDP plus carbamylated erythropoietin, carbamylated erythropoietin, and control.

In both experiments, control rats received sham i.p. injections with the CDDP solvent.

Methods of evaluation. General conditions of the animals were recorded daily, and weight was measured at the time of drug administration.

At the end of the treatment, each rat's sensory nerve conduction velocity (SNCV) was determined in the tail using a previously described method (6, 9). Briefly, antidromic SNCV was assessed by stimulating the tail nerve with ring electrodes placed 5 and 10 cm proximally to the recording ring electrodes placed distally in the tail. The latencies of the potentials recorded at the two sites after nerve stimulation were determined (peak-to-peak) and the nerve conduction velocity was calculated accordingly. All the neurophysiologic measurements were done under standard conditions in a temperature-controlled room.

At the end of the experiment, the animals were euthanized under general xylazine/ketamine anesthesia and tissue specimens were obtained.

Peripheral nerve damage was assessed on pathologic grounds by skin biopsy and an estimate of intraepidermal nerve fiber (IENF) density in the hindpaw footpad (33). Briefly, skin specimens were fixed in 2% paraformaldehyde-lysine periodate for 24 hours at 4°C , cryoprotected overnight, and serially cut with a cryostat to obtain 20 μm sections. Three sections were randomly selected and immunostained with polyclonal anti-PGP 9.5 (Biogenesis, Poole, United Kingdom) using a free-floating protocol (33). Three blinded observers counted the total number of IENF in each section under a light microscope at high magnification using a microscope-mounted video camera. Individual fibers were counted as they crossed the dermal-epidermal junction, and secondary branching within the epidermis was excluded. The length of the epidermis was measured using a computerized system (Microscience, Seattle, WA) to obtain the linear density of IENF.

Whole blood was obtained through abdominal aorta puncture, collected into a heparinated tube for hematocrit determination, and centrifuged at 2,500 rpm for 20 minutes at 4°C . Total and erythrocyte capillary tube length was measured and the hematocrit was calculated as a percentage by dividing the particulate by total length and multiplying it by 100.

Sciatic nerves, DRG, and kidneys of rats from all CDDP-treated groups were obtained, snap-frozen in liquid nitrogen, and kept at -80°C until used to determine the platinum concentration by inductively coupled plasma-tandem mass spectrometry according to the previously reported protocol (6).

Statistics. The differences between all experimental groups in body weight, SNCV, IENF density, and tissue platinum concentrations were examined by ANOVA and the Tukey-Kramer post-test.

Results

General toxicity. All the rats completed the studies, with no evidence of severe general toxicity. In each experiment, at

Table 1. Physiologic variables of rats treated with CDDP and/or cotreated with erythropoietin and carbamylated erythropoietin

Group	Experiment 1			Experiment 2		
	Baseline body weight (g)	Final body weight (g)	Hematocrit (%)	Baseline body weight (g)	Final body weight (g)	Hematocrit (%)
Control + vehicle	186 ± 2	221 ± 3	51.9 ± 1.0	184 ± 5	255 ± 9	50.0 ± 1.8
CDDP + vehicle	197 ± 3	199 ± 3*	47.5 ± 1.2	181 ± 3	212 ± 6 [†]	52.3 ± 1.1
Control + erythropoietin	196 ± 2	222 ± 3	82.4 ± 1.5 [‡]			
Control + carbamylated erythropoietin				184 ± 3	248 ± 6	56.4 ± 2.3
CDDP + erythropoietin	195 ± 3	209 ± 6	84.3 ± 1.2 [‡]	182 ± 5	229 ± 7	76.2 ± 6.3 [§]
CDDP + carbamylated erythropoietin				180 ± 5	230 ± 5	51.0 ± 1.3

NOTE: Data are mean ± SE (8 per group). Statistics by ANOVA with Tukey-Kramer HSD test for multiple comparisons.

**P* < 0.01 versus control and control + erythropoietin (experiment 1).

[†]*P* < 0.01 versus all the other groups (experiment 2).

[‡]*P* < 0.01 versus control and CDDP (experiment 1).

[§]*P* < 0.01 versus all the other groups (experiment 2).

baseline, there was no difference in body weight between groups. CDDP alone significantly reduced weight gain (Table 1); CDDP-treated rats weighed 10% and 17% less than the control groups in experiments 1 and 2, respectively (*P* < 0.01 in both cases), and the coadministration of erythropoietin or carbamylated erythropoietin reduced the difference by ~50%.

Hematocrit was significantly higher in both erythropoietin-treated groups (*P* < 0.01), whereas carbamylated erythropoietin did not produce any significant change from control rats (Table 1).

Tail nerve neurophysiologic evaluation. In each experiment, baseline SNCV did not differ in the two groups. In the first experiment, tail SNCV studies at the end of the treatment showed that the erythropoietin and control groups were very similar (Fig. 1A). This was confirmed in the second experiment, which, in addition, showed similar results for carbamylated erythropoietin (Fig. 1B). CDDP, however, reduced mean SNVC by 26% and 30% compared with control in experiments 1 and 2, respectively (*P* < 0.001). In both experiments, erythropoietin had a partial, but highly significant, protective effect (Fig. 1A and B) and SNCV compared with the CDDP group, although it remained different from control (mean of experiments 1 and 2, -15.7% and -17.8%, respectively; *P* < 0.001). Similarly, carbamylated erythropoietin partially prevented the decrease in SNVC induced by CDDP (*P* < 0.05; Fig. 1B).

IENF density. In footpad skin, both the neurotoxic effect of CDDP and the neuroprotective action of erythropoietin and carbamylated erythropoietin were confirmed at the pathologic level. Figure 2 summarizes the results of the two experiments. Erythropoietin and carbamylated erythropoietin coadministered did not affect IENF density in control rats (data not shown). CDDP significantly reduced the epidermal innervation density (mean 13.1% lower than control) and caused diffuse morphologic changes of nerve fibers, indicating axonal degeneration (Fig. 3). Erythropoietin and carbamylated erythropoietin coadministered significantly (*P* < 0.001) protected against from the loss of IENF induced by CDDP (Figs. 2 and 3).

Tissue platinum concentration. Platinum in control specimens was below the limit of detection of the inductively coupled plasma-mass spectrometry method (i.e., <0.001 μg/g tissue), but it was detectable in specimens from CDDP-treated rats. No significant differences were observed between CDDP and CDDP plus erythropoietin groups (7.64 ± 0.82 versus 9.11 ± 1.84, 1.86 ± 0.41 versus 1.67 ± 0.23, and

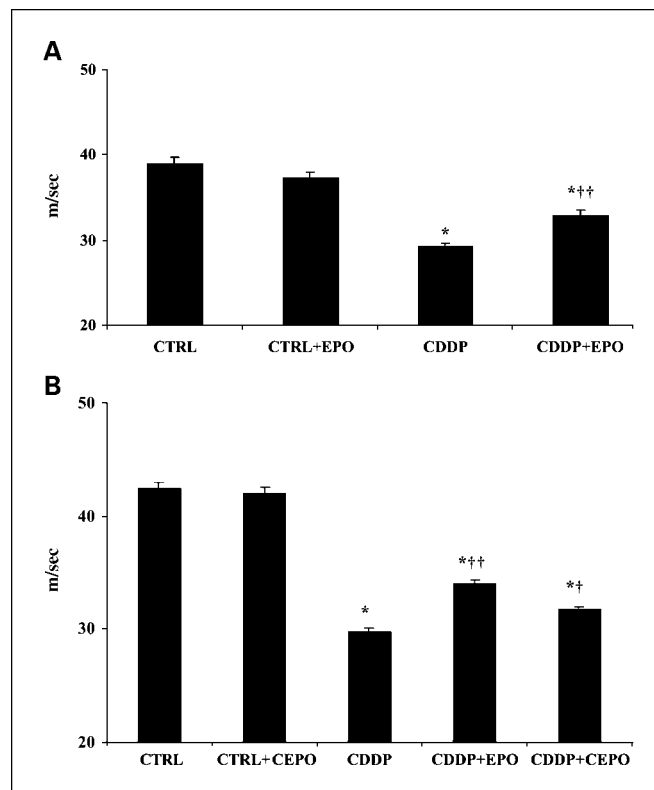


Fig. 1. SNCV at the end of the treatment. Columns, mean (in m/s) of seven to eight per group; bars, SE. A, experiment 1. *, *P* < 0.001 versus control (CTRL) and control + erythropoietin (EPO); ††, *P* < 0.01 versus CDDP. B, experiment 2. *, *P* < 0.001 versus control and control + carbamylated erythropoietin (CEPO); †, *P* < 0.05 versus CDDP; ††, *P* < 0.01 versus CDDP.

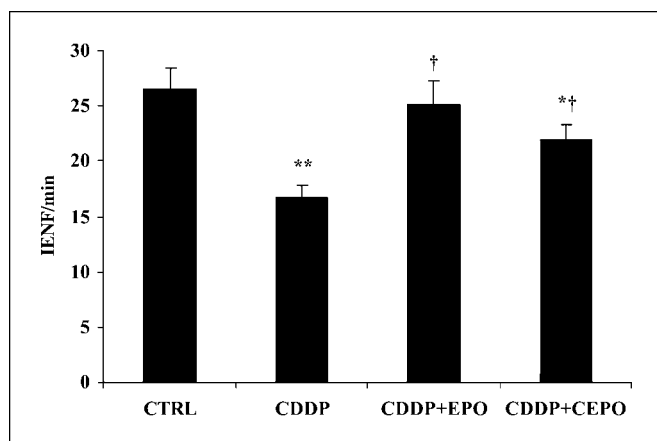


Fig. 2. Erythropoietin restores the loss of intraepidermal fibers in CDDP-treated rats. Skin biopsies were obtained at the end of the treatment. Columns, mean (number of IENF/mm) of seven to eight per group; bars, SE. *, $P < 0.05$ versus control (CTRL); **, $P < 0.001$ versus control; †, $P < 0.001$ versus CDDP.

2.38 versus 3.16 $\mu\text{g/g}$ tissue for kidney, sciatic nerves, and pooled DRG samples, respectively). Hence, the protective effect of erythropoietin was not associated with any difference in platinum concentration in DRG, sciatic nerve, or kidney specimens.

Discussion

CIPN is a major limitation in the current treatment of cancer with platinum drugs, because it frequently requires a dose reduction or even treatment withdrawal, on account of adverse effects. Therefore, effective strategies to prevent or reduce the severity of CIPN are a major goal of preclinical research, with a view to clinical application. *In vitro* studies have been frequently used for screening putative neuroprotective agents, because they are faster and cheaper than *in vivo* testing. However, the latter reproduce the clinical picture in humans more reliably, including the effects of drug metabolism and bioavailability and tissue distribution, with respect to the target organs in particular.

The peripheral neuropathy induced in rats by repeated administration of CDDP is qualitatively similar to that in humans, involving the degeneration of sensory nerve fibers caused by DRG neuronopathy. The model of CIPN used in this study has already been extensively characterized in our laboratory (4–9) and has been used to assess the effectiveness of several putative neuroprotective agents. Some were not effective (9, 31), whereas others had at least a partial effect (6, 8, 10, 12, 13). The usefulness and reliability of the preclinical results were further strengthened when one of these neuroprotectants (reduced glutathione) was evaluated in a randomized, double-blind, placebo-controlled clinical trial in ovarian cancer patients (32), which confirmed its partial protective effect against CDDP neurotoxicity, previously evidenced in the animal model.

The use of neurophysiologic tests to assess the effect of neurotoxic or neuroprotective agents is well established (6–8, 34–36). They are also largely used in clinical practice in human neuropathies. However, pathologic confirmation of the neurophysiologic results is always advisable. In our

CDDP model, we have extensively reported the pathologic features in DRG, sciatic nerves, and tibial nerves (5). In the present study, for the first time, we investigated the effect of CDDP at the pathologic level by quantifying IENF density in the footpad skin using a standardized method (33). This procedure is currently used to assess the degeneration of skin nerve fibers in human peripheral neuropathies (37). Skin biopsy for IENF density quantification is a reliable and minimally invasive technique in patients. It showed both degeneration and regeneration of nerve fibers in experimental and human diabetic neuropathies (22, 38, 39) and it might be proposed as an outcome measure in clinical trials with neurotoxic drugs. SNCV and IENF density studies explore different nerve fiber populations: SNCV evaluation mostly

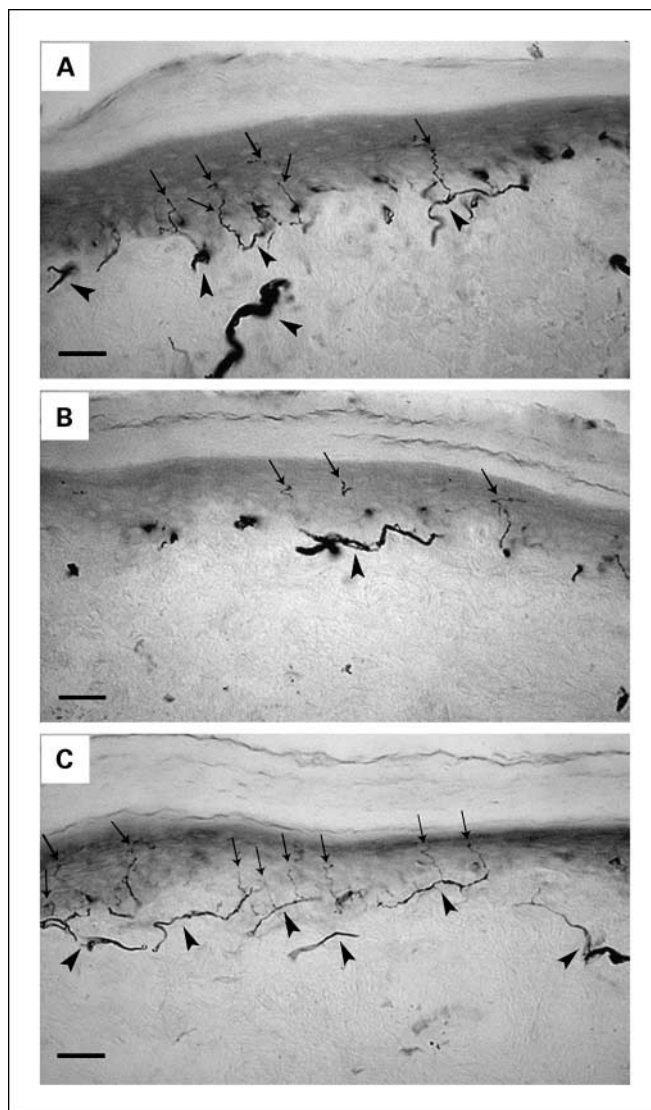


Fig. 3. Microphotographs are skin biopsies from the hindpaw footpad of Wistar rats. Sections were immunostained with the panaxonal marker PGP 9.5. Arrows, IENF; arrowheads, dermal nerve bundles. Bar, 30 μm . A, normal epidermal and dermal innervation density. B, reduced density of IENF and dermal nerve bundles in CDDP-induced neuropathy. IENF show weaker and fragmented PGP 9.5 immunoreactivity indicative of axonal degeneration. C, normal density and morphology of IENF and dermal nerve bundles in a rat cotreated with CDDP and carbamylated erythropoietin.

reflects changes in large myelinated fibers, whereas IENF density is an estimate of small-diameter fibers that are affected early in most peripheral neuropathies.

Erythropoietin, a 165-amino acid sialoglycoprotein, is essential in the regulation of erythropoiesis. Among its several clinical applications, erythropoietin is a very effective, well tolerated, and widely used treatment for anemia in cancer patients undergoing chemotherapy. However, the bone marrow is not the only target tissue of erythropoietin, and the wide expression of functional receptors for erythropoietin explains its nonerythropoietic functions, including its neuroprotective action on the injured nervous system (40). Rat models have been used to test the protective effect of erythropoietin (15, 16, 22), which was recently confirmed in an experimental model of CDDP (23). However, this rat model involved a motor, demyelinating neuropathy that did not reproduce the typical effects of CDDP in humans (i.e., sensory impairment with axonal damage in the peripheral nerves), thus raising some concern about the relevance of the positive results for potential clinical application.

Because, in rats, both DRG and peripheral nerves express the erythropoietin receptor (15, 16), our model of CDDP-induced DRG sensory neuronopathy with secondary axonal neuropathy seems adequate to assess the use of erythropoietin as a neuroprotective agent. The effect of a nonerythropoietic erythropoietin derivative, such as carbamylated erythropoietin, which showed tissue-protecting properties in several animal models of peripheral nerve damage (30), was also evaluated.

Our study shows that erythropoietin or carbamylated erythropoietin alone have no effect on the normal function of the peripheral nerves but had significant and reproducible neuroprotective effects in CDDP-induced neurotoxicity. These results were supported both by the neurophysiologic findings showing the improvement of tail SNCV and at the pathologic level by the higher density of IENF in the footpad skin.

A major concern in the use of neuroprotectant drugs to prevent CIPN is interference with the antineoplastic activity of chemotherapy. Although the effects of erythropoietin on tumor growth is still controversial, Sigounas et al. (41) suggested a synergy between erythropoietin and chemotherapeutic agents in a murine cancer model. We investigated whether erythropoietin or carbamylated erythropoietin affected the tissue distribution of CDDP by measuring the concentration of platinum in the kidney (where it accumulates after CDDP administration and CDDP-DNA adducts are present; 11) and in the peripheral nervous system. We found no difference in platinum tissue concentrations, supporting the opinion that erythropoietin and carbamylated erythropoietin do not interfere with CDDP.

The mechanism of neuroprotection of erythropoietin and carbamylated erythropoietin in our experimental paradigm is still an open issue. Based on current knowledge, a direct protective effect of erythropoietin and carbamylated erythropoietin can be suggested on sensory neurons and/or peripheral nerves through the direct binding to the erythropoietin receptor, which is widely expressed in the peripheral nervous system and overexpressed after nerve injury (16, 17). Carbamylated erythropoietin does not bind to the classic erythropoietin receptors expressed by bone marrow (30). This

affinity for the neural-type erythropoietin receptor shared by erythropoietin and carbamylated erythropoietin suggests that high circulating levels of erythropoietin or carbamylated erythropoietin might have an effect on damage prevention and/or repair. We already reported that erythropoietin reduced the severity of experimental diabetic neuropathy in two different experimental paradigms (22), thus raising the possibility that it has a "nonspecific" neuroprotective effect against different types of injury of the peripheral nervous system as shown recently *in vitro* (17).

The major point of concern in proposing of erythropoietin for neuroprotection in clinical practice is the risk of a marked increase in hematocrit with long-term treatment as observed in our experiment. However, short-term administration of erythropoietin as a neuroprotectant in clinical trials of stroke has not resulted in elevated hematocrit (42) and the optimal schedule (dose and timing) of erythropoietin treatment for the prevention of CIPN still needs to be defined. Recent data suggest that erythropoietin in combination with other growth factors (and possibly with other neuroprotectants) may synergistically activate neuroprotective pathways that allow a lower dose of erythropoietin to be used (43).

A different approach to minimize the erythropoietic effect of erythropoietin is modification of the molecule as has been done with carbamylated erythropoietin. Extensive studies of the relationship between the structure and the activity of the erythropoietin molecule identified regions and amino acids essential for its binding receptor (44) and found that several chemical modifications abolish the hematopoietic bioactivity (45, 46). Previous studies and our results are encouraging because carbamylated erythropoietin maintained its neuroprotective effect without changing the hematocrit (30). These findings open up the possibility of distinguishing between the tissue-protective action of erythropoietin and its potentially detrimental effects (e.g., endothelial activation and platelet reactivity, prothrombotic effects, and excessive erythropoiesis with chronic dosing; refs. 47, 48).

When considering the differences between erythropoietin and carbamylated erythropoietin, it is important to note that these two molecules have very similar pharmacokinetic variables and plasma half-life in particular (30), in contrast to asialo-erythropoietin that has a very short half-life (21). We have shown that, whereas erythropoietin bind the classic homodimeric erythropoietin receptor, carbamylated erythropoietin does not (30), but both molecules require the presence of the β common subunit shared by the granulocyte-macrophage colony-stimulating factor and the interleukin-3 and interleukin-5 receptors, as knockout mice deficient in this transducer do not show protective effects of either carbamylated erythropoietin or erythropoietin (49).

In conclusion, these results we obtained in a model of CIPN that reproduces the clinical features of the effects of CDDP in humans widen the spectrum of possible use of erythropoietin and carbamylated erythropoietin as neuroprotectant drugs, strongly supporting their effectiveness. However, they also indicate the need for further preclinical studies to optimize their effectiveness, to determine the exact mechanism and site of action, and to clarify the issues of long-term tolerability and safety *in vivo*, with the final aim of identifying the best strategy for clinical application.

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