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The fundamental role of morphology in experimental neurotoxicology: the example of chemotherapy-induced peripheral neurotoxicity

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Summary

The peripheral nervous system is a frequent target of toxic agents. The accurate identification of the sites of neurotoxic action through the morphological characterization of reliable in vivo models or in vitro systems can give fundamental clues when investigating the pathogenesis and interpreting the clinical features of drug-induced neuropathy.

The morphological approach has been used to investigate almost all the anticancer drugs able to induce chemotherapy-induced peripheral neurotoxicity, i.e. platinum drugs, antitubulins and proteasome inhibitors. No models have ever been described for thalidomide.

This review demonstrates that any pathogenetic study on chemotherapy-induced peripheral neurotoxicity must be based on solid morphological observations obtained in reliable animal and in vitro models. This is particularly true in this setting, since the availability of tissues of human origin is extremely limited. In fact, peripheral (generally sural) nerve biopsies are never required for diagnostic purposes in chemotherapy-treated cancer patients, and their use for a purely scientific aim, although potentially very informative, is not ethical. Moreover, several neurotoxic drugs target the dorsal root ganglia neurons, and it is very difficult to obtain high-quality specimens even from early autopsies.

It is, therefore, our opinion that an extensive morphological assessment of the in vitro and in vivo effect of any potentially neurotoxic antineoplastic drugs, as well as of neuroprotectant agents, should be taken into consideration right from the earliest stages of their development.

Key words

Dorsal root ganglia; neuropathy; chemotherapy; morphology; toxicity.

Introduction

The role of careful morphological examinations in modern experimental neurotoxicology is frequently challenged in view of the availability of new, powerful and sophisticated techniques. However, several examples could be offered in support of the still fundamental importance of this "old" assessment method, when carefully used, as a mandatory step in the overall process.

As far as the peripheral nervous system (PNS) is concerned, clear evidence is provided by the important role of morphology in the understanding of chemotherapy-

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induced peripheral neurotoxicity (CIPN) which will be discussed in this paper. The PNS is a frequent target of the neurotoxicity of several compounds and toxic agents. The accurate identification of their sites of neurotoxic action, as well as the careful study of the morphological features induced in the nervous system in in vivo models or in well-characterized in vitro systems, can allow reliable clues to be obtained when investigating the pathogenesis and interpreting the clinical features of drug-induced neuropathy.

Among the various environmental, industrial and pharmacological agents able to damage the PNS, the neurotoxic effect of several antineoplastic drugs represents a major clinical problem, given their widespread use and the potential severity of their toxicity that may be a dose-limiting side effect.

Neurotoxicity studies in this field clearly demonstrate the importance of a morphological assessment in this kind of investigation. In fact, while the clinical features of CIPN may suggest the possible target of toxicity, only morphological studies can actually demonstrate if a structure is really involved in the pathological process and to what extent. For instance, the clinical evidence that in CIPN distal sensory symptoms/signs predominate over motor impairment would seem to indicate that the primary sensory neurons located in the dorsal root ganglia (DRG) are the most frequent targets of drugs' neurotoxic action, but only a morphological examination of DRG in experimental models and in a few human specimens could confirm this hypothesis.

The specific morphological features of neuronal damage induced at different levels by platinum compounds, taxanes, vinca alkaloids and bortezomib, together with their importance in properly addressing subsequent focused research, will be described. No models have ever been described for thalidomide.

The clinical features of CIPN

The involvement of the PNS in CIPN can be clinically evidenced by the occurrence of sensory impairment with or without neuropathic pain, weakness and/or autonomic impairment. The different anticancer drugs known to induce CIPN may determine one or more of these clinical signs/symptoms, even if sensory impairment is generally more frequent and severe.

The most extensively studied neurotoxic antineoplastic drugs that have entered into clinical practice include platinum drugs (cisplatin, oxaliplatin, carboplatin), antitubulins (vincristine, taxanes, epothilones), bortezomib and thalidomide.

Although some aspects are similar among the various treatments, every class of drugs has its characteristic toxicity profile and so clinical symptoms and signs, as they generally appear during the treatment with the main antineoplastic drugs, will be described in this chapter.

Sensory impairment

As previously mentioned, sensory symptoms and signs are often isolated or largely prominent over motor impairment in most CIPN patients (Cavaletti *et al.*, 2007a, Quasthoff and Hartung, 2002). Loss of sensitivity or paresthesias, often painful, can occur early in the course of chemotherapy and may persist even after treatment with-

drawal. Sensory symptoms are usually worst in the lower limbs and they have a distal-to-proximal gradient. Sensory signs include decreased touch, pin, thermal and vibration perception. Reflexes are often lost, especially at the ankles, as an early sign (Cavaletti *et al.*, 2004).

Typically, platinum compounds produce a pattern of sensory loss consistent with primary ganglionopathy and proprioceptive loss may result in ataxia leading to severe functional impairment (Cavaletti, 2008, Cavaletti *et al.*, 2007a). Besides their toxicity to the DRG and peripheral nerves, platinum compounds can also be responsible for deafness, being toxic to the cochlea hairy cells (Schweitzer, 1993). All the various classes of antitubulins (vinca alkaloids, taxanes, epothilones) induce sensorimotor neuropathy, but distal sensory impairment with reduced pain/thermal perception and touch hypoesthesia with non-painful paresthesia are generally more severe than motor impairment (Cavaletti and Marmiroli, 2010, Cavaletti and Marmiroli, 2004). By contrast, pure sensory impairment in pain, thermal and touch perception are the most common clinical features experienced by multiple myeloma patients treated with bortezomib and/or thalidomide (Cavaletti and Marmiroli, 2010).

Lhermitte's phenomenon (indicating the involvement of the centripetal branch of the sensory pathway within the spinal cord) has occasionally been reported after cisplatin and oxaliplatin and also after taxane (paclitaxel or docetaxel) administration (Taieb *et al.*, 2002, van den Bent *et al.*, 1998, Inbar *et al.*, 1992).

Neuropathic pain

Neuropathic pain is a prominent and important side effect for many treated subjects. Patients often report this symptom during the administration of the platinum drugs, paclitaxel and vincristine (Siau *et al.*, 2006), and in the case of bortezomib its severity may be so great as to be dose-limiting particularly in the treatment of multiple myeloma (Cavaletti and Nobile-Orazio, 2007).

Motor impairment

In most cases the motor symptoms and signs are minor. Weakness is generally clinically evident in the distal muscle groups; it may be detected only on examination or it may be more severe with foot drop and distal weakness. Muscular weakness due to motor neuropathy is more frequently observed after the administration of antitubulin drugs (taxanes, epothilones or vinca alkaloids).

Muscle cramps are a common, underestimated symptom particularly in patients undergoing oxaliplatin administration and myalgias are frequent in taxane-treated patients.

Autonomic impairment

This may result in a wide spectrum of symptoms, including orthostatic hypotension, constipation and dysfunction of sexual organs and micturition. In general, autonomic symptoms are relatively infrequent in CIPN, but they can be dose-limiting in vincristine-treated subjects (Swain and Arezzo, 2008).

Incidence of CIPN

The real incidence of CIPN has not been clearly established due to marked differences in the assessment methods and to the difficulty in demonstrating the presence of pre-existing neuropathy in patients undergoing second-line treatments after a potentially neurotoxic first-line chemotherapy. Moreover, several neurotoxic antineoplastic drugs are used together in polychemotherapy schedules for the treatment of selected kinds of solid or hematological cancers, with a subsequent possible increase in the incidence and severity of CIPN and/or the appearance of combined neurotoxicity.

The observed incidence of CIPN in patients receiving a platinum drug + taxanes chemotherapy may approach 70% (Argyriou *et al.*, 2007, Argyriou *et al.*, 2005), with severe CIPN in at least 10% of patients. A similarly high incidence of CIPN may also be detected in bortezomib-treated patients, particularly when the drug is combined with thalidomide (Chaudhry *et al.*, 2008, Lanzani *et al.*, 2008, Argyriou *et al.*, 2008).

The role of morphological studies in the understanding of CIPN pathophysiology

As already observed, based on the clinical features of CIPN, it is very likely that the primary sensory neurons located in the DRG are the most probable target of drugs neurotoxic action. This hypothesis is supported by several well established observation regarding the anatomy of DRG neurons. Firstly, primary sensory neurons lie outside the blood-brain barrier (BBB) and are supplied by capillaries with fenestrated walls that allow the free passage of molecules between the bloodstream and the extracellular fluid. Moreover, the axons of these cells are among the longest of the entire nervous system and, therefore, are more susceptible to any agent that interferes with the energy metabolism or the structural basis of axonal transport (e.g. drugs interfering with the normal cytoskeleton structure or damaging mitochondrial activity thus impairing axonal transport due to energy failure). Finally, neurons rely on programmed cell death pathways that are particularly sensitive to DNA damage, a primary target of several antineoplastic chemotherapeutic agents.

In spite of these interesting hypotheses, knowledge regarding the mechanism of the neurotoxic action of chemotherapeutic drugs is still largely incomplete.

In an attempt to increase what is known about CIPN pathogenesis, several preclinical models have been established and used to perform extended morphological and morphometric evaluations. These studies have made it possible to collect much of the most relevant information regarding CIPN pathophysiology and to generate hypotheses for subsequent focused investigations.

Although in vivo animal models have obvious advantages in terms of similarities with clinical conditions, they are expensive, time-consuming and the interpretation of their results is often difficult at a molecular level due to the great number of variables to be analysed.

Therefore, reliable *in vitro* models based on the morphological examination of DRG or isolated sensory neurons cultures have also been developed.

In vivo studies

Several animal models of CIPN have been developed in recent years and have been used to examine DRG as well as peripheral nerve changes induced by treatment and to compare the results with functional assessments, e.g. neurophysiological or behavioral investigations. The sciatic nerve can be effectively used to assess the involvement of large, rather proximal, nerve fibers while caudal or digital nerves can be used to study the most distal parts of the nerve, where the earliest signs of toxicity generally ensue (Canta *et al.*, 2010). Very recently, skin biopsies have also been used with the aim of investigating the most distal parts of sensory nerves and of applying a method already available in clinical practice for studying distal polyneuropathies (Bianchi *et al.*, 2006, Lauria *et al.*, 2005).

The molecular changes induced by antineoplastic drugs at different sites (i.e. DRG, peripheral nerves) have also been studied at the pathological level with immunolocalization studies (Barajon *et al.*, 1996).

In vitro studies

CIPN has been investigated in vitro using various cellular approaches. Several data have been obtained by employing tumoral cell lines such as SH-SY5Y human neuroblastoma and rat PC12 pheochromocytoma cells. Both cell lines have the disadvantage of not being "true" neurons but they can be differentiated in culture into "neuronal-like" cells. SH-SY5Y cells are human cells, they express neuronal genes and may be considered as being neuroblasts at a different stage of differentiation. In addition, SH-SY5Y cells differentiate and develop neurites after retinoic acid (RA) treatment without the addition of neurotrophic factors. PC12 cells are rat cells and their differentiation is dependent on Nerve Growth Factor (NGF); this is a limitation in the use of the PC12 cell line in the study of growth factors' neuroprotection. In both cellular models CIPN has been studied by measuring neurite length. Furthermore, both cellular models are suitable for studying the molecular mechanisms implicated in CIPN (Villa *et al.*, 2005, Rigolio *et al.*, 2005, Nicolini *et al.*, 2003, Nicolini *et al.*, 2001).

Embryonic (E15) or adult rat DRG explants are also suitable models for studying CIPN. In fact, DRG are the main target of several antineoplastic drugs and from DRG it is possible to establish both organotypic cultures and primary cultures (neurons or satellite cells). E15 or adult rat DRG harbor post mitotic sensory neurons that are able to elongate neurites after NGF addition. Moreover, rat DRG primary cultures allow the effect of antineoplastic drugs on myelination to be studied (Podratz *et al.*, 2004). Neurotoxicity in DRG organotypic cultures can be evaluated by measuring the length of the longest neurite of each DRG, by analysing the intricacy of the neurite tree and the number of branches (Radio and Mundy, 2008) or by quantifying the density of neurite arborisation (Shah *et al.*, 2004) (Fig. 1).

Neurotoxicity of the different agents

Platinum drugs

As already mentioned, the selective vulnerability of sensory neurons is thought to be secondary to the higher concentration of neurotoxic drugs able to reach the DRG.

Control
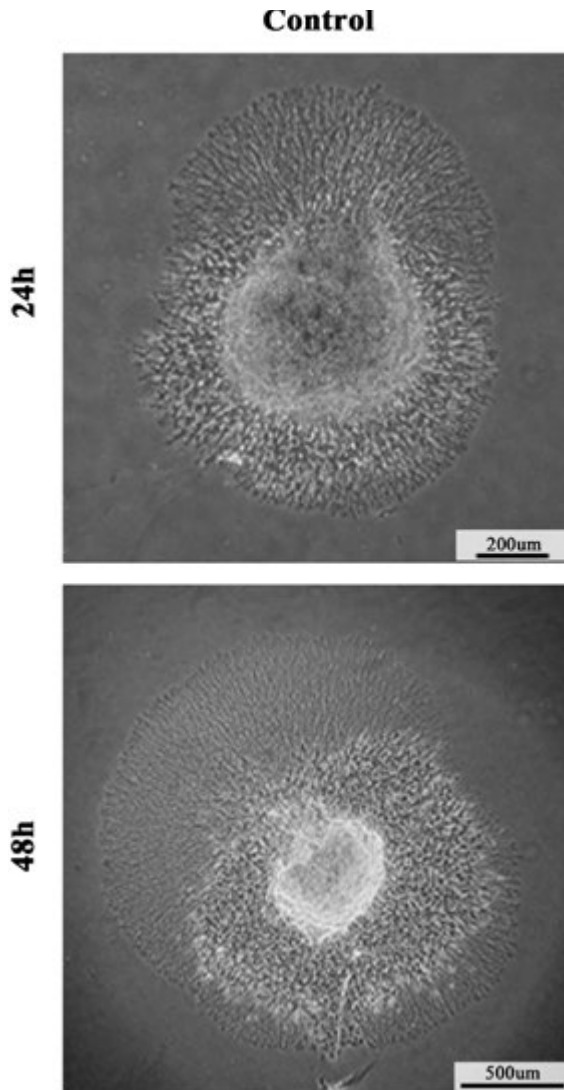
Figure 1 – Phase contrast light micrographs showing neurite outgrowth in dorsal root ganglia explants after 24 h (top) and 48 h (bottom) NGF exposure.

In agreement with this suggestion, platinum or paclitaxel concentrations measured in DRG are very close to those achievable in tumor tissue, while much lower concentrations can be detected in the BBB-protected central nervous system (Thompson *et al.*, 1984, Gregg *et al.*, 1992, Cavaletti *et al.*, 1990). Using antibodies able to recognize platinum-DNA adducts, it has subsequently been demonstrated that platinum binds avidly to DRG neuronal DNA during treatment with both cisplatin and oxaliplatin (McDonald *et al.*, 2005, Ta *et al.*, 2006, Meijer *et al.*, 1999).

The first reliable and extended pathological report of cisplatin-induced DRG damage appeared in 1986 (Tomiwa *et al.*, 1986) and it described the effect of the acute administration of cisplatin. In that model cisplatin treatment produced a segregation of the dense fibrillar from the granular component in DRG neurons nucleoli. A disorganization of ribosomes was also found in more severely intoxicated animals, with shrinkage of the Nissl substance and an increase in neurofilaments. Hypertrophy of the satellite cells with an increase in the perineuronal intercellular spaces, often associated with irregular, scalloped nuclear and cell outlines, suggested that neuron shrinkage had occurred (Tomiwa *et al.*, 1986). A few years later, similar features were demonstrated in our laboratory using chronic, long-lasting schedules of cisplatin administration developed to mimic more closely the long-term administration of the drug in clinical practice (Pisano *et al.*, 2003, Cavaletti *et al.*, 2001, Tredici *et al.*, 1998, Cavaletti *et al.*, 2002b, Cavaletti *et al.*, 2002a, Cavaletti *et al.*, 1992, Cavaletti *et al.*, 1998, Cavaletti *et al.*, 1990, Cavaletti *et al.*, 1994, Bianchi *et al.*, 2006). Also in our animals an irregular nuclear profile was frequently observed, although the nucleoplasm was of normal density. However, the most striking changes occurred in the nucleolus. Although multinucleolated neurons and nucleolar eccentricity were present also in healthy rats, their incidence was markedly increased after platinum administration. Moreover, several nucleoli underwent a segregation of their components (Fig. 2) and the damage could be so severe that focal clearing of the nucleolus occurred in some neurons. The satellite cells were much less involved than neurons although changes were evident in the nucleus, where a thinning of the chromatin layer was frequently observed at the ultrastructural examination. The morphometric determination of the somatic, nuclear and nucleolar size performed on DRG neurons demonstrated a significant and dose-dependent cellular atrophy after chronic platinum administration in comparison with healthy age-matched rats. Formal morphometric assessment of the number of neurons present in the lumbar DRG did not evidence any neuronal loss due to treatment, which is in agreement with the results obtained in the DRG with the TUNEL method that did not show apoptotic nuclei in the neurons (Tredici *et al.*, 1999).

In contrast with the evident morphological and morphometric changes demonstrated in the DRG, the pathological changes in the peripheral nerves of platinum-intoxicated rats are very mild (Cavaletti *et al.*, 1992). In the sciatic nerve a significant decrease in the mean diameter of myelinated fibers was evident, mainly affecting the fibers over 10 μm in diameter, and it was associated with a shift to the left of the fiber size distribution histogram indicating an increase in the density of smaller fibers. In agreement with the absence of neuronal loss in the DRG, the total number of myelinated fibers (which was directly measured in the saphenous nerve) was unchanged after cisplatin treatment, as were the overall density and the estimated number of myelinated fibers in the sciatic nerve (Tredici *et al.*, 1999).

The same morphological changes observed in cisplatin-treated animals were subsequently described also in carboplatin- or oxaliplatin-treated rats (Cavaletti *et al.*, 1998, Cavaletti *et al.*, 2002a, Cavaletti *et al.*, 2001) and correlated with evident neurophysiological changes in nerve conduction velocity in the tail nerve (Tredici *et al.*, 1994).

Cisplatin and oxaliplatin toxicity *in vitro* has been evaluated in SH-SY5Y human neuroblastoma cells in our laboratory (Cece *et al.*, 1995; Nicolini *et al.*, 1998; Donzelli *et al.*, 2004). Morphological studies demonstrated that both cisplatin and oxaliplatin induced apoptotic death in SH-SY5Y undifferentiated cells. In fact cisplatin- or oxali-

**Control**
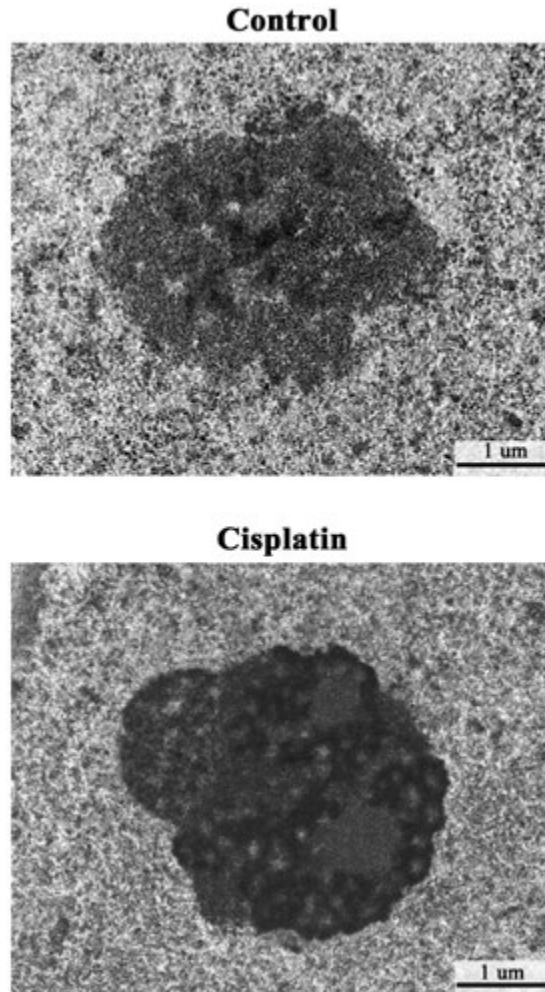
Cisplatin

Figure 2 – High power photomicrographs at the electron microscope showing the nucleolar segregation induced by chronic cisplatin administration in a rat dorsal root ganglia neuron (bottom) in comparison with an untreated rat (top).

platin-treated SH-SY5Y cells appeared positive to TUNEL staining (Fig. 3) and in the same cells caspases 3 and 7 were activated. Moreover, platinum-treated SH-SY5Y cells detached from the substrate and presented shrunken cell bodies, chromatin condensation, nuclear blebbing and cytoplasmic degeneration.

Cisplatin *cytotoxic* effect on RA-differentiated SH-SY5Y cells is much lower compared to the effect on undifferentiated cells. By contrast, it is important to underline the *neurotoxic* effect of the drug on RA-differentiated SH-SY5Y cells. RA-differentiated cells treated with cisplatin (for 24 hours) show a significant reduction in mean neurite length directly correlated with the drug concentration. Cisplatin determines also a
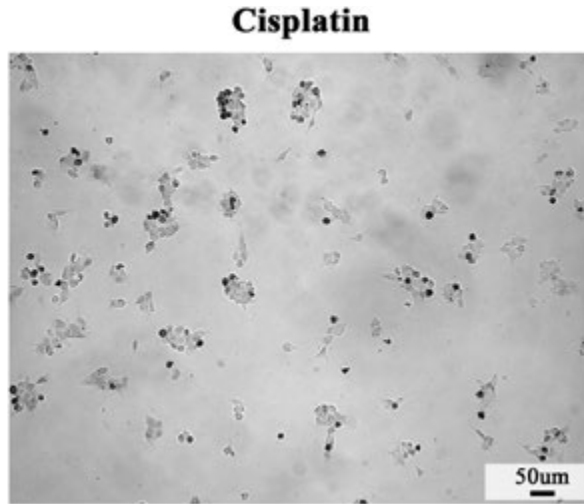
Cisplatin

Figure 3 – TUNEL staining of SH-SY5Y undifferentiated neuroblastoma cells after cisplatin exposure.

reduction in the percentage of differentiated SH-SY5Y cells, namely those with a neurite longer than 50 μm, i.e mean + 2SD of the longest neurite previously determined in non-differentiated cultures (Fig. 4).

Cisplatin and oxaliplatin toxicity *in vitro* has also been evaluated in E15 rat DRG neurons and apoptosis has been demonstrated (Fig. 5) (Scuteri *et al.*, 2009). This apparent discrepancy with most of the *in vivo* results, which frequently failed to evidence apoptosis in DRG neurons, is probably due to the general toxicity of platinum drugs in rat and mice that prevents the high doses being reached that are required to achieve these results using long-term treatments. However, apoptosis in DRG neurons has been demonstrated also *in vivo* using short-term, high-dose schedules (Fischer *et al.*, 2001).

Antitubulin drugs

The family of neurotoxic antitubulin drugs includes three main classes of compounds: vinca alkaloids, taxanes and epothilones. All these drugs interfere with microtubule assembly and mitotic spindle formation, although through different mechanisms. The vinca alkaloids act by inhibiting the assembly and promoting the disassembly of microtubules (Himes *et al.*, 1976). By contrast, the taxanes and the epothilones hyperstabilize microtubule subunit cross-linking. The antitubulin activities of the latter drugs has two well-established effects: a) the increased stability of microtubules decreases the ability of the cell to dynamically reorganize the cytoskeleton and b) the increased cross-linking results in the formation of crystalline arrays of microtubule subunits in the cell body or axon (Apfel, 2000). However, alternative toxic mechanisms have been suggested by recent animal studies demonstrating mitochondrial damage in the distal parts of peripheral nerves after paclitaxel administration (Xiao *et al.*, 2009, Jin *et al.*, 2008).

SH-SY5Y Control

SH-SY5Y RA 48h
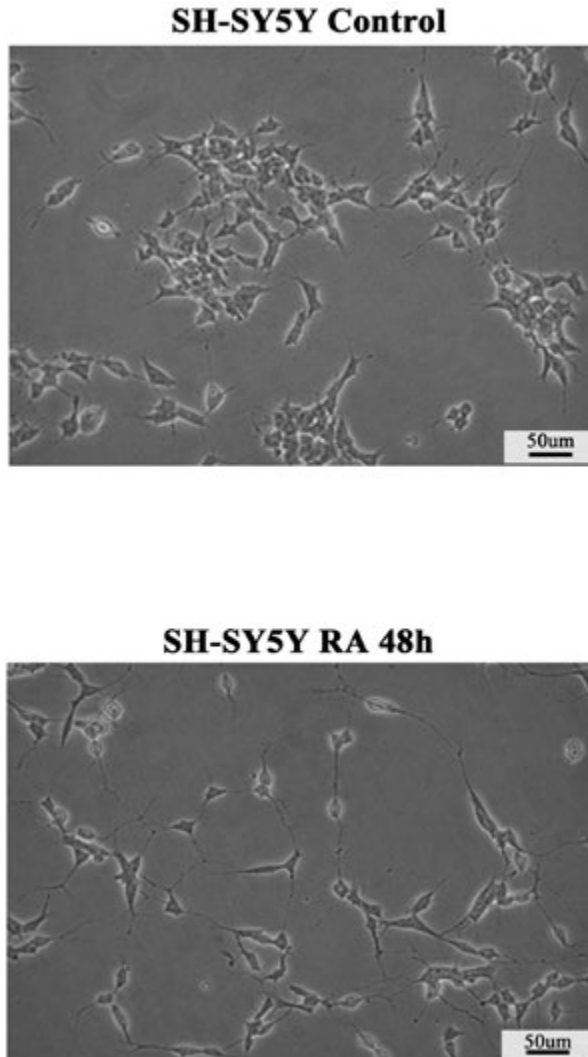
Figure 4 – Phase contrast undifferentiated (top) and retinoic acid (RA)-treated SH-SY5Y neuroblastoma cells.

Among the vinca alkaloids vincristine is the drug that has been most extensively examined. Data generated by *in vivo* animal studies support localized axonal toxicity as a cause of distal axonal degeneration due to vincristine and are consistent with the largely predominant axonal damage which has been described in human sural nerve biopsy pathological examinations (McLeod and Penny, 1969, Postma *et al.*, 1993, Authier *et al.*, 2003, Authier *et al.*, 1999, Higuera and Luo, 2004, Sahenk *et al.*, 1987). In these models, axonopathy with Wallerian-like degeneration of myelinated and unmyelinated fibers has been clearly evidenced, and the pathological changes are correlat-
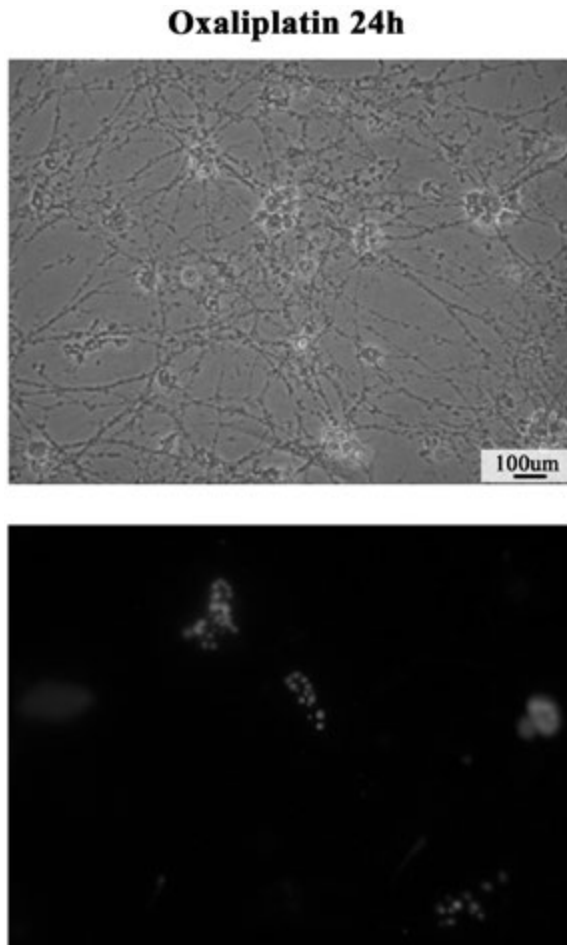
Oxaliplatin 24h

Figure 5 – Phase contrast photomicrograph showing dorsal root ganglia neuron primary cultures after NGF differentiation followed by 24 h oxaliplatin treatment (top). DNA fragmentation in neuron nuclei is demonstrated by Hochest 33342 staining (bottom).

ed with neuropathic pain assessed with behavioral tests (Ghirardi *et al.*, 2005). Even if autonomic involvement may be dose-limiting in vincristine treatment, as already observed, at present no reliable model of vincristine-induced autonomic impairment is available.

While several models of paclitaxel neurotoxicity have been reported in past years, docetaxel models of chronic administration *in vivo* have been reported only very recently by our group (Persohn *et al.*, 2005). The earliest studies performed in the 1980s reported that the direct intraneural injection of paclitaxel into the sciatic nerve induced axonal reactions and degeneration that are causally related to the slow progressive accumulation of microtubules and other axoplasmic constituents. This culmi-
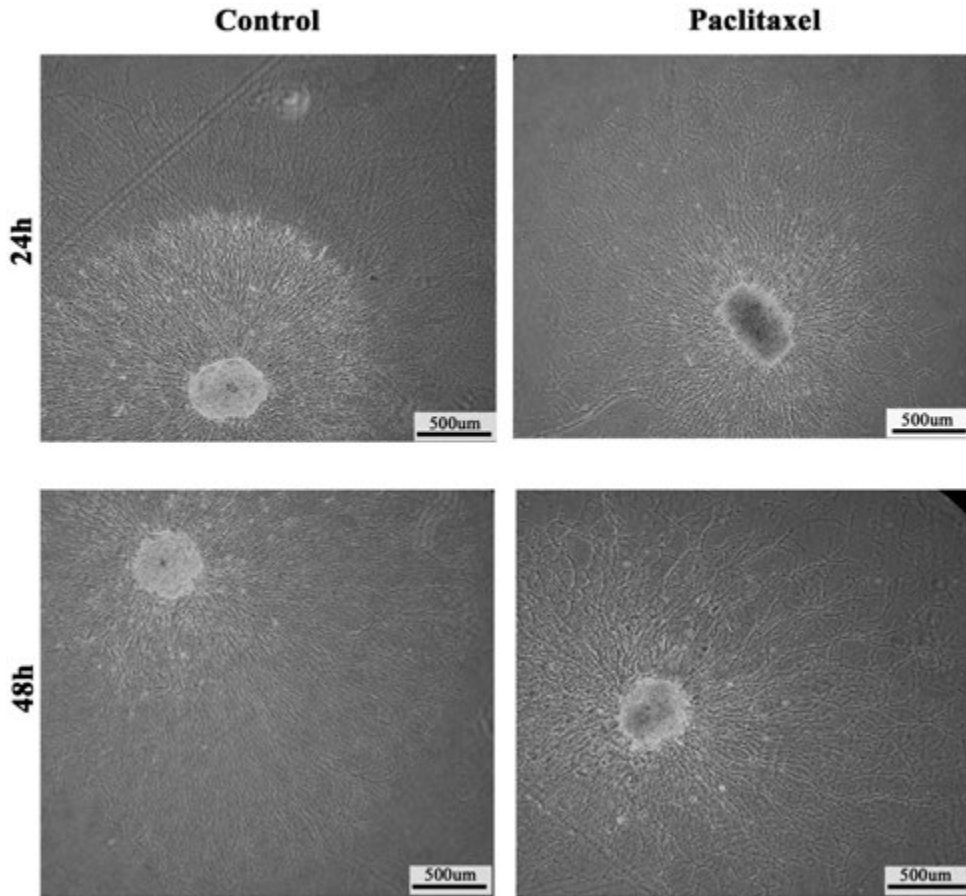
Control
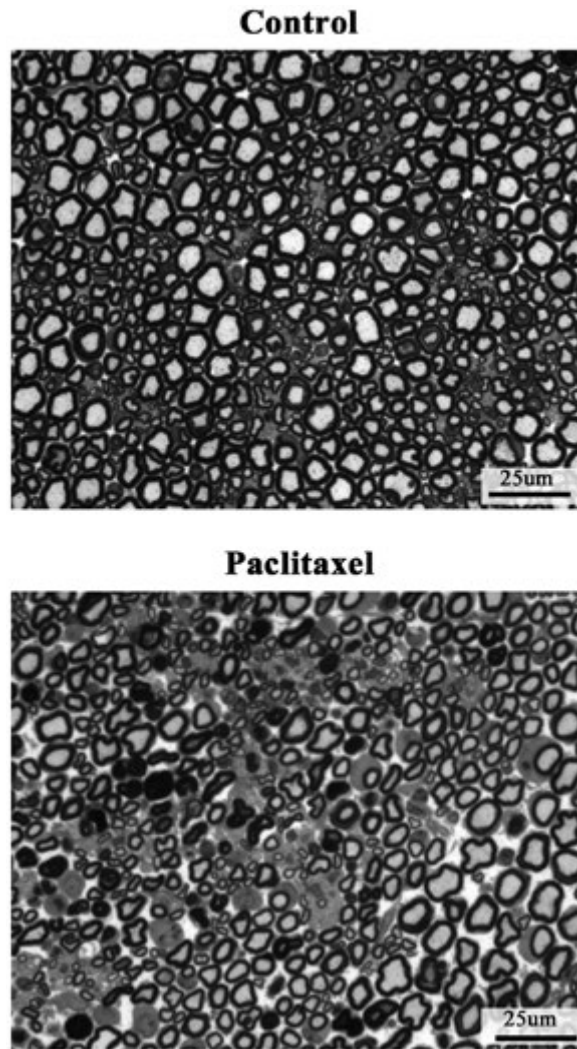
Paclitaxel

Figure 6 – Toluidine blue-stained light photomicrographs obtained from a transverse sections of sciatic nerve in an untreated (top) and a paclitaxel-treated (bottom) rat.

nated in the appearance of giant axonal spheroids and profiles similar to the retraction bulbs of Wallerian degeneration. Microtubule anomalies were also visualized in the distal portion of affected fibers and in regenerating sprouts. Schwann cells displayed microtubule abnormalities only at the site of the lesion where excessive microtubule polymerization caused the displacement of ribosomes from rough endoplasmic reticulum (Roytta and Raine, 1986). However, this model was based on a modality of administration that was able to overcome any anatomical protective barrier of the peripheral nerves.
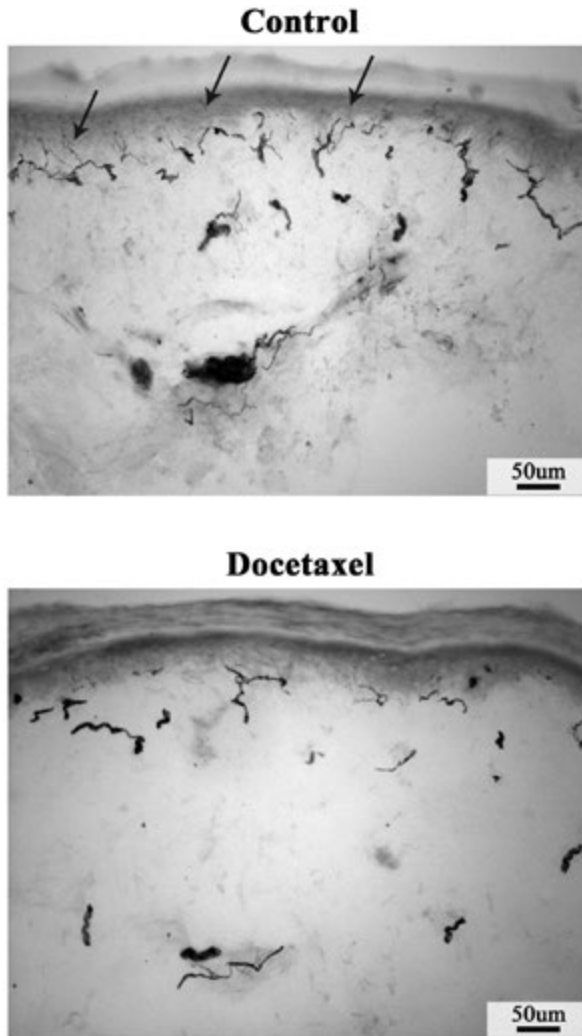
Figure 7 – Light micrographs of skin biopsies stained with PGP 9.5 to evidence the dermal and intra-epidermal (arrows) nerve fibers. A dramatic reduction in the intra-epidermal innervation is induced by docetaxel administration (bottom) if compared with the normal pattern (top).

We, therefore, characterized at the histological and ultrastructural level the effect of intraperitoneal and intravenous administrations of paclitaxel (Cavaletti *et al.*, 2000, Cavaletti *et al.*, 1997, Cavaletti *et al.*, 1995) and compared the effect of docetaxel treatment using different schedules (Persohn *et al.*, 2005) of chronic treatment. The examination at the light microscope of nerve sections from paclitaxel- or docetaxel-treated animals evidenced that most of the myelinated fibers were of normal aspect, although some fibers with axonal degeneration, including collapse and fragmentation

Control   **Paclitaxel**

Figure 8 – Phase contrast photomicrographs showing the effect of paclitaxel (right) after 24 h (top) and 48 h (bottom) exposure in NGF fully differentiated dorsal root ganglia explants in comparison with controls (left).

of myelin sheaths, could be observed (Fig. 6). The morphometric evaluation showed a significant decrease in g-ratio (i.e. the ratio between axonal diameter and the entire fiber diameter) in paclitaxel-treated animals, confirming the occurrence of axonopathy. The mean fiber diameter was significantly decreased in paclitaxel-treated animals and, accordingly, there was a significant increase in the percentage of myelinated fibers with a diameter smaller than 5.5 μm when compared with the controls (Persohn *et al.*, 2005). Electron microscopic examination of the sciatic nerve from treated animals confirmed primary axonal degeneration. Moreover, Schwann cells with condensed chromatin and "nucleolus-like" formations or enlarged intracytoplasmic organelles were observed. Occasionally, an accumulation of microtubules was observed within the axons of animals treated with both drugs. Dose-dependent changes in tail nerve conduction velocity were demonstrated with paclitaxel and docetexel (Persohn *et al.*, 2005). The use of injury markers such as activating transcription factor-3

Control   Bortezomib
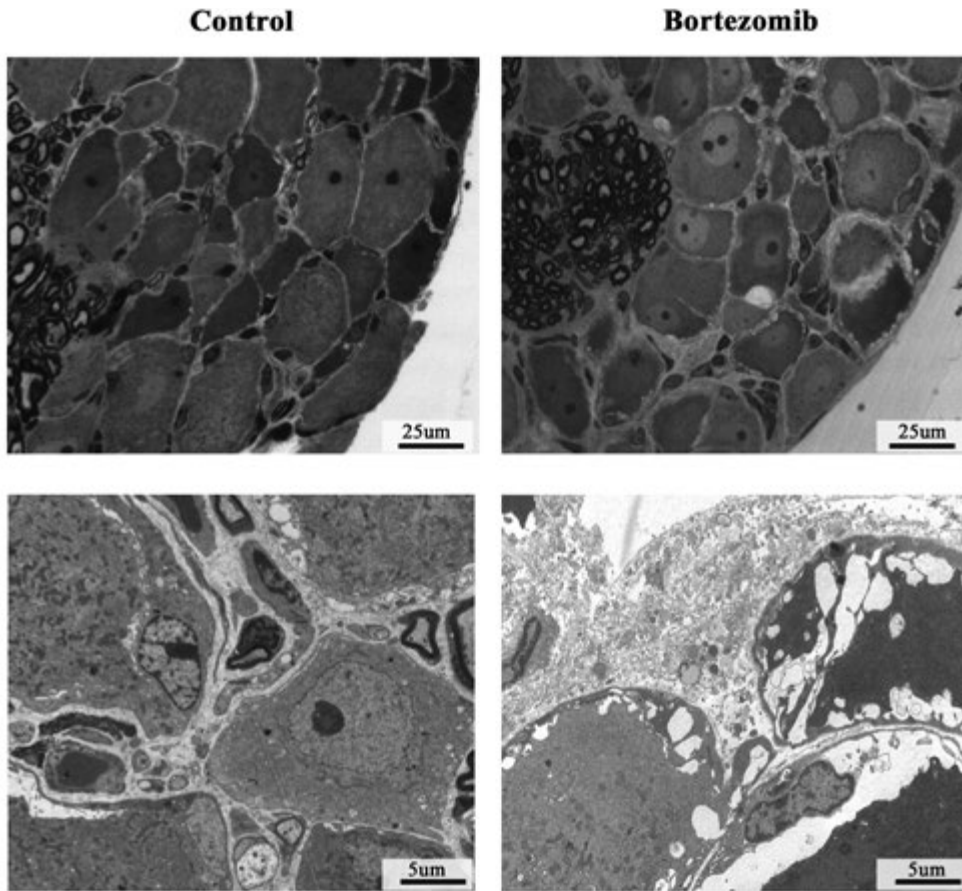
Figure 9 – Light (top) and electron microscope (bottom) findings in dorsal root ganglia specimens obtained from untreated (left) and bortezomib-treated (right) rats. Evident vacuolation of satellite cells is shown in the treated animals, while neurons are of normal appearance.

(ATF3) in DRG neurons after taxanes treatment made it possible to confirm that paclitaxel is toxic also at this cellular level (Flatters and Bennett, 2006). Accordingly, a high concentration of paclitaxel was demonstrated in the DRG (Cavaletti *et al.*, 2000). In a different rodent model of paclitaxel neuropathy axonal damage was also demonstrated in dorsal (sensory) but not in ventral (motor) spinal roots (Wang *et al.*, 2004).

When we assessed the intraepidermal nerve fiber density in skin biopsies, a diagnostic procedure already used in humans (Lauria *et al.*, 2005), to evaluate the most distal changes occurring in taxane-treated animals, a decrease in fiber density was demonstrated and the correlation with the neurophysiological assessment was highly significant (Fig. 7).

The first animal model of epothilone B peripheral neuropathy has recently been established in our laboratory (Chiorazzi *et al.*, 2009). The pathological examination

at the light and electron microscope of the peripheral nerves and DRG neurons gave results very similar to those obtained in taxanes models.

In *in vitro* studies 1 mM paclitaxel induces apoptosis in the SH-SY5Y human neuroblastoma cell line (Nicolini *et al.*, 2001; Nicolini *et al.*, 2003). The treatment causes chromatin condensation, DNA laddering and phosphatidylserine translocation. These morphological data indicating ongoing apoptosis are in agreement with molecular results that demonstrate Bcl-2 inactivation and caspase 7 activation. By contrast, 1 mM paclitaxel induces necrotic death in E15 rat DRG neurons, while no apoptotic features have been demonstrated (Scuteri *et al.*, 2006). In this case morphological and ultrastructural studies have demonstrated that neurons die through a necrotic process. Paclitaxel shows a dose and time-related effect on neurite length on NGF-differentiating E15 rat DRG, while it does not affect neurite length but only the morphology and the number of the processes of fully differentiated E15 rat DRG (Fig. 8). Similar results have been obtained by Theiss and Meller (2000) in primary cultures of sensitive neurons from 11-day-old chick embryos.

Paclitaxel neurotoxicity has also been demonstrated in NGF differentiated PC12 cells (Nuydens *et al.*, 2000; Pisano *et al.*, 2003). Paclitaxel in this *in vitro* model induces a reduction in neurite outgrowth and in the number of neurite-bearing cells.

According to in vivo results, in vitro data indicate a reduction in neurite length in E15 rat DRG treated also with epothilone B 0.1 and 0.5 nM. Epothilone B induces a significant reduction in neurite length at 24 hours that is reversed at 48 hours after the treatment, while concentrations higher than 5 nM determine neurite length reductions both at 24 and 48 hours of exposure.

Bortezomib

Bortezomib is a polycyclic derivative of boronic acid that inhibits the mammalian 26S proteasome. The proteasome degrades the intracellular inhibitor of NFkB (IkB) and so bortezomib increases the level of the inhibitor and decreases the activity of NFkB. The drug also binds to the tumor necrosis factor receptor type 1 (TNFR1). Through these mechanisms, bortezomib appears to sensitize cancer cell to inducers of apoptosis and to reduce the rate of cell division (Tobinai, 2007, O'Connor, 2005, Schwartz and Davidson, 2004). The mechanism underlying its peripheral neurotoxicity is unknown, but enhanced tubulin polymeration has been observed in cancer cells and neurons (Poruchynsky *et al.*, 2008).

At the current time only a few rat models of bortezomib-induced peripheral neurotoxicity have been reported (Carozzi *et al.*, 2010, Meregalli *et al.*, 2009, Cavaletti *et al.*, 2007b, Bruna *et al.*, 2010) and various schedules have been compared. At the light and electron microscope, sciatic nerve examination demonstrated the earliest changes mostly in the Schwann cells and myelin, but neuropathy due to axonal degeneration (the most typical effect of bortezomib administration in clinical use) was observed only after prolonged drug administration. At the ultrastructural level the pathological changes in Schwann cells were generally represented by mild vacuolation but, in the most severe cases of Schwann cell damage, cytoplasmatic involvement was more evident and the vacuoles were larger. In these cases, some of the vacuoles were found to be damaged mitochondria, while others were due to locally enlarged endoplasmic reticulum. In these *in vivo* models bortezomib-treated rats showed a significant

increase in the g-ratio, indicating an overall reduced thickness of the myelin sheath which was in agreement with the pathological observations (Meregalli *et al.*, 2009).

Also in the DRG, satellite cells were generally more severely damaged than neurons, with marked intracytoplasmatic vacuolization (Fig. 9). At the ultrastructural examination some of these vacuoles were clearly represented by damaged mitochondria, while the largest structures were due to enlarged endoplasmic reticulum. Only rarely did DRG neurons display pathological features at the light microscope: in these cases the cytoplasm showed a dark appearance with clear vacuoles occurring inside. These observations which point to mitochondrial and endoplasmic reticulum damage as a major effect of chronic bortezomib treatment in the sciatic nerve Schwann cells and DRG are intriguing, particularly in view of some of the mechanisms of action which have recently been suggested for bortezomib in cancer cells. In fact, several studies have evidenced that bortezomib is particularly effective in killing myeloma cells because it is able to induce the activation of the mitochondrial-based ("intrinsic") apoptotic pathway. In most cases, no nuclear changes were observed in satellite cells or in neurons, although very rarely mild segregation of the granular and fibrillar components of the nucleolus was observed in neurons. The morphometric examination did not evidence any significant difference in DRG neuron somatic, nuclear or nucleolar size.

In vitro studies on E15 rat DRG demonstrated a very steep dose response curve, suggesting a limited "therapeutic window" range between non neurotoxic concentrations and highly cytotoxic ones.

Conclusions

Based on the clinical features and on the symptoms and signs most frequently reported by patients suffering from CIPN, several morphological studies have been performed in animal models of CIPN and in *in vitro* studies in order to investigate the neurotoxicity of different antineoplastic drugs.

Animal models have been established for the majority of the most important anticancer drugs and their main pathological changes in the PNS have been described in this report, together with *in vitro* observations.

These studies are very important in this particular setting, since the availability of tissues of human origin is extremely limited. In fact, peripheral (generally sural) nerve biopsies are no longer required for diagnostic purposes in CIPN patients, and their use for a purely scientific aim, although potentially very informative, is not ethical. Moreover, as suggested by the clinical findings and confirmed by the preclinical studies, several neurotoxic drugs target the DRG neurons, and it is very difficult to obtain high-quality specimens even from early autopsies. In any case, even when these types of post-mortem analysis have been carried out, it is necessary to bear in mind that most of the cancer patients are treated with polychemotherapy schedules, thus making the interpretation of the results very uncertain.

When the morphological data were compared with neurophysiological and behavioral studies, the results were confirmed and, overall, they have undoubtedly been very useful for understanding some of the mechanisms of action of the neurotoxic antineoplastic drugs. Thus, it appears absolutely clear that, despite the implementa-

tion of sophisticated methods of investigation, the basis for any pathogenetic study on the toxic effect of anticancer drugs must still rely on solid morphological observations obtained in reliable animal and in vitro models.

It is, therefore, our opinion that, given the amount and the relevance of the information available, a focused morphological assessment of the in vitro and in vivo effects of any potentially neurotoxic antineoplastic drug, as well as of putative neuroprotective agents, should be included in the preclinical phase of any new drug right from the earliest stages of its development - an approach the importance of which tends to be frequently underestimated.

Acknowledgments

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