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Morphological and morphometric analysis of paclitaxel and docetaxel-induced peripheral neuropathy in rats

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Abstract

The experimentally induced neurotoxic effects of paclitaxel and docetaxel have never been compared, since no animal models of docetaxel peripheral neurotoxicity have yet been reported. In this experiment, we examined the effect of the chronic administration of these two taxanes in the Wistar rat using neurophysiological, neuropathological and morphometrical methods.

Our results showed that both paclitaxel and docetaxel induced a significant, equally severe and dose-dependent reduction in nerve conduction velocity. On the contrary, the morphometric examination demonstrated that the effect on the nerve fibres was more severe after paclitaxel administration when the same schedule was used. However, the overall severity of the pathological changes was milder than expected on the basis of the neurophysiological results.

Our results support the hypothesis that taxanes (and particularly docetaxel) may exert their neurotoxic effect not only on the microtubular system of the peripheral nerves, but also on other less obvious targets.

Keywords: Paclitaxel; Docetaxel; Peripheral neuropathy; Neurophysiology; Pathology; Morphometry; Rat

1. Introduction

Taxanes are among the most effective antineoplastic drugs, but their use is limited by peripheral neurotoxicity [1,2]. The mechanism of taxane neurotoxicity is not yet completely understood [3] and one of the reasons for this is the absence of a complete characterisation of the modifications induced in the peripheral nerves on pathological grounds. In fact, very few pathological studies have been performed in humans [4,5]. Moreover, only paclitaxel has been studied in animal models [6–14], while no animal models of docetaxel neuropathy have been reported so far.

In our experiment, we compared the peripheral neurotoxicity of different schedules of paclitaxel and docetaxel administration in rats. In addition, we carried out a detailed neuropathological and morphometrical comparison of the effects of paclitaxel and docetaxel. Finally, we investigated the possibility that nerve growth factor (NGF) changes may play a role in the course of taxane-induced peripheral neurotoxicity [12,13,15–20], by measuring NGF circulating levels after treatment.

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2. Materials and methods

A total of 80 adult female Wistar rats were used for the experiment. Different schedules of intravenous (i.v.) paclitaxel or docetaxel administration were used in two experiments (see Table 1), while untreated rats, animals treated with vehicle alone or with paclitaxel 16 mg/kg intraperitoneal (i.p.) were used as references [8]. Both drugs were dissolved in absolute ethanol/Tween 20/saline (5/5/90%), and this solvent alone was i.v. injected in one of the control groups which was used as vehicle control. In all groups (i.p. and i.v.), the experimental drugs were administered weekly for four weeks (q7d × 4) and in the i.v. treated groups a catheter was placed in the jugular vein and left *in situ* throughout the entire experiment.

The care and husbandry of animals were in conformity with the institutional guidelines in compliance with national (D.L. n. 116, *Gazzetta Ufficiale della Repubblica Italiana*, suppl. 40, February 18, 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

2.1. Neurophysiological assessment

Before starting the treatment and after the end of the treatment period, each animal underwent the determination of sensory nerve conduction velocity in the tail as previously described [21].

2.2. Sacrifice

At the end of the experiment, according to the different experimental protocols, the animals were sacrificed under general xylazine/ketamine anesthesia and used for biological sampling.

2.3. Neuropathological examination

Three rats from the control group and three rats from the 10 mg/kg i.v. paclitaxel and docetaxel groups were perfused with 2% glutaraldehyde in 0.12 M phosphate buffer. Specimens of the sciatic nerve were obtained, were further fixed by immersion with 2% glutaraldehyde in 0.12 mM phosphate buffer, pH 7.2, and kept overnight at 4 °C in the fixative. Post-fixation was performed with 2% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, for 2 h at 4 °C. The specimens were then dehydrated in graded acetone solutions and embedded in Epon.

Semithin sections were prepared from at least two tissue blocks for each animal. The sections were stained with toluidine blue and examined with a Zeiss light microscope. Based on the light microscopic findings, ultrathin sections were prepared from selected tissue blocks, counterstained with uranyl acetate and lead citrate and examined with a Philips CM 10 transmission electron microscope.

Table 1
Summary of the experimental groups

Treatment	Paclitaxel experiment	Docetaxel experiment
Untreated controls	8 animals	6 animals
16 mg/kg i.p.	8 animals	–
5 mg/kg i.v.	8 animals	8 animals
10 mg/kg i.v.	8 animals	10 animals
12.5 mg/kg i.v.	8 animals	8 animals
Vehicle	–	8 animals

2.4. Morphometric evaluation of g-ratio and axon density

Evaluation of axon density and *g*-ratio was performed [22], using a Cellenger® image analysis system (Definiens Company, München) and a Zeiss Axioplan light microscope.

Counting of myelinated fibres was performed automatically with a module of the Cellenger® system on toluidine blue stained semithin sections at a light microscope magnification of 1000× from 17 images representing a total area of 0.16 mm^2 (i.e. approximately 2000 fibres/animal). At the same time, the axoplasm area and myelin area were measured and the *g*-ratio (i.e. the ratio between the axonal diameter divided by the diameter of the axon with myelin, an index of axonal atrophy or of myelin thickness changes) was calculated. Since on the cross sections the shape of the axons was not always round, we measured the area and calculated the mean diameter from this value, assuming that the ideal shape would be a circle.

2.5. Morphometric evaluation of microtubule density

Evaluation of microtubule density was performed according to the method described by Friede and Samorjsky [23], but using an analySIS® image analysis system (Soft Imaging System GmbH, Münster) and a Philips CM 10 transmission electron microscope.

Axons were divided into three categories: (1) ''small'' myelinated axons with areas from 2.25 to 9.5 μm^2, (2) ''large'' myelinated axons with areas from 10 to 75.0 μm^2, and (3) unmyelinated axons. For each category, microtubules from 20 axons (one picture per axon = 3 μm^2) representing a total area of 60 μm^2 (category 1 and 2), or (one picture per axon = 1 μm^2) representing a total area of 20 μm^2 (category 3), were counted.

Counting of microtubules was performed automatically with a module of the analySIS® system at an electron microscopic magnification of 39,000×. At the same

time the axonal area was measured. Only well preserved fibres in which most of the microtubules and neurofilaments were cut perpendicularly to their axis were selected. For each animal and axon category, at least one of the automatic counting was also counted manually in order to check the accuracy of the result.

2.6. NGF assay

The levels of circulating NGF were determined from blood samples drawn from the abdominal aorta during general anesthesia at the sacrifice performed at the end of treatment [12,17].

2.7. Statistical analysis

The differences between all the experimental groups in bodyweight, tail nerve conduction velocity and NGF circulating level results were statistically evaluated using analysis of variance (ANOVA) and the Tukey–Kramer post-test. Since normality of the morphometric data could not be assumed for all responses, a transformation was applied: log transformation for myelin sheath area and square root transformation for small myelinated axons, large myelinated axons, unmyelinated axons, axonal area without myelin and mean myelinated fibre diameter. Subsequently, ANOVA was used, modelling the treatment group as a fixed effect and the animal as a random effect. For the percentages, a generalised linear model was applied. All calculations were carried out on the Novartis AH Development Biometrics IT infrastructure, PC AHCHBS-W10128, using SAS®, Version 8.2 software (SAS Institute, Inc, Cary, NC). Significance level was always set at $P < 0.05$.

3. Results

3.1. General toxicity

Four rats in the i.p. paclitaxel group died after 14, 16 and 18 days of treatment [2], respectively. One rat in the i.v. paclitaxel 5 mg/kg group died after the first injection, probably for reasons unrelated to the treatment. Three rats in the i.v. docetaxel 12.5 mg/kg group died after 6, 8 and 13 days, respectively.

In the i.p. paclitaxel group, all the surviving rats had evident ascites, as already observed in previous experiments [8]. The i.v. administration of the test compounds was generally well tolerated at the lowest dose; the highest doses were frequently associated with pilorection and reduced motility immediately after the administration. The dose of 12.5 mg/kg q7d × 4 used for docetaxel was associated with severe general toxicity (besides the three deaths, two more rats in this group were very sick by the end of treatment). Both with paclitaxel and docetaxel (but particularly with the latter), the highest dose schedules were associated not only with more evident behavioural and morphological changes, but also with a markedly reduced weight gain *vs.* the lowest dose schedules (Fig. 1).

No details of weight changes in i.p. treated rats are given since ascites prevented its accurate evaluation.

3.2. Neurophysiological examination

No difference between groups was observed at the baseline determination. The administration of paclitaxel i.p. (used as a reference for the toxicity of chronic administration of this drug) [8] induced a significant reduction in tail nerve conduction velocity ($P < 0.001$ *vs.* controls and vehicle-treated rats), while vehicle administration had no effect. All the schedules of i.v. paclitaxel or docetaxel administration induced a significant and dose-dependent reduction in tail nerve conduction velocity *vs.* controls and vehicle-treated rats ($P < 0.001$ in all cases).

The results of the neurophysiological examination obtained at the end of the treatment period are reported in Fig. 2.

3.3. Histopathological examination

Light micrographs of 1-μm thick semithin sections from paclitaxel- or docetaxel-treated animals showed that most of the myelinated fibres were of normal histology, although some fibres with axonal degeneration, including collapse and fragmentation of myelin sheaths, were observed.

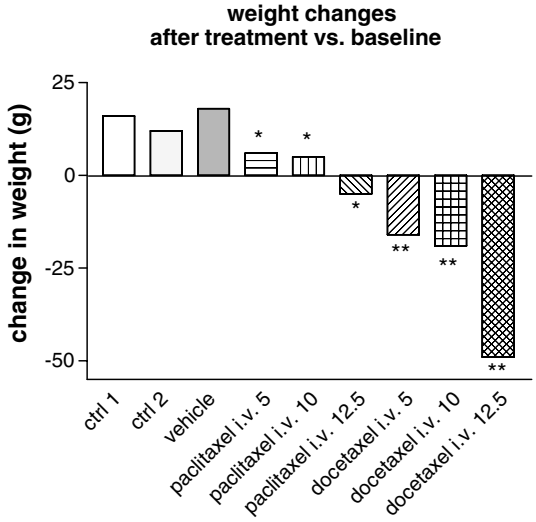

Fig. 1. Summary of the mean weight changes observed in each experimental group at the end of the treatment period. Asterisk indicates results significantly different from the control group of each experiment: *$P < 0.01$, **$P < 0.001$.

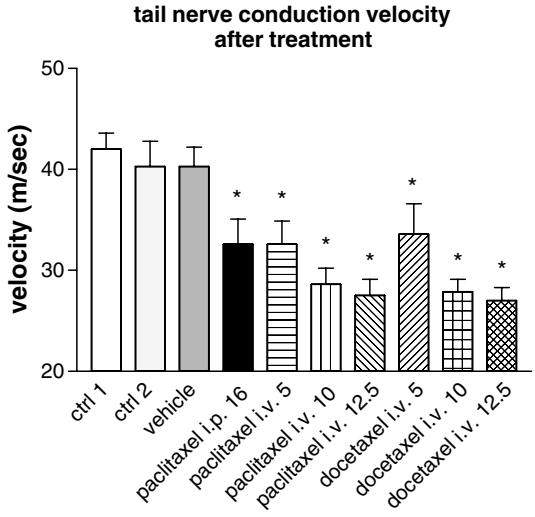

Fig. 2. Summary of the results (means ± SD) observed in the neurophysiological determinations performed in each group after treatment. Asterisk indicates results significantly different from the control group of each experiment: $*P < 0.001$.

The morphometric evaluation performed at the light microscope, summarised in Table 2, showed a significant decrease in *g*-ratio in paclitaxel-treated animals. The myelinated fibre density was increased for both paclitaxel- and docetaxel-treated rats *vs.* controls, but only paclitaxel induced a significant modification *vs.* control rats. The mean fibre diameter was significantly decreased in paclitaxel-treated animals and, accordingly, there was a significant increase in the percentage of myelinated fibres with a diameter smaller than 5.5 μm when compared to the controls. By contrast, the percentage of myelinated fibres with a diameter larger than 10 μm decreased after paclitaxel treatment, but this reduction, although marked (−27% in mean value), was only close to statistical significance ($P = 0.055$). In agreement with the *g*-ratio determination, the myelin sheath area was unchanged in both paclitaxel- and docetaxel-treated animals, while the axonal area was decreased only in paclitaxel-treated animals.

Electron microscopic examination of the sciatic nerve from treated animals showed occasional axonal degeneration, including collapse and fragmentation of the myelin sheath of some axons. The morphometric evaluation performed at the ultrastructural level (summarised in Table 2) showed that only in the smallest myelinated axons was the number of microtubules per μm^2 increased after docetaxel treatment *vs.* controls (Fig. 3).

3.4. NGF circulating levels assay

Circulating NGF levels were determined in controls and paclitaxel or docetaxel-treated rats (10 mg/kg i.v. in both cases). No statistically significant difference between groups was observed in NGF levels in treated rats *vs.* controls (controls mean ± SD = 23.82 ± 4.94 μg/ml, paclitaxel-treated = 24.25 ± 4.75 μg/ml, docetaxel-treated = 20.76 ± 6.52 μg/ml).

4. Discussion

The use of animal models is a well-established method for investigating several aspects of antineoplastic drug-induced peripheral neurotoxicity [8–10,12–14,24–27]. On the contrary, the semi-synthetic taxane docetaxel, which is potentially less neurotoxic than paclitaxel [1,28], has never been investigated in pre-clinical animal models of peripheral neurotoxicity. In this experiment, we performed a dose-finding study to assess the best schedule to be used to obtain a reliable model of paclitaxel and docetaxel peripheral neurotoxicity in rats and

Table 2
Summary of the morphometric results obtained from light and electron microscopy

Drug dose (mg/kg i.v. q7d × 4)	Controls		Paclitaxel			Docetaxel		
	0		10			10		
	Mean	SD	Mean	SD	*P vs.* controls	Mean	SD	*P vs.* controls
Mean myelinated fibres diameter (μm)	6.4	2.4	6.1	2.3	0.033	6.2	2.4	0.228
Myelinated fibres (%)								
(∅ ⩽ 5.5 μm)	31.2	0.6	35.2	2.3	<0.001	34.5	4.6	0.106
(∅ ⩾ 10 μm)	12.8	1.4	9.41	3.13	0.055	12.6	1.5	0.852
Fibre density (myelinated fibres/mm^2)	15161	2372	17279	2341	0.022	16737	2503	0.088
g-ratio	0.61	0.07	0.57	0.06	0.027	0.61	0.07	0.926
Myelin sheath area (μm^2)	22.8	15.5	21.7	14.6	0.345	21.5	14.9	0.143
axonal area (μm^2)	13.8	10.6	11.5	9.0	0.021	13.5	10.9	0.614
Microtubule density (number per μm^2)								
Small myelinated axons	25.77	5.92	30.17	7.40	0.117	34.52	9.92	0.003
Large myelinated axons	15.81	4.09	17.02	3.89	0.618	19.44	4.03	0.158
Unmyelinated axons	70.73	13.41	76.10	11.23	0.180	68.38	12.19	0.584

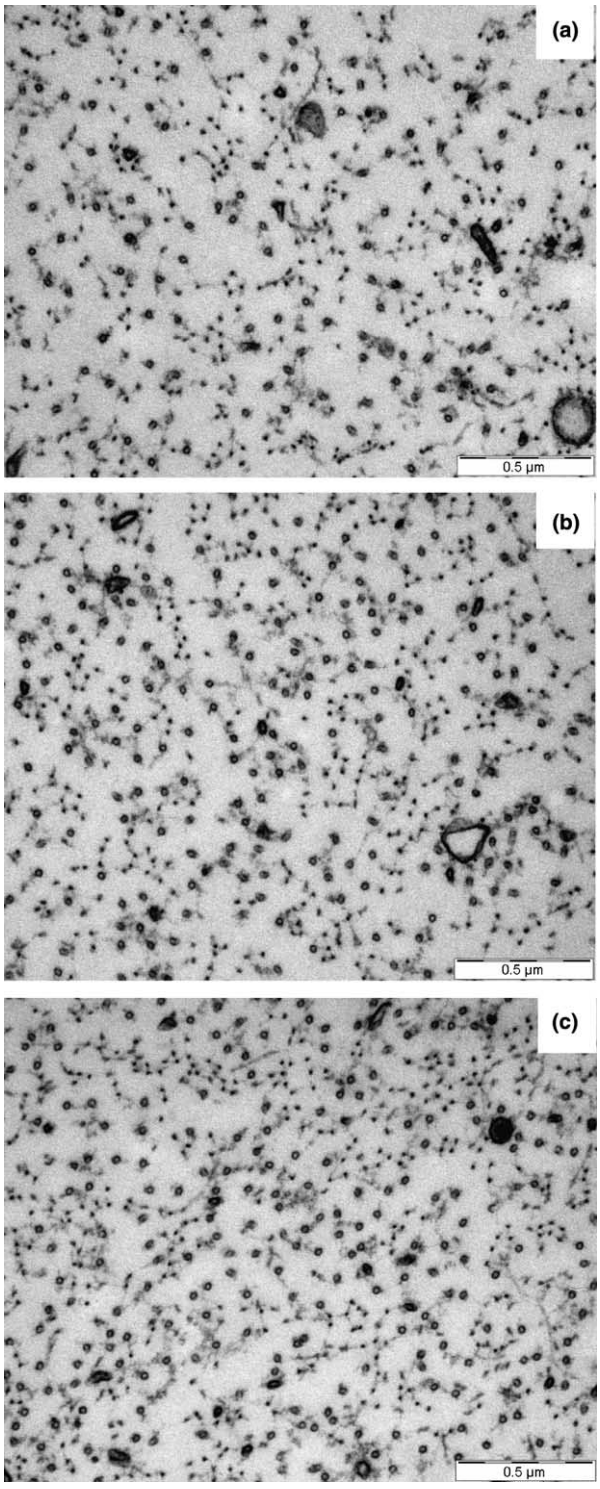

Fig. 3. Transmission electron micrograph of small myelinated axons from sciatic nerve of a: (a) control animal, (b) an animal treated with paclitaxel 10 mg/kg i.v., or (c) docetaxel 10 mg/kg i.v. Increased density of microtubules (confirmed by morphometric analysis) is induced by docetaxel treatment (lead citrate and uranyl acetate, original magnification 39,000×). Microtubules were identified as circular profiles 25 nm in diameter with electron-lucent centers. Neurofilaments were apparent as black profiles 10 nm in diameter.

to define a possible role for neurotrophin NGF which seems to be involved in the onset of platinum-based chemotherapy-induced peripheral neurotoxicity [12,15–17,29]. Moreover, we performed an extensive morphological and morphometrical examination on the sciatic nerves of paclitaxel- and docetaxel-treated rats. Our results suggest that docetaxel, used at the same dose and with the same schedule, has a more severe general toxicity than paclitaxel.

Regarding neurotoxicity, at the neurophysiological level, both paclitaxel and docetaxel induced a significant reduction in tail nerve conduction velocity and this effect was evident throughout the entire range of doses used (i.e. from 5 to 12.5 mg/kg i.v. q7d × 4). A clear dose-dependency of the neurophysiological changes was observed with both drugs and no significant difference could be evidenced between paclitaxel and docetaxel at each dose.

In contrast with the results obtained with platinum-derived drugs [12,15–17,29], no change in NGF circulating levels was observed using a schedule (i.e. 10 mg/kg q7d × 4) which induced significant neurophysiological changes and was selected for the neuropathological examination.

At the pathological level, the administration of paclitaxel or docetaxel induced mild changes in both cases, mainly represented by axonal degeneration of myelinated fibres. However, the morphometrical assessment of the *g*-ratio and of the axonal area in the sciatic nerve demonstrated, in agreement with previous results obtained in models of i.p. administration [8,27], a significant axonal atrophy in paclitaxel-treated rats. On the contrary, axonal atrophy was not observed in animals treated with the same dose of docetaxel. Another demonstration of a different effect of the two drugs on myelinated fibres is represented by the shift towards a smaller size of the fibres observed with paclitaxel but not with docetaxel, a pathological change that has already been reported for the i.p. administration of paclitaxel [8,27].

The most obvious target of taxanes in the peripheral nerve fibres is represented by the microtubular system, and for this reason a detailed morphometric analysis was performed on different fibre populations: large and small myelinated fibres and unmyelinated fibres. Rather surprisingly, despite both drugs tending to induce an increase in microtubule density in myelinated fibres, due to the variability of the microtubule density in the axons, only the increase induced by docetaxel treatment in the small myelinated fibres was statistically significant.

In conclusion, the results of this experimental study allowed new models of taxanes' peripheral neurotoxicity to be characterised and defined using chronic schedules of administration and confirmed that the intravenous

rather than the intraperitoneal method of administration (i.e. that used in clinical practice) is more suitable for treatment. The changes induced by identical doses of paclitaxel or docetaxel were marked and they were equally severe when the neurotoxic effect was assessed with neurophysiological methods. At the neuropathological level, it was confirmed that the large myelinated fibres are the main target of both drugs. However, a detailed morphometric examination of the sciatic nerves demonstrated that docetaxel is less neurotoxic than paclitaxel on the peripheral nerves when they are administered according to the same schedule. Our observations, reveal a discrepancy between the marked neurophysiological effect and the rather milder pathological changes induced by taxanes at doses which have a very modest general toxicity. Our data lend support to the possibility that taxanes exert a major neurotoxic effect not only on their most obvious target (i.e. the axonal microtubular system), but also on other targets such as the dorsal root ganglia [6–8,13,30] where paclitaxel accumulates after chronic treatment [7,30] and induces neuropeptide changes [13], or the spinal roots where microtubular accumulation was reported [6,8]. When the possible biological marker of taxane peripheral neurotoxicity (i.e. NGF circulating levels) was investigated [12,15–17,29], no differences *vs.* control rats were found.

We believe that these new models of chronic taxanes' peripheral neurotoxicity can be reliably used in further studies in order to obtain more detailed knowledge of other possible mechanisms of neurological damage caused by these antineoplastic drugs and, moreover, they may also be useful for testing the effect of putative neuroprotectant drugs or for comparing the effect of new antitubulinic agents in *in vivo* systems.

Conflict of interest statement

None declared. Two authors are from Norvartis Pharma AG and Hoffman LaRoche.

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