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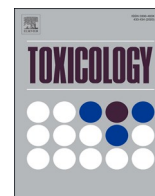
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The challenge to identify sensitive safety biomarkers of peripheral neurotoxicity in the rat: A collaborative effort across industry and academia (IMI NeuroDeRisk project)

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ABSTRACT

Peripheral nervous system (PNS) toxicity assessment in non-clinical safety studies is challenging and relies mostly on histopathological assessment. The present work aims to identify blood-based biomarkers that could detect peripheral neuropathy in rats upon exposure to neurotoxic compounds. Three anticancer agents (oxaliplatin, cisplatin, paclitaxel) and a developmental compound (NVS-1) were assessed in male rats (Wistar Han). Clinical and/or functional endpoints (i.e., electronic Von Frey, Cold Plate, and Paw Pressure tests) and blood biomarkers (i.e., neurofilament light chain (NFL), neurofilament heavy chain (NF-H), microtubule-associated protein Tau (Tau), neuron specific enolase (NSE), vascular endothelial growth factor A (VEGFA), and glial fibrillary acidic protein (GFAP)) were assessed. Drug exposure and histopathological evaluations were conducted on selected nervous tissues. Oxaliplatin, cisplatin and paclitaxel treatment resulted in a significant decrease of nociceptive thresholds. Clinical signs suggestive of PNS toxicity were observed with NVS-1. NFL was consistently increased in the NVS-1 study and correlated with moderate microscopic findings in dorsal root ganglia (DRG). Only minimal microscopic findings were observed in oxaliplatin-treated animals, whereas no treatment-related microscopic findings were observed in animals treated with cisplatin and paclitaxel. For all compounds, exposure was confirmed in the PNS tissues. Clinical and functional changes were observed with all the compounds evaluated. NFL levels in plasma proved to be the most sensitive indicator of PNS toxicities, capturing moderate

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nervous degeneration in DRG. A combined approach that includes both functional assessments and biomarker measurements offers a more comprehensive evaluation than histopathological analysis alone when monitoring drug-induced neurotoxicity in rat models.

1. Introduction

The current *in-vitro* and *in-vivo* nonclinical safety assessment studies conducted in the early stages of drug development are not effective at predicting adverse effects on peripheral nervous system (PNS). The NeuroDeRisk project, part of Innovative Medicines Initiative, focused on enhancing the nonclinical prediction of neurological adverse effects caused by pharmaceuticals, aiming to mitigate risks associated with early-stage drug candidates during discovery and nonclinical testing phases. Moreover, given that PNS toxicity can result in debilitating and potentially irreversible effects in humans, there is an urgent need to improve its detection. Circulating biomarkers stand out as one of the most promising endpoints due to the ease of measurements potential translatability into clinical practice.

In humans, PNS toxicity or drug-induced peripheral neuropathy, comprises a small subset of neuropathies, accounting for only 2–4 % of all neuropathy cases (de Anda-Jáuregui et al., 2019; Jain, 2012), whereas more than 60 % of patients treated with anticancer drugs (e.g. platinum derivatives, taxanes, vinca alkaloids, bortezomib, and thalidomide) will develop chemotherapy-induced peripheral neuropathy (CIPN) (Seretny et al., 2014). CIPN is a prominent adverse effect following the administration of anticancer drugs (Argyriou et al., 2012), manifested mainly through sensory abnormalities and histological alterations, altering the quality of life of patients and lasting several years after the end of treatments (Kerckhove et al., 2017). Both in clinical trials and in medical practice, the methods of CIPN evaluation are based on clinical examination, patient self-administered questionnaires, and neurophysiological functionality assessments (Jordan et al., 2020). These types of measures still present feasibility problems in the clinical setting, due to the need for special instruments, qualified personnel, and time-consuming assessments (Park et al., 2019). Balancing the risks of CIPN with the benefits of treatment is a crucial aspect of the oncological practice, with an impelling need to develop simple and effective strategies for the identification of neuropathy before its clinical manifestation (Burgess et al., 2021).

Neurofilament light chain (NfL), a major component of the axonal cytoskeleton identified as marker of neuronal damage has emerged in the last years as a potentially useful translational blood biomarker of CIPN (Meregalli et al., 2018; Mortensen et al., 2023) and peripheral neuropathies (Fundaun et al., 2022). Besides NfL, additional neurological biomarkers have been explored as putative translational biomarkers of central nervous system (CNS) and PNS toxicity. Vascular endothelial growth factor A (VEGFA) is a pro-nociceptive factor of key relevance in the onset and maintenance of neuropathic pain (Micheli et al., 2021). Tau is a microtubule-associated protein that stabilizes neuronal microtubules under normal physiological conditions. Accumulation of hyperphosphorylated Tau leads to the formation of intracellular aberrant aggregates called neurofibrillary tangles, which are common histopathological hallmark of a set of neurodegenerative diseases (Sebastián-Serrano et al., 2018). Neuron specific enolase (NSE) is a glycolytic enzyme almost exclusive in neurons and neuroendocrine cells which blood concentration is correlated with the degree of neuronal loss (Haque et al., 2018). Glial fibrillary acidic protein (GFAP), a glial cytoskeletal protein, is released in the blood following neuroaxonal damage (Notturmo et al., 2009). Ubiquitin C-terminal hydrolase L1 (UCH-L1), is a cytoplasmic neuron-specific protein found in brain and neuroendocrine cells that is important for neuroaxonal stability and repair after brain injury (Nawaz et al., 2020).

In the present study, we utilized three anticancer agents (oxaliplatin, cisplatin, and paclitaxel) alongside a developmental compound (NVS-1)

to elicit varying levels of PNS toxicity in the rats. We then correlated behavioral signs of injury, histopathological changes, and plasma protein concentrations to determine the most predictive biomarker for PNS toxicity.

2. Materials and methods

2.1. Research centers involved in the study

The *in vivo* studies were conducted in three different research centers, University Clermont Auvergne (UCA, France), University of Florence (UNIFI, Italy), and Novartis Pharma AG (NOVA, Switzerland and USA). Histopathological assessments were conducted by Sanofi laboratories (France) and NOVA; analyses of tissue exposure to compounds were conducted by Uppsala University (Sweden) and UCA; plasma biomarkers were analyzed by UNIFI, NOVA and Merck Research Laboratories (USA).

2.2. Study designs

Study designs for the different compounds are presented in Fig. 1. Animals received repeated injections of oxaliplatin (UCA), cisplatin (UNIFI), paclitaxel (UCA and UNIFI), or NVS-1 (NOVA) as described in the “Compounds and treatments” section.

Behavioral assays were performed throughout the duration of the studies to explore functional changes in animals. At the indicated days of euthanasia in Fig. 1, nervous tissues (brain, spinal cord, dorsal root ganglia (DRG), sciatic and saphenous nerves) were collected for microscopic assessment of histopathological changes and measurement of drug exposure. Plasma was collected for biomarkers analysis (i.e., NfL, NF-H, TAU, NSE, GFAP, and VEGFA).

2.3. Animals

Oxaliplatin and paclitaxel studies performed at UCA were conducted in male Wistar rats (5–7 weeks old upon arrival, Charles River Labs; n=6 per group). Animals were housed in the Unité de stabulation animale (Faculté de Pharmacie, UCA) and acclimated for at least 6 days. Four rats were housed per cage with water and food *ad libitum*, a non-reverse 12-h light/dark cycle, and 40–75 % hygrometry. These experiments received ethical approval from the Animal Care and Use Committee of Auvergne (C2EA-02) and from the French Ministry of Higher Education, Research and Innovation (Ministère de l'Enseignement supérieur, de la Recherche et de l'Innovation), with the following agreement number: oxaliplatin study APAFIS#22542–2019102211127150 v2 (January 08, 2020), and paclitaxel study APAFIS#28498–2019102211127150 v3 (January 19, 2021).

Cisplatin and paclitaxel studies were performed at UNIFI in male Wistar rats (Envigo - Varese; n=6 per group) weighing approximately 200–250 g at the beginning of the experimental procedure. Animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and acclimated for 1 week. Four rats were housed per cage with a 12-h light/dark cycle and were fed standard laboratory diet and tap water *ad libitum*. Formal approval to conduct the experiments described was obtained from the Italian Ministry of Health (No. 300/2021-PR) and from the Animal Subjects Review Board of the University of Florence.

The NVS-1 study was performed at NOVA, East Hanover facility, New Jersey 07936 (USA) in male Wistar rats (Charles River Laboratories, Raleigh, USA; n=8 per group). The animals were received from

the supplier surgically implanted (approximately 3–5 days prior to arrival) with a single port (femoral) for infusion and with a molded elastomeric harness to facilitated attachment to a quick-connect tethered infusion system. At the initiation of dosing animals were approximately 10 weeks of age and weighed 262 g to 346 g. The NVS-1 experiments performed in USA were conducted in accordance with the NOVA Animal Care and Use Committee (NACUC) generic protocol TX 4039.

All animal studies performed in Europe were carried out according to the Directive 2010/63/EU of the European parliament and of the European Union council (22 September 2010) on the protection of animals used for scientific purposes. The results are reported according to the ARRIVE guidelines for animal research (Kilkenny et al., 2010).

The animals were randomly assigned to control or treated groups. All the experimental evaluations were performed by operators blinded to the treatment groups. The health status of the animals was assessed every day, monitoring weight, fur condition and mobility. Animals that showed weight reduction greater than 20 %, marked piloerection, immobility for more than 24 hours, abdominal ascites, anemia would have been excluded from the study and euthanized. None of the animals was excluded.

The sample size was determined using the 3Rs principle of reduction, based on body weight variability in male rats. This approach was adopted in anticipation of toxicokinetic assays, where body and tissue weight variations can influence kinetic parameters.

2.4. Compounds and treatments

Oxaliplatin (Leancare, UK) was administered by intravenous route (i. v.) at 1, 2 and 4 mg/kg/dose, twice a week for 4 weeks, for a total of 8 injections (cumulative doses: 8, 16, and 32 mg/kg, respectively). Control animals received an equal amount of vehicle (5 % glucose). Doses and methods were derived from previous studies in rat (Ling et al., 2007). The animals were sacrificed from day 29–32 via decapitation after deep isoflurane 4 % anesthesia.

Paclitaxel at UCA site (Leancare, UK) was solubilized in a stock solution at 6 mg/mL (Cremophor EL/Ethanol (1/1)); paclitaxel at UNIFI site was supplied as solution (Mylan generics, Italy; 6 mg/mL in Cremophor EL/Ethanol in 1:1 ratio). Both were thereafter diluted in 0.9 % NaCl, in order to prepare a dosing solution of 5 mg/mL. Paclitaxel was administered at 5 mg/kg/dose, i.v., once a week for either 2 or 4 weeks for a total of 2 and 4 injections. Control animals received an equal amount of vehicle. At UCA, animals treated for two weeks were sacrificed from day 15–17, those treated for four weeks were sacrificed on days 29 and 30 via decapitation after deep isoflurane 4 % anesthesia. At UNIFI, animals treated for two weeks were sacrificed on day 15, those treated for four weeks were sacrificed on day 30 via decapitation after deep isoflurane 4 % anesthesia.

Cisplatin (Teva, Italy) was diluted in 0.9 % NaCl solution and administered intraperitoneally (i.p.) on days 1, 4, 8 and 11 at the 1, 2, and 4 mg/kg dose. Animals were sacrificed via decapitation after deep isoflurane 4 % anesthesia at the end of the behavioral measurements (day 15). Control animals were treated with vehicle (0.9 % NaCl solution).

NVS-1 compound was administered to the rats via a 2-hour i.v. infusion through femoral port as a solution in vehicle (10 % (w/v) hydroxypropyl- β -cyclodextrin and 3.3 % (w/v) glucose prepared in sterile water for injection, at 30 mg/kg/dose and 6 mL/kg on selected dosing days. Animals were sacrificed by deep isoflurane anesthesia followed by abdominal aorta sectioning and exsanguination. Control animals received the vehicle at an equivalent dose volume.

2.5. Behavioral assays

Behavioral tests to measure mechanical and thermal sensitivities were performed at the frequency depicted in Fig. 1. The analysis performed for each compound were as follows. For oxaliplatin and paclitaxel assessments performed in UCA, mechanical sensitivity thresholds were assessed using an electronic Von Frey test (Bioseb, France) (Ferrier et al., 2016). Three days before the beginning of drug injections, each rat

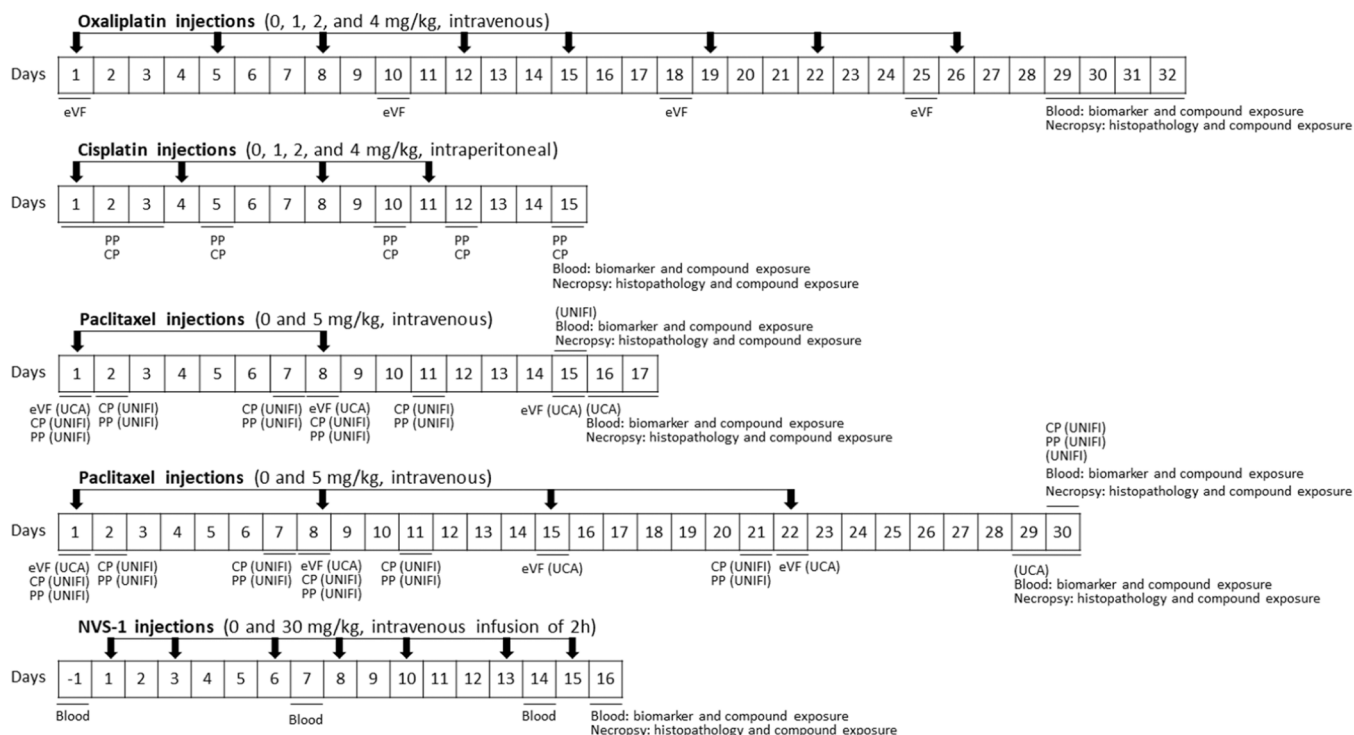


Fig. 1. Overview of the design of studies. CP: Cold Plate test; eVF: Electronic Von Frey test; PP: Paw Pressure test; Blood: collection of blood samples for measurements of biomarkers.

was habituated to be in the plastic compartments to avoid any fear or stress during the experiment. Rats were placed individually in plastic compartments on an elevated wire floor and left for habituation for 15 min before each experiment. The Von Frey apparatus, consisting of a plastic tip fitted in a hand-held force transducer, was applied perpendicularly to the animal's right hind paw from below and the force applied was gradually increased until paw withdrawal. The maximum force applied (expressed in grams) to induce paw withdrawal was recorded automatically. The two measurements not differing by more than 10 g were averaged and assigned as the nociceptive threshold (Ferrier et al., 2016). For cisplatin and paclitaxel studies performed at UNIFI, measurements of mechanical and thermal sensitivity were performed. Thermal allodynia was evaluated using the Cold Plate test. With minimum animal-operator interaction, the animals were removed from their cages and placed on the surface of the cold Plate (Ugo Basile, Varese, Italy) maintained at a constant temperature of $4 \pm 1^\circ\text{C}$. The movement was reduced by a cylindrical chamber in Plexiglas (diameter: 10 cm, height: 15 cm), with the upper surface open. Using a timer, the latency time to paw licking (seconds) of the animal was measured from the moment it was placed on the surface of the plate. The cut-off for paw licking was set at 30 s (Baptista-de-Souza et al., 2014). Mechanical sensitivity was determined with an analgesimeter (Ugo Basile, Varese, Italy) according to the method described by Leighton and colleagues (Leighton et al., 1988). Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the hind paw using a blunt conical mechanical probe. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. An arbitrary cutoff value of 100 g was adopted.

No behavioral testing was performed in the NVS-1 study. However clinical observations were recorded during the study and after each NVS-1 dosing.

2.6. Microscopic evaluations

At necropsy, nervous tissue, including the sciatic (sensorimotor), and saphenous (sensory) nerves, dorsal root ganglion (DRG) from lumbar spinal cord levels (L3 to L5), brain and spinal cord (cervical, thoracic and lumbar levels) was collected for histopathological examination. For NVS-1, nervous tissue from both the right and left sides were collected for histopathology evaluations. For paclitaxel, cisplatin and oxaliplatin study, only the left side of each tissue was collected for histopathology evaluations, while the right side was collected for drug exposure evaluations.

Tissues collected were immersion-fixed in 10 % neutral buffered formalin and embedded in paraffin. Sections of 4 μm thickness were cut and stained with hematoxylin and eosin based on best practice guidelines recommended by the Society of Toxicologic Pathology (Bolon et al., 2013).

Stained tissue sections were examined microscopically by board certified veterinary pathologists, and histopathology severity grading was performed according to the suggested INHAND toxicological pathology nomenclature (Mann et al., 2012) and Society of Toxicologic Pathology 'Points to Consider' document (Schafer et al., 2018). The numerical scoring system applied in the studies was indicative of the degree of severity and used a grade description as follow. Grade 0: no observable effect; grade 1 (minimal): slight changes, often reversible, with no significant impact; grade 2 (mild): observable changes with minimal impact on function or structure; grade 3 (moderate): clear changes with moderate impact on function or structure; grade 4 (severe): pronounced changes with significant impact on function or structure.

Sciatic/saphenous nerves samples were additionally collected from animals of the NVS-1 study for morphometric evaluation. Sciatic/saphenous nerves were immersion fixed in modified Karnovsky's solution (Electron Microscopy Sciences Inc, Hatfield, PA, USA), and epoxy

resin-embedded for semi-thin sectioning and toluidine blue staining according to established test site laboratory methods at the Sponsor Electron Microscopy and Digital Imaging Laboratory (Novartis Pharmaceuticals Inc, East Hanover NJ, USA).

Semi-thin (approximately 70 nm) sections of stained resin embedded nerve tissues were scanned using an Aperio scanner (ScanScope and ImageScope software, V12.1.0.5029; Leica Biosystems, Milton Keynes, UK). The image tiles were then subjected to morphometric evaluation of g-ratio and axon density using the ASTORIA platform (Novartis in-house developed image analysis software) for automated counting of myelinated fibers and assessment of axoplasm and myelin areas. In addition, the g-ratio was calculated using the 1-dimensional g-ratio parameter (defined by the length projection of a cell in the direction of its minimal diameter) and the 2-dimensional area-based g-ratio parameter, defined as the ratio of the axoplasm area over axoplasm + myelin area (Raimondo et al., 2009). For the treatment group analysis, the median of the g-ratio values over all analyzed cells per sample was used to represent an individual animal's g-ratio.

On DRG samples from animals treated with NVS-1 compound, immunohistochemistry staining with an anti-neurofilament light chain (NFL) antibody (rabbit polyclonal antibody cat#AB9568, provided by Sigma-Aldrich, Switzerland) was performed on 3 μm thickness tissue sections using the Ventana Discovery XT autostainer (Roche Diagnostics AG, Switzerland). After deparaffinization, the slides were rehydrated and demasked by heat retrieval cycles before incubation of the primary antibody diluted at 1/8000 for 3 hours. Detection was performed using a biotin-conjugated donkey anti-rabbit secondary antibody (cat# 711-065-152, Jackson ImmunoResearch Inc) revealed by a chromogenic DABMap® kit (cat#05266360001, Roche Diagnostics AG, Switzerland) following the provider instructions. Staining results were assessed by visual examination of the digital files generated at 40x on the Nanozoomer 2.0HT scanner instrument (Nikon Europe B.V, Amsterdam, Netherlands).

2.7. Drug exposure assessment

Drug levels in CNS (brain and spinal cord) and in PNS (sciatic and saphenous nerves, and DRG from L3 to L5 lumbar spinal cord levels) were assessed to confirm the drug exposure of nervous tissues. After collection, each specimen (tissues) was weighted and placed in appropriate polypropylene tubes before being frozen at -80°C until analysis.

For oxaliplatin and cisplatin, total platinum (Pt) concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS) using a NexION 350xx ICP-MS (Perkin-Elmer, France). The detailed method is reported in the [supplementary materials](#).

NVS-1 drug exposure assessments were performed in previous rat studies and demonstrated no NVS-1 exposure in the brain (Novartis Pharma AG internal studies, data not shown). Exposure in spinal cord was not performed assuming that the distribution of orally administered drugs should be comparable between CNS and spinal cord based on pharmacokinetic principles and similarities of the blood-brain barrier and blood-spinal cord barrier properties (Partridge, 2012).

2.8. Plasma biomarker assessments

In all studies, NFL, Tau and GFAP were measured in plasma at the end of the study as outlined in Fig. 1. NSE and VEGFA were also measured in plasma of animals treated with oxaliplatin, cisplatin and paclitaxel. NF-H and UCH-L1 were examined only in the NVS-1 study. For oxaliplatin, cisplatin and paclitaxel blood was collected via cardiac puncture under deep anesthesia with isoflurane 4 %. For NVS-1, blood samples were collected from abdominal aorta under deep isoflurane anesthesia. Blood collection directly after decapitation was an excluded methodology since the baseline level of all tested biomarkers was substantially increased in comparison to that measured in blood samples collected by exsanguination method (cardiac puncture or from abdominal aorta)

under isoflurane anesthesia (data not shown). This evidence suggests that decapitation method leads to contamination of blood by nervous system proteins which results in increased and variable concentrations of nervous biomarkers in blood samples. The detailed method is reported in the [supplementary materials](#).

2.9. Statistical analyses

Each value in graphs represents the mean \pm S.E.M. The statistical analysis was conducted through the ANOVA method followed by procedures of post hoc comparison. Data were analyzed using the “Origin 9” software (OriginLab, Northampton, USA), XLSTAT (v2022.5.1, Addinsoft) or GraphPad Prism (version 9.5.1 (733)).

At UNIFI, the behavioral results and body weight monitoring were expressed as means \pm S.E.M. and the analysis of variance was performed by ANOVA. A Bonferroni’s significant difference procedure was used as *post-hoc* comparison of treatment groups. Data were analyzed using the “Origin 9.1” software.

At UCA, the compound concentrations in nervous tissues (quantitative variables) were expressed as means \pm S.E.M. The comparisons of tissue concentrations between different compound doses (non-repeated quantitative variables of independent groups) were performed using the Mann-Whitney test followed by a *post hoc* Dunn test. The comparisons of tissue concentrations for the same compound dose (non-repeated quantitative variables of paired variables within the same group) were performed using the Friedman test followed by a *post hoc* Nemenyi procedure. Plasma concentrations of biomarkers were compared between groups using the Kruskal-Wallis test followed by a *post hoc* Dunn test for multiple comparisons (4 doses/conditions of oxaliplatin and cisplatin assays), and using the Mann-Whitney test for single comparisons (2 doses/ conditions for paclitaxel 2-week, paclitaxel 4-week, and NVS-1). For all these analyses, two-tailed tests were run. Statistical analyses were performed using XLSTAT (v2022.5.1, Addinsoft).

At NOVA, statistical analysis results were expressed as means \pm S.E.M. Statistical significance was measured by running two-tailed tests using GraphPad Prism (version 9.5.1 (733)).

Total plasma concentration of paclitaxel was compared between different nervous tissues, using a one-way ANOVA followed by Tukey’s multiple comparisons test performed with GraphPad Prism 9.1 for Windows (GraphPad Software, San Diego, CA, USA). A $p < 0.05$ was considered to be the first statistically significant threshold for all tests.

3. Results

3.1. Body weight monitoring

In comparison to control animals, oxaliplatin significantly reduced the body weight gain of animals and in a dose dependent manner, starting on day 12 for the 4 mg/kg dose and on day 15 for the 2 mg/kg dose, and until the end of the treatment. No decrease in body weight gain was seen in animals treated with the 1 mg/kg oxaliplatin dose ([Supplementary Figure S1a](#)).

Cisplatin significantly reduced the body weight of the animals in a dose-dependent manner in comparison to the control group treated with vehicle. The body weight decreased on day 5 (after 2 cisplatin injections with dose 4 mg/kg) remaining stably lower than control up to day 15. Cisplatin 2 mg/kg significantly affected the body weight of the animals after 3 injections (from day 10 to the end of the experiment) while the lowest dose did not influence this parameter ([Supplementary Figure S1b](#)).

Four injections of paclitaxel 5 mg/kg significantly reduced the body weight gain of the animals in comparison to the control group treated with vehicle, from day 21 till the end of the experiment (day 30). Two paclitaxel injections did not significantly affect the body weight of animals ([Supplementary Figure S1c](#)).

NVS-1 compound treatment reduced body weight gain (-5.9 % to

-51.9 %) on days 4–15, respectively, when compared to the concurrent controls ([Supplementary Figure S1d](#)).

3.2. Behavioral assessment

All three doses of oxaliplatin decreased the mechanical hypersensitivity thresholds in comparison to control, starting on day 10 (after 3 oxaliplatin injections) up to day 25 (after 7 oxaliplatin injections). Animals treated with the 4 mg/kg oxaliplatin dose showed the lowest threshold on day 10 in comparison to 1 and 2 mg/kg dose groups. On days 18 and 25, mechanical thresholds of all oxaliplatin treated animals reached a plateau ([Fig. 2a](#)).

Stimulating animals with a thermal stimulus (Cold Plate tests), cisplatin treatment significantly decreased the nociceptive thresholds in a dose-dependent manner. This alteration was significant after 3 cisplatin injections (day 10) with no differences between the doses of 2 mg/kg and 4 mg/kg. Subsequently, the animal’s nociceptive thresholds remained stable up to the end of the experiment. The lowest dose did not evoke these effects ([Fig. 2b](#)).

Cisplatin significantly decreased the nociceptive thresholds of the rats in a dose-dependent manner when stimulated by a noxious mechanical stimulus (Paw Pressure test; [Fig. 2c](#)). The nociceptive effect was observed after the first cisplatin injection (day 3) at the high dose (4 mg/kg). Subsequently, the animal’s pain thresholds continued to decline after the following two treatments to remain stable up to the end of the experiment (day 15) after the fourth injection. Cisplatin at 2 mg/kg and 1 mg/kg doses evoked hypersensitivity that lasted up to the end of the experiment after 2 and 4 injections, respectively.

The Cold Plate test revealed the hypersensitivity induced by paclitaxel in rats when stimulated by a thermal stimulus ([Fig. 2d](#)).

Paclitaxel significantly decreased the nociceptive thresholds when stimulated by a noxious mechanical stimulus (Paw Pressure test; [Fig. 2e](#)). The effect appeared after one injection and progressively increased after the second treatment to remain stable up to the end of the experiment (day 30) following the third and fourth doses. The nociceptive effect emerged the next day after the first injection and remained stable during days and after the following administrations of the drug.

The results obtained by UNIFI following paclitaxel treatment were similar to results obtained by UCA, shown in [Supplementary Figure S2](#).

In NVS-1-treated animals, clinical signs of ptosis (1/8 animals on days 14–15), abnormal gait (1/8 animals on day 16), ataxia (4/8 animals on days 14–16), impaired righting reflex (1/8 animals on day 15), decreased motor activity (4/8 animals on days 14–16) and reduced muscle tone (1/8 animals on days 14–15) were observed at 30 mg/kg/dose. No behavioral assessment was performed for the NVS-1 study.

3.3. Microscopic evaluations

The detailed microscopic assessment results are available in [Supplementary Table S3](#).

In the oxaliplatin study, minimal compound-related findings were observed with a dose-dependent increase in incidence in nervous structures (axons) in peripheral nerves, DRG, and spinal cord. These findings consisted of axonal degeneration including vacuolation and rare cellular infiltrations by macrophages (formation of digestion chambers), hypereosinophilic and/or fragmented axons. Axonal degeneration was also observed in the DRG of a single control animal. Tissues from brain and saphenous nerve did not show any findings.

In the cisplatin study, no compound-related microscopic findings were observed in the brain, spinal cord, DRG, or in the sciatic and saphenous nerves.

In the paclitaxel study (UCA), no compound-related microscopic findings were observed in the brain, spinal cord, DRG, or in the peripheral nerves (sciatic and saphenous). Some low incidence and low severity degenerative findings were noticed in some tissues (sciatic nerve and lumbar/thoracic spinal cord) with a similar incidence and

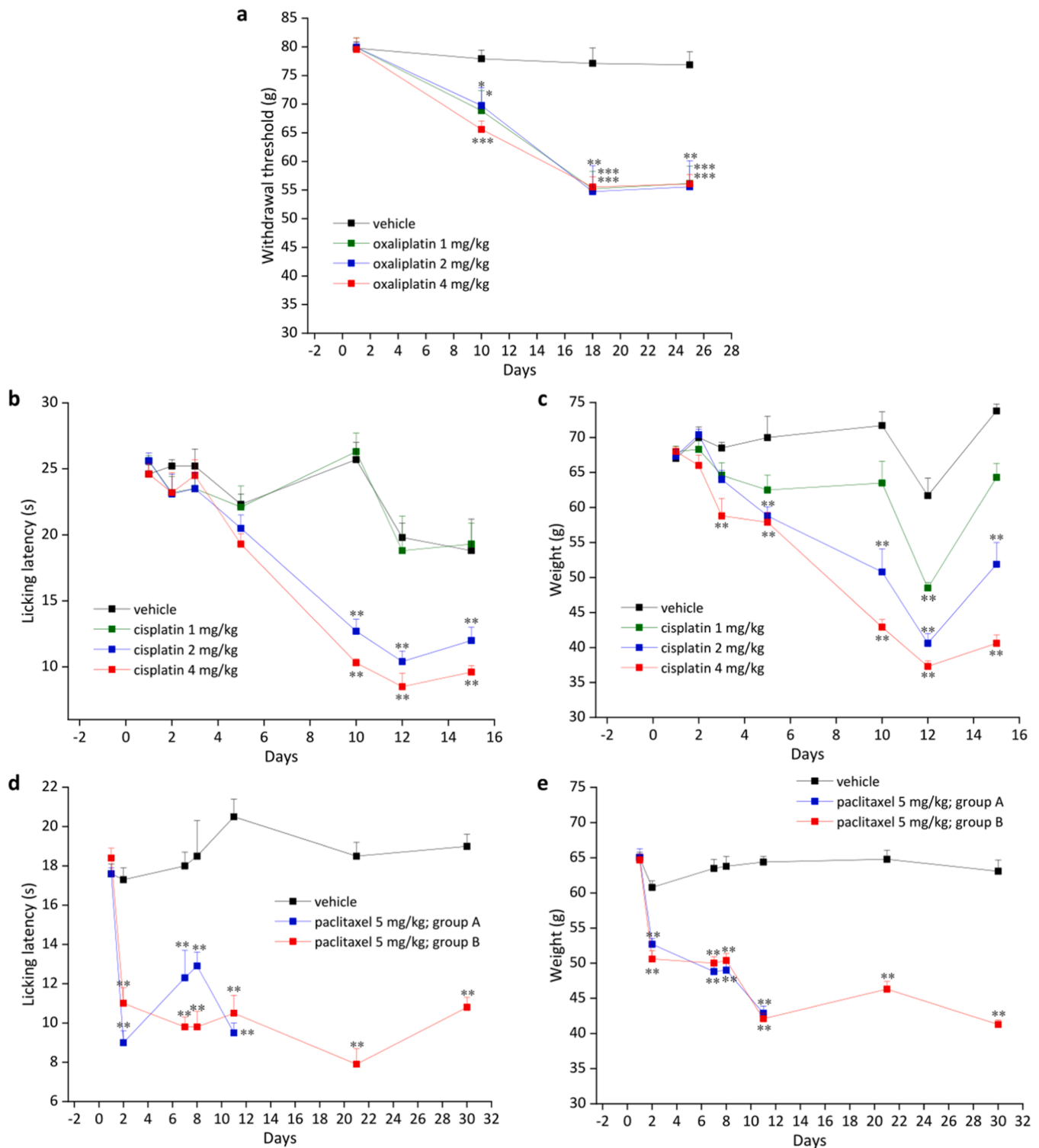


Fig. 2. Behavioral alterations. a) Oxaliplatin. Evaluation of mechanical sensitivity was performed using the electronic Von Frey test, recording the withdrawal threshold (g). Each value represents the mean \pm S.E.M. of 6 animals per group. * $P < 0.05$ vs vehicle, ** $P < 0.01$ vs vehicle, *** $P < 0.001$ vs vehicle. b and c) Cisplatin. Evaluation of thermal sensitivity (b) was performed using the Cold Plate test, measuring the paw licking latency (s); Evaluation of mechanical sensitivity was performed using the Paw Pressure test (c), recording the threshold (in grams). Each value represents the mean \pm S.E.M. of 6 animals per group. ** $P < 0.01$ vs vehicle. d and e) Paclitaxel. Evaluation of thermal sensitivity was performed using the Cold Plate test (d), measuring the paw licking latency (s); Evaluation of mechanical sensitivity was performed using the Paw Pressure test (e), recording the threshold (g). Each value represents the mean \pm S.E.M. of 6 animals per group. ** $P < 0.01$ vs vehicle.

severity in the vehicle treated animals compared to treated animals. Therefore, these findings were considered not treatment-related.

In the NVS-1 study, drug-related microscopic findings were observed in the DRG (lumbar) and in the saphenous and sciatic nerves. NVS-1-related microscopic findings included minimal to mild neuronal degeneration in the DRGs. Neuron degeneration was characterized by one or more of the following: varying cell profiles ranging from shrunken to swollen cell bodies, cytoplasmic small vacuoles, pale basophilic cytoplasm (uncommonly hypereosinophilic cytoplasm) that lacked Nissl substance (chromatolysis), and peripherally displaced or absent nuclei. In addition, there were increased numbers of satellite cells (satellitosis). The majority of the myelinated saphenous and sciatic nerve fibers in NVS-1-treated animals presented with normal histology, although a few fibers exhibited minimal axonal degeneration characterized by fragmentation of myelin sheaths and digestion chambers in approximately half of the animals. A representative example of sciatic and saphenous nerve with minimal nerve fiber degeneration is shown in

Fig. 4. Additionally, when performing a morphometric analysis of the g-ratio in the saphenous nerve samples of these NVS-1 treated animals, a not statistically significant increased g-ratio in axons within size bins of 1400 pixels or more was seen when compared to g-ratio in the vehicle group. The change is indicating that the myelin sheaths in saphenous nerve tend to become thinner in large size axons and that a demyelination process is occurring. As comparison, the g-ratio measured in sciatic nerve samples from the same animals did not reveal any axon size dependent g-ratio change (Fig. 3).

For further characterization of the microscopic findings of axonal degeneration in the DRGs, immunostaining using an anti-NfL antibody was applied. In the DRGs of vehicle treated animals, the NfL immunostaining was cytoplasmic with varying degrees of intensity. NfL immunostaining was present in all neuronal cell bodies and axonal bundles of the DRGs. In the DRGs of NVS-1 treated animals, the NfL immunostaining was overall less pronounced in the nerve fibers and neurons, which is suggestive of depletion of NfL and release into circulation.

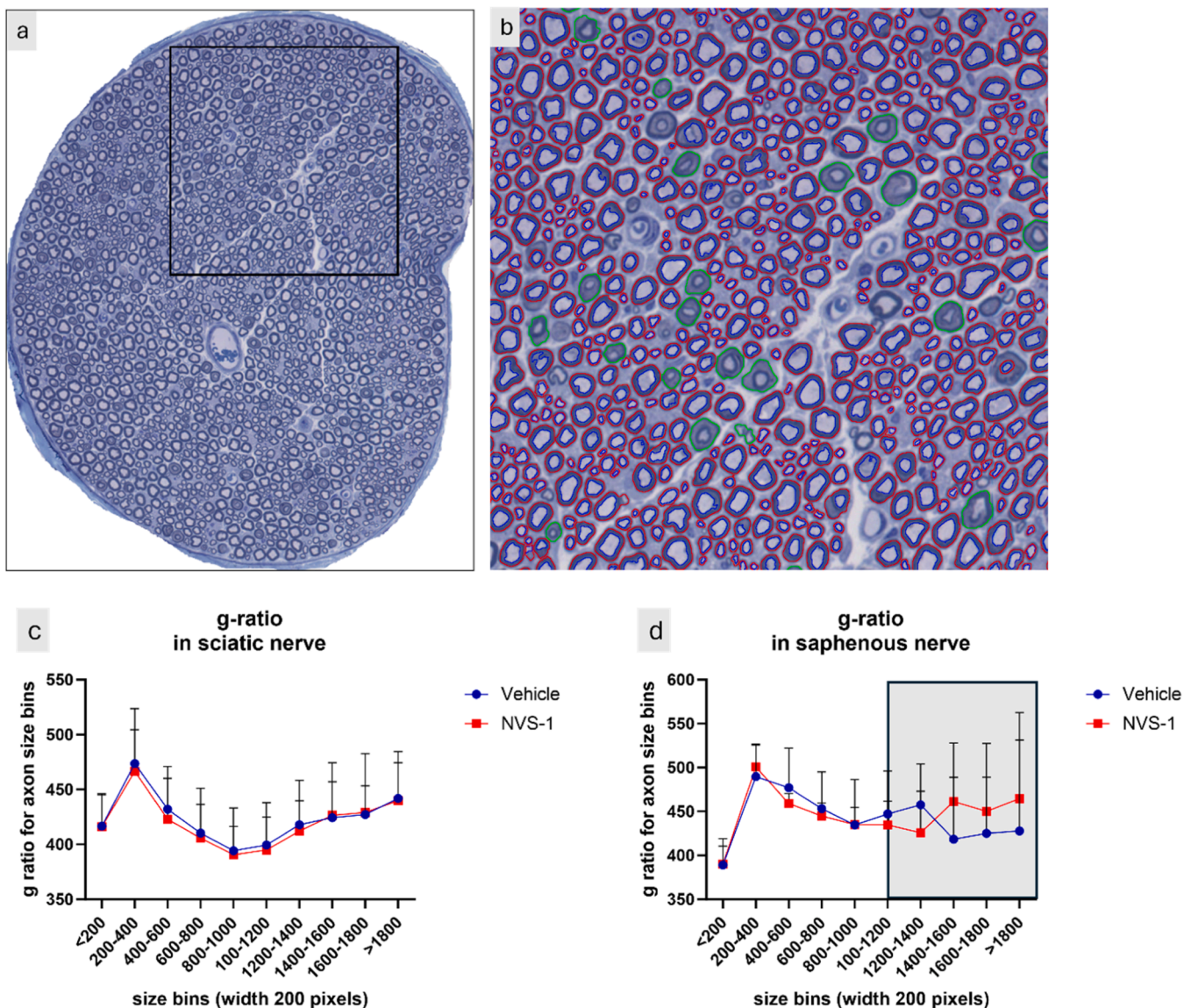


Fig. 3. Representative photomicrograph showing toluidine blue staining of sciatic nerve of a vehicle-treated animal prepared for morphometric analysis. Graphical representation of g-ratio results in sciatic (c) and saphenous nerves (d) following NVS-1 treatment. Morphometric g-ratio has been used for the assessment of axonal myelination after NVS-1 treatment. Semi-thin sections and toluidine blue staining was performed on sciatic and saphenous nerves samples collected at the end on the study. The g-ratio was measured as ratio of the inner-to-outer diameter of myelinated axons in both nerves. The morphometric g ratio showed a no statistically significant increase (p value > 0.05 at all size bins by unpaired student T-test) in large size axons of the saphenous nerve but not in sciatic nerve after treatment with NVS-1.

Small NfL positive aggregates were observed among the nerve fibers (Fig. 4).

3.4. Drug exposure

Drug exposures of nervous tissues following treatment with oxaliplatin, cisplatin and paclitaxel are presented in Fig. 5, and Supplementary Tables S4, S5, and S6. Platinum accumulation increased dose-dependently in nervous tissues of animals treated by oxaliplatin and cisplatin. Similar results were reported to lower extent for paclitaxel treated animals after 4 weeks in comparison to 2 weeks (Fig. 5). For oxaliplatin-treated animals, platinum accumulation was higher in peripheral nerves than tissues from the CNS (brain and spinal cord) (Table S4). For cisplatin-treated animals, platinum accumulation was higher in saphenous nerves than DRG (Table S5). For paclitaxel-treated animals, drug accumulation was higher in the DRG than in tissues from CNS and peripheral nerves (Table S6).

3.5. Plasma biomarkers

Across all studies, NfL, Tau and GFAP were measured in plasma at the end of the study as outlined in Fig. 1. NSE and VEGFA were also measured in plasma of animals treated with oxaliplatin, cisplatin and paclitaxel. NF-H and UCH-L1 were measured only in NVS-1 study.

Any measurements below the lower level of quantification were replaced by limits of detection value (Keizer et al., 2015). The detailed results for all biomarkers are reported in the supplementary (Table S7-S10).

In the studies where VEGFA was measured using MSD assay, there were no changes in this biomarker. Results from the Bio-Rad assay were difficult to interpret due to their very low levels.

The levels of NfL measured in plasma remained unchanged after oxaliplatin treatment compared to the vehicle group. The levels of Tau

showed a trend to decrease but only high dose oxaliplatin-treated animals compared to vehicle-treated controls (60.0 ± 24.0 pg/mL versus 9.0 ± 4.4 pg/mL; a 6.7-fold change) showed a statistically significant decrease. Changes of NSE levels in blood were not dose-dependent. VEGFA showed some potential changes in plasma of oxaliplatin treated animals, but levels were overall very low and very close to the detection limit of the assay. GFAP measurements were below the lower limit of quantification. The levels of NfL measured in plasma were very low and statistically significant changes were observed only following 4 mg/kg cisplatin treatment.

The NSE and the VEGFA levels in plasma did not show any significant change in cisplatin-treated animals compared to vehicle-treated animals. Tau and GFAP results were below lower limit of quantification for all the experimental groups. The measured levels of NfL in plasma of paclitaxel treated animals were slightly elevated (no statistical significance) at day 14 but tendency of increased NfL concentrations was not confirmed at day 28. VEGFA results would point toward a statistically significant increase (p value < 0.001) in plasma levels after paclitaxel treatment at 5 mg/kg on day 28 (9.88 ± 1.61 pg/mL; 5.88-fold change increase). Nevertheless, the VEGFA levels in most control animals were below the lower limit of quantification and were replaced by the value of the limit of quantification, introducing a potential statistical bias. The other biomarkers assessed showed either no significant changes (Tau and NSE) or were below lower limit of quantification (GFAP). The levels of NfL in plasma samples of NVS-1 treated animals showed a significant increase (p value $p < 0.05$) compared to vehicle treated animals. Also, a statistically significant increased level of Neurofilament heavy chain (NF-H) was measured after treatment. The other evaluated biomarkers (Tau, GFAP and UCH-L1) remained within the range of vehicle controls and were not showing any NVS-1 treatment related change.

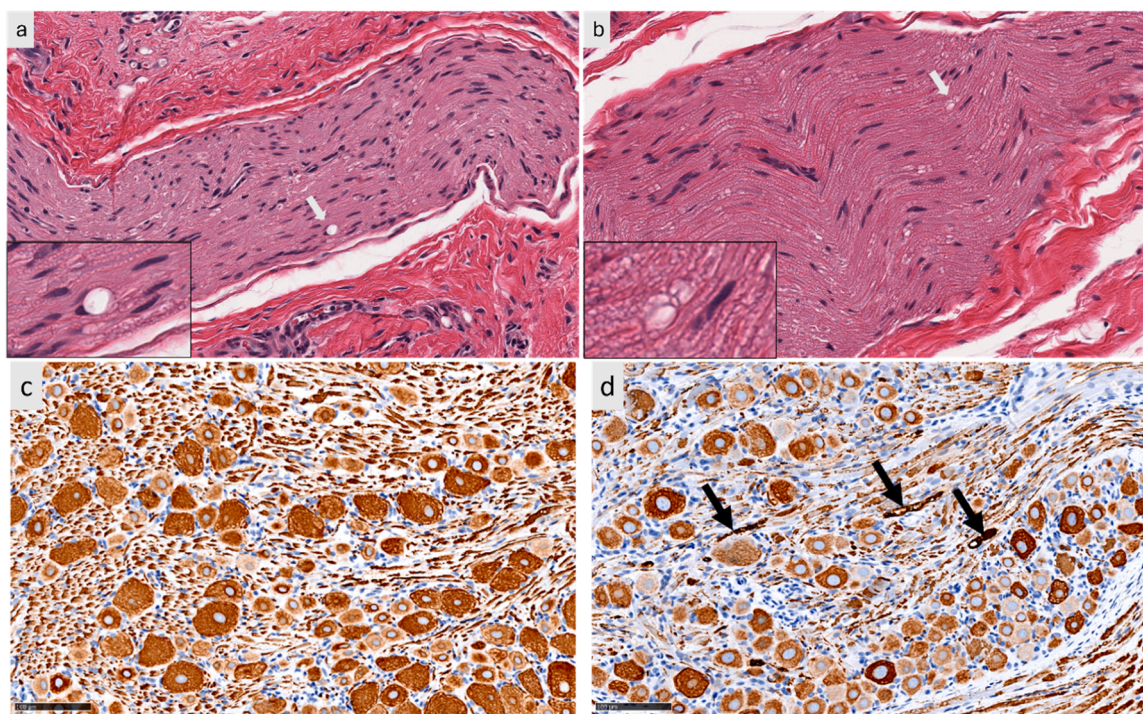


Fig. 4. Histopathological findings (minimal grade) in saphenous (a) and sciatic nerve (b) of NVS-1 treated animals and NfL immunostaining in DRGs of vehicle (c) and NVS-1 (d) treated animals. In sciatic and saphenous nerve of NVS-1 treated animals, minimal axonal degeneration characterized by fragmentation of myelin sheaths and digestion chambers was observed (white arrows). An immunostaining with an anti-NfL antibody highlighted perturbation of NfL in DRG with a homogenous NfL staining pattern in vehicle treated animals (c) and patchy less intense staining pattern in NVS-1 treated animals (d), with NfL positive aggregates visible in axonal bundles after NVS-1 treatment (black arrows). Magnification x40 (a,b) and x20 (c,d).

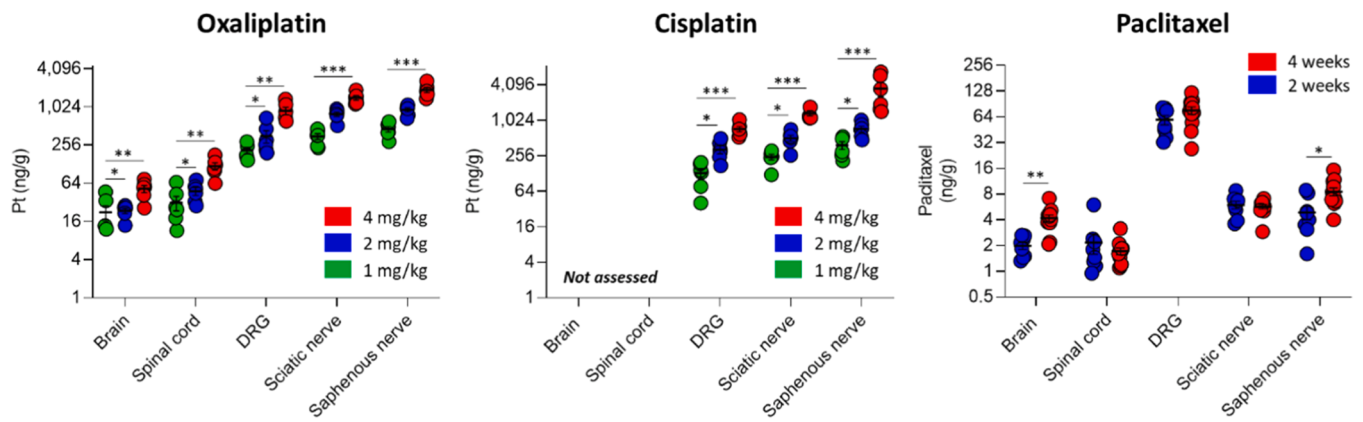


Fig. 5. Drug exposure in nervous tissues. Results are presented by the mean \pm S.E.M, with a semilogarithmic scale. Tissues were collected 3–6 days after the last oxaliplatin injection (days 29–32), 4 days after the last cisplatin injection (day 15), and 7 days after the last paclitaxel injection (days 16–17 for 2-week treatment, and days 29–30 for 4-week treatment). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.6. Relation of NfL plasma levels with histopathology

The NfL plasma levels measured after treatment with oxaliplatin, cisplatin, paclitaxel and NVS-1 were compared to the histopathological

score results in DRG tissue. The results expressed in NfL fold changes (FC) and the color-coded histopathology results were graphically plotted in Fig. 6.

For oxaliplatin, individual histopathological score results in DRG

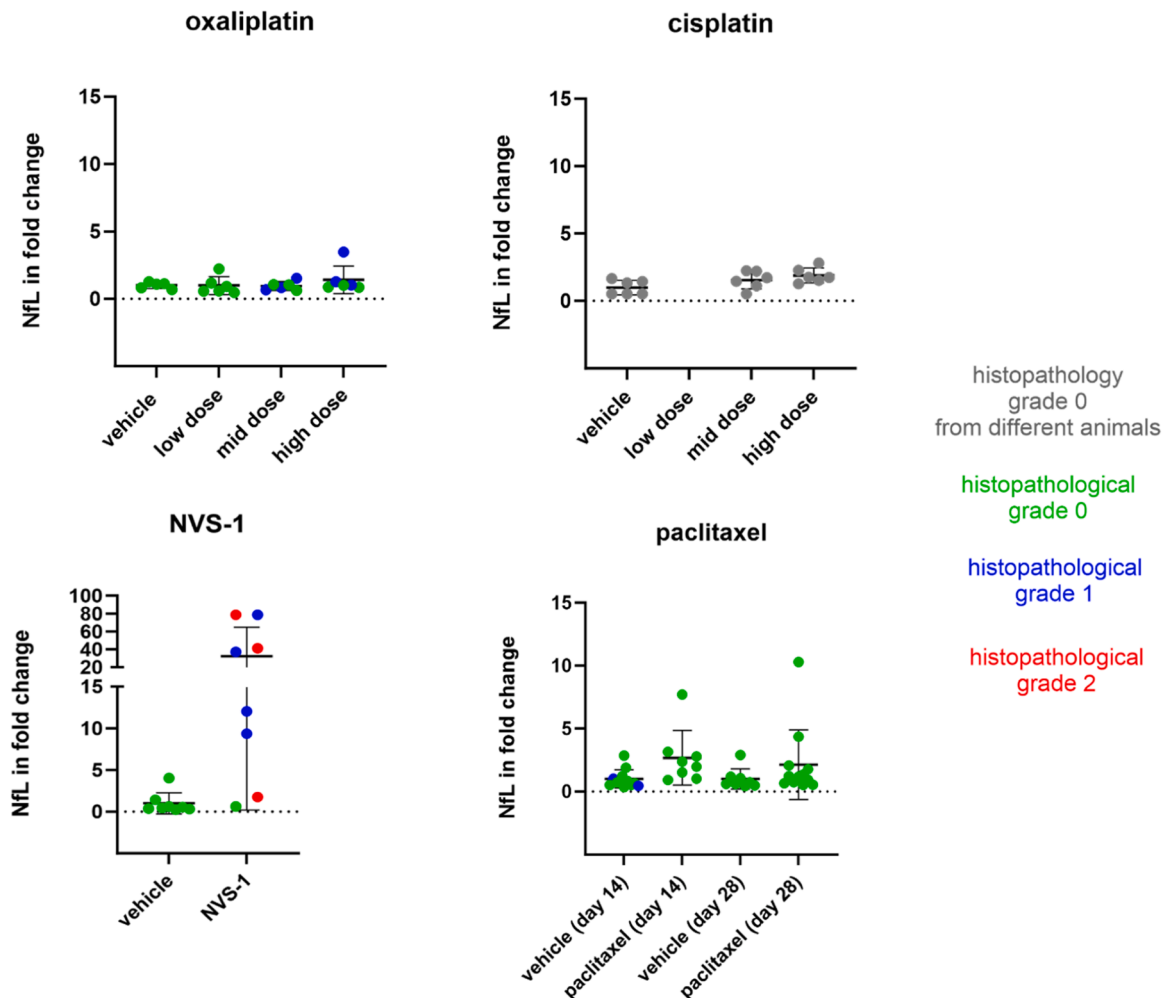


Fig. 6. Color-coded relationship of NfL plasma levels (in fold change) of rats administrated with oxaliplatin, cisplatin, NVS-1 or paclitaxel and histopathological grading results in DRG tissue. The comparison of the individual histopathology score results in DRG tissue of NVS-1 treated rat with the NfL levels in plasma showed a clear correlation with the degree of microscopic alteration. Minimal microscopic findings observed in DRG of Oxaliplatin treated rats were not reflected by a NfL change. No consistent change of NfL levels could be measured with paclitaxel or cisplatin treatment in line with the absence of histopathological changes.

were minimal (grade 1) after treatment with 2 mg/kg or 4 mg/kg doses of oxaliplatin. The comparison with the NfL levels in plasma showed that the histopathological lesions were not associated with an increase of NfL levels in the peripheral circulation.

For cisplatin, the negative individual histopathological score results in DRG (grade 0) were aligned with an absence of NfL elevation.

For paclitaxel, the absence of histopathological changes detected in DRG tissues at any time point are reflected by an unchanged fold change of NfL in the plasma of treated animals.

The NVS-1 compound was the only treatment that associate increased histopathological score results (grade 1 and 2) indicative of tissue lesions in DRG with increased peripheral levels of NfL in plasma. It is interesting to note that one animal with mild (grade 2) tissue lesions had no increased levels of the NfL.

3.7. Overview of the achieved experimental endpoints

Table 1 summarized the different endpoints measured in rat treated with several PNS toxicants.

4. Discussion

This study represents an investigation using *in vivo* models of peripheral neuropathy induced by pharmaceutical compounds with different degrees of PNS toxicity. The male Wistar Han rat strain was selected as it is the best characterized and the most commonly used for toxicology studies to support the assessment of nonclinical safety in drug development (Weber et al., 2011).

We selected seven blood biomarkers with potential to monitor the severity degrees of peripheral neuropathy with a potential future translational value for the clinical setting. Neuropathy models were selected to reproduce neurotoxicity induced by marketed anticancer drugs. In addition, a tool compound (NVS-1) was included, known to induce a peripheral neuropathy confirmed with microscopic findings in the PNS tissues (NOVA internal data, not published).

In all animal models, compound exposure was confirmed in the PNS tissues. Each compound had a specific distribution, e.g., paclitaxel exposure more pronounced in DRG while platinum exposure (oxaliplatin and cisplatin) more pronounced in nerves. Only very small amounts were detected in the brain, which is in line with the published literature for the three tested molecules (paclitaxel (Cavaletti et al., 2000; Hu et al., 2024) and platinum derivatives (Screnci et al., 2000)).

For behavioral assays, nociceptive disorders have been explored, since it is one of the most relevant clinical symptoms of peripheral neuropathy in patients, correlating with its severity (e.g., CIPN) (Selvy et al., 2020). Nociceptive-related behaviors were measured using thermal and mechanical sensitivity testing for oxaliplatin-, cisplatin-, and paclitaxel-treated animals. All three compounds revealed significant decreases of nociceptive thresholds in comparison to vehicle-treated animals, as previously described (Authier et al., 2009).

No axonal degeneration was observed in the nervous tissues assessed (PNS and CNS) of paclitaxel- and cisplatin-treated animals. Minimal axonal degeneration was observed in oxaliplatin-treated animals. As expected, minimal to moderate axonal degeneration was observed with NVS-1 compound in the peripheral nerves (with large fiber demyelination occurring in saphenous nerve as measured by g-ratio morphometry) and neuronal degeneration in the DRGs (NOVA internal data, not shown).

As a matter of fact, of all the blood biomarkers assessed, only blood concentrations of NfL and NfH were increased with PNS toxicity. Increased NfL and NF-H concentrations in plasma were observed with NVS-1, when clinical neurological signs were present and when microscopic findings were minimal to moderate in severity. Local NfL depletion could also be observed in DRG neurons as showed by IHC using an anti-NfL antibody on DRG tissues sections, possibly due to an increased release of NfL in blood circulation.

This is in line with published data by Meregalli et al. (Meregalli et al., 2020, 2018), who highlighted a correlation between nerve damages and increase in serum NfL levels in female rats treated with vincristine (Meregalli et al., 2018) and found that NfL correlates with the severity of nerve fiber degeneration in female rats treated with cisplatin and paclitaxel (Meregalli et al., 2020).

Our findings in rats treated with cisplatin and paclitaxel differ from those reported by the group of Meregalli et al. Despite clear behavioral functional alterations, no increase of NfL was observed in plasma and no morphological changes were observed in DRGs. This discrepancy may be related to difference of treatment regimens, which in our conditions were able to induce nociceptive hypersensitivity and only minor or absent microscopic lesions. For example, the cumulative dose for paclitaxel in the Meregalli et al.'s study was 40 mg/kg, whereas in our study the cumulative dose for paclitaxel was 10 mg/kg (2-week treatment) and 20 mg/kg (4-week treatment). However, the cumulative dose of cisplatin in the Meregalli et al.'s work (Meregalli et al., 2020) was equal to our studies (16 mg/kg), but the frequency of administration

Table 1
Summary end points results with PNS toxicants.

Compound	Nociceptive disorder		Microscopic changes		Biomarkers alteration						
	Mechanical	Thermal	PNS	CNS	NfL	VEGF-A	Tau	NSE	GFAP	UCLH-1	NFH
Oxaliplatin	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Cisplatin	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Paclitaxel	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
NVS-1	Modified motor ability		NA	NA	NA	NA	NA	NA	NA	NA	NA

Definition (Nociceptive disorder / microscopic change / biomarker)

NA	Not assessed
	Not detectable (for biomarker only)
NA	No nociceptive disorder / no microscopic finding / unchanged biomarker level
NA	Nociceptive disorder / minimal microscopy finding / increased biomarker but no statistical significance
NA	Nociceptive disorder at all doses / mild microscopy finding / statistically significant increase of biomarker

was higher in the Meregalli et al.'s work in comparison to our study (2 mg/kg twice a week vs. 4 mg/kg once a week). The higher cumulative dose or administration frequency may explain the different results between these two studies confirming the relevance of the dosage regimen, and consequently of the nerve damage and on NfL concentrations in plasma. These last points may underline the limit of NfL as an early blood biomarker of PNS lesions, since blood concentrations of NfL rise only with confirmed histological lesions of the PNS, as reported by the NeuroDeRisk consortium in mice (Balayssac et al., 2023).

In the present rat studies, other biomarker candidates of particular relevance in the field of neurotoxicity were also measured, in particular, NF-H, VEGFA, Tau, NSE, GFAP, and UCH-L1.

The plasma levels of GFAP, UCH-L1 and NSE were unchanged or BLOQ. These markers may better correlate with a clear CNS toxicity mainly when dosed in the cerebrospinal fluid (Vlasakova et al., 2024). Some changes were observed for NF-H in NVS-1-treated *versus* control animals, and for Tau in oxaliplatin-treated *versus* control animals. VEGFA increased in a statistically significant manner in oxaliplatin-treated rats and in paclitaxel-treated ones (without dose response), nonetheless the absolute values were very low and close to assay sensibility making the results difficult to interpret. Overall, these responses could be related to the lack of sensitivity of these assays.

The results of this collaborative effort across industry and academia to identify sensitive safety biomarkers highlighted NfL as currently best performing biomarker. We have also shown, that assessing functional and biomarker endpoints can add value to histopathologic assessment alone. Since functional assays are not standardly run in the pharma industry, the implementation of NfL blood measurement in preclinical safety studies should be taken in consideration, as it remains a good indicator of neurotoxicity, and not only in rat but also in non-rodent species (Theil et al., 2023). However, for a more in-depth evaluation of PNS toxicity, addition of behavioral assessments may represent a good option.

Incorporating NfL into preclinical safety assessment during R&D process can inform go/no-go decisions, helping to prioritize the compounds with the best safety profile for further development. The evaluation of plasma levels of NfL as an index of ongoing tissue injury would allow monitoring the progression of the damage in animal models through multiple sampling on the same animals, allowing the reduction of animal number per study. Due to its translational potential (Cavaletti et al., 2023; Huehnchen et al., 2022), NfL can bridge the gap between preclinical studies and clinical trials, refining and increasing the likelihood of predicting human responses accurately.

In patients, the increase in blood NfL concentrations in relation to CIPN severity is well documented (for a review, see (Andersen et al., 2024)). Notably, the authors of the review highlighted that while blood NfL concentrations may serve as a diagnostic biomarker for CIPN, its potential as a prognostic marker to predict CIPN requires further prospective evaluation. Importantly, only one clinical study has compared different neurotoxic anticancer drugs (Velasco et al., 2024). This study found a lower correlation between CIPN severity (assessed by the total neuropathy score) and NfL concentrations for oxaliplatin ($r = 0.341$, $p = 0.01$) compared to spindle poisons such as brentuximab vedotin ($r = 0.556$, $p < 0.001$) and paclitaxel ($r = 0.551$, $p < 0.001$). These results suggest that the sensitivity of blood NfL concentrations may vary depending on the neurotoxic agent (Velasco et al., 2024). To our knowledge, no study has been conducted to compare interspecies specificity, such as between rodents and humans. Moreover, there remains a significant gap between clinical and non-clinical studies in terms of CIPN assessment. No gold standard exists for patient evaluation beyond the combination of a clinical exam and self-administered questionnaires (Jordan et al., 2020). In animal models, outcome measures are typically focused on nociceptive assays, histopathological evaluations, and electrophysiological tests (Andersen et al., 2024), which are rarely performed routinely in patients. In this context, blood biomarkers of neurotoxicity, such as those for CIPN, hold great promise and could

help bridge the gap between patient assessments and animal models.

Some limitations can be raised and discussed. Only male animals were used, whereas these anticancer drugs are used both in males and females for colorectal and lung cancers and using both male and female could therefore improve the translationality. Although CIPN is described as more severe in females than in male patients (Mizrabi et al., 2021; Selvy et al., 2020), the results in animals are less clear, with some sex differences being more pronounced in male animals than in female ones, and *vice versa* (Warncke et al., 2021). However, exploratory safety studies are commonly performed in male animals. Young adult animals were used, whereas these anticancer drugs are used for malignancies affecting adults (e.g., colorectal and lung cancers). Sensitivity of peripheral nerves may vary with age of animals, and recovery capabilities decrease with age of animals (Bruna et al., 2020). Same studies could be repeated in older animals to improve the translationality of the results, nevertheless may represent a financial burden for the non-clinical safety studies.

In vivo models of PNS toxicity were assessed in this study with the goal to identify blood biomarkers able to monitor different degrees of peripheral neuropathy. An increased nociceptive sensitivity remains the first detectable sign. However, increased levels of circulating NfL were observed when minimal axonal degeneration at multiple sites or moderate axonal or neuronal degeneration was present in DRGs. Therefore, results presented in this paper delineate NfL as a promising candidate biomarker to monitor axonal damage in the PNS.

The ideal biomarker candidate should be able to give an early indication of the neurotoxic processes before the tissue alteration becomes a symptom (peripheral neuropathic signs or neuropathic pain). Future research efforts will concentrate on the in-depth investigation of the molecular mechanisms underlying the onset of CIPN in order to provide additional effective biomarkers of clinical relevance.

CRediT authorship contribution statement

Jérôme Busserolles: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Olivier Perrault:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Laetitia Prival:** Investigation. **Irena Loryan:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Cristelle Dalbos:** Investigation. **Yang Hu:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Formal analysis, Conceptualization. **Diethilde Theil:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Warren E. Glaab:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **David Balayssac:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Valerie Dubost:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Katerina Vlasakova:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Laura Micheli:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Dominique Brees:** Writing – review & editing. **Carla Ghelardini:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition. **Kelley Penrrat:** Writing – review & editing. **Clara Ciampi:** Writing – original draft, Methodology, Investigation, Formal analysis,

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Some authors are employees in the companies involved in the studies and may have access to stock/share options. In particular Kuno Wuersch, Eric Johnson, Wilfried Friauff, Kelley Penratt, Dominique Brees, Valerie Dubost and Diethilde Theil in Novartis; Warren E. Glaab, Katerina Vlasakova in Merck & Co., Inc., Rahway, NJ, United States; Olivier Perrault and Mohamed Slaoui in Sanofi.

The other authors declare no competing interests.

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ARRIVE statement

The results presented in this paper are reported according to the ARRIVE guidelines for animal research

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.tox.2024.153998](https://doi.org/10.1016/j.tox.2024.153998).

Data availability

Data will be made available on request.

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