Cancer Chemother Pharmacol (1999) 44: 29–38 © Springer-Verlag 1999

ORIGINAL ARTICLE

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Comparative neurotoxicity of oxaliplatin, ormaplatin, and their biotransformation products utilizing a rat dorsal root ganglia in vitro explant culture model

Received: 12 June 1998 / Accepted: 22 October 1998

Abstract *Purpose*: Neurotoxicity is one of the major toxicities of platinum-based anticancer drugs, especially oxaliplatin and ormaplatin. It has been postulated that biotransformation products are likely to be responsible for the toxicity of platinum drugs. In our preceding pharmacokinetic study, both oxaliplatin and ormaplatin were observed to produce the same types of major plasma biotransformation products. However, while the plasma concentration of ormaplatin was much lower than that of oxaliplatin at an equimolar dose, one of their common biotransformation products, $Pt(dach)Cl_2$, was present at 29-fold higher concentrations in the plasma following the i.v. injection of ormaplatin than of oxaliplatin. Because ormaplatin has severe neurotoxicity and $Pt(dach)Cl_2$ is very cytotoxic, we have postulated that $Pt(dach)Cl_2$ is likely to be responsible for the differences in neurotoxicity between ormaplatin and oxaliplatin. In order to test this hypothesis, we compared the neurotoxicity of oxaliplatin, ormaplatin, and their biotransformation products. Since the dorsal root ganglia (DRGs) have been suggested to be the likely targtet for platinum drugs and in vitro DRG explant cultures have been suggested to be a valid model for studying cisplatin-associated neurotoxicity, our comparative neurotoxicity study was conducted with DRG explant cultures in vitro. *Methods*: Based on the previous studies of cisplatin neurotoxicity, we established our in vitro DRG explant culture utilizing DRGs dissected from E-19 embryonic rats. Rat DRGs were incubated for 30 min with different platinum compounds to mimic in vivo exposure conditions; this was by followed by a 48-h incubation in culture medium at 37 °C. At the end of the incubation, the neurites were fixed and stained with toluidine blue, and neurite outgrowth was quantitated by phase-contrast microscopy. The inhibition of neurite outgrowth by platinum compounds was used as an indicator of in vitro neurotoxicity. Since an in vivo study has indicated that the order of neurotoxicity is ormaplatin > cisplatin ≥ oxaliplatin > carboplatin as measured by morphometric changes to rat DRGs, we initially validated our DRG explant culture model by comparing the in vitro neurotoxicity of ormaplatin, cisplatin, oxaliplatin, and carboplatin. After observing the same neurotoxicity rank between this study and a previous in vivo study, we further compared the neurotoxicity of oxaliplatin, ormaplatin, and their biotransformation products including $Pt(dach)Cl_2$, $Pt(dach)(H_2O)Cl$, $Pt(dach)(H_2O)_2$, Pt(dach)(Met), and Pt(dach)(GSH) utilizing the DRG explant culture model. *Results*: Our study indicated that $Pt(dach)Cl_2$ and its hydrolysis products were more potent at inhibiting neurite outgrowth than the parent drugs oxaliplatin and ormaplatin. In contrast, no detectable inhibition of neurite outgrowth was observed for DRGs dosed with Pt(dach)(Met) and Pt(dach)(GSH). *Conclusion*: This study suggests that biotransformation products such as $Pt(dach)Cl_2$ and its hydrolysis products are more neurotoxic than the parent drugs oxaliplatin and ormaplatin. The different neurotoxicity profiles of oxaliplatin and ormaplatin are more likely due to the different plasma concentrations of their common biotransformation product $Pt(dach)Cl_2$ than to differences in their intrinsic neurotoxicity.

Key words Neurotoxicity · Oxaliplatin · Ormaplatin · Biotransformation products · Rat dorsal root ganglia

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Introduction

Cisplatin (*cis*-diamminedichloroplatinum(II)) is a widely used antineoplastic agent for the treatment of solid tumors such as cancer of the ovary, testes, and bladder [32]. However, there are a variety of side effects associated with this drug including nausea, vomiting, nephrotoxicity, and neurotoxicity. Clinically, the nephrotoxicity is ameliorated by hydration, while the neurotoxicity is currently the dose-limiting side effect [21].

Oxaliplatin (*trans*-L-1,2-diaminocyclohexaneoxalatoplatinum(II)), a cisplatin analog, has recently been approved in France for the treatment of colorectal cancer and is currently being evaluated in phase II clinical trials in the USA. This drug is as effective as cisplatin in the treatment of a variety of tumors [18, 24, 26]. However, it shows no significant nephrotoxicity and myelotoxicity. The dose-limiting toxicity of oxaliplatin is peripheral sensory neuropathy, which is observed at high cumulative doses [13, 24].

Ormaplatin (trans-D,L-1,2-diaminocyclohexanetetrachloroplatinum(IV)), another cisplatin analog with the 1,2-diaminocyclohexane (dach) carrier ligand, is also an anticancer drug that has been selected for clincial development due to its efficacy against cisplatin-resistant tumors [1, 34, 48] and its reduced nephrotoxicity [1, 12, 41]. As with oxaliplatin and cisplatin, the dose-limiting toxicity for ormaplatin is neurotoxicity [9, 30, 31]. However, the neurotoxicity associated with ormaplatin is severe and poorly reversible [9, 30, 31, 37], which has halted the clinical development of ormaplatin.

Of these neurotoxicities, that of cisplatin has been best characterized in humans. The initial symptoms of neurotoxicity are paresthesias and numbness [27, 47]. The peripheral neuropathy which develops at high cumulative doses features the loss of vibratory sensation, loss of deep tendon reflexes, and marked sensory ataxia [27, 28, 47]. Neurophysiologically, cisplatin causes a decrease in sensory nerve conduction velocity, but has no effect on the motor nerve conduction velocity [28]. Pathologically, there is a loss of large myelinated fibers, especially in the distal part of the nerve, and abnormalities in the myelin sheath of medium fibers [28, 43]. Among the nerve tissues, it has been found that the Pt concentrations are greatest in the dorsal root ganglia (DRGs), followed by the dorsal root and peripheral nerves [43].

Cisplatin neurotoxicity has also been well studied in rats. Profound morphometric changes are observed in rat DRGs including shrinkage of neurons and an increase in the number of abnormal nucleoli in the neuronal cell bodies [3, 7, 29, 44]. However, morphometric changes are subtle in peripheral nerves, and no morphometric changes have been observed in spinal cord neurons. Therefore, the DRGs have been suggested to be a primary site of damage by platinum drugs. Based on the available data, the rat appears to be a good model system for studying the neurotoxicity of platinum drugs.

Compared to cisplatin, the neurotoxicity of oxaliplatin and ormaplatin is less well characterized. It has been observed that the neurotoxicity associated with ormaplatin is primarily peripheral sensory neuropathy [30, 31, 37]. Oxaliplatin causes cold-induced paresthesia at low cumulative doses, which is rapidly reversible and is distinct from the neurotoxicity symptoms of cisplatin and ormaplatin. However, oxaliplatin causes peripheral sensory neuropathy at high cumulative doses, which is more slowly reversible and is similar to the symptoms associated with cisplatin and ormaplatin neurotoxicity. Recently, the neurotoxicities of cisplatin, oxaliplatin, and ormaplatin have been quantitatively compared in a Wistar rat model system in vivo [15]. The neurotoxicity order was ormaplatin $>$ cisplatin $\geq$ oxaliplatin. Carboplatin shows no detectable neurotoxicity in that system. Among these platinum drugs, ormaplatin is the most toxic, but is taken up by DRGs to the least extent.

It is possible that the observed neurotoxicity order is due to intrinsic differences in neurotoxicity among these platinum drugs. However, our recent pharmacokinetic study [22] has shown that while both ormaplatin and oxaliplatin produce the same types of biotransformation products, the plasma concentration of one of their common biotransformation products, $Pt(dach)Cl_2$, is 29-fold higher with ormaplatin than with oxaliplatin. In contrast, ormaplatin itself is present at a 19-fold lower concentration than oxaliplatin following equimolar drug dosing. Because of the unusually severe neurotoxicity of ormaplatin, we have hypothesized that the difference in neurotoxicity between ormaplatin and oxaliplatin is likely due to different plasma concentrations of their common biotransformation product, $Pt(dach)Cl_2$.

Embroyonic rat DRG explant cultures have been proposed as an in vitro model to study neurotoxins [49], and have been used in evaluating the direct neurotoxic effects of cisplatin [4, 14]. Based on the DRG explant culture protocol used in the previous studies of cisplatin neurotoxicity, we have established a DRG explant culture utilizing DRGs dissected from E-19 embryonic Wistar rats. In order to assess whether there were any intrinsic neurotoxicity differences among cisplatin, oxaliplatin, ormaplatin, and carboplatin, we compared their neurotoxicities utilizing this in vitro rat DRG explant culture model. Since the biotransformation products of platinum drugs could also be responsible for the different neurotoxicities of the parent drugs, we also compared the neurotoxicities of oxaliplatin and ormaplatin with those of their biotransformation products $Pt(dach)Cl_2$, $Pt(dach)(H_2O)Cl$, $Pt(dach)(H_2O)_2$, Pt(dach)(Met), and Pt(dach)(GSH). Nerve growth factor (NGF) has been observed to prevent cisplatin-induced neurotoxicity in rats in vivo [2, 16]. However, no protection occurs in the in vitro DRG model [50]. In order to clarify this issue, we also determined whether NGF prevents the neurotoxicity of oxaliplatin and ormaplatin in our DRG explant culture model.

This study provided a direct neurotoxicological comparison between the parent platinum drugs cisplatin,

ormaplatin, oxaliplatin, and carboplatin as well as between the parent platinum drugs and their biotransformation products. It may also form the basis for further cellular and molecular studies on platinum drug-induced neurotoxicity. This knowledge could well lead to the alternative design of treatment protocols in which the neurotoxicity would be reduced. In addition, it may also provide some guidance for the future design of platinum drugs with reduced neurotoxicity.

Materials and methods

Drugs

^{3}H-oxaliplatin (340 mCi/mmol), ^{3}H-ormaplatin (360 mCi/mmol), ^{3}H-Pt(dach)Cl_2 (320 mCi/mmol), and Pt(dach)(Met) were synthesized by Dr. Steven Wyrick (Radiosynthesis Laboratory, Division of Medicinal Chemistry and Natural Products, School of Pharmacy, UNC-CH) [51]. Unlabeled oxaliplatin was kindly provided by Dr. Martine Bayssas (Debiopharm, Lausanne, Switzerland). Pt(dach)(Met) and Pt(dach)(GSH) were kindly provided by Dr. Martin Graham (Sanofi Research Division, Malvern, Pa.) and Dr. Dennis Greenslade (Sanofi Research Division, Alnwick, UK). Cisplatin and carboplatin were purchased from Sigma (St. Louis, Mo.). Oxaliplatin (2.0 m*M*), Pt(dach)(Met) (10 m*M*), and Pt(dach)(GSH) (5 m*M*) were prepared by dissolving appropriate amounts of drug in double-distilled water. Cisplatin (2.0 m*M*), ormaplatin (2.0 m*M*), Pt(dach)Cl_2 (0.5 m*M*) solutions were prepared in 150 m*M* NaCl solution. Pt(dach)(H_2O)Cl (1.0 m*M*) and Pt(dach)$(H_2O)_2$ (1.0 m*M*) solutions were prepared by mixing Pt(dach)Cl_2 with $AgNO_3$ at either a 1:1 or a 1:2 molar ratio in water followed by incubation for 12 h at 37 °C in the dark. HPLC analysis of the final incubation mixtures showed that the Pt(dach)(H_2O)Cl obtained from our preparation was a mixture of Pt(dach)Cl_2 (5.4%), Pt(dach)(H_2O)Cl (80.6%), and Pt(dach)$(H_2O)_2$ (14.0%). The Pt(dach)$(H_2O)_2$ obtained was mixture of Pt(dach)(H_2O)Cl (21.6%) and Pt(dach)$(H_2O)_2$ (78.4%). All stock solutions were aliquoted and stored at −20 °C.

DRG culture

DRGs were dissected from E-19 embryonic Wistar rats (Charles River Laboratory, Raleigh, N.C.). After being stripped of nerve roots and connective tissues, DRGs were plated in eight-well tissue culture chambers (Nunc, Naperville, Ill.) containing 200 µl culture medium and incubated at 37 °C in an atmosphere containing 5% CO_2. The culture chambers were coated with a single layer of rat-tail collagen (Sigma, St. Louis, Mo.) (10 µg/cm^2) 2 days prior to the experiments according to the coating protocol recommended by Sigma. The culture medium used in this study was Eagle's minimal essential medium (MEM) with glutamine, supplemented with 100 ng/ml 2.5S NGF, 10% fetal calf serum (FCS), 5% horse serum (HS), and antibiotics.

Drug dosing

DRGs were incubated with the parent drugs oxaliplatin, cisplatin, ormaplatin, and carboplatin at doses ranging from 20 µ*M* to 350 µ*M* in MEM (−FCS) at 37 °C for 30 min. Pt(dach)Cl_2 was dosed from 20 µ*M* to 100 µ*M* under the same conditions. Pt(dach)(Met) and Pt(dach)(GSH) were dosed at 100 µ*M* only. Pt(dach)(H_2O)Cl and Pt(dach)$(H_2O)_2$ were dosed in the same range but in a chloride-deficient MEM (−FCS). After dosing, DRGs were washed three times with phosphate-buffered saline (PBS) and incubated in 200 µl MEM supplemented with 100 ng/ml 2.5S NGF, 10% FCS, and 5% HS for 48 h in an incubator with an atmosphere containing 5% CO_2 at 37 °C.

Quantitation of neurite outgrowth

After an incubation of 48 h, the culture medium was removed, and the DRGs were washed twice with PBS and fixed in MEM (−FCS) plus 3% glutaraldehyde. Before quantitation, the DRGs were washed three times with PBS to remove residual glutaraldehyde and stained with toluidine blue in PBS. The quantitation of neurite outgrowth was conducted in the Microscopy Service Laboratory, Department of Pathology, University of North Carolina at Chapel Hill. The images of the DRGs were scanned with a IMT-2 Olympus inverted phase contrast microscope at 40× magnification connected with an Optronics DEI 750 camera and captured with a Scion LG3 Image Card. Neurite outgrowth was quantitated on a Power Macintosh 7100/80AV using the public domain NIH image program (Scion Corporation, Frederick, Md.). The circular portion of the halo was divided into eight equal parts. The neurite length was measured from the edge of the ganglion to the edge of the halo for each portion. The average neurite length was used to calculate the extent of neurite outgrowth. For each concentration, the mean and standard error of the mean were calculated. The statistical significance of the inhibition of neurite outgrowth at the different doses was determined using analysis of variance (ANOVA) in the Systat Statistical Package (Systat, Evanston, Ill.).

Preparation of chloride-deficient MEM

This medium was prepared by dissolving all inorganic salts plus glucose into double-distilled water based on the composition of MEM listed in the Gibco BRL Product Catalog. The chloride salts were replaced by equivalent amounts of nitrates. No amino acids, vitamins, bacto-peptone, or glutathione supplements were added in order to minimize the biotransformations of the aquated platinum compounds.

Platinum compound uptake

The uptake was determined using the radioactively labeled Pt compounds. The dosing procedure was the same as described above for neurite inhibition. However, after dosing, DRGs were washed three times with PBS, transferred into 500 µl PBS, and sonicated for 2 × 20 s on ice. To quantitate the uptake of Pt-dach, 400-µl aliquots of tissue sonicate were mixed with 5 ml Scinti Verse (Fisher Scientific, Pittsburgh, Pa.) and counted on an LKB 1215 liquid scintillation counter.

Results

Inhibition of neurite outgrowth by parent platinum drugs

DRGs from E-19 embryonic Wister rats were incubated with platinum drugs (20 µ*M* to 350 µ*M*) including oxaliplatin, ormaplatin, cisplatin, and carboplatin. Drug treatment was conducted in serum-free MEM for 30 min, followed by incubation for 48 h in fresh MEM supplemented with 10% FCS, 5% HS, and 100 ng/ml NGF. The DRGs were fixed with glutaraldehyde, stained with toluidine blue, and the degree of neurite extension was measured in images of the DRGs using the public domain NIH image program. The neurite outgrowth inhibition was calculated and used as an indicator of the relative neurotoxicity caused by the platinum compounds. NGF was tested initially to determine whether it afforded any protective effects on the inhibition of neurite outgrowth.

With DRGs treated with oxaliplatin and ormaplatin at 100 μ*M*, no statistically significant protective effect was observed on the inhibition of neurite outgrowth at NGF concentrations up to 100 ng/ml (data not shown). Therefore, NGF was routinely included in all DRG incubations.

In order to determine the intrinsic differences in neurotoxicity among the platinum drugs, serum-free MEM was used to prevent the protein binding of platinum drugs and the reduction of ormaplatin to Pt(dach)Cl_2. HPLC analysis has shown that biotransformations of oxaliplatin and ormaplatin are <5% for an incubation in serum-free MEM for 30 min at 37 °C (data not shown). Previous studies have shown that the $t_{1/2}$ values of cisplatin and carboplatin in rat plasma in vitro are approximately 1 h and 48 h [10, 11], which are longer than the $t_{1/2}$ of oxaliplatin (0.68 h) in rat plasma in vitro [23]. Therefore, the biotransformations of cisplatin and carboplatin were also believed to be <5% in serum-free MEM under our incubation conditions. Control studies showed that there were no significant effects on neurite outgrowth for DRGs incubated in MEM (−FCS) compared to those incubated in MEM (+FCS) for 30 min (data not shown).

Based on our recent pharmacokinetic study of oxaliplatin in rats [22], the estimated C_{max} of oxaliplatin is around 86 μ*M* following a single bolus i.v. injection (20 μmol/kg). In that study, oxaliplatin quickly decayed below 1 μ*M* within 30 min with a $t_{1/2\alpha}$ of 2.1 min. Similar values of C_{max} and $t_{1/2}$ have been estimated in a previous pharmacokinetic study of cisplatin and carboplatin in rats at similar doses [38]. Therefore, the platinum drug concentration range in this study was set from 20 to 350 μ*M* and the treatment time was set at 30 min based on the pharmacokinetic parameters of these platinum drugs. In a pilot study, the lengths of the neurites were measured at time-points of both 24 h and 48 h. Neurite outgrowth was linear for up to 48 h and the differences in neurite outgrowth at 48 h were sufficient to compare the extent of inhibition among different

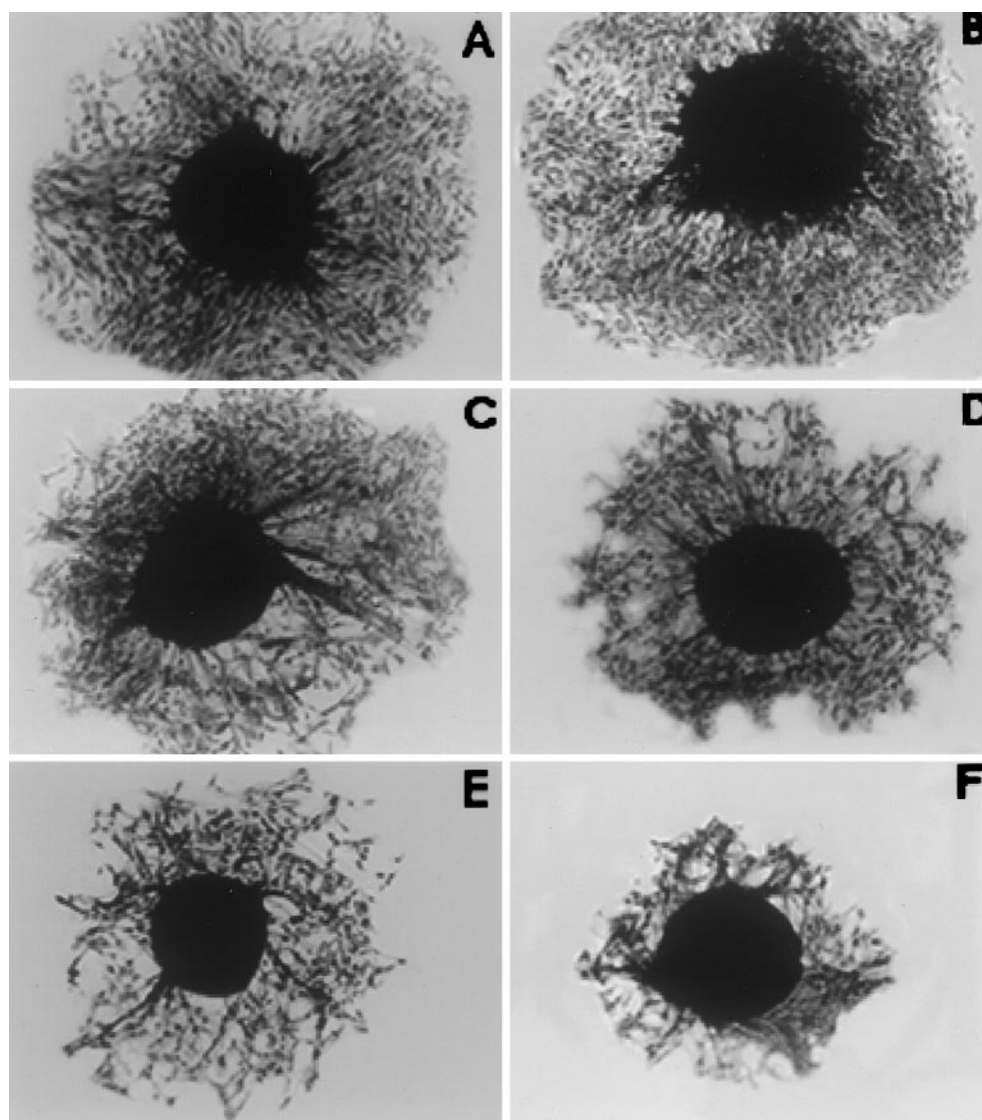

Fig. 1A–F Neurite outgrowth from E-19 rat DRGs. Neurites were fixed after the DRGs were dosed with Pt compounds at 200 μ*M* followed by incubation for 48 h in MEM containing 10% FCS, 5% HS, and 100 ng/ml NGF. Radial outgrowth of neurites was measured on the images of the DRGs taken with a camera at 40× magnification (**A** saline control, **B** carboplatin, **C** oxaliplatin, **D** cisplatin, **E** ormaplatin, **F** Pt(dach)Cl_2)

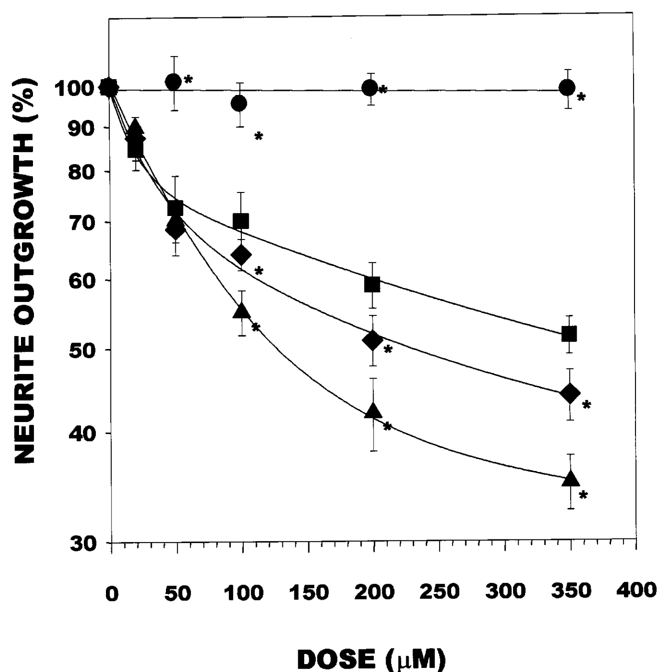

Fig. 2 Inhibition of neurite outgrowth by parent platinum compounds. Inhibition is expressed as the percentage of the average radius of the halo of DRGs dosed with Pt compounds in relation to that of the control (● carboplatin, ■ oxaliplatin, ◆ cisplatin, ▲ ormaplatin). Each point represents the mean (±SE) from six to eight DRGs. $^{*}P \leq 0.05$ vs oxaliplatin

Table 1 Neurite outgrowth inhibition (*n.i.* no inhibition detected)

Pt compound	IC^{a}_{50} (μ*M*)	Fold increase in neurotoxicity compared to oxaliplatin[b]
Carboplatin	n.i.	n.i.
Oxaliplatin	358	1.0
Cisplatin	256	1.4
Ormaplatin	139	2.6
$Pt(dach)Cl_2$	94	3.8
$Pt(dach)(H_2O)Cl$	80	4.5
$Pt(dach)(H_2O)_2$	58	6.2
Pt(dach)(Met)	n.i.	n.i.
Pt(dach)(GSH)	n.i.	n.i.

[a] Concentration required to cause 50% inhibition of neurite outgrowth
[b] Ratio of IC_{50} oxaliplatin to IC_{50} of other compound

platinum drugs. Therefore, DRGs were incubated for 48 h after drug dosing in all our assays.

Neurite outgrowth was significantly inhibited by cisplatin, oxaliplatin, and ormaplatin at 50–350 μ*M* (Figs. 1 and 2 and Table 1). However, carboplatin caused no detectable inhibition at up to 350 μ*M*. A plot of neurite outgrowth versus drug concentration is shown in Fig. 2. Biphasic dose responses were observed for cisplatin, oxaliplatin, and ormaplatin. Through the initial phase (< 50 μ*M*), the inhibition of neurite outgrowth did not exceed 20% of the control and differences in the neurite outgrowth inhibition by cisplatin, oxaliplatin, and ormaplatin were not significant. At higher drug concentrations (> 50 μ*M*), substantial inhibitions of neurite outgrowth were observed (Fig. 2), and the differences in the neurite outgrowth inhibition by platinum drugs were statistically significant. The neurotoxicity was ranked as ormaplatin > cisplatin > oxaliplatin > carboplatin (Table 1). From the second phase of the concentration response curve, IC_{50} was estimated as 139 μ*M* for ormaplatin, 256 μ*M* for cisplatin, and 358 μ*M* for oxaliplatin (Table 1). Based on either IC_{50} or neurite outgrowth inhibition at concentrations higher than 50 μ*M*, the in vitro neurotoxicity order observed in our study was consistent with that reported in the previous in vivo neurotoxicity study [15].

Inhibition of neurite outgrowth by the biotransformation products of oxaliplatin and ormaplatin

It was hypothesized that differences in the neurotoxicity of ormaplatin and oxaliplatin could be due to the different plasma concentrations of their active biotransformation products. This hypothesis was based on the significantly different plasma concentrations of $Pt(dach)Cl_2$ derived from ormaplatin and from oxaliplatin in a pharmacokinetic study following equimolar drug dosing [22]. The estimated C_{max} for $Pt(dach)Cl_2$ is 49 μ*M* from ormaplatin and only 1.7 μ*M* from oxaliplatin while the C_{max} is ≤0.1 μ*M* for all other biotransformation products [22]. Therefore, the same assay conditions were used for the biotransformation products as for their parent drugs except that a lower concentration range was used. Because previous studies have suggested that Pt(dach)(Met) and Pt(dach)(GSH) are inactive biotransformation products, their neurotoxicity was assayed at 100 μ*M* only. For all other biotransformation products including $Pt(dach)Cl_2$, $Pt(dach)(H_2O)Cl$, and $Pt(dach)(H_2O)_2$, the neurotoxicity was measured at concentrations ranging from 20 μ*M* to 100 μ*M*. Owing to the fact that Pt(dach) $(H_2O)Cl$ and $Pt(dach)(H_2O)_2$ are unstable in MEM, DRGs were treated with these two compounds in chloride-deficient MEM (details of the preparation in Materials and methods). HPLC analysis showed that these two platinum compounds are stable in chloride-deficient MEM for a 30-min incubation (data not shown). Control experiments also showed no detectable difference in neurite outgrowth for DRGs treated in normal MEM or in chloride-deficient MEM for 30 min (data not shown).

Neurite outgrowth was significantly inhibited by $Pt(dach)Cl_2$, $Pt(dach)(H_2O)Cl$, and $Pt(dach)(H_2O)_2$ at concentrations of 50 μ*M* or above (Fig. 3 and Table 1). However, Pt(dach)(Met) and Pt(dach)(GSH) showed no detectable neurite outgrowth inhibition at a concentration of 100 μ*M* which is 1000-fold higher than their C_{max} values achived in vivo [22]. $Pt(dach)Cl_2$ and its hydrolysis products inhibited neurite outgrowth signficantly more than the parent drugs at comparable doses (Table 1).

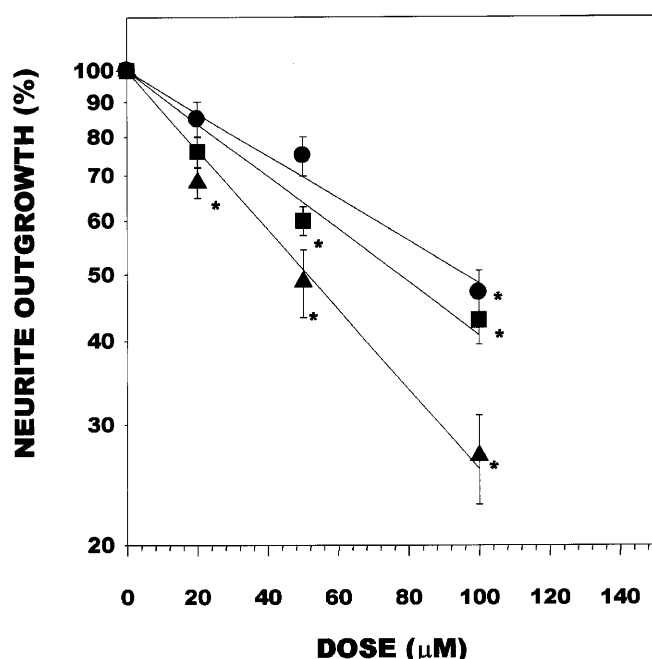

Fig. 3 Inhibition of neurite outgrowth by Pt-dach biotransformation products. Inhibition is expressed as the percentage of the average radius of the halo of DRGs dosed with Pt compounds in relation to that of the control (● $Pt(dach)Cl_2$, ■ $Pt(dach)(H_2O)Cl$, ▲ $Pt(dach)(H_2O)_2$). Each point represents the mean (±SE) from six to eight DRGs. *$P \leq 0.05$ vs oxaliplatin

There was no significant difference in neurite outgrowth inhibition between $Pt(dach)Cl_2$ and $Pt(dach)(H_2O)Cl$ with the concentration range used in these experiments, but $Pt(dach)(H_2O)_2$ was significantly more potent at inhibiting neurite outgrowth than either $Pt(dach)Cl_2$ or $Pt(dach)(H_2O)Cl$. The estimated IC_{50} values were 98 μ*M* for $Pt(dach)Cl_2$, 92 μ*M* for $Pt(dach)(H_2O)Cl$, and 45 μ*M* for $Pt(dach)(H_2O)_2$ (Table 1).

DRG uptake of parent platinum drugs and their biotransformation products

The differences in the observed inhibition of neurite outgrowth could be due to either intrinsic differences in the neurotoxicity of the platinum compounds tested or due to the differences in their uptake by DRGs. Therefore, the DRG uptake of platinum compounds was also determined in this study. E-19 embryonic DRGs were incubated with tritium-labeled platinum compounds including oxaliplatin, ormaplatin, and their biotransformation products $Pt(dach)Cl_2$, $Pt(dach)(H_2O)Cl$, and $Pt(dach)(H_2O)_2$. The same incubation conditions were used as in the neurite outgrowth inhibition assay except that DRGs were harvested immediately after treatment. The uptake was linear over 30 min and was linear with drug concentrations up to 100 μ*M* (data not shown). The initial uptake rates were therefore determined from the uptake over 30 min at 100 μ*M* drug concentration (Table 2). As for the neurite outgrowth inhibition experiments, statistical analysis was also performed for DRG uptake (Table 2). It was found that ormaplatin was taken up 2.8-fold more readily than oxaliplatin. The uptake of $Pt(dach)Cl_2$ was slightly higher than that of oxaliplatin, but 2.0-fold less than that of ormaplatin. The hydrolysis products of $Pt(dach)Cl_2$ were taken up 5–8-fold more readily than oxaliplatin and 2–3-fold more readily than ormaplatin.

Discussion

Rat DRG explant cultures have been proposed as a useful in vitro assay for the neurotoxicity induced by platinum antitumor agents [50]. However, this assay has not been used previously to compare the neurotoxicity of different platinum compounds. Therefore, it was important to validate this assay by comparing the results obtained with those obtained following in vivo exposure of rats to the same platinum compounds. The in vivo neurotoxicity of cisplatin, oxaliplatin, and ormaplatin have been compared in Wister rats utilizing morphometric alteration of DRGs L4–L6 as an indicator of neurotoxicity [15]. In that study, it was observed that ormaplatin caused significant reduction in cell area, nuclear area, and a significant increase in the percentage of cells with multiple nucleoli in DRG neurons. Cisplatin only caused significant changes in the percentage of cells with multiple nucleoli, while oxaliplatin caused nonsignificant changes in all three parameters [15]. Carboplatin was not included in the final study because it had shown no detectable neurotoxicity in a prelimi-

Table 2 DRG uptake of Pt-dach compounds

Pt compound	Initial rate of uptake[a] (pmol/DRG/min)		Fold increase compared to oxaliplatin[b]
	Mean ± SE	*P*-value	
Saline control	0		0
Oxaliplatin	83 ± 3	0.001	1.0
Ormaplatin	217 ± 20	0.001	2.6
$Pt(dach)Cl_2$	124 ± 10	0.01	1.5
$Pt(dach)(H_2O)Cl$	523 ± 10	0.001	6.3
$Pt(dach)(H_2O)_2$	670 ± 38	0.01	8.1

[a] The initial rate of uptake was calculated at a Pt compound dose of 100 μ*M*
[b] The ratio of the initial uptake rate of oxaliplatin to that of other Pt compounds

nary study [15]. These data are also consistent with clinical reports on the neurotoxicity of platinum drugs. All four platinum drugs were tested in our study. Ormaplatin caused the greatest inhibition of DRG neurite outgrowth, cisplatin caused an intermediate inhibition, and oxaliplatin caused the least inhibition. Carboplatin caused no detectable inhibition of neurite outgrowth (Table 1 and Figs. 1 and 2). The neurotoxicity rank observed in our in vitro assay was in agreement with that reported in the in vivo neurotoxicity study in Wistar rats for these platinum drugs [15]. These data suggest that the in vitro rat DRG explant culture can be used to compare the relative neurotoxicity of different platinum compounds.

DRGs are thought to be the major damage site for platinum-induced neurotoxicity in vivo [2, 7, 29, 43, 44]. Therefore, we designed our in vitro DRG exposure conditions to mimic the exposure of DRG to platinum compounds in vivo, i.e. we used a very short exposure time (30 min) at concentrations in the range of the platinum compound C_{max} values achieved in vivo. We then compared the IC_{50} values for in vitro neurotoxicity with the C_{max} values achived in vivo to predict the relative neurotoxicity for oxaliplatin, ormaplatin, and their biotransformation products in vivo (Table 3). We recognize that this comparison assumes that the in vitro model is valid for predicting in vivo neurotoxicity and that the pharmacokinetic parameters in plasma are good estimates of the DRG exposure to the platinum compounds in vivo. We also recognize that even for the most neurotoxic platinum compound, its IC_{50} is still severalfold greater than its in vivo C_{max} (Table 3). However, it should be kept in mind that the in vitro assay is limited to a single platinum exposure, while significant neurotoxicity in vivo requires multiple exposures [15]. Therefore, the IC_{50} value for in vitro neurotoxicity of any platinum compound in this assay is likely to be somewhat higher than the C_{max} for that compound in vivo.

In a pharmacokinetic comparison between oxaliplatin and ormaplatin at equimolar doses [22], the C_{max} of $Pt(dach)Cl_2$ was observed to be 29-fold higher for ormaplatin than for oxaliplatin. All other biotransformation products of oxaliplatin and ormaplatin have similar

Table 3 Comparison of the in vitro neurotoxicity with the C_{max} for Pt-dach compounds in vivo (*n.i.* no inhibition)

Pt compound	IC_{50}[a]	C_{max}(μM)[b]	
	(μM)	Oxaliplatin	Ormaplatin
Oxaliplatin	358	86.0	–
Ormaplatin	139	–	4.6
$Pt(dach)Cl_2$	94	1.67	49.1
$Pt(dach)(H_2O)Cl$	80	< 0.06	< 0.11
$Pt(dach)(H_2O)_2$	58	< 0.06	< 0.07
Pt(dach)(Met)	n.i.	0.13	0.07
Pt(dach)(GSH)	n.i.	0.04	0.02

[a] Values of IC_{50} determined in this study
[b] Values of C_{max} determined in previous pharmacokinetic study [22]

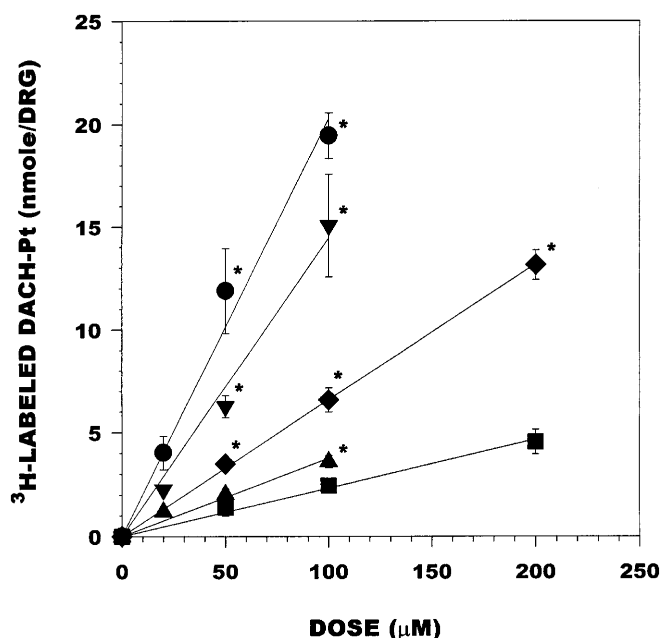

Fig. 4 Platinum compound uptake by rat DRGs. DRGs were dosed under the same conditions as for the neurite outgrowth inhibition assay. Following a 30-min incubation with the indicated dose of Pt compounds, DRGs were harvested in 0.5 ml PBS and sonicated for 2 × 20 s. Aliquots of 0.4 ml were used to quantitate the uptake of Pt by the DRGs (● $Pt(dach)(H_2O)_2$, ▼ $Pt(dach)(H_2O)Cl$, ◆ ormaplatin, ▲ $Pt(dach)Cl_2$, ■ oxaliplatin). Each point represents the mean (+SE) from five or six DRGs. $*P \leq 0.05$ vs oxaliplatin

plasma concentrations. In contrast, the C_{max} is 19-fold less for ormaplatin than for oxaliplatin. Because ormaplatin is more neurotoxic than oxaliplatin in vivo [15], $Pt(dach)Cl_2$ is suspected to be a critical biotransformation product that might be responsible for the neurotoxicity of these two platinum compounds. Since $Pt(dach)Cl_2$ can be further hydrolyzed to the more cytotoxic aquated species $Pt(dach)(H_2O)Cl$ and $Pt(dach)(H_2O)_2$ [8], we compared the in vitro neurotoxicity of $Pt(dach)Cl_2$ and its hydrolysis products with that of oxaliplatin and ormaplatin in this study. We found that $Pt(dach)Cl_2$ and its hydrolysis products were 3.8–6.2-fold more neurotoxic than the parent drugs. Because of the relatively low plasma concentration of ormaplatin observed in vivo [22], $Pt(dach)Cl_2$ is likely to be responsible for the majority of the in vivo neurotoxicity of ormaplatin, even though ormaplatin itself was also found to be neurotoxic in our in vitro study (Table 3). This is consistent with the observed correlation of the neurotoxicity of ormaplatin with the plasma concentrations of $Pt(dach)Cl_2$ in a previous clinical study [36]. In contrast, the C_{max} of $Pt(dach)Cl_2$ is 51-fold lower than that of oxaliplatin following i.v. adminsitration of oxaliplatin. Thus, even though $Pt(dach)Cl_2$ was 3.8-fold more neurotoxic than oxaliplatin in our in vitro assay, it probably contributes relatively little to the oxaliplatin neurotoxicity in vivo (Table 3). Thus, it is likely that the different neurotoxicity profiles of oxaliplatin and

ormaplatin result at least partially from the different plasma concentrations of $Pt(dach)Cl_2$ following i.v. infusions of oxaliplatin and ormaplatin. While the acquated biotransformation products $Pt(dach)(H_2O)Cl$ and $Pt(dach)(H_2O)_2$ are more neurotoxic than $Pt(dach)Cl_2$ in this assay, the concentrations of these two compounds required to cause 50% inhibition of neurite outgrowth are 400–1000-fold greater than their C_{max} values achieved in vivo [22]. Therefore, $Pt(dach)(H_2O)Cl$ and $Pt(dach)(H_2O)_2$ in the plasma are unlikely to make significant contributions to the neurotoxicity of either ormaplatin or oxaliplatin in vivo. In addition, stable biotransformation products such as Pt(dach)(Met) and Pt(dach)(GSH) have no detectable neurotoxicity at 100 μM, which is 1000-fold higher than their estimated C_{max} values in vivo. Therefore, they also are unlikely to contribute to the neurotoxicity of oxaliplatin or ormaplatin in vivo.

The rate of uptake of platinum compounds by rat DRGs was measured to determine whether the observed differences in neurite outgrowth inhibition represent intrinsic differences in the neurotoxicity of platinum drugs or differences in their uptake by DRGs. This can be assessed by comparing the neurotoxicity (relative to oxaliplatin based on the IC_{50} values for inhibition of neurite outgrowth, Table 1) to the DRG uptake (relative to oxaliplatin, Table 2). For most of the platinum drugs tested, the neurotoxicity was approximately proportional to their DRG uptakes. However, $Pt(dach)Cl_2$ was significantly more neurotoxic (3.8-fold relative to oxaliplatin) than one would predict based on its DRG uptake (1.3-fold relative to oxaliplatin). This suggests that $Pt(dach)Cl_2$ is uniquely neurotoxic, perhaps because it can be readily hydrolyzed into more neurotoxic $Pt(dach)(H_2O)Cl$ and $Pt(dach)(H_2O)_2$ complexes after being taken up by cells in the DRG. At the plasma concentration of chloride ion (108 mM), $Pt(dach)Cl_2$ is probably not hydrolyzed into aquated species in the plasma to a significant extent. However, once it is taken up by cells, $Pt(dach)Cl_2$ will be more readily converted into acquated species due to the lower intracellular concentration of chloride ion (3 mM) [25]. Under the same conditions, oxaliplatin is converted into aquated species at a much lower rate than $Pt(dach)Cl_2$ [23]. This may explain why $Pt(dach)Cl_2$ was significantly more neurotoxic than oxaliplatin in our assay.

Ormaplatin can be reduced to $Pt(dach)Cl_2$ rapidly in the plasma. However, the conversion of oxaliplatin to $Pt(dach)Cl_2$ is relatively slow (an S_N2 displacement of the leaving ligand) [33]. Carboplatin is also converted to cisplatin by a simple chloride displacement of the leaving ligand. However, the displacement of the leaving ligand by chloride is even slower for carboplatin than for oxaliplatin [46]. Thus, it appears that the neurotoxicity of platinum drugs may be correlated with their ability to form the platinum(II) complexes which contain chloro leaving ligands in the plasma, e.g. ormaplatin ($Pt(dach)Cl_2$) is more neurotoxic than oxaliplatin and cisplatin is more neurotoxic than carboplatin. Since $Pt(dach)Cl_2$ is more neurotoxic than cisplatin, and oxaliplatin is more neurotoxic than carboplatin, the nature of the carrier ligand (dach versus *cis*-diammine) may also influence the neurotoxicity of platinum drugs. These data could be useful for the future design of Pt-dach anticancer drugs with reduced neurotoxicity.

Pt-dach drugs are unique because they display minimal nephrotoxicity, and myelotoxicity, and increased activity against cisplatin-resistant tumors [5, 13, 24, 35, 37, 42, 45]. Therefore, it would be logical to keep the Pt-dach moiety for future drug design, while the axial and/or leaving ligands of Pt-dach drugs could be modified to reduce their neurotoxicity by minimizing the formation of $Pt(dach)Cl_2$ in plasma. For Pt(II)-dach complexes, this would require replacing the oxalate ligand with a leaving ligand which would be displaced more slowly than oxalate by chloride ions. For example, both the malonate and 1,1-cyclobutanedicarboxylate leaving ligands are displaced more slowly by chloride ions. However, these Pt-dach compounds suffer from poor solubility and low efficacy [19]. The situation with Pt(IV)-dach compounds may be somewhat more promising. Ormaplatin probably represents an extreme case of Pt(IV)-dach compounds, owing to its rapid reduction to the more neurotoxic $Pt(dach)Cl_2$ in the plasma ($t_{1/2}$ = 3 s). It has been shown that the reduction of ormaplatin can be slowed by modifying its axial leaving ligands [20]. It is possible that the neurotoxicity of ormaplatin could also be modulated postreduction by changing its equatorial ligands (chlorides) into ones (malonate and 1,1-cyclobutanedicarboxylate) that would be very slowly displaced by chloride ions once the Pt(IV)-dach compound had been reduced to the Pt(II)-dach analog. Some of these Pt(IV)-dach compounds have been tested in a cisplatin-resistant L1210 tumor model in vivo [17, 39, 40], and no cross-resistance with cisplatin has been observed. It would be of interest to evaluate the neurotoxicity of these compounds.

In summary, our in vitro rat DRG explant model has produced the same neurotoxicity pattern as the in vivo rat model. It is the first time that the neurotoxicities of different platinum drugs and their biotransformation products have been compared using this assay. These data strengthen the justification for using rat DRG explant cultures as an alternative method for screening the neurotoxicity of platinum drugs. Our study also suggests that the biotransformation product $Pt(dach)Cl_2$ and its hydrolysis products are more neurotoxic than the parent drugs oxaliplatin and ormaplatin. Thus, the different neurotoxicity profiles of oxaliplatin and ormaplatin are likely related to the different plasma concentration of their biotransformation product $Pt(dach)Cl_2$. The available pharmacokinetic and biotransformation data in both rats [6, 22] and humans [36] suggest that $Pt(dach)Cl_2$ is likely to make a major contribution to the neurotoxicity associated with ormaplatin treatment. Our pharmacokinetic data [22] also suggest that the plasma concentration of $Pt(dach)Cl_2$ is too low following oxaliplatin infusion to contribute significantly to the oxali-

platin neurotoxicity in rats. However, the relative plasma concentrations of oxaliplatin and $Pt(dach)Cl_2$ during and following oxaliplatin infusion in humans is unknown. If the plasma concentrations of $Pt(dach)Cl_2$ are significantly higher relative to oxaliplatin in humans than in rats, the monitoring of the plasma concentration of $Pt(dach)Cl_2$ might be helpful for predicting the neurotoxicity of oxaliplatin in future clinical trials.

Acknowledgements We would like to thank Dr. Martine Bayssas (Debiopharm S.A., Lausanne, Switzerland) for providing us with unlabeled oxaliplatin, Dr. Martin Graham (Sanofi Research Division, Malvern, Pa.) and Dr. Dennis Greenslade (Sanofi Research Division, Alnwick, UK) for providing us with Pt(dach)(GSH). We would also like to thank Dr. Robert Bagnell, Victoria Madden (Department of Pathology, UNC-CH) and Jason Stanko for technical support in the quantitation of neurite outgrowth and DRG dissection. This research was supported by NIH Grant CA 55326.

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