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Preclinical study

Neurotoxic effect of cisplatin and the cisplatin–procaine complex DPR studied in organotypic cultures of chick embryonic dorsal root ganglia

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Neurotoxic effects of cisplatin and the cisplatin–procaine complex *cis*-diaminechloro-[2-(diethylamino)ethyl 4-aminobenzoate, *N*4]-chloridoplatinum(II) monohydrochloride monohydrate (DPR) were compared in organotypic cultures of chick embryonic dorsal root ganglia maintained in a semi-solid (soft agar) culture medium. The changes of two characteristics of the neurite outgrowth, the mean radial length of neuritic processes growing out from the ganglia and the area of neurite outgrowth around the ganglion, were used as parameters to evaluate the toxic effect of both compounds. The drugs were administered to the cultures at concentrations ranging from 13 to 120 μM. The half-maximum inhibition concentration (IC_{50}) was determined from the concentration–response curves for both the mean radial length of neurites and the area of neurite outgrowth. An analysis of these parameters revealed that DPR was significantly less neurotoxic than cisplatin. In fact, considering the mean radial length of neurite processes, the IC_{50}s of cisplatin were 56, 65 and 66 μM after 24, 48 and 72 h of exposure, respectively. By contrast, for DPR the IC_{50}s were 116 μM after 24 h, and greater than 120 μM after 48 and 72 h of exposure. When we considered the area index (i.e. the area of neurite outgrowth normalized for the area of the ganglia), the IC_{50}s for cisplatin were 41, 52 and 55 μM after 24, 48 and 72 h of exposure, respectively, whereas for DPR the IC_{50}s were 59 μM after 24 h, and greater than 120 μM after 48 and 72 h of exposure. Our results support previous findings of lower toxicity of DPR to non-neoplastic tissues, as compared to cisplatin. [© 1998 Lippincott Williams & Wilkins.]

Key words: Cisplatin, DPR, neurotoxicity, spinal ganglia.

Introduction

Cisplatin is a potent antitumor drug highly effective in the therapy of ovarian and testicular malignancies, and head and neck cancers.[1-3] The dosage of this drug is limited because of side effects of nephrologic, neurologic, gastrointestinal and haematological origin. Neurotoxicity of cisplatin is considered one of the main dose-limiting factors.[4,5] A considerable effort has been made to eliminate, postpone or decrease toxic side effects of cisplatin. Neurotoxicity of cisplatin was successfully limited by several neuroprotective factors, derived from ACTH.[6,7] Nimodipine and WR-2721 were also shown to have protective effects either in neurotoxicity models[8] or in patients.[4]

Another possibility to circumvent cisplatin neurotoxicity is the synthesis of less toxic cisplatin analogs[9] or cisplatin complexes. DPR was introduced several years ago as a platinum triamine complex obtained by the synthesis of *cis*-diamminedichloroplatinum(II) and procaine.[10] This complex reveals outstanding anticancer activity, comparable with the original drug cisplatin, but its toxicity to non-neoplastic renal tissue studied under *in vitro* and *in vivo* experimental conditions was considerably lower.[10,11]

The aim of the present study was to compare the neurotoxic effect of DPR and cisplatin in a newly developed sensitive *in vitro* method, utilizing organotypic cultures of chick embryonic dorsal root

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ganglia (DRG) maintained in a semi-solid culture medium.

Materials and methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), and penicillin and streptomycin were obtained from HyClone Europe (Cramlington, UK), fetal calf serum (FCS) from PAA-Labor (Linz, Austria), and NGF (7S Nerve Growth Factor) from Calbiochem (La Jolla, CA). Agar (Difco Agar Noble) was purchased from Difco (Detroit, MI). Sterile plastic ware was purchased from Falcon (Lincoln Park, NJ).

Culture experiments

A two-layer soft agar culture medium culture system, as previously described,[12] was used for the cultivation. The lower agar medium layer consisted of culture medium supplemented with 0.5% agar. This medium (1.5 ml) was pipetted into 35 mm Petri dishes. Freshly isolated DRG from 12-day-old chick embryos (HH stage 37–38) were placed on top of this layer. Then the upper agar medium layer, consisting of 1 ml culture medium with 0.25% agar, was added so that the DRGs were completely embedded in agar. At least eight ganglia were used for each condition in four independent experiments. The culture medium consisted of DMEM supplemented with 10% FCS, 50 U/ml penicillin/ 100 μg/ml streptomycin sulfate and 25 ng/ml NGF. The pH of culture medium was adjusted to 7.2–7.4 with a 7.5% solution of $NaHCO_3$. The Petri dishes were kept in a humidified atmosphere of 5% CO_2 in air at 37°C.

Cisplatin and DPR treatment

Cisplatin and DPR were added separately in individual experiments to the culture medium in both the lower and upper layer. The final concentration of DPR ranged from 13 to 120 μM, while that of cisplatin ranged from 13 to 80 μM.

Morphology and morphometry of the cultures

The cultures were evaluated during three consecutive culture days using a Leitz DIAVERT inverted microscope (Leitz, Wetzlar, Germany). Morphological changes of neuritic processes growing out from the DRGs were observed using phase contrast. To determine the changes of neuritic outgrowth, pictures of cultured DRG were made by tracing the contours on a plastic sheet directly from the screen of a video monitor. Obtained schematic drawings were than scanned with Scan Maker (MICROTEK) and consequently evaluated using a Macintosh Power PC 8100 and TIFF Software imaging. Two parameters of neuritic outgrowth were evaluated: the radial length of the processes and the area of outgrowth. Obtained values were used to calculate the area index of neuritic outgrowth, defined as area of outgrowth divided by area of the ganglion, to correct for the size of the ganglion.

Statistical analysis

To evaluate the significance of changes in neurite length and area index as a function of time and concentration of tested drugs, a non-parametric Mann-Whitney test was used.

Results

The effect of cisplatin and DPR on the morphology of neurites

The first influence on morphological appearance of neuritic processes growing out of the ganglia was seen at concentrations of 13 μM cisplatin or 40 μM DPR. At these concentrations, the processes grew out around the whole perimeter of the ganglia, but their shape was slightly irregular and their density was smaller than in control cultures. More prominent morphological changes were present in the cultures influenced by 60 and 80 μM cisplatin and 80 and 120 μM DPR (Figure 1a–c).

The effect of cisplatin and DPR on the growth parameters of neurites

Statistical analysis showed that both drugs had a significant inhibitory effect on outgrowth, reflected by both parameters used, i.e. neurite length (Figure 2) and area index (Figure 3).

The approximate median inhibitory concentrations (IC_{50} values) expressed as percent inhibition in comparison with control cultures revealed significant differences depending on the drug as well as the exposure time. Cisplatin exhibited a much higher toxicity than DPR, especially when the exposure time

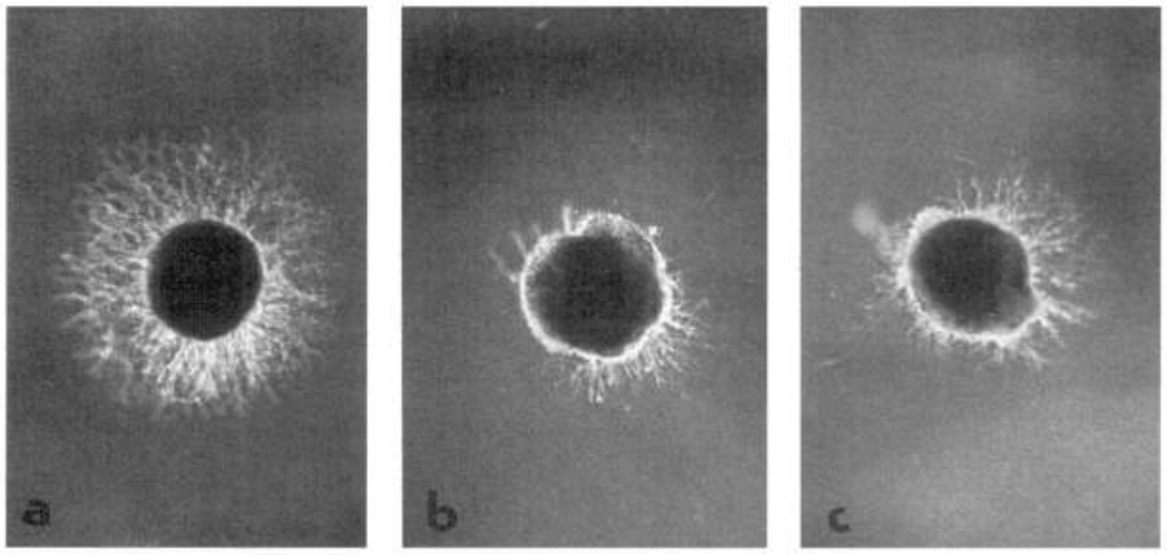

Figure 1. Toxic effect on neuritic outgrowth of cultured chick embryonic DRG (×40). Untreated (a), treated with 80 μM cisplatin (b) and treated with 80 μM DPR (c). Chick embryonic DRG were cultured in the presence of platinum compounds for 48 h.

was prolonged to 48 and 72 h. Considering the length of neurites, the IC_{50}s for cisplatin were 56, 65 and 66 μM after 24, 48 and 72 h of exposure, respectively. After the same exposure, the IC_{50}s for DPR were higher, being 116 μM (after 24 h), and larger than 120 μM after 48 and 72 h of exposure. When we consider the parameter area index, the IC_{50}s for cisplatin were 41, 52 and 55 μM after 24, 48 and 72 h of exposure, respectively. By contrast, for DPR the IC_{50}s were 59 μM after 24 h, and larger than 120 μM after 48 and 72 h of exposure.

Discussion

DPR is a new platinum triamine complex containing procaine synthesized several years ago.[10] Apart from its good antitumor activity demonstrated in previous studies,[10,12,13] a significantly lower nephrotoxicity of DPR compared to cisplatin was found both with *in vitro* and *in vivo* experimental models.[10,11] Furthermore, both *in vitro* and *in vivo* synergism between DPR and several anticancer drugs with different mechanisms of action was found.[12,14]

Organotypic cultures of chick DRG maintained with a semi-solid (soft agar) culture medium represent a highly sensitive model to study organ-specific toxic mechanisms within the peripheral nervous system.[15,16] In this model, internal organization of DRG is preserved and intracellular contacts are kept. From this point of view, this culture system resembles quite closely the *in vivo* situation.[17-19] It enables the evaluation of indicators specific for nervous tissue, especially with regard to potential to form and extend neuritic processes.

In the present study we compared neurotoxicity of DPR and cisplatin in organotypic cultures of chick embryonic DRG. In accordance with previous studies,[15,20] cisplatin caused toxic changes of the ganglia, expressed by morphological irregularities and the depression of growth parameters of neurites growing in the vicinity of cultured ganglia.

Compared to cisplatin, the neurotoxic effect of DPR differed substantially in our culture system. DPR caused morphological changes and the reduction of growth parameters of neurites at higher concentrations than cisplatin. Moreover, the effect of DPR did not progress with the prolongation of exposure, as did the effect of cisplatin, but rather the superiority of DPR was more prominent when the exposure time was prolonged to 48 and 72 h.

Why cisplatin causes neurotoxicity is not very clear yet. Non-neuronal cells, especially satellite and Schwann cells, represent probably the main target for the toxic action of this drug;[7,21,22] nevertheless, neuronal damage was also observed in some experiments.[23,24] The toxic action of different drugs within the peripheral nervous system was successfully diminished by the administration of neurotrophic factors, such as the nerve growth factor NGF[25] or by

ACTH-related peptides.[6-8] These factors influence predominantly the neurons within the DRG and could play a role in the replacement of trophic signals in the situation where non-neuronal cells are damaged.

As previously reported in *in vitro* studies,[10] the mechanistic possibility that DPR may exert its action *in vivo* after a previous decomposition into its parent forms appears to be unlikely. It might be argued from these findings that the procaine moiety in the DPR complex plays a crucial role in its mechanism of

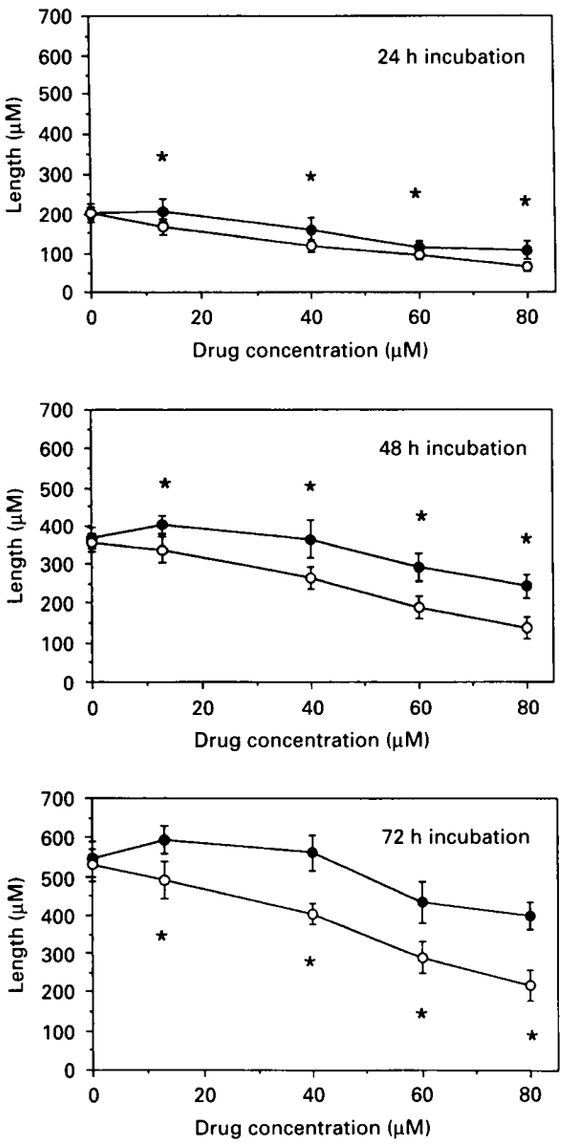

Figure 2. Toxic effect of different concentrations of cisplatin (○) and DPR (●) on neuritic outgrowth of cultured chick embryonic DRG. Mean radial length of the neurites is given in μm. The error bars indicate the SD. Differences between DPR and cisplatin treatment were evaluated by non-parametric Mann–Whitney test. p values: 24 h exposure, *$p \leq 0.02$; 48 h exposure, *$p \leq 0.002$; 72 h exposure, *$p \leq 0.002$.

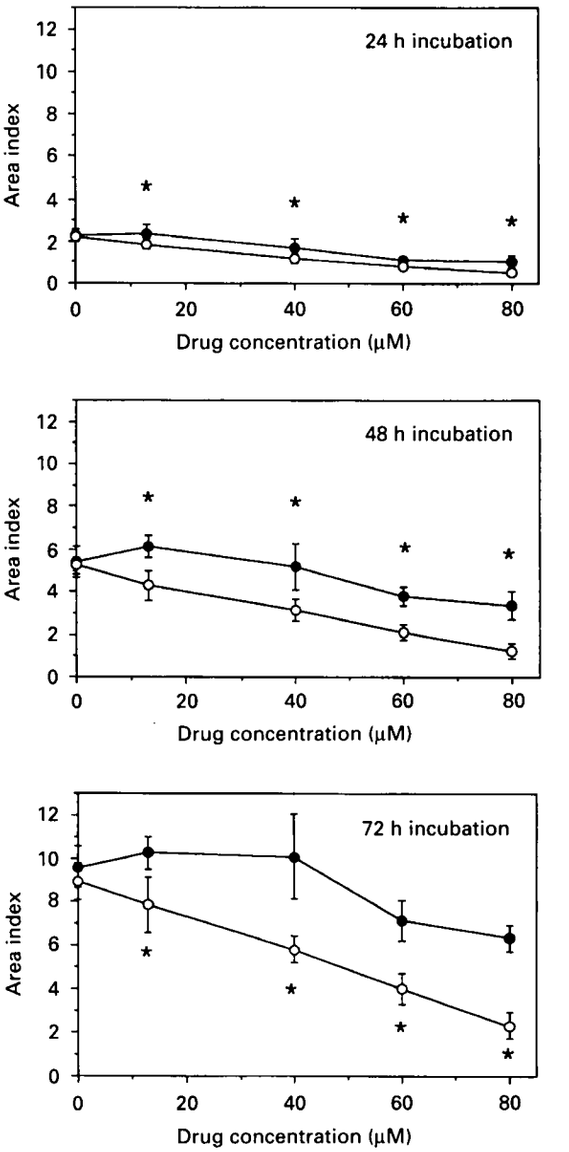

Figure 3. Toxic effect of different concentrations of cisplatin (○) and DPR (●) on neuritic outgrowth of cultured chick embryonic DRG. Area index is calculated as the ratio between area of outgrowth (area around the ganglion covered with processes) and area of ganglion. The error bars indicate the SD. For each drug concentration the differences between DPR and cisplatin treatment were calculated by non-parametric Mann–Whitney test. p values: 24 h exposure, *$p \leq 0.02$; 48 h exposure, *$p \leq 0.002$; 72 h exposure, *$p \leq 0.002$.

action. It has been reported that procaine inhibits cisplatin-induced lipid peroxidation *in vitro*[26,27] and preliminary data from our laboratory suggest that the binding of DPR to DNA results in a release of procaine. Therefore, the hypothesis that the local anesthetic may protect neurons and non-neuronal cells against lipid peroxidation, thus improving their viability, cannot be excluded. Moreover, this could also explain the observation that the toxic effect of DPR did not strictly progress accordingly with the prolongation of exposure time, as was found for cisplatin. This phenomenon together with the lower neurotoxicity of DPR compared to cisplatin could be very important in further clinical application of DPR.

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