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A 5-HT_3 receptor antagonist fails to prevent cisplatin-induced toxicity in immature rat spinal cord

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Abstract

The use of high doses of cisplatin in treating cancers has been limited by two major adverse effects – emesis and peripheral neuropathies. The emesis has become largely controlled by the introduction of a new class of drugs – the 5-HT_3 receptor antagonists. The current study was undertaken to determine if these drugs would also prevent cisplatin-induced neuropathy. We have used a developing rat as an animal model and determined the effects of cisplatin on morphology (loss of spinal cord calcitonin gene-related peptide (CGRP)-containing neurons) and behavior (gait abnormalities and pain perception). Rat pups from the age of 5 days were treated twice weekly for 4 weeks with cisplatin (1 mg/kg), the 5-HT_3 antagonist MDL 72222 (3 mg/kg) or both. The animals were tested for pain perception (using tail-flick latencies) at 17 and 21 days of age and for a gait abnormality at 24 days of age. At 34 days of age, the animals were perfused and the lumbar region of the spinal cords stained immunocytochemically for CGRP. Our results show that cisplatin treatment resulted in a dramatic loss of CGRP neurons in the dorsal horn of the spinal cord and a corresponding increase in the animals' threshold for pain. In addition, the animals showed a pronounced gait abnormality, characterized by 'toeing-in'. Treatment with MDL 72222 not only failed to protect against the loss of CGRP neurons but also worsened the gait abnormalities seen after cisplatin treatment alone. These studies confirm and extend the list of morphological and functional adverse effects of cisplatin treatment. As well, our results raise the concern that co-administration of a 5-HT_3 antagonist may potentiate some adverse effects.

Keywords: Cisplatin; Peripheral neuropathy; CGRP (calcitonin gene-related peptide); 5-HT_3 antagonist

1. Introduction

Cisplatin is used in the treatment of neoplastic diseases, due to its cytotoxic properties. However, the therapeutic usefulness of this agent is significantly inhibited by its major adverse effects: nausea, vomiting and peripheral neuropathy. The peripheral neuropathy induced by cisplatin is primarily sensory in nature. There is a slowing of nerve conductance velocities and a subsequent loss in pain perception and proprioception. Clinically, this is described as acrodysaesthesiae, absent tendon reflexes, gait abnormalities and a positive Romberg sign (MacDonald, 1991: LoMonaco et al., 1992). In animal models of cisplatin neuropathy, outcome measures typically include the tail-flick response (for pain perception), the roto-rod test (Apfel et al., 1992) and measurements of sensory nerve conduction velocity (Gispen et al., 1992). As well, radioimmunoassays for calcitonin-gene-related peptide (CGRP) have shown a decrease in the content of this peptide in sensory ganglia after cisplatin treatment (Apfel et al., 1992).

In the past few years, drugs selectively antagonistic to the 5-HT_3 receptor (5-HT_3 antagonists), such as ondansetron, have proven useful in preventing the emesis (Milne and Heel, 1991; Costall and Naylor, 1992). This observation has led to the conclusion that the emesis is caused by an effect of cisplatin on the serotonergic system, and it has been recently shown that in some intestinal tissues cisplatin can release serotonin (Schworer et al., 1991). Interestingly, the serotonin system has an influence on the content of spinal cord CGRP (Fone, 1992).

In spite of the extensive research on the effects of 5-HT_3 antagonists on cisplatin-induced emesis, little is known about the potential of these drugs for preventing the peripheral neuropathy. There is also a lack of

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information concerning the mechanism by which toxicity is produced in the spinal cord. Although there is no direct evidence to suggest that the serotonergic system may be involved in this adverse effect of cisplatin, we have previously shown that serotonergic receptors, in particular 5-HT_3 receptors, play a role in regulating the development of pain pathways in the spinal cord (Bell et al., 1991). Since developmental signals are often also involved in neuroplasticity and neuroprotection, it is possible that 5-HT_3 receptor antagonists would also prevent cisplatin-induced spinal cord neuropathy. Moreover, 5-HT_3 receptors have been localized to the dorsal root ganglia (Tecott et al., 1992), the site of highest cisplatin accumulation and subsequent neuropathy (Gregg et al., 1992).

In this study, we have used a model of cisplatin-induced peripheral neuropathy in a developing rat pup, from postnatal day 5 to 33. We chose to use this model since toxic and trophic events are in general more robust and the treatment time would be less. Thus, the cisplatin-induced neuropathy could be induced after only 4 weeks of treatment in the young animals, while adult models of peripheral neuropathy typically require 8–10 weeks of treatment (Hamers et al., 1991; Apfel et al., 1992; Gispen et al., 1992; Hamers et al., 1993). For the 5-HT_3 antagonist we used MDL 72222, a drug which we have previously found to influence spinal cord development (Bell et al., 1991).

2. Materials and methods

2.1. Animal treatment

32 male Sprague-Dawley rats, from eight different litters, were cross-fostered to four mothers in equal groups of eight at birth. At 5 days of age, the pups were assigned to one of four groups (i.e. each litter had all four treatment groups – two pups to a group). The four groups were: vehicle (dimethylsulfoxide; DMSO) control, cisplatin (1 mg/kg), MDL 72222 (3 mg/kg), and both drugs in a single injection. The pups were injected subcutaneously twice weekly for 4 weeks.

2.2. Behavioral measures

Animals were tested between 09:00 and 14:00 h on each of the postnatal days designated for testing – day 17 and 21 for pain perception and day 24 for gait abnormalities. None of these days was an injection day. Prior to testing each pup was weighed and placed with its littermates in a holding cage.

The tail-flick latency test was used to document analgesic response. During testing, the torso of each animal was placed on a section of gauze which was previously rubbed on the maternal dam and hand held over the apparatus. Prior to each trial, the rat pup's tail was adjusted to cover the aperture of the heat source. The trial was started only when the pup displayed momentary quiescence. A trial consisted of the time elapsed from the introduction of a noxious stimulus (heat produced by a beam of light focused on the distal end of the tail) to the reflexive lateral movement of the tail away from the source. Tail movement was not considered an actual flick if it occurred concurrently with limb or body movements. The mean of three trials was taken for the tail-flick latency of each animal and group means and standard errors of the means calculated.

Gait abnormality was quantified by a method designed in our laboratory. Each pup was placed in a lighted corridor 12 cm wide and 80 cm long with an additional 20 cm of darkened area at the opposite end. After one trial, to ensure that the pup would walk, the bottom of the corridor was lined with white absorbent paper and the rear feet were blackened with ink. The pup was again placed in the corridor, this time leaving marks on the paper. The degree of gait abnormality was determined by measuring the distance between the toes of the feet and subtracting the distance between the heels. Five pairs of prints were measured for each animal by a rater blind to the treatment. The mean distance for each animal was calculated and the group means and standard errors of the means determined. Fig. 2 shows representative tracings with the method for calculation.

2.3. Immunocytochemistry

At 34 days of age, four rats from each group were anaesthetized with a combination of 4 mg xylazine and 20 mg ketamine, then transcardially perfused with 300 ml of normal saline followed by 300 ml with 4% paraformaldehyde. The spinal cords were removed and post-fixed overnight in 20% sucrose in 0.1 M phosphate buffer. The lumbar region of the spinal cords was sectioned at 15 μm on a cryostat and collected into 0.1 M phosphate buffer. Free-floating sections from each group were incubated for 24 h with a primary antibody raised against calcitonin gene-related peptide (CGRP; 1/4000; Chemicon International, rabbit polyclonal in antibody diluting buffer consisting of 0.1% triton, 0.1% normal goat serum in phosphate-buffered saline). After rinsing 3 times, 10 min each, in PBS, the sections were incubated in anti-rabbit IgG (1/200 in antibody diluting buffer; Sigma Chemical Corp.) for 2 h. After three more 10-min PBS rinses, the sections were developed using the ABC Elite reaction (Vectastain) following the manufacturer's directions with diaminobenzidine (DAB; 5 mg/50 ml) as the chromogen. The sections were mounted on gelatin-coated slides (60 sections/slide) and visualized using an Olympus BH2 microscope at a 100 × magnification. Each section was photographed on color slide film and the number of

cells in each section counted by a rater blind to the treatments. Final results were calculated by taking the mean number of cells per section for each animal and calculating the group means and standard errors.

2.4. Statistical analysis

One-way analysis of variance was performed, with a post-hoc Scheffé test for specific between-group comparisons.

3. Results

3.1. Weight gain

Animals receiving cisplatin or MDL 72222 alone, gained weight more rapidly than DMSO-treated controls. However, animals receiving both drugs gained weight at the same rate as controls. These results are given in Table 1.

3.2. Tail-flick latency

Postnatal day 17: overall analysis of variance was significant ($P < 0.005$; DF = 3: $F = 5.382$). Post-hoc tests showed cisplatin caused a significant increase in tail-flick latency (cisplatin 13.2 ± 2.2 vs. 4.6 ± 0.9 s, $P < 0.05$) which was not prevented by MDL 72222 (MDL 72222/cisplatin 11.3 ± 1.9 s.). Postnatal day 21: overall analysis of variance was significant ($P < 0.006$; DF = 3; $F = 5.30$). Post-hoc tests showed cisplatin caused a significant increase in tail-flick latency (cisplatin 22.8 ± 4.9 vs. 4.6 ± 0.7 s., $P < 0.05$) which was not prevented by MDL 72222 (MDL-72222/cisplatin 15.6 ± 4.2 s.). MDL 72222 had no significant effect on tail-flick latency when given alone at either time. These data are summarized in Fig. 1.

3.3. Gait abnormalities

By subtracting the interheel distance from the intertoe distance, the degree of toeing-in could be determined. Overall analysis of variance was significant ($P < 0.001$; DF = 3; $F = 9.84$). Post-hoc analysis showed that animals treated with cisplatin had a significant decrease in the toe-heel measure (1.15 ± 0.17 cm. vs. 1.61 ± 0.17 cm for DMSO, $P < 0.02$), as did the MDL 72222-treated animals (1.17 ± 0.09 cm, $P < 0.02$). The combination of treatment with both drugs caused an

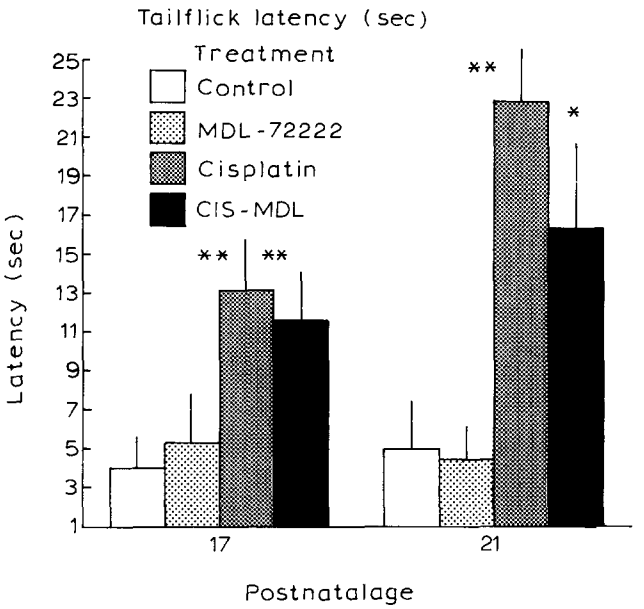

Fig. 1. Tail-flick latency in treated animals. The data are expressed as the means of eight animals, each of which were tested 3 times at each timepoint. The error bars represent standard errors of the means. ** $P < 0.05$ compared to control: * $P < 0.02$ compared to control.

Table 1
Weight (in g ± standard deviation) of treated pups

Treatment	Age (days)			
	5	8	11	32
DMSO	14.2±1.3	16.4±0.9	24.3±1.2	114.4± 6.7
MDL 72222	14.3±0.4	23.1±0.7 [a]	31.7±0.6 [a]	133.2± 9.8 [a]
Cisplatin	14.3±0.4	21.4±0.7 [a]	30.2±0.9 [a]	135.7±10.5 [a]
Cis/MDL	13.8±1.2	18.1±2.0	23.3±3.1	110.3± 9.5

[a] $P < 0.001$, compared to DMSO.

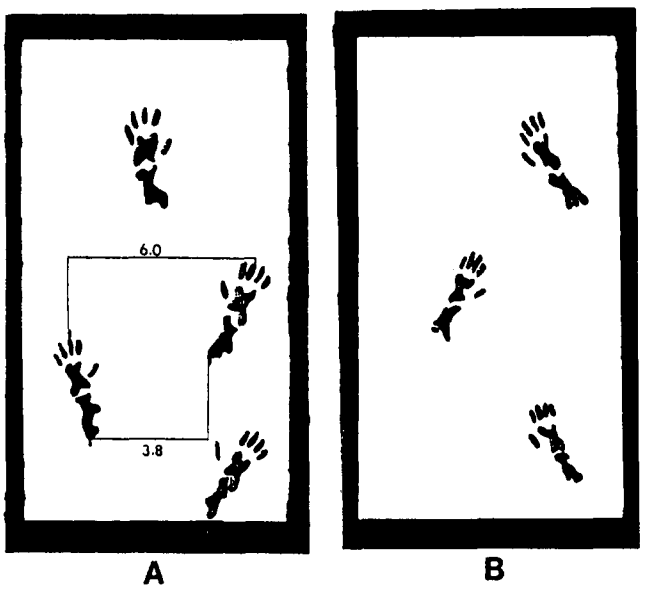

Fig. 2. Representative footprints, showing the method of calculating the gait abnormality in the control (A) animal. Panel B is a tracing from an animal treated with both cisplatin and MDL-72222.

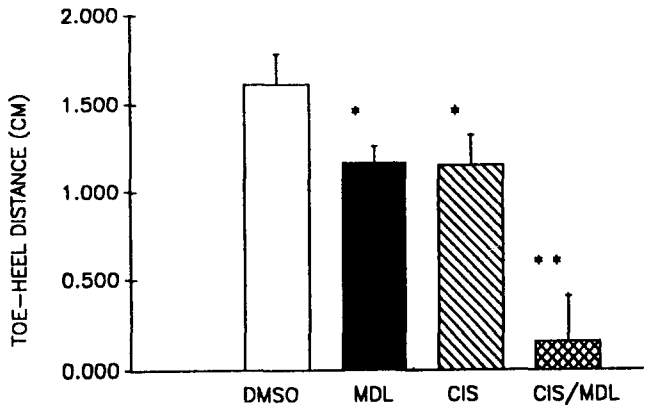

Fig. 3. Gait abnormalities. The data are expressed as the means of eight animals with five pairs of footprints measured for each animal. The error bars represent standard errors of the means. ** $P < 0.002$ compared to control: * $P < 0.02$ compared to control.

even greater decrease in the difference (0.16 ± 0.25 cm, $P < 0.002$). Fig. 2 shows representative tracings from animals. The data are given in Fig. 3.

3.4. CGRP immunoreactive cell count

Overall analysis of variance was significant ($P < 0.008$; DF = 3; $F = 6.28$). Post-hoc analysis showed cisplatin caused a significant decrease in the number of CGRP immunoreactive cells (cisplatin 23.8 ± 2.1 vs. 34.9 ± 4.3, $P < 0.02$). MDL 72222 had no significant effect on the number of CGRP immunoreactive cells when given alone (28.3 ± 2.7), nor did it prevent the effects of cisplatin (MDL 72222/cisplatin 23.5 ± 1.7 vs. cisplatin 23.8 ± 2.1). These data are summarized in Fig. 4.

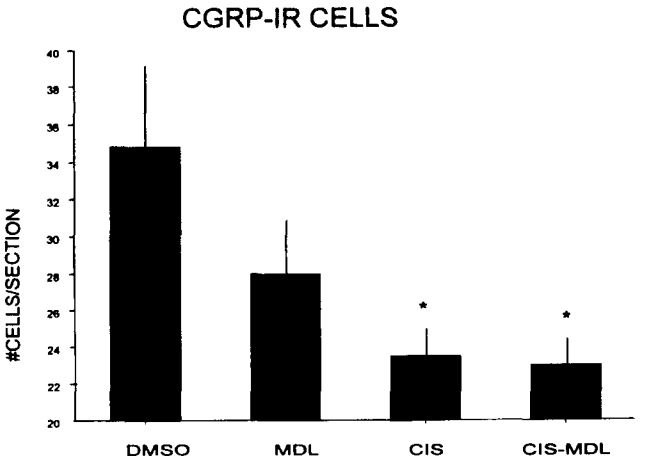

Fig. 4. CGRP immunoreactive cell count. Each bar is the mean number of CGRP-immunoreactive cells in sections taken from the L1 to L5 region of spinal cord of four animals. At least 60 sections were counted for each animal. The error bars indicate standard errors of the means. * $P < 0.02$ compared to control.

4. Discussion

Our findings have shown that in a developing rat pup model, cisplatin induces gait abnormalities and an increase in pain threshold. The increase in pain threshold may correlate with the loss of CGRP-containing neurons in the spinal cord.

Although there was reason to expect that the 5-HT_3 antagonist MDL 72222 would prevent the peripheral neuropathy produced by cisplatin, our results have shown this to not be the case. Moreover, our results suggest that some measures of cisplatin-induced toxicity might in fact be made worse by co-treatment.

In addition to 5-HT_3 antagonists inhibiting cisplatin-induced emesis, there are other arguments leading to the expectation that MDL 72222 should prevent cisplatin peripheral neuropathy. In a study of cisplatin effects on serotonin, using the isolated guinea pig small intestine, cisplatin was found to release serotonin via 5-HT_3 receptors (Schworer et al., 1991). In our developmental studies, we have found that 5-HT_3 receptors influence development of pain pathways (as measured by the tail-flick method; Bell et al., 1991). Thus, the observed changes in tail-flick latencies (Apfel et al., 1992) previously reported in cisplatin-treated animals could well be through 5-HT_3 receptors, which should be blocked by MDL 72222. In spinal cord, serotonin regulates CGRP immunoreactivity. In animals lesioned with a selective serotonergic toxin, CGRP immunoreactivity is markedly increased (Fone, 1992). Serotonin can cause release of CGRP from primary sensory areas through a 5-HT_3 receptor mechanism (Saria et al., 1991). The cisplatin-induced loss of CGRP immunoreactivity, therefore, could be expected to be via a 5-HT_3 mechanism and should thus also have been blocked by MDL 72222. There are a number of possible explanations why the 5-HT_3 antagonist did not block the cisplatin toxicity.

First, the dose of the antagonist which we used could have been too low. This does not seem likely, as this is a dose known to elicit behavioral changes and is the dose used to induce developmental changes (Bell et al., 1991). Moreover, the dose required in humans to prevent emesis is approximately 1 mg/kg (Logue et al., 1991). Finally, this dose was sufficient to induce some changes in our animals – for example the animals treated with MDL 72222 alone had significant increases in weight gain and showed gait abnormalities.

Another explanation is that we have used a new model for peripheral neuropathy – the developing rat pup. However, this appears to be a valid model, since we have found changes in CGRP content and in tail-flick latency, both of which have been described for adult mice (Apfel et al., 1992). The shortened time for treatment until changes are seen (12 days as opposed to 4–8 weeks in adult animals) and the decreased

animal costs are both advantages to this model. One disadvantage of this model may be that the blood brain barrier was not intact until halfway through the treatment time. Thus there may have been higher levels of cisplatin in the CNS than would occur in the adult model.

The final explanation appears to be the most valid – that is, cisplatin-induced peripheral neuropathy does not take place through a 5-HT_3 receptor mechanism. Our data suggest that 5-HT_3 antagonists will not prevent all the adverse effects of cisplatin therapy. Moreover, some drugs from this class may increase side effects, as seen in the data on gait abnormalities. A recent clinical study has shown that a 5-HT_3 antagonist increases the nephrotoxicity produced by an antineoplastic agent related to cisplatin (Aamdal, 1992). Together, these observations suggest some caution may be necessary in the use of these drugs in preventing cisplatin-induced emesis, as the second major adverse effect of cisplatin, peripheral neuropathy, may be made worse.

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