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ORIGINAL ARTICLE

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The experimental neuroprotectant leukaemia inhibitory factor (LIF) does not compromise antitumour activity of paclitaxel, cisplatin and carboplatin

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Abstract *Purpose*: Peripheral neuropathy caused by the anticancer agents cisplatin and paclitaxel is a significant dose-limiting toxicity of these drugs. The growth factor leukaemia inhibitory factor (LIF) has neuroprotectant activity in preclinical models of nerve injury and degeneration and is now in a phase II trial in chemotherapy-induced peripheral neuropathy (CIPN). It is therefore important to ensure that LIF neither inhibits the antitumour activity of these drugs, nor stimulates tumour growth. *Methods*: Mature female Dark Agouti rats were implanted subcutaneously with a mammary carcinoma, DAMA. It was confirmed that the tumour expressed LIF receptors by reverse transcriptase polymerase chain reaction. Paclitaxel was administered at a dose of 5 mg/kg daily for 6 days, cisplatin at a dose of 3 mg/kg twice weekly and carboplatin at a dose of 10 mg/kg twice weekly. The effect of LIF on tumour growth and response to chemotherapy was assessed at two doses (2 and 10 μg/kg per day). Peripheral neuropathy was assessed in terms of gait disturbance and tail-flick threshold. *Results*: Neither dose of LIF stimulated growth of control tumours. Mean tumour volumes were lower on day 14 in all paclitaxel-, cisplatin- and carboplatin-treated groups, compared to controls (ANOVA $P < 0.001$). LIF did not interfere with this antitumour effect. Cisplatin- and paclitaxel-treated groups had developed increasing tail-flick thresholds by day 14. These manifestations of sensory neuropathy were prevented by LIF administration. *Conclusions*: These results suggest that LIF may be safely used in human trials as a neuroprotectant for patients receiving cisplatin, paclitaxel and carboplatin without concern for impairment of antitumour effect.

Keywords Neurotoxicity · Leukaemia inhibitory factor · Paclitaxel · Carboplatin · Cisplatin

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Introduction

Peripheral neuropathy caused by cytotoxic drugs is an increasingly recognized problem in cancer treatment. Cisplatin and paclitaxel, which are amongst the most active agents currently used in the treatment of the common solid tumours, cause dose-related neurotoxicity alone, in sequence and particularly in combination [6, 7, 35]. Recovery is variable, and persistent neuropathy may limit the quality of life of cancer survivors [6, 21].

Paclitaxel suppresses microtubule dynamics, causing mitotic arrest in dividing cells [12]. Similar effects in axonal microtubules lead to interference with axonal transport [35]. The resulting neuropathy predominantly affects small sensory fibres, but at higher doses motor dysfunction occurs [17]. The mechanism of nerve injury caused by the alkylating agents cisplatin and carboplatin is less well understood. The drugs accumulate in dorsal root ganglia (DRG) [36], leading to nucleolar damage and alterations in peptide content. In cultured DRG, inhibition of neurite regeneration, a process requiring microtubule assembly, has been demonstrated [29], but a direct effect of cisplatin on microtubules has not been described. Cisplatin causes sensory neuropathy, with large proprioceptive fibres being particularly sensitive [7, 27].

Attempts to avoid neuropathy by drug substitution have been successful in part. The combination of pac-

litaxel with carboplatin in ovarian cancer leads to a somewhat lower incidence of neurotoxicity than seen with paclitaxel/cisplatin [16], without apparent loss of antitumour activity. Further exploitation of these drugs in combination or in the dose-escalated setting, with potential for enhanced cure rates, might be possible if safe and effective neuroprotectants were available. The ideal neuroprotectant would be selective for nerves and not interfere with cytotoxic activity, and be without intrinsic toxicity.

Leukaemia inhibitory factor (LIF) is a 180-amino acid single-chain protein, named after its effect on haematopoietic cells [19]. LIF belongs to a group of cytokines which includes ciliary neurotrophic factor, interleukin-6 (IL-6), IL-11, cardiotrophin-1 and oncostatin M. LIF has been described as a pleiotropic cytokine, by which it is meant that it has effects on many different cell types and its activities are not restricted to cells of one lineage.

LIF acts through the LIF cell-surface receptor complex with two components (LIFR and gp130) [20, 23]. Many types of cells express LIF receptors, including neurones, megakaryocytes, macrophages, adipocytes, hepatocytes, osteoblasts, myoblasts, kidney and breast epithelium [22, 38]. Additionally, LIF's messenger RNA has been identified in many kinds of tissues, including but not limited to those of the nervous system, skeletal muscle and the myocardium [32, 39]. There are no grounds for considering LIF to be mutagenic, but it can promote cell proliferation. The production of LIF and LIF receptor mRNA has been observed in human tumour cells, including melanoma, leukaemia and carcinoma cells [11, 13, 18, 26].

A number of studies over the last 6–8 years have shown LIF to have potent neuromuscular activity. In vitro and in vivo studies, on axotomy and nerve crush models, have demonstrated a powerful effect of LIF. LIF enhances the survival of both motor and sensory neurones, while reducing denervation-induced muscle atrophy [8, 9, 15, 25, 30, 37]. LIF has also been shown to retard progression of motor neurone disease in the *wobbler* mouse, a model of axonopathy [24].

AMRAD is developing recombinant human LIF (otherwise known by its international non-proprietary name, emfilermin) as a potential treatment for neuromuscular disorders. A phase II efficacy study in chemotherapy-induced peripheral neuropathy (CIPN) is now underway in Australia [28]. Therefore it is appropriate to examine possible tumour-promoting activity of LIF in vivo, and to rule out any potential deleterious effect of LIF on the antitumour effect of the agents to be used in human trials. This was performed using a spontaneously arising mammary adenocarcinoma in Dark Agouti rats.

The secondary aim of this study was to use neurophysiological tests as dynamic models of acute paclitaxel and cisplatin neuropathy in rats, analogous to high-dose chemotherapy in humans. We sought to determine whether LIF had efficacy as a neuroprotectant for these agents in these acute models. Carboplatin neuropathy was not formally assessed as preliminary experiments had shown that at the dose used in this study neuropathy was unlikely (given its lower potency as an inducer of CIPN).

Materials and methods

Animals

Dark Agouti rats at 10 weeks of age were housed six to a cage at the Gore Hill Research Laboratories. They were maintained on a 12-h light/dark cycle and allowed free access to standard chow and water. Weight was assessed daily and any animal losing 20% of baseline weight was withdrawn form the study. All techniques used were approved by the RNSH Animal Care and Ethics Committee.

Cell line

A spontaneously arising mammary adenocarcinoma in Dark Agouti rats (DAMA) was obtained from Dr. Alan Rofe, Adelaide [10]. The cell line was maintained in passage through animals. It was assessed for the presence of LIF receptors as described below.

Reverse transcriptase polymerase chain reaction for LIF receptors

Total RNA was extracted from snap-frozen DAMA tissue as previously described [5]. First-strand cDNA synthesis was performed on 1 μg of total RNA. Reverse transcription was carried out at 42°C for 60 min in a 20-ml reaction containing 50 m*M* Tris-HCl, pH 8.3, 20 m*M* KCl, 10 m*M* $MgCl_2$, 5 m*M* dithiothreitol, 1 m*M* of each dNTP, 20 μg/ml oligo(dT) and 12.5 U AMV reverse transcriptase (Boehringer Mannheim, Germany). The reverse transcription reaction mixture was diluted to 100 μl with water and 5 μl was used for each PCR reaction.

PCR reactions were carried out in a 50-μl reaction buffer (Boehringer Mannheim) containing 200 μ*M* of each dNTP, 1 μ*M* of each primer and 2.5 U Taq polymerase (Boehringer Mannheim). After an initial denaturation of 2 min at 96°C, PCR was performed for 30 cycles in a PTC-100 Programmable Thermal Controller (MJ Research, Waltham, Mass.). Each cycle consisted of 30 s at 96°C, 30 s at 60°C and 2 min at 72°C. A 20-μl aliquot of the reaction mixture was electrophoresed on a 1% (w/v) agarose gel, transferred to a nylon membrane (hybond-N+, Amersham). Southern blots were performed as previously described [34]. Hybridization was carried out with end-labelled oligonucleotides internal to the respective cDNA sequences as previously described [13]. Positive controls were PC3 prostate carcinoma cells, and negative controls SW1222 colon carcinoma cells and water.

Tumour growth

Tumours were implanted subcutaneously (s.c.) in the right flank on day 0. Under inhalational anaesthesia with 2% halothane, nitrous oxide and oxygen, 0.2 ml of a 20% solution of DAMA cells in tissue culture medium (RPMI) was injected. A tumour nodule was measurable by day 7. Tumour volume was calculated from bi-dimensional measurements taken with vernier callipers daily on days 7–14 after implantation, using the formula $0.5 \times \text{length} \times \text{width}^2$.

Drug administration

All drug injections were performed under inhalational anaesthesia as described above. The observer performing tumour measurements was blinded to the injection contents.

Paclitaxel (Anzatax; Faulding, Adelaide, Australia) was administered intraperitoneally (i.p.) at 5 mg/kg for 6 days on days 7–12 after implantation of tumour. Control animals received a similar concentration of the vehicle (Cremophor EL) mixed with dextrose. Cisplatin (David Bull, Australia) was administered at a dose of 3 mg/kg i.p. twice weekly on days 7 and 10. Cisplatin was diluted in saline to allow for additional hydration, with up to 2 ml total volume being administered. Carboplatin (David Bull, Australia) was administered at a dose of 10 mg/kg i.p. twice weekly on days 7 and 10. Controls received a similar volume of saline.

Recombinant murine LIF (mLIF; AMRAD Corporation, Melbourne, Australia) was administered by daily s.c. injection at either 2 μg/kg or 10 μg/kg on days 7–12 after implantation of the tumour. This was first performed on two control groups to assess any effects of LIF on tumour growth. To assess effects of LIF on the antitumour effect of paclitaxel, cisplatin and carboplatin, LIF was then administered at the same doses to two paclitaxel-treated groups on days 7–12, to two cisplatin-treated groups on days 7–14, and to two carboplatin treated groups on days 7–14. In each case, animals receiving cytotoxics alone (cytotoxic controls) received similar volumes of phosphate buffered saline (PBS) as daily s.c. injections to maintain blinding.

Neurophysiological testing

Neurophysiological testing was performed at baseline, and after six doses of paclitaxel (30 mg/kg total dose) and two doses of cisplatin (6 mg/kg total dose). Methods have been previously described [1, 4].

Gait disturbance consisted of toe-walking with an arched back, scored as a positive result [3]. Thermal thresholds were measured with a tail-flick test (Ugo Basile, Varese, Italy), adapted as previously described [4]. The unanaesthetized rat was lightly restrained with its tail draped over a photocell, onto which a light was focused. When the infrared source was activated, the time taken for the rat to flick its tail out of the beam was recorded. Even in the presence of motor weakness this test has been validated as a measure of peripheral nocioceptive reflexes mediated by small sensory fibres [33].

Statistics

Tumour volumes on the final day were compared with ANOVA, supplemented by Tukey-Kramer tests for pair-wise comparisons. Changes in tail-flick times within groups between baseline and end of the study were compared with *t*-tests ($2P < 0.05$ considered significant).

Results

Receptor expression on rat mammary adenocarcinoma

Figure 1 shows the result of the PCR analysis and shows that the rat tumour expresses both LIFR and gp130 mRNA. The presence of both receptor components indicates that exogenous mLIF has the opportunity to bind to a functioning LIF receptor complex.

Tumour growth

Figure 2A shows the effect of the two different doses of mLIF on tumour growth. Neither dose of LIF stimulated or inhibited the growth of control tumours. On day 13 mean tumour volumes were similar in all three groups (ANOVA $P = 0.4780$). Figure 2B shows the effect of LIF on the antitumour effect of paclitaxel, cisplatin and carboplatin. In each case, the LIF-treated groups were indistinguishable from those treated with cytotoxic alone, whilst control tumours were significantly larger than tumours from the treated groups (ANOVA $P < 0.001$ in each case). The addition of LIF did not interfere with the antitumour activity of these drugs.

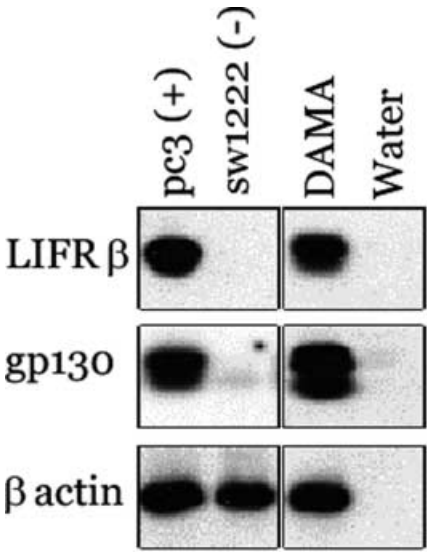

Fig. 1 Analysis of LIFRβ and gp130 expression in DAMA by RT-PCR. Autoradigraph of PCR products obtained from rat adenocarcinoma (DAMA) cell total RNA. PCR products were transferred to nylon membranes prior to probing with a ^{32}P-labelled oligonucleotide corresponding to the indicated receptor. Positive control (PC3 prostate carcinoma cell line) demonstrated both LIFRβ and gp130 expression. Negative control (SW1222 colon carcinoma cell line) gave no signal for either component

Neuropathy

Gait disturbance was detected in one paclitaxel-treated animal on day 9 and in two animals (33%) on day 14. In each of the LIF cotreatment groups, mild abnormalities were noted in 33% of animals on day 14. The tail-flick threshold rose in paclitaxel-treated animals from a baseline value of 2.45 ± 0.12 s to 3.6 ± 0.35 s ($2P < 0.05$) on day 14, as shown in Fig. 3A. This is indicative of sensory neuropathy. The threshold was unchanged in controls and in both LIF treatment groups ($2P$ all > 0.05).

Gait disturbance was not observed in animals from any of the cisplatin treatment groups. The tail-flick threshold rose in animals treated with cisplatin alone from a baseline value of 2.7 ± 0.23 s to 3.5 ± 0.30 s ($2P = 0.007$). Animals cotreated with LIF (2 or 10 μg/kg per day) demonstrated no significant change from baseline (Fig. 3B).

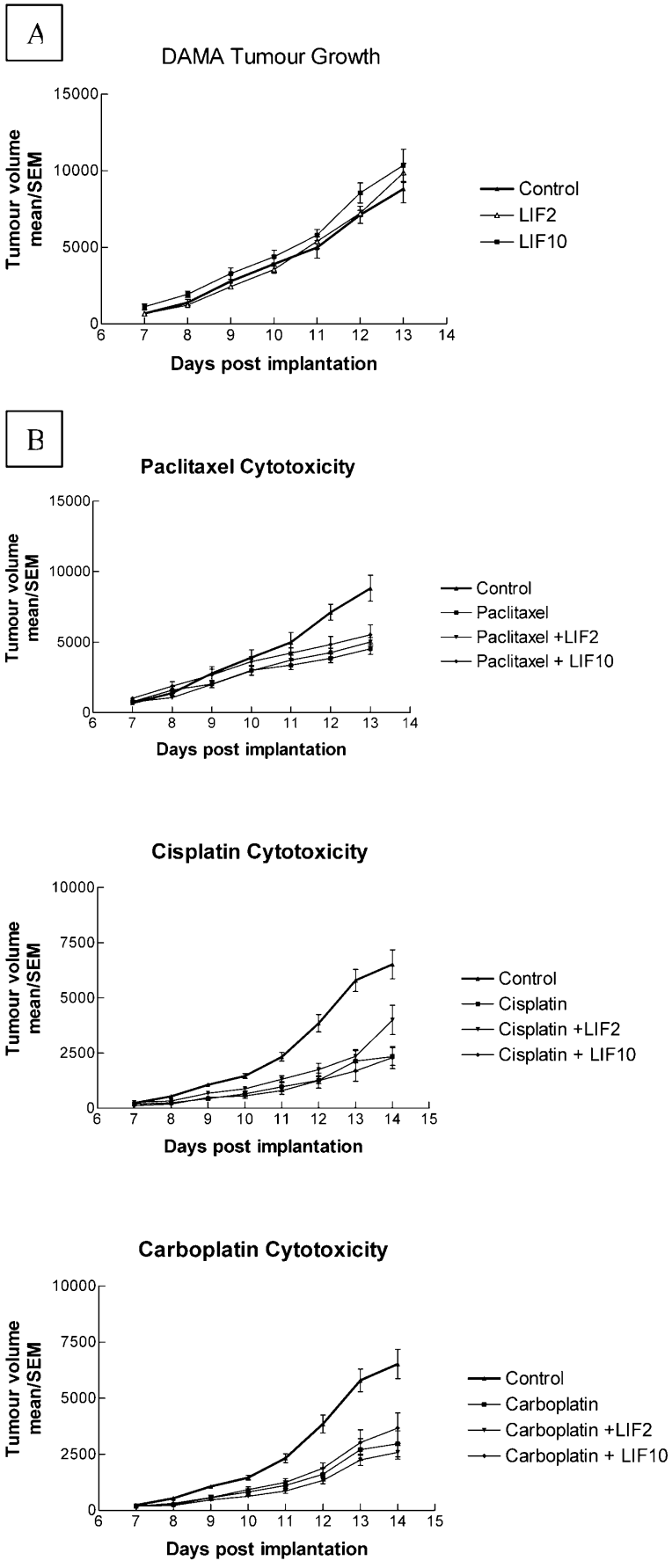

Fig. 2 **A** Growth of DAMA tumour was not stimulated by daily s.c. injections of mLIF at 2 μg/kg or 10 μg/kg. **B** Anti-tumour effects of paclitaxel, cisplatin and carboplatin ($P < 0.001$ vs control on day 14) were not inhibited by the administration of daily s.c. injections of mLIF ($n = 6$ per group)

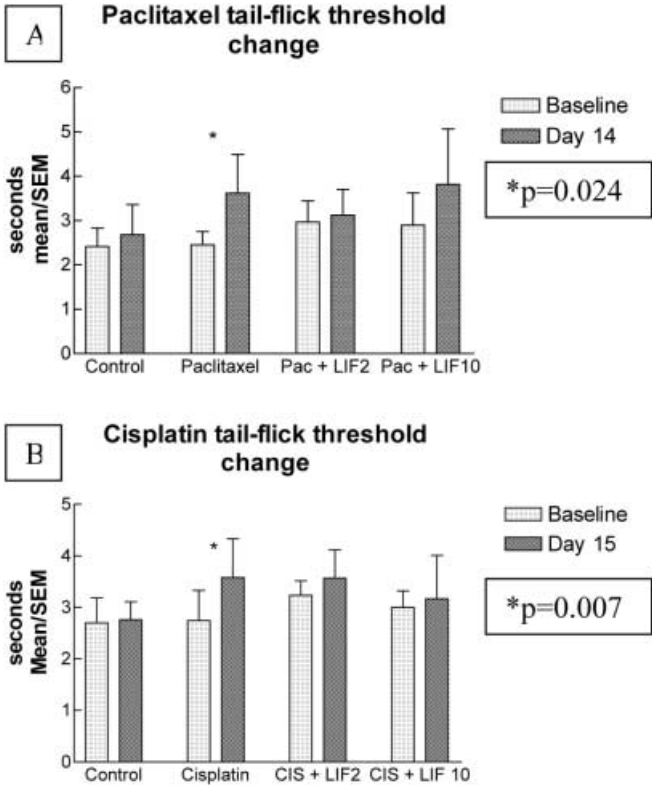

Fig. 3A, B Paclitaxel and cisplatin induced a significant rise in tail-flick threshold ($P < 0.05$ vs baseline), which was not observed in controls or in animals cotreated with LIF (2 or 10 μg/kg per day; $n = 6$ per group)

Discussion

LIF and LIFRβ are expressed by a number of breast and other tumour lines [11, 13, 26, 38]. Thus it is possible that tumours could respond to exogenous LIF. However, there are several factors that would need to be considered when evaluating the role of LIF in tumour development. Firstly, the responsiveness of the target cell to LIF would depend on its state of differentiation. A second factor is the microenvironment of the target cell, and the interaction with other cytokines.

All previous studies of LIF in tumour cells have been restricted to in vitro characterization of LIF and LIF receptor expression or the ability of tumour cell lines to respond to LIF under various cell culture conditions. LIF has been shown to inhibit proliferation and induce differentiation of some breast cancer cell lines in vitro [13]. This study provides the first direct in vivo evidence that LIF will not stimulate nor inhibit the growth of a primary tumour that expresses the LIF receptor. Recombinant murine LIF was used in these studies and mLIF has been shown to be biologically active in both mouse and rat and will bind to the LIF receptor from both species and activate signal transduction pathways. It is probable that the exogenous LIF bound to the LIF receptor complex on the tumour cells, but LIF did not cause cell proliferation or increase the tumour mass, or if there was a proliferative effect, it was masked by the natural tumour progression.

In addition LIF did not alter the antitumour effect of paclitaxel, cisplatin or carboplatin in an animal model. There was also no evidence to suggest that the coadministration of LIF with the cytotoxics induced drug resistance. These findings support those of a recent in vitro study in which LIF was found not to alter the cytotoxic action of paclitaxel or cisplatin on a number of cancer cell lines [31].

This study provided further evidence of the neuroprotective action of LIF on peripheral sensory nerves. The tail-flick threshold abnormalities demonstrated in animals receiving paclitaxel or cisplatin are evidence of sensory impairment. This is milder than that previously demonstrated with chronic administration of lower doses [4], a methodology which allows for a higher cumulative dose to be reached. Systemically administered LIF was able to prevent the development of sensory impairment in this model with the low dose of LIF as effective as the high dose. This neuroprotective action has been observed previously by Ikeda et al. [25] using similar doses of LIF. That study used a rat model of nerve axotomy, and LIF (1 and 10 μg/kg per day i.p.) was effective in promoting neuronal survival over a 14-day period. In our study the action of LIF was on otherwise intact peripheral nerves. The precise mechanism of action of LIF on injured nerves has not yet been determined. However, LIF receptors are found on sensory neurons of the dorsal root ganglion as well as on the Schwann cells surrounding axons [2, 14]. This would allow exogenous LIF to signal through its specific cell surface receptor and mediate a neurotrophic or neuroprotective effect.

The observation that the antitumour activity of paclitaxel, cisplatin and carboplatin was not inhibited by LIF in an animal model has important clinical implications, as the current phase II trial of emfilermin involves the concurrent use of these drugs. The additional evidence of a neuroprotective effect of LIF in this model provides a rationale for examining LIF as a potential treatment for the peripheral neuropathy that occurs as a result of high-dose chemotherapy.

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