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Cisplatin induced sensory neuropathy is prevented by vascular endothelial growth factor-A

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Conflict of interests:

LFD is a founder shareholder in Exonate Ltd, a company focused on the development of splicing inhibitors as treatment for various conditions including cancer and pain. All other authors have no conflicts to disclose.

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Abstract

Increased patient survival is a mark of modern anti-cancer therapy success. Unfortunately treatment side-effects such as neurotoxicity are a major long term concern. Sensory neuropathy is one of the common toxicities that can arise during platinum based chemotherapy. In many cases the current poor understanding of the neurological degeneration and lack of suitable analgesia has led to high incidences of patient drop out of treatment. VEGF-A is a prominent neuroprotective agent thus it was hypothesised to prevent cisplatin induced neuropathy. Systemic cisplatin treatment (lasting 3 weeks biweekly) resulted in mechanical allodynia and heat hyperalgesia in mice when compared to vehicle control. PGP9.5 sensory nerve fibre innervation was reduced in the plantar skin in the cisplatin treated group versus vehicle control mice. The cisplatin induced sensory neurodegeneration was associated with increased cleaved caspase 3 expression as well as a reduction in Activating Transcription Factor 3 and pan VEGF-A expression in sensory neurons. VEGF-A_{165}b expression was unaltered between vehicle and cisplatin treatment. rhVEGF-A_{165}a and rhVEGF-A_{165}b both prevented cisplatin induced sensory neurodegeneration. Cisplatin exposure blunts the regenerative properties of sensory neurons thus leading to sensory neuropathy. However, here it is identified that administration of VEGF-A isoform subtypes induce regeneration and prevent cell death and are therefore a possible adjunct therapy for chemotherapy induced neuropathy.

Keywords; VEGF-A, chemotherapy, neuropathy.

Introduction

Chemotherapy is routinely used alone or in combination with other therapies to treat patients following cancer diagnosis. However, chemotherapy-induced peripheral neuropathy (CIPN) is a major dose limiting effect of many chemotherapeutic agents, contributing to the further deterioration of the quality of life for cancer patients. CIPN is typically seen in about 40% [1-3] (reported in up to 95%) of patients treated with platinum based treatments, including cisplatin [1]. These typically lead to severe neurotoxicity which results in sensory and motor neurodegeneration, and is classified as pain associated with touch, 'pins and needles' and numbness as well as loss of motor coordination [3]. These symmetrical sensory complications are routinely identified in the extremities e.g. fingers and toes, with symptoms persisting from weeks/months to many years. Moreover, the symptoms of CIPN may remain after discontinuation of treatment with platinum compounds [1, 4, 5]. Currently, there are limited treatments available for CIPN. Therefore it is of great importance to identify potential therapeutic agents that would help in either preventing or treating the CIPN.

Cisplatin belongs to the first generation of platinum drugs and has been in use for the last 40 years in the treatment of a number of advanced and metastatic cancers. Platinum based compounds lead to DNA cross linkage ultimately preventing mitosis, thus suppressing tumour growth. However, cisplatin also causes damage to other cell types such as sensory neurons. It is now widely regarded that such treatments cause the described sensory symptoms, although the mechanisms by which these cause neuropathy are still undefined. A number of rodent models (including oxaliplatin [6, 7] and cisplatin [8, 9] administration) have been developed, which display classical hallmarks of human chemotherapy-induced sensory neurodegeneration.

It is widely accepted that upon a nervous insult such as a nerve injury, a number of neurotrophins and growth factors are up-regulated due to their integral role in neuronal survival and regeneration [10-12]. Recent evidence has identified that the vascular endothelial growth factor family-A (VEGF-A) family has a strong role in neuroprotection. This family consists of two sister protein groups that are unique, differing solely in the final 6 amino acids of the C terminus, which give distinct functional roles to each isoform family [13]. The neuroprotective actions of VEGF-A have been demonstrated extensively in motor degenerative disease [14, 15]. In addition, VEGF-A_{xxx}a is strongly associated with sensory neuronal development, protection and regeneration [16-19], with increased expression in dorsal root ganglia (DRG) sensory neurons upon traumatic nerve injury [20]. Recent work now highlights the role of VEGF-A_{xxx}b in neuroprotection [12].

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This study tested the hypothesis that there is a reduction in the regenerative ability of sensory neurons upon chemotherapy exposure, which can be reversed by treatment with neurotrophic VEGF isoforms. The experimental aims were to target the impact of cisplatin on sensory neurons and determine the role of the VEGF-A family in response to such an insult. Cisplatin led to a reduction in regenerative markers including VEGF-A. Treatment with either VEGF-A_{165}a or VEGF-A_{165}b prevented both cisplatin induced cell death and reduction in neurite growth.

Materials and methods

Studies in mice treated with or without cisplatin

Adult C57bl6 male (~30g, 10 total) mice were used. All procedures were performed according to UK Home Office legislation in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and associated guidelines (2012), and were approved by the University of Nottingham AWERB. Animals were provided food and water ab libitum.

Nociceptive behavioral assays

All treatments administered were given via intraperitoneal (i.p.) injection. Treatments were biweekly i.p. injections of either vehicle (phosphate buffered saline) or cisplatin (2mg/kg [21, 22]). Animal weight was monitored regularly. The experiment was terminated at the end of week 3. Prior to onset of nociceptive behavioral testing, mice were habituated to the testing procedure which involved transport and handling, and to the testing environment including mesh and glass-floored Perspex boxes, for 10 minutes before the start of testing. Mechanical withdrawal thresholds were defined as previously described [23]. A total of five applications of each von Frey hair (Linton) were applied to the plantar surface of both hind paws. Von Frey forces applied were used to obtain data ranging from 0% to 100% nociceptive withdrawal responses, which were then used to construct force response curves to determine 50% withdrawal threshold. Nociceptive responses to heat were also determined using the Hargreaves test [20]. Both hind paws were tested three times to provide a mean withdrawal latency. A rest period between each hind paw stimulation was enforced to prevent sensitization to the heat stimulation. These parameters were recorded at weekly intervals for duration of the study.

Cell culture

An immortalized embryonic sensory neuronal cell line, 50B11s, was provided as a gift from Drs Ahmet Hoke and Damon Lowes. The cells were prepared and cultured to 80% confluence in a T75 flask as previously described [24] in neurobasal media (Gibco) with Fetal bovine serum (Sigma-Aldrich), 2% B27 (Life Technologies), 0.2% L-glutamine (Sigma-Aldrich), 0.2% (11mM) glucose (Fisher) as supplements. Prior to differentiation and experimentation the cells were left for 24hrs in the culture plate. For neuronal growth and cleaved caspase III assays cells were plated at low density (5000 cells per well) on ethanol

sterilized coverslips in a 24 well plate. 75μM forskolin (Abcam) was used to differentiate the 50B11s and was applied after 24hrs along with other experimental agents; vehicle (phosphate buffered saline), 2.5nM recombinant human (rh) VEGF-A_{165}a (R&D systems), 2.5 nM rhVEGF-A_{165}b (R&D systems), 1nM nerve growth factor (NGF), cisplatin (a range of doses as outlined in figure legends given in μg/ml). Following initial experiments to determine dose, 5μg/ml cisplatin was used. Cells were incubated under experimental conditions for 24 hours, after which they were fixed with 4% paraformaldehyde (PFA) prior to imaging.

Neurite growth

For neurite growth measurements cells grown in 24 well plates and were imaged using a Nikon Eclipse Ti microscope using differential interference contrast. Each well was imaged with an x20 objective and for each well 5 random fields of view were captured and used for analysis. Image J was used to analyze images to determine neurite growth [25].

Protein expression

Immunofluorescence

Plantar skin from the hind paws of all mice was taken and placed into 4% PFA overnight, cryoprotected in 30% sucrose solution overnight, all at 4^{O}C. Tissue was frozen in OCT and stored at -80^{O}C until processing. Thin sections (20μm) of plantar skin from hind paws of the mice were cut on a cryostat and placed onto slides (VWR international). Sections were washed once in phosphate-buffered saline (PBS) for 10 minutes. The skin sections were then subjected to a 10 minute wash in PBS-0.2% Triton X-100 after which they were incubated in blocking buffer (10% FBS, 5% BSA, 0.2% PBS-Triton) for 1hr at room temperature. The primary antibody (anti-rabbit anti-polyclonal protein gene product 9.5 (PGP 9.5), 1 in 100, Ultraclone) was added in blocking solution and incubated at 4^{O}C overnight. Three PBS washes were performed followed by incubation in PBS 0.2%-Triton X-100 containing biotinylated anti-rabbit IgG (1 in 500, Jackson) at room temperature for 2hrs. Three further washes were performed and streptavidin Alexa Fluor 555 anti-rabbit antibody (Invitrogen) at a concentration of 1:1000 in 0.2% PBS-Triton was added for 1 hr at room temperature. Samples were then subjected to three 5 minute washes with PBS, coverslipped with Fluorsave (Calbiochem) and imaged on a Leica TCS SPE confocal microscope using Leica application suite. 5 random images were taken per skin sample (per field of view from x40 objective/25600μm^2). A mean was taken from the number of PGP9.5+ve nerve

terminals identified at the dermal:epidermal border of each image to provide a mean value per animal. These were then used for subsequent analysis for treatment groups

Immunocytochemistry

Cultured cells were treated with 4% PFA for 15 minutes and as with the above protocol (Immunofluorescence) were prepared in block solution. Cells were incubated in primary antibody (rabbit anti-cleaved caspase 3, 1:500, Cell Signalling Technology) at 4^{o}C overnight, and with secondary antibody (Alexa Fluor 555 conjugated anti-rabbit antibody, 1:1000, (Invitrogen) in 0.2% PBS-Triton X-100 for 1 h at RT. Cell nuclei were labeled with DAPI (1:2000, Invitrogen) for five minutes at room temperature. Washes and cell imaging were performed as described above. The slides were then imaged using a fluorescent microscope (Nikon Eclipse Ti). Five random images of immunofluorescence were taken per well.

Protein extraction and quantification

50B11 cells were seeded in 10 cm petri dishes and grown to 70% confluence after which they were differentiated with 75μM Forskolin ± 5μg/ml cisplatin. After 24 hours of treatment, protein was extracted from the cells. The media from each dish was removed and centrifuged. The supernatant was discarded and the resulting pellet was kept. 150μl lysis buffer (Radioimmuno Precipitation Assay (RIPA) buffer supplemented with 1mM phenylmethylsulfonyl fluoride (PMSF), 20μg/ml Protease Inhibitor cocktail, 50mM sodium fluoride and 1mM sodium orthovanodate (Na_3VO_4), all reagents from Sigma-Aldrich, UK), was added to each dish, and the cells were collected. The lysis buffer was collected in Eppendorf tubes and left on ice for 30 minutes with occasional agitation. The samples were sonicated on ice for 15 minutes and the protein concentration was subsequently quantified using a Bradford assay (Bioad).

Western blotting

100μg of each protein was loaded in a 4% stacking/12% separating gel and separated by SDS-PAGE at 70V until the ladder reached the resolving gel, then at 120 V. The proteins were then transferred onto polyvinyldifluoride (PVDF, Biorad) membrane by wet transfer at 90V for 1 hour 30 minutes. The membrane was incubated in 5% milk powder in Tris buffered saline (TBS)-Tween 0.1% for 30 minutes at room temperature with agitation. Primary antibodies, rabbit anti-cleaved caspase 3 (1:1000 Cell Signalling Technology; #9664), rabbit anti-total caspase III (Cell Signaling Technology; #9662), rabbit anti-Pan VEGF (A20,

1µg/ml, Santa Cruz Bio Technology; sc-152), mouse anti-$VEGF_{165b}$ (2µg/ml, Abcam; ab-14994), rabbit anti-Activating Transcription factor 3 (ATF3; 2µg/ml, Santa Cruz Bio Technology; sc-128) and rabbit anti-α/β tubulin (1:500 Cell Signalling; #2148) antibodies were diluted in blocking solution. The membrane was incubated in the primary antibodies overnight at 4^0C with agitation. The membranes were washed three times in TBST-0.1% before incubation with secondary antibodies (Licor donkey anti-rabbit and anti-mouse antibodies 1:10000) in TBST-0.1% 1% BSA. After 90 minutes incubation in the secondary antibodies, the membranes were washed thrice with TBST-0.1% before visualizing on the Licor Odessey.

Statistical analysis

Data were acquired and analysed using Microsoft Excel, Image J and GraphPad Prism 6. The results obtained are presented as means with standard error of the mean. Behavioural assays were recorded and analysed as outlined in the methods. No significant difference was observed between the hind paws within groups ($p= 0.1724$, two way ANOVA between hind paws and groups), therefore in all instances both hind paws per animal were used as replicates in the analysis. A two way ANOVA with post-hoc Sidak's multiple comparison tests was used to determine any statistical difference of treatment over time. For quantification of CC3+ve cells the total number of cleaved caspase 3 positive cells (CC3+ve) was counted based upon colocalision with DAPI stain. For neurite length analysis, Image J was calibrated to a microscope graticule and neurite length was manually determined using Image J measuring tools. For both *in vitro* cell based assays and *in vivo* IENF PGP9.5+ve nerve innervation (per field of view from x40 objective/25600µm^2), values were determined from 5 random images acquired per sample (i.e. well or tissue sample) and a mean determined. Western blot densitometry was measured using Image J gel plugin. Differences in protein loading were corrected using densitometry of the control protein (i.e. tubulin or actin). Data obtained in *in vitro* assays, PGP9.5+ve nerve fibre innervation and western blot densitometry were analysed using Mann Whitney and Kruskal Wallis tests for two and multiple group comparisons respectively.

Results

Biweekly administration of cisplatin in mice led to a neuropathic pain phenotype (**Figure1**). Compared to gender/age matched sham (vehicle treatment) injected mice, those treated with biweekly i.p. 2mg/kg cisplatin showed a significant drop in mechanical withdrawal threshold (**Figure1A**; Week 3 Sham=1.59g ± 0.09 vs. Cisplatin = 1.23g ± 0.08) and a reduction in heat nociceptive response latencies (**Figure 1B**; Week 3 Sham = 9.46s ± 0.17 vs. Cisplatin = 6.11s ± 0.63). Cisplatin did not induce weight loss in the treatment group compared to the sham group (**Figure1C**; Week 3 Sham = 28.08g ± 0.79 vs. Cisplatin = 25.42g ± 0.6). Furthermore, there was a significant reduction in intra-epidermal sensory nerve innervation (**Figure 2A**; Week 3 Sham = 6.14 ± 0.39 PGP9.5+ve terminals/field of view vs. Cisplatin = 2.38 ± 0.8 PGP9.5+ve terminals/field of view) in the cisplatin group (**Figure 2C**) compared to the vehicle injected mice (**Figure 2B**).

To isolate the actions of cisplatin on sensory neurons, an immortalised nociceptive sensory neuronal cell line, 50B11s, was used [24]. To determine *in vitro* markers of sensory neurodegeneration, the degree of cell stress/death (cleaved caspase 3) and neurite length, 50B11s were treated with cisplatin in increasing concentrations (0µg/ml-10µg/ml; **Figure 3**) for 24hrs. Increasing concentrations of cisplatin led to a dose dependent increase in cleaved caspase 3 expression (**Figure 3A**, **E**&**F**; 0µg/ml = 0.0% ± 0.0, 10µg/ml = 72.06% ± 3.09). This was accompanied by a reduction in neurite growth, with decreased total neurite growth per cell (**Figure 3B**; 0µg/ml = 234.4µm ± 20.47 vs. 10µg/ml = 30.57 µm ± 12.3), a reduction in mean neurite length (**Figure 3C**; 0µg/ml = 108.9 µm ± 3.65 vs. 10µg/ml = 20.85 µm ± 5.86) and the percentage of cells with neurites (**Figure 3D**; 0µg/ml = 63.37 ± 2.8 vs. 10µg/ml = 2.96 ± 1.25). All subsequent experiments were carried at sub-maximal dose of 5 µg/ml as there was also a significant loss of neuronal growth and high degree of neuronal compromise.

In protein extracted from cells treated for 24hrs with cisplatin (sub-maximal dose 5µg/ml) there were also elevated levels of cleaved caspase 3/ total caspase (**Fig.4A&B**). Additionally, cisplatin treatment significantly reduced ATF3 expression compared to the vehicle group (**Fig.4C&D**), and was associated with the suppression of regenerative capacity as shown by reduced neurite outgrowth (**Figure 3**). The expression of pan-VEGF-A_{xxx} (total VEGF-A isoforms) was significantly reduced compared to the vehicle treated group (**Figure 4E&F**; VEGF-A_{165}) in the cisplatin group (5µg/ml), but expression of the alternatively spliced variant VEGF-A_{165}b was unaltered by cisplatin treatment (**Fig.4E&F**).

Treatment of 50B11 cells with exogenous rhVEGF-A_{165}a alone (without cisplatin) for 24hrs led to robust neurite growth compared to vehicle treated cells (**Figure 5A**; Vehicle =

117.1μm ± 4.88 vs. rhVEGF-A_{165}a = 211μm ± 12.1; representative images **C&E**), as did the archetypal sensory neuronal growth factor NGF (**Figure 5A**; positive control NGF = 157.4μm ± 10.8; representative images D). Furthermore, rhVEGF-A_{165}a led to a significant increase in total neurite outgrowth compared to NGF (**Figure 5A**). Treatment with rhVEGF-A_{165}b did not induce increased total neurite growth compared to vehicle treated cells (Fig.5B; vehicle = 145.1μm ± 4.72 vs. rhVEGF-A_{165}b = 158.2μm ± 6.48; representative images F). Incubation with either rhVEGF-A_{165}a or rhVEGF-A_{165}b did not affect cleaved caspase 3 expression (**Figure 6**; vehicle = 5.94 ± 1.55% vs. rhVEGF-A_{165}a = 7.64 ± 1.48% vs. rhVEGF-A_{165}b = 3.65 ± 1.08%) versus vehicle treated cells. Thus $VEGF_{165}$a acted as a growth factor for 50B11 cells, whereas $VEGF_{165}$b did not.

To determine whether exogenous VEGF-A (either VEGF-A_{165}a or VEGF-A_{165}b) could prevent cisplatin-induced neuronal damage *in vitro*, rhVEGF-A_{165}a or rhVEGF-A_{165}b were co-administered with cisplatin for 24hrs. Cisplatin treatment (5μg/ml) again led to a significant reduction in neurite outgrowth (**Figure 7A**; control (untreated normal media) = 181.6μm ± 7.92 vs. Cisplatin 5μg/ml + Vehicle = 97.72μm±6.79) and increased caspase 3 expression (**Figure7B**; control (untreated normal media) = 9.29% ± 2.7 vs. Cisplatin 5μg/ml + Vehicle = 76.22% ± 4.15). Administration of rhVEGF-A_{165}a or rhVEGF-A_{165}b to cisplatin treated cells led to pronounced neuroprotection, preventing both the cisplatin-induced reduction in total nevurite length (**Figure 7A**; Cisplatin 5μg/ml + rhVEGF-A_{165}a = 180.7μm ± 9.74 vs. Cisplatin 5μg/ml + rh VEGF-A_{165}b = 163.6μm ± 9.58) and cleaved caspase expression (Fig.7B; Cisplatin 5μg/ml + rhVEGF-A_{165}a = 31.34% ± 5.89 vs. Cisplatin 5μg/ml + rhVEGF-A_{165}b = 36.21% ± 5.92). However, neither rhVEGF-A_{165}a (**Figure 7E**) or rhVEGF-A_{165}b (**Figure 7F**) treatment completely prevented cisplatin induced cleaved caspase 3 expression (**Figure 7**B), with slight increases above control conditions.

Discussion

Chemotherapy induced neuropathy is highly prevalent in cancer patients and survivors [3] and is a dose limiting side effect that can impact upon the continuation of treatment regimes, ultimately on patient survival and on cancer survivor quality of life. We show here that cisplatin treatment results in chronic pain phenotypes in mice, with evidence of mechanical allodynia and heat hyperalgesia. Cisplatin-induced neuropathic pain may occur due to suppression of the endogenous capacity for neuroregeneration and neuroprotection of the peripheral sensory neuron, as endogenous growth factor (VEGF-A_{xxx}a) and regeneration marker (ATF3) expression are reduced by cisplatin in sensory neurons *in vitro*. Administration of VEGF-A isoforms can provide neuroprotection and neurotrophic support to these neurons, inhibiting the effects of cisplatin.

Sensory neuropathy is induced by a host of anti-cancer treatments e.g. platinum derived agents, vinca alkaloids. Symptoms range from exacerbated evoked painful sensations, and pain resulting from touch or non-noxious temperature change, through to pins and needles and numbness (paresthesia). The onset of neuropathy can occur immediately following the start of chemotherapy treatment, and most unfortunately, the symptoms can persist not only for the duration of treatment but also past it's termination, which impacts greatly on the survivors quality of life. Critically, despite not being directly related to the disease, chemotherapy-induced neuropathy weighs heavily on the patient, carers and family. The additional and on-going burden of an iatrogenic medical complication can lead to emotional disturbances arising as a result of reduced quality of life often resulting from limited mobility and reduced sleep quality [26, 27]. These contribute to the well-documented increased incidence of depression and anxiety in cancer patients, with patient catastrophization, leading to enhanced perceived pain [28, 29]. Furthermore, there are very few available analgesic drugs that can cisplatin-induced pain in the majority of patients [30].

Typically the sensations associated with peripheral sensory neurodegeneration are as described above, but the degeneration itself can also be demonstrated by loss of intra-epidermal sensory nerve fibre (IENF) innervation in the skin [31] and by alterations in sensory nerve conduction velocity [32]. These hallmarks of peripheral sensory neuropathy are apparent in humans but to allow further investigation of the mechanisms of these changes, a number of rodent models are now being characterised [8]. Cisplatin administration in rodents leads to prominent sensory alterations including mechanical and cold hypersensitivity [33, 34] with progression to hypoalgesia as neurodegeneration occurs. Our findings demonstrate that this regime of cisplatin leads to a pronounced sensory neuropathy in adult mice as previously reported [21, 22], including IENF terminal loss and

altered nociceptive behaviour, comparable to reports from previous studies [8] providing a suitable model for the study of novel therapies.

Platinum-based chemotherapy treatment results in significant neuronal damage and degeneration in the peripheral sensory nervous system, with many reports of sensory nerve regression and axonal dysfunction with accompanying neuropathic pain [35]. However, the underlying mechanisms and causes of neuropathy are still unclear. Platinum based treatments induce neuropathy in rodent models but there are contrasting data showing that DRG sensory neurons do not necessarily express all common markers of sensory neuronal damage or regeneration (e.g. ATF3) in response to this cellular stress or insult. Oxaliplatin induces cleaved caspase 3 expression in DRG sensory neurons [12] and regression of neurite outgrowth [36], and pharmacological inhibition of caspase activity blunted pain behaviours induced by oxaliplatin and cisplatin [7]. We demonstrate a pronounced reduction in the expression of the widely accepted neuronal damage marker and inducer of sensory neuronal regeneration, ATF3 [10, 37] following cisplatin treatment. In contrast, ATF3 expression in DRG neurons is increased in response to cisplatin treatment [9] or unchanged following exposure to paclitaxel and oxaliplatin [6, 38], in animals that displayed on-going neuropathic pain behaviours and alterations in sensory neuronal function typified by increased ongoing neuronal activity and alterations in conduction velocity [39]. In general, previous *in vitro* data are consistent with our *in vivo* observations of epidermal sensory nerve regression indicating that sensory neurons lose their normal neuroregenerative capability in response to cisplatin induced peripheral nerve damage.

Here we demonstrate that cisplatin treatment induced a significant neurodegeneration *in vivo* and *in vitro*. In vitro, this was accompanied by a reduction of total VEGF-A expression, a potent neuro-protective and regenerative agent [12, 18, 40]. Studies to date have demonstrated that VEGF-A has prominent actions on neurons, promoting survival and protection. In a number of rodent models of neuropathic pain (traumatic [18] and diabetic neuropathy [40]) VEGF-A can prevent sensory neuropathy. Interestingly, *in vitro*, total expression of VEGF-A was reduced, but expression of VEGF-A_{xxx}b was unchanged following cisplatin treatment, which suggests a reduction in the relative expression of VEGF-A_{xxx}a isoforms. Administration of a specific VEGF-A_{xxx}b neutralising antibody exacerbated oxaliplatin-induced neuronal toxicity *in vitro* [12] indicating that endogenous VEGF-A_{xxx}b levels may be maintained during chemotherapy exposure, and may offer a degree of neuroprotection, as supported by our finding that exogenous VEGF-A_{165}b was neuroprotective . Consideration of the independent neuroprotective functions of alternatively spliced isoforms is of great importance, as many well-established anti-cancer drugs are targeted against all VEGF-A isoforms or the functional receptor, VEGF receptor 2. These

drugs were developed to inhibit VEGF-A_{165}a-mediated angiogenesis and tumour growth [41], but also block the actions of VEGF-A_{xxx}b isoforms, which have different physiological and pathological actions [20]. Blockade of VEGF-A signalling leads to the suppression of tumour growth and improved survival in some cancers, however these patients also demonstrate pronounced neuropathy [42, 43]. This implies that the effects of these treatments on important VEGF-A-mediated neural signalling pathways may contribute to the advancing neuropathy that ultimately results in termination of treatment [44]. Consideration of the function and expression of known and emerging neuroprotective and regenerative growth factors such as VEGF-A_{165}b, is now crucial when designing and using such anti-cancer therapy, especially in combination with platinum-based chemotherapy. Understanding the functions that loss of endogenous growth factor support has in the adverse effects of such treatments may provide avenues to nullify those treatment-limiting side effects.

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Conflicts of interest

LFD is a founder shareholder in Exonate Ltd, a company focused on the development of splicing inhibitors as treatment for various conditions including cancer and pain. The authors have no other conflicts of interest to declare.

Figure legends

Figure 1

Cisplatin induces neuropathic pain behaviour.

[A] Intraperitoneal administration of cisplatin (biweekly 2mg/kg) to adult male mice led to a significant reduction in mechanical nociceptive withdrawal threshold indicative of mechanical allodynia and [B] decrease in withdrawal latency to heat (heat hyperalgesia) versus the control group (i.p. vehicle) (*$p<0.05$, **$p<0.01$ two way ANOVA with post-hoc Bonferroni tests, n=5 per group). [C] Cisplatin treatment did not lead to a significant reduction in body weight when compared to the age/gender matched sham group.

Figure 2

Intraepidermal nerve fibre innervation is lost as a result of cisplatin treatment.

[A] Three weeks of cisplatin treatment resulted in a significant reduction in intra-epidermal nerve fibre innervation in the plantar skin/per field of view (PGP9.5 = red, *$p<0.05$ Mann Whitney U test).[B & C] Representative examples of PGP 9.5 positive nerve terminal staining in [B] control and [C] cisplatin groups (scale bar = 20μm).

Figure 3

Cisplatin treatment inhibits neurite outgrowth and increases cleaved caspase in immortalised sensory neurons.

[A] Increasing doses of cisplatin (given in μg/ml) induced a significant increase in cleaved caspase 3 expression. [B] Cisplatin prevented neurite growth leading to a reduction in total neurite growth per cell, [C] reduced average neurite length per cell and [D] reduced percentage of cells with neurites. [E] Representative examples of cleaved caspase 3 expression (CC3=Red, DAPI=Blue) in control conditions (0μg/ml) and [F] the presence of 10 μg/ml cisplatin. (**$p<0.01$, ***$p<0.001$ one way ANOVA with post-hoc Bonferroni tests, scale bar = 100μm).

Figure 4

Sensory neurons have altered expression of pro- and anti-degenerative molecules following cisplatin treatment.

[A] The immortalised sensory neuronal cell line (50B11) showed increased expression of cleaved caspase 3 (corrected for total caspase 3 expression) following cisplatin (5μg/ml) treatment. [B] Representative Western blot of cleaved caspase expression. [C] 50B11 cells showed reduced expression of the neuroregenerative marker Activating Transcription Factor 3 (ATF3) following cisplatin (corrected for loading against actin expression). [D] Representative Western blot of ATF3 expression. [E] Total (pan) Vascular Endothelial Growth factor-A expression was decreased by cisplatin treatment, but VEGF-A_{165}b levels did not change (corrected for loading against tubulin. [F] Representative Western blots of pan-VEGF and VEGF-A_{165}b expression. (A, C and D all *$p<0.05$ Mann Whitney test, n=4).

Figure 5

Vascular Endothelial Growth Factor-A isoforms induce sensory neuronal neurite growth.

[A] VEGF-A_{165}a administration resulted in a greater total neurite outgrowth per cell than either the archetypal trophic factor, NGF or vehicle. [B] In contrast, VEGF-A_{165}b did not lead to increased neurite growth in 50B11 neurons. [C-F] Representative images of 50B11 neurons grown in the presence of [C] vehicle, [D] NGF, [E] VEGF-A_{165}a and [F] VEGF-A_{165}b. (**$p<0.01$, *** $p<0.001$, one way ANOVA with post-hoc Bonferroni tests, scale bar = 50μm).

Figure 6

Vascular endothelial growth factor-A isoforms does not affect cleaved caspase 3 expression in immortalised sensory neurons.

[A] There was no effect of VEGF-A isoforms on cleaved caspase 3 expression in 50B11 neurons. Representative images of 50B11 neurons cultured with either [B] vehicle, [C] VEGF-A_{165}a and [D] VEGF-A_{165}b. (CC3=Red, DAPI=Blue, scale bar = 100μm)

Figure 7. VEGF-A isoforms reverse the cisplatin-induced changes in neurite outgrowth and cleaved caspase expression.

50B11 neurons were cultured in 5μg/ml cisplatin for 24hrs, which led to a reduction in [A] total neurite growth per cell and [B] increased cleaved caspase 3 (CC3=Red, DAPI=Blue) expression (representative images [C] normal media and [D] cisplatin 5μg/ml). Administration of either VEGF-A_{165}a or VEGF-A_{165}b prevented cisplatin induced (5μg/ml) [A] reduction in total neurite growth. In addition, [E=representative image VEGF-A_{165}a] or [F=representative image VEGF-A_{165}b] treatment attenuated cisplatin induced cleaved caspase 3 expression [B] (***$p<0.001$ one way ANOVA with post-hoc Bonferroni test). However, neither [E] VEGF-A_{165}a nor [F] VEGF-A_{165}b completely prevented cisplatin induced cell death compared to normal media alone (one way ANOVA with post-hoc Bonferroni test, *$p<0.05$, **$p<0.01$, ***$p<0.001$, scale bar = 100μm).

References

[1] Windebank A and Grisold W. Chemotherapy-induced neuropathy. J Peripher Nerv Syst 2008; 13: 27-46.

[2] Pace A, Savarese A, Picardo M, Maresca V, Pacetti U, Del Monte G, Biroccio A, Leonetti C, Jandolo B, Cognetti F and Bove L. Neuroprotective effect of vitamin E supplementation in patients treated with cisplatin chemotherapy. J Clin Oncol. 2003; 21: 927-931.

[3] Paice JA. Chronic treatment-related pain in cancer survivors. Pain 2011; 152: S84-89.

[4] van den Bent M. Prevention of chemotherapy-induced neuropathy: leukemia inhibitory factor. Clin Cancer Res. 2005; 11: 1691-1693.

[5] Hilkens P, Planting A, van der Burg M, Moll J, van Putten W, Vecht C and van den Bent M. Clinical course and risk factors of neurotoxicity following cisplatin in an intensive dosing schedule. Eur J Neurol. 1994; 11: 45-50.

[6] Xiao WH, Zheng H and Bennett GJ. Characterization of oxaliplatin-induced chronic painful peripheral neuropathy in the rat and comparison with the neuropathy induced by paclitaxel. Neuroscience 2012; 203: 194-206.

[7] Joseph E and Levine J. Comparison of oxaliplatin- and cisplatin-induced painful peripheral neuropathy in the rat. J Pain 2009; 10: 534-541.

[8] Mao-Ying QL, Kavelaars A, Krukowski K, Huo XJ, Zhou W, Price TJ, Cleeland C and Heijnen CJ. The anti-diabetic drug metformin protects against chemotherapy-induced peripheral neuropathy in a mouse model. PLoS One 2014; 9: e100701.

[9] Park H, Stokes J, Pirie E, Skahen J, Shtaerman Y and Yaksh T. Persistent hyperalgesia in the cisplatin-treated mouse as defined by threshold measures, the conditioned place preference paradigm, and changes in dorsal root ganglia activated transcription factor 3: the effects of gabapentin, ketorolac, and etanercept. Anesth Analg 2013; 116: 224-231.

[10] Seijffers R, Mills C and Woolf C. ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. J Neuro 2007; 27: 7911-7920.

[11] Hulse R, Wynick D and Donaldson L. Characterization of a novel neuropathic pain model in mice. Neuroreport 2008; 19: 825-829.

[12] Beazley-Long N, Hua J, Jehle T, Hulse RP, Dersch R, Lehrling C, Bevan H, Qiu Y, Lagreze WA, Wynick D, Churchill AJ, Kehoe P, Harper SJ, Bates DO and Donaldson LF. VEGF-A165b Is an Endogenous Neuroprotective Splice Isoform of Vascular Endothelial Growth Factor A in Vivo and in Vitro. Am J Pathol 2013; 183: 918-929.

[13] Harper S and Bates D. VEGF-A splicing: the key to anti-angiogenic therapeutics? Nat Rev Cancer 2008; 8: 880-887.

[14] Tovar-Y-Romo L, Zepeda A and Tapia R. Vascular endothelial growth factor prevents paralysis and motoneuron death in a rat model of excitotoxic spinal cord neurodegeneration. J Neuropathol Exp Neurol. 2007; 66: 913-922.

[15] Storkebaum E, Lambrechts D and Carmeliet P. VEGF: once regarded as a specific angiogenic factor, now implicated in neuroprotection. Bioessays. 2004; 26: 943-954.

[16] Sondell M LG, Kanje M. Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. J Neurosci. 1999; 19: 5731-5740.

[17] Sondell M SF, Kanje M. Vascular endothelial growth factor is a neurotrophic factor which stimulates axonal outgrowth through the flk-1 receptor. Eur J Neurosci. 2000; 12: 4243-4254.

[18] Verheyen A, Peeraer E, Nuydens R, Dhondt J, Poesen K, Pintelon I, Daniels A, Timmermans JP, Meert T, Carmeliet P and Lambrechts D. Systemic anti-vascular endothelial growth factor therapies induce a painful sensory neuropathy. Brain 2012; 135: 2629-2641.

[19] Verheyen A PE, Lambrechts D, Poesen K, Carmeliet P, Shibuya M, Pintelon I, Timmermans JP, Nuydens R, Meert T. Therapeutic potential of VEGF and VEGF-derived peptide in peripheral neuropathies. Neuroscience 2013; 244: 77-89.

[20] Hulse RP, Beazley-Long N, Hua J, Kennedy H, Prager J, Bevan H, Qiu Y, Fernandes ES, Gammons MV, Ballmer-Hofer K, Gittenberger de Groot AC, Churchill AJ, Harper SJ, Brain SD, Bates DO and Donaldson LF. Regulation of alternative VEGF-A mRNA splicing is a therapeutic target for analgesia. Neurobiol Dis 2014; 71: 245-259

[21] Ozturk G and Tonge DA. Effects of leukemia inhibitory factor on galanin expression and on axonal growth in adult dorsal root ganglion neurons in vitro. Exp Neurol 2001; 169: 376-385.

[22] Authier N. An animal model of nociceptive peripheral neuropathy following repeated cisplatin injections. Experimental Neurology 2003; 182: 12-20.

[23] Hulse R, Wynick D and Donaldson L. Activation of the galanin receptor 2 in the periphery reverses nerve injury-induced allodynia. Mol Pain 2011; 7: 26

[24] Chen W, Mi R, Haughey N, Oz M and Hoke A. Immortalization and characterization of a nociceptive dorsal root ganglion sensory neuronal line. Journal of the peripheral nervous system 2007; 12: 121-130.

[25] Schneider C, Rasband W and Eliceiri K. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012; 9: 671-675.

[26] Schaefer C, Mann R, Sadosky A, Daniel S, Parsons B, Nalamachu S, Stacey B, Tuchman M, Anschel A and Nieshoff E. Health status, function, productivity, and costs among individuals with idiopathic painful peripheral neuropathy with small fiber involvement in the United States: results from a retrospective chart review and cross-sectional survey. J Med Econ 2014; 17: 394-407.

[27] Pachman D, Barton D, Swetz K and Loprinzi C. Troublesome symptoms in cancer survivors: fatigue, insomnia, neuropathy, and pain. J Clin Oncol. 2012; 30: 3687-3696.

[28] Khan R, Ahmed K, Blakeway E, Skapinakis P, Nihoyannopoulos L, Macleod K, Sevdalis N, Ashrafian H, Platt M, Darzi A and Athanasiou T. Catastrophizing: a predictive factor for postoperative pain. Am J Surg 2011; 201: 122-131.

[29] Schreiber K, Martel M, Shnol H, Shaffer J, Greco C, Viray N, Taylor L, McLaughlin M, Brufsky A, Ahrendt G, Bovbjerg D, Edwards R and Belfer I. Persistent pain in postmastectomy patients: comparison of psychophysical, medical, surgical, and psychosocial characteristics between patients with and without pain. Pain 2013; 154: 660-668.

[30] Han F, Wyse B and Smith M. Optimization and pharmacological characterization of a refined cisplatin-induced rat model of peripheral neuropathic pain. Behav Pharmacol. 2014; 25: 732-740.

[31] Boyette-Davis J, Cata J, Zhang H, Driver L, Wendelschafer-Crabb G, Kennedy W and Dougherty P. Follow-up psychophysical studies in bortezomib-related chemoneuropathy patients. J Pain 2011; 12: 1017-1024.

[32] Krarup-Hansen A, Helweg-Larsen S, Schmalbruch H, Rorth M and Krarup C. Neuronal involvement in cisplatin neuropathy: prospective clinical and neurophysiological studies. Brain 2007; 130: 1076-1088.

[33] Carozzi V, Chiorazzi A, Canta A, Meregalli C, Oggioni N, Cavaletti G and Marmiroli P. Chemotherapy-induced peripheral neurotoxicity in immune-deficient mice: New useful ready-to-use animal models. Exp Neurol. 2014; 264c: 92-102.

[34] Park H, Stokes J, Corr M and Yaksh T. Toll-like receptor signaling regulates cisplatin-induced mechanical allodynia in mice. Cancer Chemother Pharmacol. 2014; 73: 25-34.

[35] Carozzi V, Canta A, Chiorazzi A and Cavaletti G. Chemotherapy-induced peripheral neuropathy: What do we know about mechanisms? Neurosci Lett 2015; 596: 90-107.

[36] Rodriguez-Menendez V, Gilardini A, Bossi M, Canta A, Oggioni N, Carozzi V, Tremolizzo L and Cavaletti G. Valproate protective effects on cisplatin-induced peripheral neuropathy: an in vitro and in vivo study. Anticancer Res 2008; 28: 335-342.

[37] Seijffers R, Allchorne A and Woolf C. The transcription factor ATF-3 promotes neurite outgrowth. Mol Cell Neurosci 2006; 32: 143-154.
[38] Flatters SJ and Bennett GJ. Studies of peripheral sensory nerves in paclitaxel-induced painful peripheral neuropathy: evidence for mitochondrial dysfunction. Pain 2006; 122: 245-257.
[39] Joseph E, Chen X, Bogen O and Levine J. Oxaliplatin acts on IB4-positive nociceptors to induce an oxidative stress-dependent acute painful peripheral neuropathy. J Pain 2008; 9: 463-472.
[40] Pawson EJ, Duran-Jimenez B, Surosky R, Brooke HE, Spratt SK, Tomlinson DR and Gardiner NJ. Engineered zinc finger protein-mediated VEGF-a activation restores deficient VEGF-a in sensory neurons in experimental diabetes. Diabetes 2010; 59: 509-518.
[41] Bates DO CP, Symonds KE, Varey AH, Ramani P, O'Dwyer PJ, Giantonio BJ, Meropol NJ, Benson AB, Harper SJ. Association between VEGF splice isoforms and progression-free survival in metastatic colorectal cancer patients treated with bevacizumab. Clin Cancer Res 2012; 18: 6384-6391.
[42] Burger RA, Sill MW, Monk BJ, Greer BE and Sorosky JI. Phase II trial of bevacizumab in persistent or recurrent epithelial ovarian cancer or primary peritoneal cancer: a Gynecologic Oncology Group Study. J Clin Oncol 2007; 25: 5165-5171.
[43] Chiorean EG, Malireddy S, Younger AE, Jones DR, Waddell MJ, Sloop MI, Yu M, Hall SD, Schneider B and Sweeney CJ. A phase I dose escalation and pharmacokinetic study of vatalanib (PTK787/ZK 222584) in combination with paclitaxel in patients with advanced solid tumors. Cancer Chemother Pharmacol 2010; 66: 441-448.
[44] Mina L, Yu M, Johnson C, Burkhardt C, Miller K and Zon R. A phase II study of combined VEGF inhibitor (bevacizumab+sorafenib) in patients with metastatic breast cancer: Hoosier Oncology Group Study BRE06-109. Invest New Drugs 2013; 31: 1307-1310.

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Figure 1

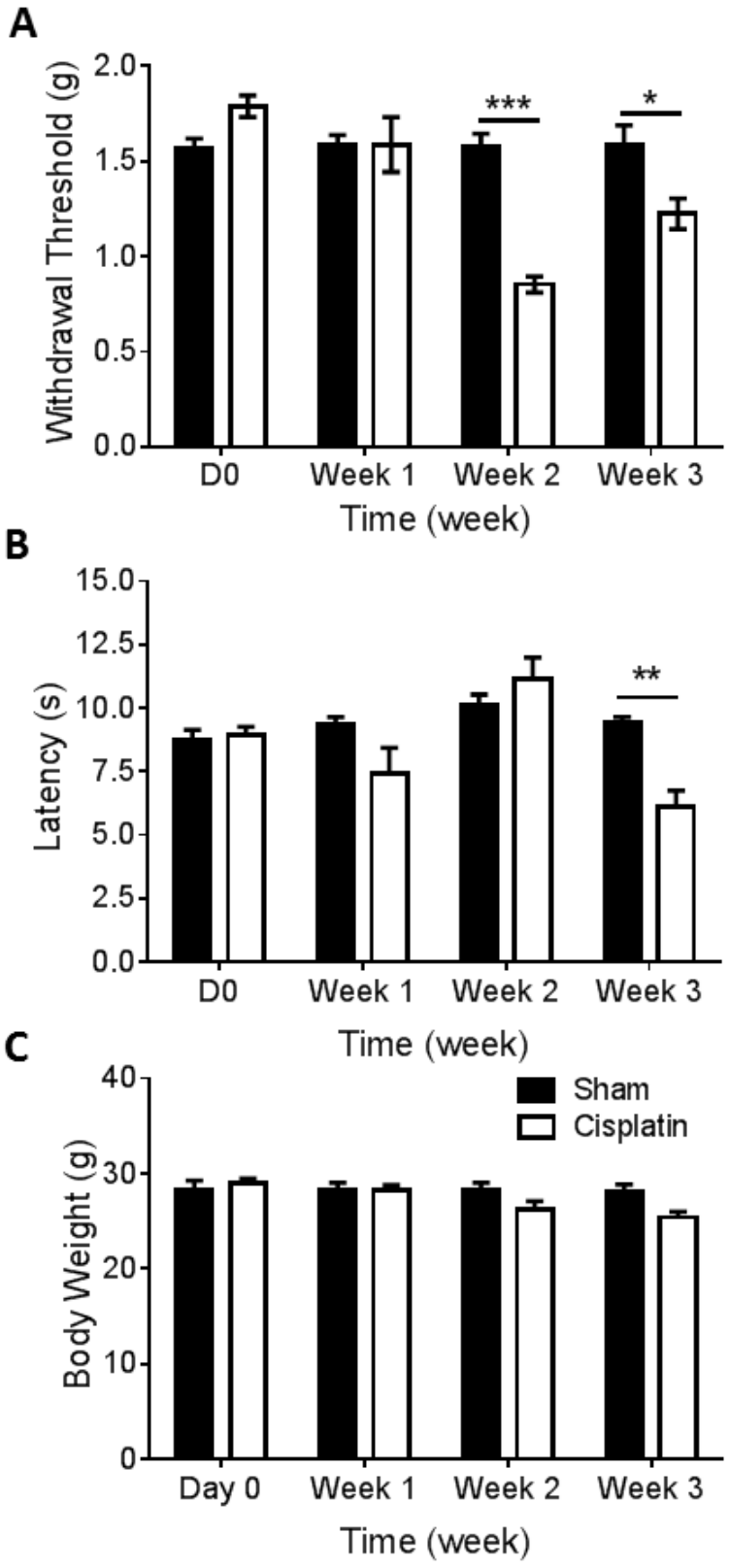

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Figure 2

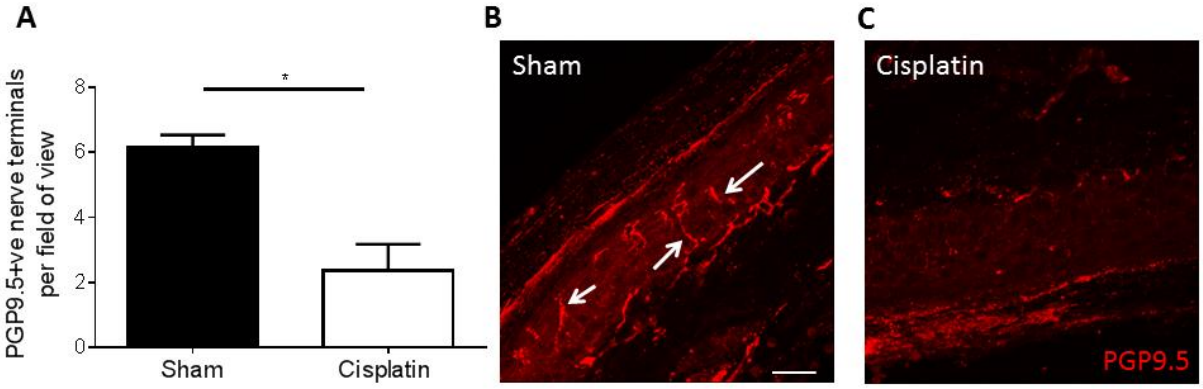

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Figure 3

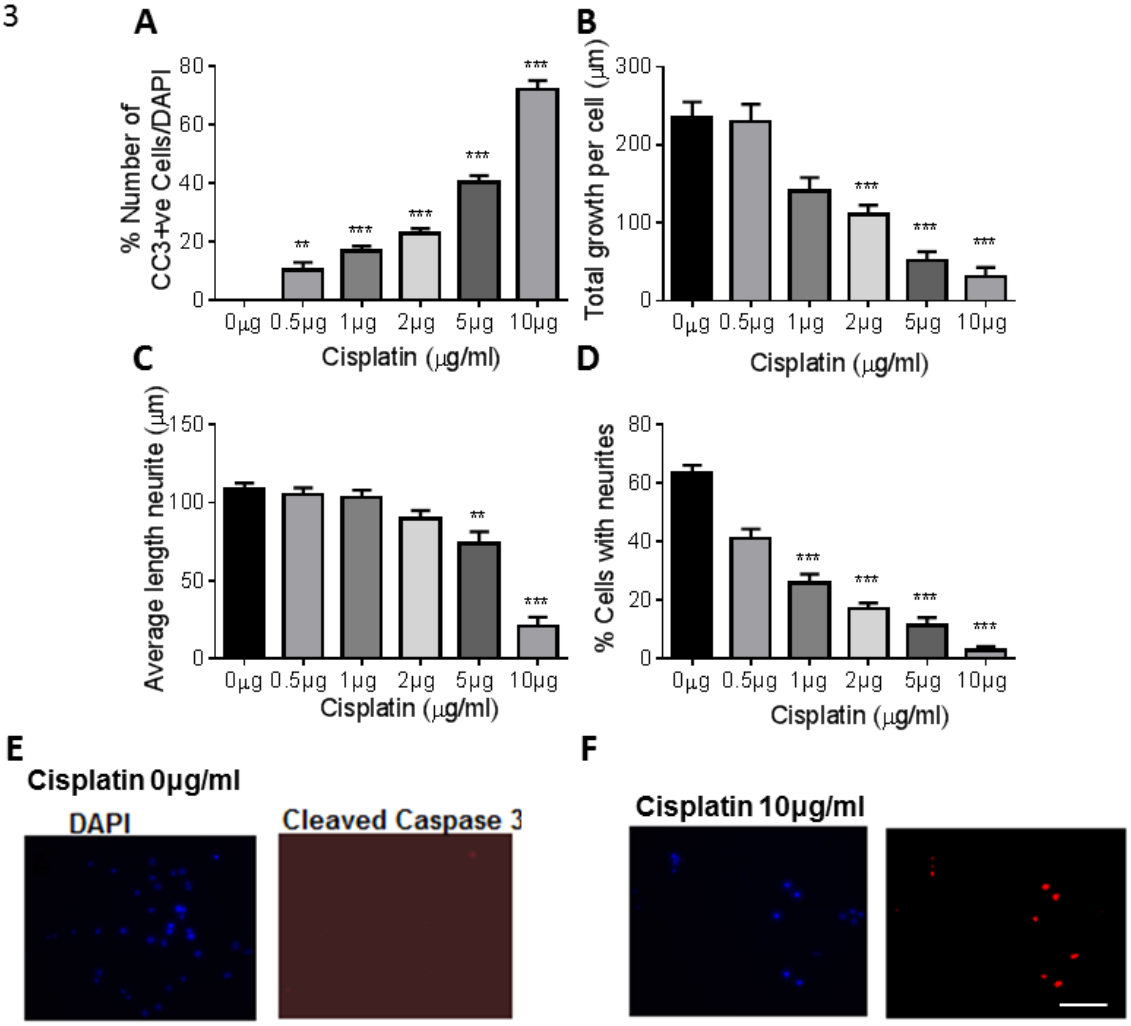

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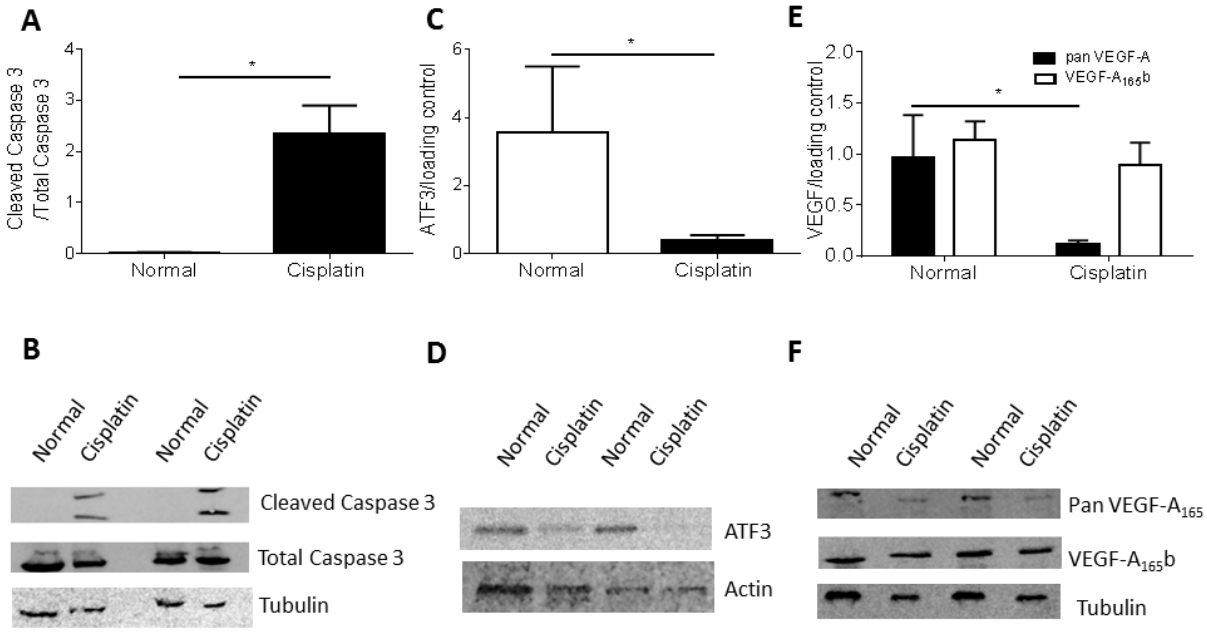
Figure 4



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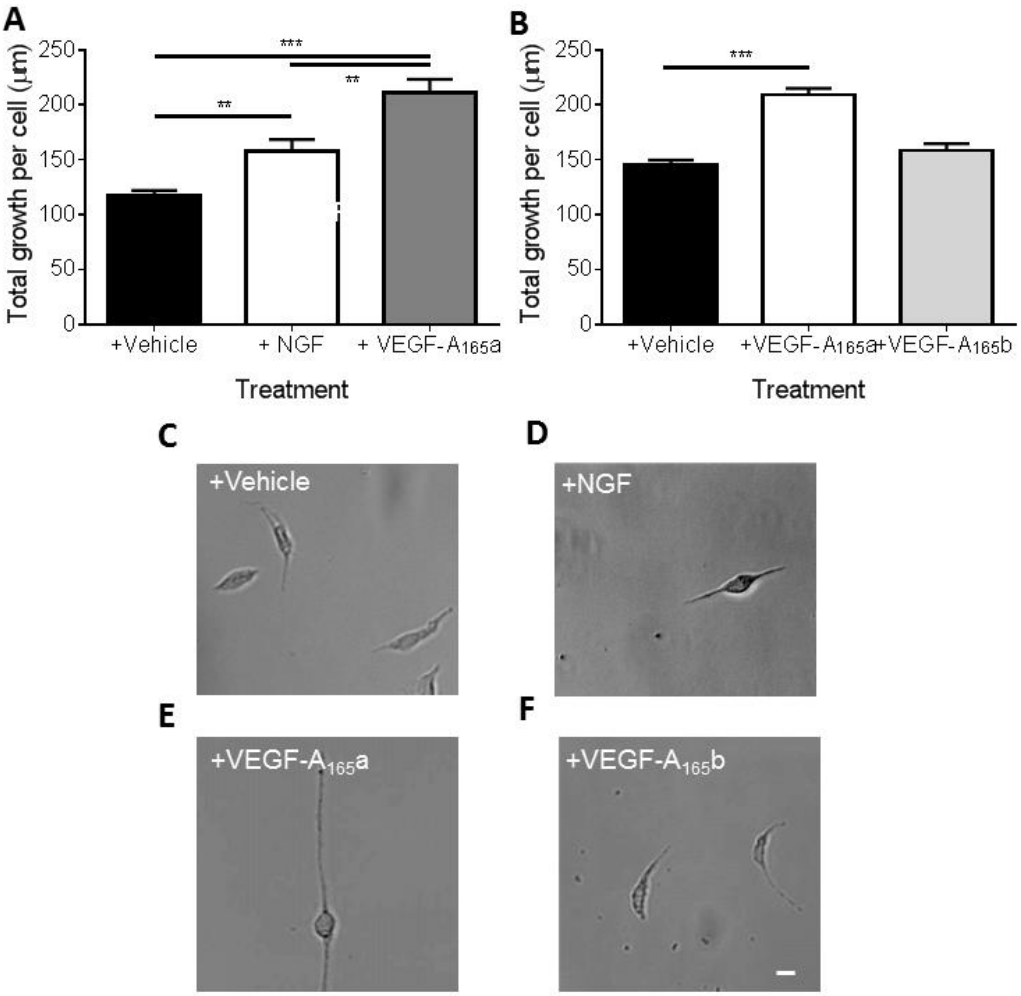
A
Total growth per cell (μm)
250
200
150
100
50
0
***
**
**
+Vehicle
+ NGF
+ VEGF-A165a
Treatment
B
Total growth per cell (μm)
250
200
150
100
50
0
***
+Vehicle
+VEGF-A165a
+VEGF-A165b
Treatment
C
+Vehicle
D
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F
+VEGF-A165b

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Figure 6

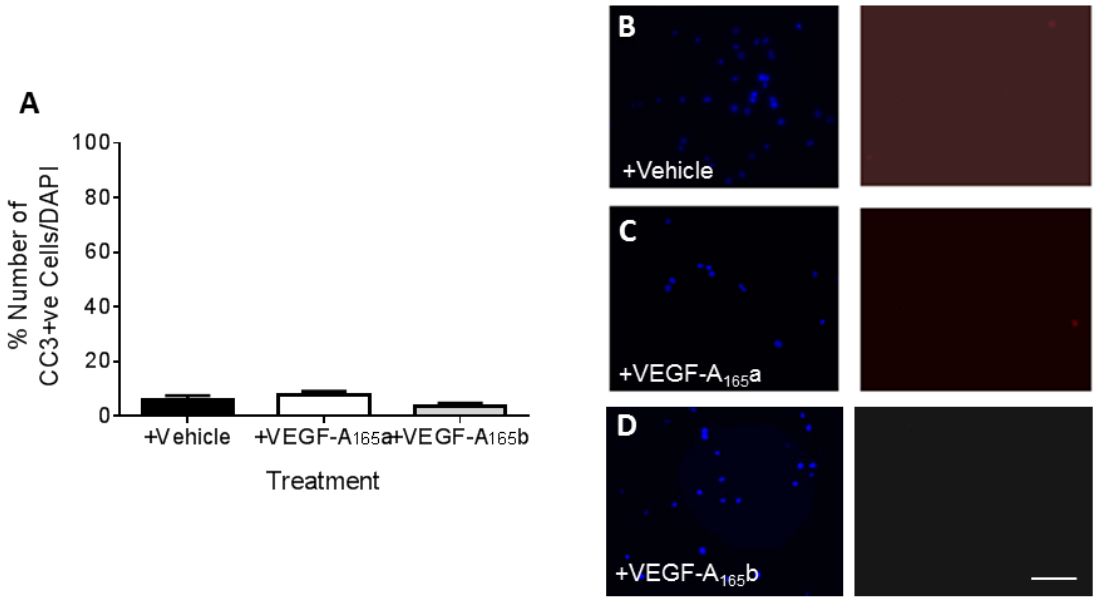
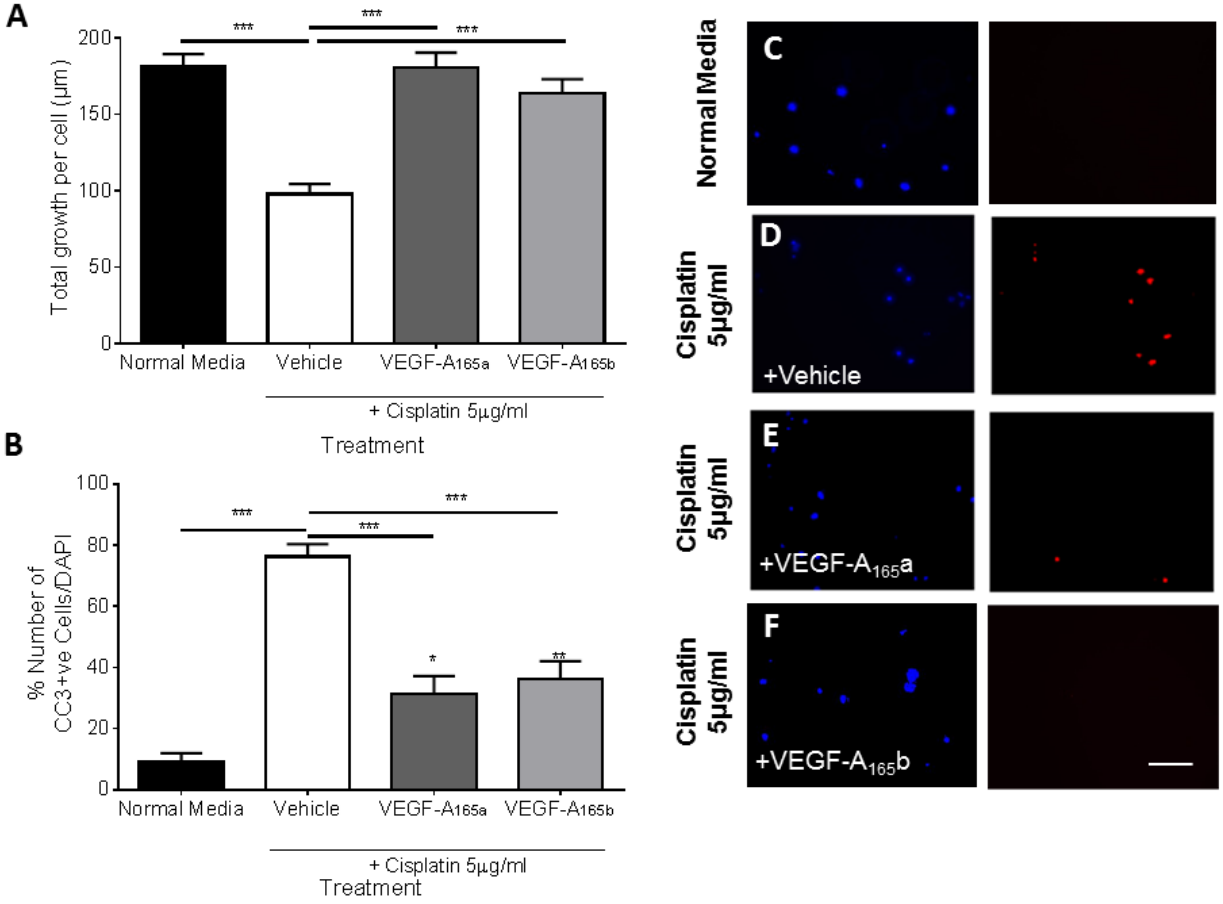


Figure 7


A
Total growth per cell (µm)
200
150
100
50
0
***
***
***
Normal Media
Vehicle
VEGF-A165a
VEGF-A165b
+ Cisplatin 5µg/ml
Treatment
B
% Number of CC3+ve Cells/DAPI
100
80
60
40
20
0
***
***
***
*
**
Normal Media
Vehicle
VEGF-A165a
VEGF-A165b
+ Cisplatin 5µg/ml
Treatment
C
Normal Media
D
Cisplatin 5µg/ml
+Vehicle
E
Cisplatin 5µg/ml
+VEGF-A165a
F
Cisplatin 5µg/ml
+VEGF-A165b