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The Expression of Copper Transporters in Rat Dorsal Root Ganglion and its Role in Oxaliplatin Neurotoxicity

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Pharmacology and Clinical Pharmacology, The University of Auckland, 2011
Abstract

Oxaliplatin is a platinum-based anticancer agent used for the treatment of colorectal cancer. Oxaliplatin chemotherapy is associated with peripheral neuropathy and dorsal root ganglia neurons are the putative target, however the mechanism of toxicity is currently unknown. Recent evidence suggests that copper transporters may be implemented in the transport and neurotoxicity of platinum drugs. We hypothesis that platinum drugs induce neurotoxicity by mechanisms involving copper transporters.

To examine the mechanisms by which dorsal root ganglia neurons are damaged by oxaliplatin, the mRNA and protein expressions of copper transporters were investigated in healthy and oxaliplatin treated adult rats. Adult rat dorsal root ganglion tissue exhibited a specific pattern of expression of copper transporters with distinct subsets of neurons intensely expressing either ATP7A or CTR1, but not both or ATP7B. Neurons expressing the copper influx transporter, CTR1, were more susceptible to oxaliplatin-induced neurotoxicity compared to neurons expressing the copper efflux transporter ATP7A.

Based on the abovementioned findings, a series of compounds used for the treatment of Menkes and Wilson’s disease were tested in a rat model of oxaliplatin-induced neurotoxicity. Copper histidine (0.02 – 20 mg/kg) and ammonium tetrathiomolybdate (1 - 30 mg/kg) showed initial protection in a pilot study but did not significantly reduce the neurotoxicity of oxaliplatin, based on DRG morphometric changes, in a subsequent definitive study. Copper treatment did not increase the systemic copper level or reduce the neurotoxicity of oxaliplatin in the current in vivo rat model.

Primary cultures of dorsal root ganglion neurons were employed to investigate the expressions of copper transporters in untreated and in oxaliplatin treated neurons. It was found that CTR1 and ATP7A were expressed in distinct sub-population of cultured rat DRG neurons. Oxaliplatin caused atrophy in the neurons expressing CTR1 and a loss of membrane staining frequency without altering the number of neurons. Neurons expressing ATP7A was not affected by oxaliplatin treatment.
In conclusion, we speculate that oxaliplatin-induced neurotoxicity in dorsal root ganglion neurons may involve copper transporter via mediating oxaliplatin uptake and/or the disruption of copper homeostasis.
Acknowledgements

During my time doing this project I have learnt, shed tears of joy, fell, picked myself up and more. I have learnt as much science as I have life. There are a lot of people who has contributed to this and were on this ride with me. They brought joy, good humour, wisdom and warmth into my PhD life. I sincerely appreciate each and every single one of you. Thank you so much for being there. I really couldn’t have asked for more.

Firstly, I wholehearted thank my primary supervisor, Associate Professor Mark McKeage. Mark has given me light during the darkest hours, through his optimistic outlook, wisdom in science and guidance on the project. This would not have been possible without him. Thank you so much Mark.

I would like to thank my co-supervisor, Dr Johnson Liu, who took me under his wings from the very first day I set foot into the lab and taught me so much. Johnson helped me built the foundations that made carrying out the experiments possible. Without him, I wouldn’t have the skills needed to complete this project.

I would like to extend my gratitude to the McKeagers; Deanna, Steve, Nancy, Josh, Yaeseul, Prashi and Fang; you guys have been my second family for the past 7 years. I appreciate all the laughter we have shared together. Thanks for all the encouragements and conversations.

I really appreciate the scholarship offered to me from the Cancer Society of Auckland who has provided me with financial assistance for 3 years.

The ADMET team/family, past and present members, has helped me so much, brought me so much joy and given me valuable feedbacks. Malcolm, Nuala, James and Yan, thanks for the wisdom, insights and chats we have had. Thanks for all the cakes/birthdays we have shared together Mike, Maggers, Wingers, Joyce, Raymond, Mini, Marcus, Jez, Taryn, Benedict, Katie, JP and Lulu. The chats about doing research and having someone who can empathise with me were great. You are a truly delightful bunch and the memories we have shared together will be treasured by me.
I want to thank my best friends; Nancy, Tom, Sharon, Cat, Sam, Felix, Sharyn, Catherine, Victor, Angela and Chien. You guys have been there for me to hear about life’s joy as well as the sniffles. You guys kept me sane, truly cared for me and were never far from me despite the distance.

I want to thank my extended family, my late Grandpa, Aunt Elsie, Aunt Jenny, Aunt Grace, and Aunt Veronica. I am so thankful to have such a great extended family network that never stopped giving from the day I meet you.

I wish to thank my family, Dad, Mum, Jimmy and Ann. They were always there for me and cared for me. They were always thoughtful and provided both emotional and practical support that made doing this PhD possible. Thanks for showing me so much love through actions; you continue to inspire me every day. I am so grateful for having a family that devotes so much to me.

Lastly, I would like to thank my partner of 13 years, Tony. Thank you so much for your love, devotion and everything that you have given me and experienced with me. I appreciate all that we have shared.
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<th>Description</th>
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<tbody>
<tr>
<td>$A_{260}$</td>
<td>Absorption at a wavelength of 260 nm</td>
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<tr>
<td>$A_{280}$</td>
<td>Absorption at a wavelength of 280 nm</td>
</tr>
<tr>
<td>ADME</td>
<td>Absorption, distribution, metabolism and elimination</td>
</tr>
<tr>
<td>Ag</td>
<td>Silver</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATF-3</td>
<td>Activating transcription factor - 3</td>
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<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>ATP7A, B</td>
<td>P-type ATPases A, B</td>
</tr>
<tr>
<td>Atox1</td>
<td>Antioxidant protein 1</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under concentration-time curve</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CCS</td>
<td>Copper chaperone for superoxide dismutase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related polypeptide</td>
</tr>
<tr>
<td>CH</td>
<td>Copper histidine</td>
</tr>
<tr>
<td>Cl`</td>
<td>Chloride ion</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>Peak concentration</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Conc</td>
<td>Concentration</td>
</tr>
<tr>
<td>COX17</td>
<td>Cytochrome c oxidase 17</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold cycle values</td>
</tr>
<tr>
<td>CTCAE</td>
<td>Common terminology criteria for adverse events</td>
</tr>
<tr>
<td>CTR1</td>
<td>Copper transporter 1</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3`-diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DACH</td>
<td>Diaminocyclohexane</td>
</tr>
<tr>
<td>DAPI</td>
<td>6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DDTC</td>
<td>Diethyldithiocarbamate</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglia</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>g</td>
<td>Relative centrifuge force</td>
</tr>
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<td>Gram</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HCO₃⁻</td>
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</tr>
<tr>
<td>hr</td>
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</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>H₂PO₄⁻</td>
<td>Dihydrogen phosphate ion</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectrometry</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>ip</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IR</td>
<td>Immuno-reactive</td>
</tr>
<tr>
<td>iv</td>
<td>Intravenous</td>
</tr>
<tr>
<td>kg</td>
<td>Kilo-gram</td>
</tr>
<tr>
<td>L3, L4, L5, L6</td>
<td>Lumbar 3, 4, 5, 6</td>
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<tr>
<td>LV</td>
<td>Leucovorin</td>
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<tr>
<td>M</td>
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<td>mM</td>
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<tr>
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<tr>
<td>µm</td>
<td>Mirco-meter</td>
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<tr>
<td>µm²</td>
<td>Square micron</td>
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min  Minute
mRNA  Messenger ribonucleic acid
MBD  Metal binding domain
MNCV  Motor nerve conduction velocity
MTD  Maximum tolerated dose
Na+  Sodium ion
NCI-CTC  National Cancer Institute-Common Toxicity Criteria
NFH  Neurofilament heavy subunit
NGF  Nerve growth factor
ng  Nano-gram
NH2  Amino
nm  Nano-metre
NS  Not significant
O2−  oxide ion
OCT compound  Optimal cutting temperature compound
OCTs  Organic cation transporters
P  P-value
PBS  Phosphate buffered saline
PC12  Pheochromacytoma 12 cells
PCR  Polymerase chain reaction
PFA  Paraformaldehyde
P-GP  P-glycoprotein
po  Orally
ppb  Parts-per-billion
pNF-H  Phosphorylated heavy neurofilament subunit
PNS  Peripheral nervous system
PSG  Penicillin-streptomycin-glutamine
Pt  Platinum
qPCR  Real-time polymerase chain reaction
r2  Coefficient of determination
rDNA  Ribosomal deoxyribonucleic acid
RNA  Ribonucleic acid
<table>
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<tr>
<td>rRNA</td>
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</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase - polymerase chain reaction</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>$T_{\text{max}}$</td>
<td>Time to reach peak concentration</td>
</tr>
<tr>
<td>TTM</td>
<td>Ammonium tetrathiomolybdate</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>18S</td>
<td>18S ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
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<tr>
<td>$\Delta\text{Ct}$</td>
<td>Delta threshold cycle values</td>
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| Extent of contribution by PhD candidate (%) | 20%

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Chapter 3/3.3.1/p 71-89

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Extent of contribution by PhD candidate (%)
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Chapter 3/Figure 3.8/p 85

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Extant of contribution by PhD candidate (%): 5

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CHAPTER ONE

GENERAL INTRODUCTION

This review will focus on five major topics. These topics are: the background on oxaliplatin, the available known information on oxaliplatin-induced peripheral sensory neurotoxicity, dorsal root ganglion neurons as the tissue of insult by platinum drug-induced neurotoxicity, copper transporters and its association to platinum drug-induced neurotoxicity, and lastly the evidence of platinum drug transport by copper transporters will be presented. Since the start of this project new information has been published which supports the ideas being presented in this review involving the tissue expressions of copper transporters and the association between transporters discussed with platinum drug-induced neuropathy. Because these new information are highly relevant therefore they will also be covered in this review.

1.1. Oxaliplatin

Oxaliplatin is a third generation platinum-based anticancer agent, with a different structure and metabolic intermediates from that of the first and second generations, i.e. cisplatin and carboplatin. Oxaliplatin consists of a platinum atom, an oxalate ligand leaving group and a non-hydrolysable DACH ligand in the trans-l form (Figure 1.1) (Francesco et al., 2002).

Unlike cisplatin and carboplatin, oxaliplatin does not share a common cis-diammine stable carrier ligand, but it contains a diaminocyclohexane (DACH) carrier ligand that is maintained intact in the final reactive cytotoxic metabolites of the drug (Raymond
Figure 1.1: The chemical structure of cisplatin (A), carboplatin (B) and oxaliplatin (C).
et al., 1998). The different chemical structure of the DACH-platinum adducts formed from oxaliplatin appear to contribute to its lack of cross-resistance with cisplatin (Francesco et al., 2002). Although the molecular background for this lack of cross-resistance is incompletely known, it has been reported that oxaliplatin-DNA adducts bypass mismatch repair and replicative bypass processes more readily than cisplatin-DNA adducts (Ahmad et al., 2010).

1.1.1. Therapeutic Application

At present, oxaliplatin is the only platinum complex showing activity toward colorectal cancer, where cisplatin and carboplatin have no significant clinical activity (O'Dwyer et al., 2000). Oxaliplatin is currently used in the combination chemotherapy for advanced metastatic colorectal cancer. For many decades 5-fluorouracil (5-FU) used alone or in combination with leucovorin (LV) had been the first choice of treatment for metastatic colorectal cancer, but recently the addition of oxaliplatin have been shown to enhance patient response and survival significantly following treatment (de Gramont et al., 2000). Oxaliplatin became the key component of several combination chemotherapy regimens for the treatment of advanced metastatic colorectal cancer including FOLFOX (5-fluorouracil/folinic acid and oxaliplatin) (de Gramont et al., 2000), FOLFOXIRI (5-fluorouracil/folinic acid, oxaliplatin and irinotecan) (Montagnani et al., 2011) and XELOX (capecitabine and oxaliplatin) (Cassidy et al., 2004). Oxaliplatin is now routinely used as first- and second-line treatment as a standard treatment for metastatic colorectal cancer (Giacchetti et al., 2000, Tournigand et al., 2004).

1.1.2. Mechanism of Action

The mechanism of action of oxaliplatin is similar to that of other members of the class of platinum anticancer drugs such as cisplatin and carboplatin. It is generally accepted that platinum drugs exert their anti-proliferative effects by forming DNA-Pt adducts predominantly at the N7 position of guanosine (Woynarowski et al., 1998). Oxaliplatin induces the formation of GG and AG intrastrand cross-links. The sequence- and region-specificity, and number of DNA lesions induced in both naked
and cellular DNA have been reported to be similar between oxaliplatin and cisplatin (Woynarowski et al., 1998). The cellular and molecular aspects of the mechanism of actions of oxaliplatin have not been fully elucidated. It is known however, that the platinum atom forms a 1,2-intrastrand cross-link and bends the double helix by approximately 30° toward the major groove (Spingler et al., 2003). The major effect of the formation of these DNA adducts is the inhibition of DNA replication, cell cycle arrest and apoptosis (Roberts et al., 1979).

The spectrum of antitumor activity of oxaliplatin against tumour models differs from that of cisplatin and carboplatin. Oxaliplatin is particularly effective in cisplatin-resistant colon carcinoma cells (Brabec et al., 2005). The presence of the cyclohexane ring is the putative reason for this difference. The projection of the 1,2-DACH ring into the major groove of DNA enable it to escape recognition by the mismatch repair enzyme complex (Fink et al., 1996). In addition, it is speculated that the bulky DACH carrier ligand of oxaliplatin contributes to the differential replicative bypass and binding of damage-recognition protein on the platinated DNA, thereby conferring less cross resistance and enhanced activity compared to cisplatin (Chaney et al., 2004, Jung et al., 2003, Raymond et al., 2002, Vaisman et al., 1999). It has been hypothesized that both the lack of recognition by mismatch repair enzyme and replicative bypass offered by oxaliplatin contributes to a higher efficiency of DNA synthesis inhibition compared to cisplatin at equimolar concentrations, despite inducing fewer lesions in naked and cellular DNA (Saris et al., 1996, Schmidt et al., 1993, Woynarowski et al., 1998, Woynarowski et al., 2000).

1.1.3. Pharmacokinetics

Oxaliplatin is administered through the intravenous route and undergoes extensive non-enzymatic biotransformation to numerous platinum-containing transient intermediates. In a chloride rich setting such as the extracellular environment, the biotransformation of oxaliplatin is facilitated through the displacement of the oxalate group by water and endogenous nucleophiles such as Cl⁻, HCO₃⁻ and H₂PO₄⁻ ions. The transient reactive species formed are monoaqua-1,2-DACH-monochloroplatinum, 1,2-DACH-platinum dichloride, and 1,2-DACH diaquo platinum.
It is the replacement of oxalate group by water that yield the highly reactive intermediate which reacts with DNA (Raymond et al., 2000). These reactive platinum intermediates bind extensively and irreversibly to plasma protein and erythrocytes (Allain et al., 2000, Graham et al., 2000). The distribution of ultrafilterable platinum following oxaliplatin administration appears to be triphasic, comprising of two initial short distribution phases, α and β averaging 0.43 h and 16.8 h respectively, followed by a long terminal elimination phase γ averaging 391 h (Graham et al., 2000). The biotransformation products of oxaliplatin are principally excreted in the urine (Graham et al., 2000).

1.1.4. Toxicities

Oxaliplatin causes toxicities to several normal tissues, but the major dose-limiting toxicity is peripheral sensory neurotoxicity (Culy et al., 2000). Other common toxicities are often mild to moderate, and resolve quickly after treatment, including nausea and vomiting, diarrhoea, neutropenia and thrombocytopenia. Patients may also experience some forms of rare toxicity such as laryngospasm. Unlike cisplatin and carboplatin, oxaliplatin is associated with no significant renal or auditory toxicities (Culy et al., 2000). The adverse effects associated with oxaliplatin are more significant and frequent when it is used in combination with other chemotherapeutic agents. Nonetheless, except neurotoxicity, the tolerability of other adverse effects is usually acceptable by patients without the necessity of dosage reductions of other agents that are used below recommended doses established for single-agent therapy (Culy et al., 2000).

1.2. Oxaliplatin-induced Peripheral Sensory Neurotoxicity

1.2.1. Clinical Manifestation

Oxaliplatin-induced sensory neuropathy takes two forms; the first, an acute, early onset, transient and cold-induced dysesthaesia, and the second which is a dose-limiting, late-onset peripheral sensory neuropathy that is related to total cumulative dose received (Quasthoff et al., 2002). The incidence of occurrence of chronic
neurotoxicity varies from 65% to 98% of patients receiving oxaliplatin (Becouarn et al., 1998, de Gramont et al., 2000, Kemeny et al., 2004). The incidence for acute neurotoxicity has been reported in almost all patients treated with oxaliplatin (Lehky et al., 2004, Wilson et al., 2002). There is high inter-individual variability in severity of oxaliplatin-induced chronic neuropathy. In some patients oxaliplatin treatment can be administered for as long as 18 months with no signs of functional impairment (Cassidy et al., 2002, Gamelin et al., 2002).

The symptoms of the acute neurotoxicity include distal and perioral paresthesia/dysesthesia, muscular cramps, stiffness of the hands and feet, and contractions of the jaw, which is exacerbated by exposure to cold (Argyriou et al., 2008, Quasthoff et al., 2002). It has been hypothesized that acute neuropathy is attributable to motor and sensory nerves hyperexcitability resulted from blockage of sodium and potassium currents in the myelinated axons (Adelsberger et al., 2000, Grolleau et al., 2001). The acute form of oxaliplatin-induced neuropathy, although unpleasant, resolves within hours or days after infusion (Argyriou et al., 2008, Quasthoff et al., 2002).

As oxaliplatin treatment continues and total cumulative drug dose increases, a sensory axonal neuropathy develops. The cumulative, chronic form of sensory neuropathy induced by oxaliplatin is dose-limiting and disruptive to normal life. It manifests as persistence dysesthesia and paraesthesia of the extremities, impaired sensory ataxia and loss of fine sensory motor coordination that interrupts the performance of day to day activities like writing or holding objects (Argyriou et al., 2008, Cassidy et al., 2002, Quasthoff et al., 2002). The chronic neuropathy associated with oxaliplatin prominently affects sensory functions with little or no involvement of motor functions. Sensory nerve conduction velocity is reduced during oxaliplatin-induced peripheral sensory neuropathy in patients (Lehky et al., 2003, Lomonaco et al., 1992, Pirovano et al., 1992, Thompson et al., 1984) and animal models (Boyle et al., 1999, Cavaletti et al., 1992, Cavaletti et al., 2001, Screnci et al., 2000), without affecting the motor sensory conduction velocity.
1.2.2. Mechanisms and Site of Damage

The mechanisms of peripheral neurotoxicity induced by oxaliplatin are not fully elucidated, although the putative site of damage of oxaliplatin-induced neuropathy is the dorsal root ganglion (DRG) neurons. After oxaliplatin treatment it has been found consistently that preferential platinum accumulation occurs within the DRG neurons in both human (Gregg et al., 1992, Krarup-Hansen et al., 1999, Thompson et al., 1984) and animal models (Cavaletti et al., 1998, Cavaletti et al., 2001, Holmes et al., 1998, Jamieson et al., 2005, Luo et al., 1999a, McKeage et al., 2001). High levels of platinum binding to DNA has been reported in DRG neurons after platinum drug treatment in a rat model (McDonald et al., 2005, Ta et al., 2006), and it was positively correlated to the severity of neurotoxicity observed (Ta et al., 2006). In addition, morphological changes in the form of DRG neuronal cell body and nucleolus atrophy have been commonly observed after oxaliplatin treatment (Cavaletti et al., 1998, Cavaletti et al., 2001, Holmes et al., 1998, Jamieson et al., 2005, Krarup-Hansen et al., 1999, Luo et al., 1999, McKeage et al., 2001).

The reason for selective platinum accumulation and damage to DRG neurons in platinum drug induced neuropathy is unknown. The post mitotic nature of DRG neurons does not adequately explain the selective damage induced by platinum drugs, which mainly exert their pharmacological effect through inhibiting DNA replication and cell cycle arrest. In addition, the tissue accumulation of platinum does not explain the mechanisms of neurotoxicity because the amount of platinum accumulated in DRG does not correlate to the severity of neurotoxicity (Holmes et al., 1998, Screnci et al., 1997, Screnci et al., 2000). However it is worth to noting that these DRG neurons are situated in the peripheries and lack the protection of the blood-brain-barrier making them vulnerable to exposure to molecules in the circulation.

The characteristics of peripheral neuropathy are reflective of damages to DRG neurons. DRG neurons convey sensory information from the periphery into the spinal cord and brain. The cell bodies of DRG neurons lie bilaterally beside the vertebral column on each spinal nerve dorsal root. Sensory nerve conduction
velocity is related to cell body area of DRG neurons (Harper et al., 1985), and the slowing of sensory nerve conduction velocity and the atrophy in cell body area of DRG induced by platinum drugs supports this point.

Several speculative mechanisms of neurotoxicity have been proposed. For example, the putative mechanism of oxaliplatin-induced acute neurotoxicity involves the intermediate metabolite of oxaliplatin, oxalate, which chelates calcium and alters functioning of voltage-gated sodium channel (Grolleau et al., 2001). In addition, emerging evidence suggests that platinum drug induced neuropathy is associated with oxidative stress. Platinum drug have shown to induce toxicity in the peripheral nervous system and other organs through inducing oxidative stress (Ajith et al., 2007, Altun et al., 2010, Custodio et al., 2009, Joseph et al., 2008, Nassini et al., 2011 Thomas et al., 2006). Another suggested mechanism of neurotoxicity is the apoptotic cell death of DRG neurons involving the release of cytochrome c by the mitochondria (McDonald et al., 2002). Drug transporters were also linked to the peripheral neurotoxicity induced by platinum drugs such as multidrug resistance associated proteins, P-glycoprotein, organic cation transporters and copper transporters; this was discussed in more details in section 1.3.6.

There is a pressing need to understand why DRG neurons are selectively damaged by oxaliplatin. Appreciation of the mechanism of toxicity of oxaliplatin-induced neurotoxicity may offer novel insights into strategies for preventing this troubling adverse effect of oxaliplatin. An increase in the understanding of the pathophysiology of oxaliplatin induced neurotoxicity may aid to develop an effective neuro-protector against oxaliplatin induced neuropathy.

1.2.3. Neuro-protective Agents for the Treatment of Peripheral Sensory Neuropathy Associated with Platinum Drugs

There is no clinically-approved treatment currently available for preventing or managing symptoms of oxaliplatin-induced neuropathy. Many compounds have been tested for alleviating this toxicity preclinically in animal models and clinically in patients, but could not achieve apparent beneficial effects. Perhaps the lack of
efficacy and the use of various un-validated neurotoxicity scales to assess benefits attributed to the failure of these compounds to be successfully translated into clinical use. Important criteria for an effective neuro-protective agent include the amelioration of neurotoxicity without compromising the treatment benefits of oxaliplatin and minimal adverse effects. Many neuro-protective agents have been assessed against platinum drugs induced neuropathy and reported in the literature but only those that progressed into human patients in will be discussed in this section.

1.2.3.1 Calcium and Magnesium Infusion

The use of pre and post-treatment of calcium and magnesium infusions to ameliorate oxaliplatin induced acute neurotoxicity has been shown to improve symptoms in patients (Gamelin et al., 2004). The putative mechanism of oxaliplatin-induced acute neurotoxicity involves the intermediate metabolite of oxaliplatin, oxalate, which chelates calcium and alters functioning of voltage-gated sodium channel. The hypothesized mechanism of protection is that calcium and magnesium are divalent cations and have the ability to modify voltage-gated sodium channels and replaces calcium ions in the extracellular environment to restore the function of voltage-gated sodium channels and reduce the neurotoxicity of oxaliplatin (Grolleau et al., 2001). Magnesium has also shown to prevent cisplatin induced hypomagnesaemia (Lajer et al., 1999). However, the use of calcium and magnesium for the reduction in oxaliplatin induced neuropathy has been controversial and fate of this treatment remains uncertain. In a clinical trial named CONcePT (Combined Oxaliplatin Neurotoxicity Prevention Trial), prior to the completion of trial, reported that the addition of calcium and magnesium reduced the response rate of cancer to oxaliplatin treatment (Hochester et al., 2007). This resulted in early termination of the CONcePT trial and the NCCTG trial that was investigating the effect of calcium and magnesium for the treatment of oxaliplatin induced neurotoxicity in adjuvant colon cancer (Grothey et al., 2011). However, later reports have emerged claiming that the infusion of calcium and magnesium did not interfere with the patients’ responsiveness to oxaliplatin (Gamelin et al., 2008, Grothey et al., 2011).
Nevertheless, the utility of calcium and magnesium in preventing or alleviating neuropathy in patients receiving oxaliplatin requires further investigation.

1.2.3.2 Amifostine

Amifostine is an organic thiophosphate compound with a proposed mechanism of protection through its active thiol metabolite binding to platinum metabolites and the ability to scavenge reactive oxygen species generated by platinum drug radiation therapy (Grdina et al., 2000, Treskes et al., 1992). Amifostine has been assessed for providing benefits against cisplatin or carboplatin induced neuropathy. Available data shows conflicting results of the neuroprotective effects of amifostine against platinum drug induced neuropathy. For example, in a phase III trial involving 242 patients receiving cisplatin 100 mg/m² plus cyclophosphamide 1,000 mg/m² with or without amifostine for treatment of epithelial ovarian cancer, Kemp et al reported that there was a significantly lower incidence of peripheral neuropathy among patients who receive amifostine compared to those who didn’t (Kemp et al., 1996). Subsequently another study reported about nine patients with metastatic colorectal cancer that were administrated subcutaneous amifostine (500mg) prior to receiving oxaliplatin for up to 8 cycles. Penz et al reported that patients generally showed small improvement in neurologic symptoms as assessed according to the World Health Organization grading system for neurotoxicity with oxaliplatin therapy. In addition, patients reported a one grade decrease in the symptoms of neurotoxicity experienced (Penz et al., 2001). However, Moore et al concluded from a phase II Study of 27 patients given cisplatin and 3-hour paclitaxel chemotherapy that the level of activity was insufficient to warrant further study in a phase III trial (Moore et al., 2003). Furthermore, Hipert et al reported that although amifostine improved objective neurological assessment based on the neurotoxicity evaluation according to the National Cancer Institute-Common Toxicity Criteria (NCI-CTC), but there were no differences in self-estimated specific sensory or motor symptoms (Hilpert et al., 2005). Available data shows conflicting results and in the light of inconsistent results stronger evidence would be required for the determination of the neuroprotective effects of amifostine.
1.2.3.3 Xaliproden

Xaliproden is a 5-hydroxytryptamine (5-HT) 1A receptor agonist, a non-peptide neurotrophic agent, and is administrated orally. Prior to being tested in the clinic for platinum drug induced neuropathy, xaliproden was reported to reduce in-vitro neuronal damage following incubation with oxaliplatin and 5-FU/LV (Cassidy et al., 2006). Xaliproden had been tested in a clinical trial involving 649 colorectal cancer patients receiving oxaliplatin and 5-FU/LV (Cassidy et al., 2006). In this trial, it had been shown that the addition of 1 mg xaliproden for 15 days did not impact the treatment response rate and provided a significantly lower incidence (39%) of neuropathy occurrence compared to control. As a result a phase III trial is underway to confirm these neuroprotective benefits of xaliproden (Cassidy et al., 2006).

1.2.3.4 Oxacarbazepine

Oxacarbazepine has emerged as a potential neuroprotective agent against oxaliplatin’s effect on voltage-gated sodium channels (Argyriou et al., 2006a). Oxacarbazepine modulates voltage-gated sodium channels and inhibits high-frequency firing of nerves without impairing normal impulse conduction (Willow et al., 1984). In a trial with 32 advanced colorectal cancer patients receiving oxaliplatin FOLFOX treatment, oral oxacarbazepine were administered up to a maximum dose of 600 mg twice a day and maintained for 20 weeks (Argyriou et al., 2006a). Neurotoxicity was assessed using validated clinical instruments based on neurologic symptom score and neurologic disability score. Neurophysiological measurements such as sural, superficial peroneal, and ulnar sensory action potentials and peroneal motor response were also monitored. The results from this trial suggested that oxacarbazepine showed protection against oxaliplatin induced neurotoxicity symptoms but no differences in any of the sensory nerve action potential amplitudes assessed (Argyriou et al., 2006a). Thus, the benefits reported by this trial were modest and clinical importance is weak.
Lamotrigine

Lamotrigine is an antiepileptic agent that is reported to inhibit the function of neuronal sodium channels by decreasing the release of excitatory neurotransmitters, glutamate and aspartate (Rao et al., 2008). Despite increasing the threshold to cold-induced pain in healthy volunteers it was shown to be ineffective for ameliorating platinum drug-induced neuropathic symptoms in 125 patients (Rao et al., 2008).

Gabapentin

Gabapentin is an anticonvulsant drug and was tested in patients with previously untreated metastatic colorectal cancer for the treatment of oxaliplatin-induced neurotoxicity. Mitchell et al. concluded at the end of the trial that the addition of gabapentin to a modified FOLFOX regimen (flurouracil / leucovorin / oxaliplatin) did not significantly reduce oxaliplatin-induced neurotoxicity (Mitchell et al., 2006).

Org 2766

Org 2766 is a neurotrophic peptide that has shown protection in animal models of neurotoxicity of cisplatin without reducing the anti-tumor effects in mice implanted with tumor cells from a FMa human tumor line origin (Vanderhoop et al., 1988). The mechanism of protection is unclear but it does not appear to be related to the interference with the cisplatin-DNA binding in the DRG (Terheggen et al., 1989). Due to the apparent success in the animal model Org 2766 was subsequently evaluated in several randomized trials involving cisplatin treatments. Unfortunately most studies shared the same shortcomings of having an inadequate number of participants involved in the trial and the use of non-validated scales for the evaluation of neurological symptoms and showed no protection against cisplatin induced neurotoxicity (Hovestadt et al., 1992, Vanderhoop et al., 1990, Vangerven et al., 1994). The study conducted by Roberts et al. evaluated the efficacy of 2 or 4 mg of Org 2766 in ovarian cancer patients by testing vibration perception threshold and reported a worsening response in the group receiving Org 2766 (Roberts et al., 1997). The results from these trials suggest that Org 2766 does not provide protection against cisplatin induced neurotoxicity.
1.2.3.8  Diethyldithiocarbamate (DDTC)

DDTC removes platinum from mono-guanine adducts without reactivity towards the cisplatin-bis-guanine adducts (Bodenner et al., 1986a). In other models of cisplatin induced toxicity, DDTC rescued tissues from damage in the kidneys and bone marrow in mouse, rat and beagle dog models (Bodenner et al., 1986a). Subsequently, Gandara et al investigated the ability of DDTC to protect against cisplatin induced neuropathy in 221 patients with metastatic non small-cell lung cancer and ovarian cancer (Gandara et al., 1995). The outcome of the neuroprotective effect by DDTC against cisplatin treatment was assessed based on subjective reporting of neurotoxicity symptoms without nerve conduction measurement. It was concluded that the group of patients receiving DDCT did not provide any advantage over the control group receiving only cisplatin (Gandara et al., 1995).

1.2.3.9  Anti-Oxidants

Many antioxidants have been tested in vitro and in vivo pre-clinical studies for the prevention of platinum drug induced neuropathy and showed various protections, some agents selected for clinical trials as described as below.

1.2.3.9.1  Oral Acetyl-L-Carnitine

Acetyl-L-carnitine is an L-carnitine ester that plays an important role in intermediary metabolism (De Grandis et al., 2007) and a precursor of glutathione (Carozzi et al., 2010). Many researchers have studied the protective effect of acetyl-L-carnitine against chemotherapy induced neuropathy in animals and showed positive results. Using an in vivo rat model, Pisano et al and Ghirardi et al showed an improvement of electrophysiological parameters without comprising the efficacy of cisplatin or oxaliplatin, respectively, when acetyl-L-carnitine was co-administered (Ghirardi et al., 2005, Pisano et al., 2003). A small pilot Phase II study was consequently conducted involving 25 patients with grade 3 neuropathy receiving cisplatin or paclitaxel and acetyl-L-carnitine. The group receiving acetyl-L-carnitine in addition to cisplatin or paclitaxel showed improvement in both sensory conduction velocity and amplitude
The efficacy of acetyl-L-carnitine against platinum drug induced neuropathy requires further evaluation.

1.2.3.9.2. Glutathione Infusion

Hamers et al showed partial protection on cisplatin-induced reduction in sensory conduction velocity with glutathione in a rat model (Hamers et al., 1993). There have been 2 clinical studies involving the use of 1.5 G/m² of glutathione infusion prior to oxaliplatin drug treatment. In a clinical study involving 52 advanced colorectal cancer patients the use of glutathione infusion prior to oxaliplatin treatment provided significant protection on abnormal reduction in sural sensory nerve conduction velocity without interfering with the response rate of oxaliplatin (Cascinu et al., 2002). Since then Milla et al also demonstrated similar protection of glutathione infusion without affecting the pharmacokinetic and Pt-adduct formation of oxaliplatin currently on the FOLFOX treatment involving 27 patients with colorectal cancer (Milla et al., 2009). The available data supports the further evaluation of glutathione in ameliorating platinum drug induced neuropathy but further studies using a larger number of patients are required to draw definitive conclusions.

1.2.3.9.3. Oral N-acetylcysteine

N-acetylcysteine is an anti oxidant drug that increases whole blood concentration of glutathione and has been tested in a small trial involving 14 stage 3 colon cancer patients receiving oxaliplatin treatment (Lin et al., 2006). N-acetylcysteine was reported to reduce the severity of oxaliplatin induced neurotoxicity in terms of National Cancer Institute common toxicity criteria (NCICTC) grading. However it did not alter electrophysiological functions such as sural sensory nerve conduction velocity, sensory amplitude potentials or mean latency (Lin et al., 2006). The reported article did not appear to have provided sufficient evidence to warrant the claim that N-acetylcysteine reduced oxaliplatin induced neuropathy.

1.2.3.9.4. Oral Glutamine
Glutamine have been shown to up regulate nerve growth factor mRNA in animal models (Gwag et al., 1997). Wang et al explored the therapeutic role of glutamine in the prevention of peripheral nerve damage during oxaliplatin treatment in 86 patients with metastatic colorectal cancer (Wang et al., 2007). A reduction in incidence of grade 3-4 neuropathy and rate of oxaliplatin dose reductions were reported. In addition the efficacy of oxaliplatin did not appear compromised. To confirm the benefits of glutamine as a neuroprotector a larger trial is required.

1.2.3.9.5. **Oral Vitamin E**

Two research groups have investigated the efficacy of vitamin E against cisplatin associated neuropathy and the data available seem to be positive. Pace et al assessed the incidence of peripheral neuropathy in 27 patients with solid malignancies receiving cisplatin treatment that were given daily oral vitamin E (300mg/d) prior to cisplatin treatment and for 3 months after. A significant reduction in neurotoxicity measured by a non-validated neurotoxicity score based on clinical and neurophysiological parameters was reported in the treatment group receiving vitamin E (Pace et al., 2003). Argyrious et al found that by doubling the dose of vitamin E to 600mg/d in patients with solid or other malignancies, their relative risk of developing neurotoxicity were significantly reduced compared to the control group not receiving vitamin E (Argyrious et al., 2006b). In a more recent study Pace et al reported a significantly lower incidence of neurotoxicity in 68 patients with solid malignancies receiving 400mg/d of vitamin E in addition to cisplatin. However it did not have an effect on the sural sensory nerve amplitude (Pace et al., 2010). The use of vitamin E appeared to lower the reported incidence of neurotoxicity associated with cisplatin but further studies using a validated neurotoxicity scoring measurement is required for a convincing and reliable result.

Some of the above mentioned neuro-protective agents were first tested in models of peripheral sensory neuropathy (Bodenner et al., 1986, Evans et al., 1984, Forloni et al., 1994, Ghirardi et al., 2005, Luo et al., 2002, Verstappen et al., 2004, Windebank et al., 1994) and both in vivo and in vitro model are available.
1.2.4. Experimental Model for Studying Platinum Induced Peripheral Sensory Neuropathy

1.2.4.1 In Vivo Animal Model

Many researchers have employed animal models to study oxaliplatin induced chronic neuropathy. Several functional and morphological endpoints have been utilized to assess the severity of neurotoxicity in these animal models and some bear similarity to the toxicity in humans. For example, the platinum drugs induced changes in sensory nerve conduction velocity are similar in the rat (Hamers et al., 1991, McKeage et al., 1995, Vanderhoop et al., 1988) and human (Mollman et al., 1990). The platinum drug-induced atrophy and accumulation of platinum in the DRG neurons seen postmortem in humans (Gregg et al., 1992, Thompson et al., 1984) are also reported in the rodent models of neuropathy (Cavaletti et al., 1992, Cavaletti et al., 1998, Gregg et al., 1992, Holmes et al., 1998, McKeage et al., 2001). In addition the pharmacokinetics of platinum drugs is comparable in humans and rodent (Vanhennik et al., 1987). Other commonly used behavioral and functional endpoints for the assessment of platinum drug induced neuropathy in animal models include the von Frey Hair stimulation test, heat and cold stimulation, paw-withdrawal test and rotarod test, (Authier et al., 2003, Joseph et al., 2009, Verdu et al., 1999).

Of the animal models utilized, the Wistar rats is well validated (Cavaletti et al., 2001) and has been employed by many to study oxaliplatin induced neurotoxicity (Cavaletti et al., 2001, Holmes et al., 1998, Jamieson et al., 2005, McKeage et al., 2001, Screnci et al., 1997, Screnci et al., 2000, Jamieson et al., 2002,). Common endpoints employed to evaluate neurotoxicity the Wistar rats are changes in DRG neurons caused by oxaliplatin that are relevant to the pathogenesis of the sensory neuropathy found in man. These included DRG morphometry changes and immunohistochemical protein markers of neuronal injury in DRG neurons.

The morphometry changes in DRG are reflective of sensory nerve damage because a hallmark of oxaliplatin induced neuropathy is the reduction in sensory nerve conduction velocity (Argyriot et al., 2008), and conduction velocity is related to
DRG neuronal cell size (Harper et al., 1985). Therefore, the atrophy of DRG neurons can be used to quantitatively measure the severity of oxaliplatin induced neuropathy. In addition, the nucleolus size can also be used as an indication of oxaliplatin induced neuropathy because the nucleolus size is related to the DRG cell body size (Coggeshall et al., 1985).

Protein markers of neuronal injury in DRG neurons studied by immunohistochemistry have been identified and are likely to signify changes induced by sensory neuropathy (Jamieson et al., 2005, Jamieson et al., 2009, Liu et al., 2009, Noguchi et al., 1995). DRG neurons have been classified into sub-populations based on their different biochemical compositions and are related to their functions (Jessell et al., 1985, Kaikai et al., 1989, Ohtori et al., 2007). Thus, identifying the sub-population damaged by oxaliplatin induced neuropathy using protein markers such as phosphorylated form of the heavy neurofilament subunit (pNF-H) and copper transporter 1 (CTR1) provides an additional means of assessing neurotoxicity damage induced by platinum drugs.

Other endpoints such as platinum accumulation measurement were also utilized by researchers since the severity of neurotoxicity associated with platinum drugs correlates with the concentration of platinum in peripheral nerves (Gregg et al., 1992).

In many studies of oxaliplatin-induced neurotoxicity using the Wistar rats the administration of oxaliplatin is via the intraperitoneal route (i.p.) (Cavaleti et al., 2001, Holmes et al., 1998, Jamieson et al., 2005, McKeage et al., 2001, Screnci et al., 1997, Screnci et al., 2000, Jamieson et al., 2002,) since i.p. and intravenous (i.v.) administration of platinum drugs in the rat results in similar plasma AUCs and platinum organ distribution (Los et al., 1990).

1.2.4.2 Primary Culture of Dorsal Root Ganglion Neurons

Primary culture of DRG from the rat origin have been by many researchers to study platinum drug induced peripheral neuropathy (Gill et al., 1998, Jong et al., 2011, Luo
et al., 1999, McDonald et al., 2005, Melli et al., 2008, Scott et al., 1994, Ta et al., 2006). Primary cell cultures are defined as cells harvested directly from the organism. After which they are dissociated into single cells prior to seeding into the culture vessel and maintained in vitro for periods over 24 hr (Harry et al., 1998, Melli et al., 2009). A primary culture from dorsal root ganglia contains both neuronal and non-neuronal cells. The non-neuronal cells consist of fibroblast and Schwann cells that form a support on which these neurons can live. These dissociated postnatal DRG sensory neurons offers the possibility to study mature and completely developed neurons surrounded by cellular substrates that normally exist in the in vivo environment.

Primary culture has been employed to investigate the morphological and biochemical alterations in the DRG neurons in response to oxaliplatin treatment. It provides a means to study biological processes in a more isolated context. One advantage of using an ex-vivo system is that the time and concentration of drug exposure can be tightly controlled. Whereas in an in-vivo whole animal model other confounding factors, such as ADME can influence drug exposure. In addition, experimental investigations of primary cultures of DRG neurons are relatively simple to perform compared to in vivo models and the use of animals can be minimized.

In a tissue culture model, the cellular and molecular events leading to neuropathy in patients are comparable to the in vivo model of peripheral neuropathy. For example Gill et al used primary DRG neuronal cultures to examine the role of cell cycle regulatory elements in cisplatin-induced neurotoxicity and showed that cisplatin caused similar morphologic changes of apoptotic cell death both in primary sensory neurons in vitro and in vivo (Gill et al., 1998). Ta et al found biochemical evidence of apoptosis in cultures of DRG and in vivo morphological evidence of apoptosis suggesting that neuronal death induced by oxaliplatin was due to apoptosis in both in vitro and in vivo models (Ta et al., 2006). McDonald et al observed that after platinum drug exposure platinum preferentially accumulated in ex vivo cultured DRG neurons and in-vivo DRG tissue over other neuronal cell line and other tissues respectively (McDonald et al., 2005). The neurotoxicity pattern and severity of
different platinum drugs in *in vitro* rat DRG culture were similar to that of *in vivo* model. It was reported that the DRG morphometric alterations induced by platinum compounds and the neurotoxicity ranking in primary cultures of DRG neurons were comparable to that observed in vivo (Holmes et al., 1998, Luo et al., 1999).

1.2.4.3 Tumour Cell-lines of Neural Origin

Cell lines differ from primary cell cultures because they have been serially transplanted or sub-cultured through a number of generations and can be propagated for an extended period of time (Harry et al., 1998). Cell-lines of neural origin has been employed to study platinum drug induced toxicity such as neuroblastoma cell-lines (Schulze et al., 2011), N1E-115 (Konings et al., 1994), SH-SY5Y (Donzelli et al., 2004) and pheochromocytoma 12 cells (PC12) (Geldof et al., 1995, Kawashiri et al., 2011, McDonald et al., 2005, Pisano et al., 2003, Scuteri et al., 2009, Verstappen et al., 2004). It has been suggested that the benefits of using tumour cell-lines to study neurotoxicity provides the advantages of lessening confounding influences of mixed cultures and toxicity to non-neuronal support cells (Screnci et al., 1999).

1.3. Dorsal Root Ganglion Neurons

The dorsal root ganglion (DRG) neurons are part of the peripheral nervous system and lies within the vertebral column, outside the blood brain barrier. DRG contains the cell bodies of the afferent peripheral sensory nerves that carry information into the nervous system for processing. Each ganglion consists of individual neuronal cell bodies, together with the proximal portion of their axons, Schwann cell sheaths, a capsule of satellite cells and the connective tissue element of the peripheral nervous system. The general structure of dorsal root ganglion neurons is similar to all other cells of the body but in addition to that these neurons have axons that enable conduction of nerve impulses for communication to the central nervous system. DRG are pseudounipolar neurons and have an axon with two branches that acts as one; one axon extends to innervate a sensory receptor in the peripheral branch and the second axon projects to the central nervous system. The cell bodies of neurons
synthesize the essential components for maintaining structural components of the membrane and general cell functioning (Lieberman et al., 1976).

1.3.1 Classification

The DRG cell population is heterogeneous in many aspects. They differ in their biochemical makeup, central and peripheral connections, response to chemicals, membrane properties and size. It has been suggested that a single DRG cell harvest could represent more than 25 functionally diverse afferent populations; each with its own contributions to a variety of sensory events (Petruska et al., 2000). The classification of sub populations of DRG neurons are at large arbitrary and many have done so based on features such as expressions of functional neuropeptides (Price et al., 1985, Lawson et al., 1984, Lawson et al., 1991), sensitivity to chemical (Petruska et al., 2002), cell body size (Kawamura et al., 1978, Lawson et al., 1979), sensitivity to ATP (Li et al., 1999, Petruska et al., 2000), electrophysiological properties (Koerber et al., 1988) and fibre myelination (Tandrup et al., 1995). The basis of these classifications of DRG neurons are attempts to distinguish and group them into cell type that have similar physiological role.

1.3.2 Large Light and Small Dark DRG Neurons

There are two main types of DRG neurons defined based on morphological subtypes; the A, large light neurons and the B, small dark neurons (Lawson et al., 1979, Lieberman et al., 1976). These two subtypes were distinguished on the basis of their appearance using light and electron microscopic studies (Lawson et al., 1979, Lieberman et al., 1976). Aside from the qualitative differences there are likely to be fundamental differences between the two cell types. The A type, large light neurons are connected to large myelinated fibres. They comprise a broad diameter distribution and electrophysiologically are Aα and Aβ fibres. The B type cells are small dark neurons which generally connect to unmyelinated fibres (C fibres) and are sparsely connected to small myelinated fibres (Harper et al., 1985). Correlations between the size of the DRG neurons and the electrophysiological properties of the neurons and the axonal diameter have been made in the rat and cat (Harper et al.,
1985, Lee et al., 1986). It has been found that the size of the neuron correlates to the conduction velocity of the axon, with the large A type neurons (Aα and Aβ fibres) having a faster conduction velocity than the small B type neurons (C fibres) (Harper et al., 1985, Lee et al., 1986). In addition, it has been shown that different size neurons conduct different stimuli; large diameter sensory neurons convey information regarding proprioception, localized touch, and vibration while small diameter sensory DRG neurons conveying information about pain and temperature (Lieberman et al., 1976).

### 1.3.3 Different Response to Chemicals in a Sub-Population of DRG

Distinct sub population of the rat dorsal root ganglion neurons have varying sensitivity to external chemicals, which has shown to be related to their neuronal size. Chiba et al reported that the small neuronal sub-population expressing substance P were more susceptible to capsaicin induced mitochondria swelling (Chiba et al., 1986). On the contrary, Jamieson et al found that the larger neuronal sub-population expressing parvalbumin were more vulnerable to oxaliplatin induced neuronal atrophy (Jamieson et al., 2005). Although the underlying reason for the differences in response is not clearly known but fundamental differences appear to exist between the two cell types.

### 1.3.4 Ultrastructural Difference Between Sub-Population of DRG

Consistent ultrastructural differences between the large light cells and small dark cells have been reported. Mainly, the type A neurons (Aα and Aβ fibres) comprises prominent levels of neurofilaments that runs through aggregates of granular endoplasmic reticulum (Lawson et al., 1974, Peach et al., 1972). Aα neurons are reported to have less neurofilaments than Aβ fibre cell types (Lawson et al., 1974). The features of type B small dark cells (C fibres) are highly concentrated granular endoplasmic reticulum and prominent Golgi apparatus distributed throughout the perikaryon (Duce et al., 1977).

### 1.3.5 DRG Neurons Response to Peripheral Nerve Injury
There are several consequences described in DRG neurons in response to cell injury that appear to promote cell survival via upregulation of protective structural proteins and downregulating the production of non-essential proteins (Lieberman et al., 1971). For example, injuries to the peripheral nerve profoundly affected the production of many substances; sensory neurons increased their production of vasoactive intestinal peptide (VIP) and galanin (Anand et al., 1983, McGregor et al., 1984, Villar et al., 1989) but reduced the production of substance P (Villar et al., 1989). In addition to changes in neuropeptide levels, chromatolysis was apparent after sensory ganglion cell injuries which were hallmarked by a breakdown of large Nissl bodies and a loss of Nissl substance from the central portion of the cell. Furthermore, the nuclei are displaced from the centre of the cell to the cell periphery (Lieberman et al., 1976). Apoptosis of DRG is another outcome of peripheral nerve injury (Groves et al., 2003, Zimmermann et al., 2001).

1.3.6 Expression of Transporters in DRG and the Association with Platinum Drug-Induced Neuropathy

Recent research has implicated endogenous transporters in playing a role in platinum drug induced peripheral neuropathy. Studies have investigated the expressions and activities of transporters in DRG tissues but further research is required to elucidate the role they play in modulating the severity of platinum drug-induced neurotoxicity. New information has been published since the commencement of this study which supports the transporter of interest discussed below to be associated with platinum drug-induced neuropathy. Because these new information are highly relevant therefore they will also be covered in this review.

1.3.6.1. Multidrug Resistance Associated Proteins

The Multidrug resistance associated proteins (MRP) have been suggested to be involved in cisplatin-induced neuropathy. MRP2 have shown to transport cisplatin (Schinkel et al., 2003), and the mrp1 gene was found to be expressed at a similar level in neuronal tissues such as the brain, spinal cord and DRG (Balayssac et al., 2005, Balayssac et al., 2006). In an in vivo model of cisplatin-induced neuropathy
Balayssac *et al* reported the upregulation of mrp1 but not mrp2 gene in DRG tissue of rats after cisplatin treatment (Balayssac *et al.*, 2006). It is unknown if the upregulation of MRP genes are important in cisplatin-induced neuropathy. However the mechanism of MRP gene modulation due to exposure to cisplatin has been proposed; an increase in MRP transporter activities may be due to the induction of interleukin-6 gene expression (Ceresa *et al.*, 2011, Lee *et al.*, 2004). While the upregulation of multidrug resistance genes are commonly observed when cells are under oxidative stress (Ceresa *et al.*, 2011, Felix *et al.*, 2002).

1.3.6.2. P-Glycoprotein

Cisplatin treatment has been linked to multidrug resistant through P-glycoprotein (P-GP). After cisplatin treatment the induction of the mdr1 a P-GP encoding gene in DRG was accompanied by a significantly lower uptake of $[^{99m}Tc]$sestamibi, a substrate that is associated with the transporter activity of P-GP, suggesting that the induction of P-GP via cisplatin treatment may have conferred resistance (Balayssac *et al.*, 2006). A possible mechanism involving pregnane X receptor has been suggested; it has been reported that the gene and protein expression of P-GP were induced by cisplatin through the activation of the pregnane X receptor (Ceresa *et al.*, 2011, Masuyama *et al.*, 2005) which can stimulate the transcription of P-GP (Ceresa *et al.*, 2011, Ohga *et al.*, 1998).

1.3.6.3. Organic Cation Transporters

Yonezawa *et al* was the first to report the involvement of organic cation transporters (OCTs) in platinum drug transport by showing that both cisplatin and oxaliplatin accumulated in rOCT2 expressing cells (Yonezawa *et al.*, 2006). More information have came into light since the commencement of this project; OCTN1 and OCTN2 have been reported to be expressed in the rat DRG tissue and were implicated in modulating oxaliplatin-induced neurotoxicity (Jong *et al.*, 2011). Jong *et al* reported that the uptake of oxaliplatin was reduced in the presence of both ergothioneine and L-carnitine, which are substrates of OCTN1 and OCTN2 respectively. In addition, the cytotoxicity induced by oxaliplatin was significantly reduced in the presence of
ergothioneine. Based on these results Jong et al suggested that OCTN1 was involved in transport and toxicity of oxaliplatin in rat DRG (Jong et al., 2011).

OCTs were also linked to cisplatin-induced ototoxicity and nephrotoxicity. In a mouse in vivo study, the double knockout of OCT1 and OCT2 abolished cisplatin-induced ototoxicity and the co-administration of cimetidine, a substrate of OCT2, partly protected the wild type mice from ototoxicity and nephrotoxicity (Ciarimboli et al., 2010). This has also been demonstrated in an in vitro model where the co-incubation of cimetidine in hOCT2-HEK293 cells reduced cisplatin-induced apoptosis (Ciarimboli et al., 2005).

1.3.6.4. Copper Transporters

The expression of CTR1 has been reported in rat DRG tissue (Liu et al., 2009). There are ample of evidence to support the proposition that copper transporters are involved in the transport of platinum drugs resulting in an alteration of cell sensitivity to platinum drugs. These were discussed in more details under Section 1.5.

1.4. Copper Transporters

1.4.1 Copper Physiology

Copper (Cu) is an essential element for the survival of mammals but it can also be potentially toxic to life. Copper is a transition metal with the ability to adopt either oxidized or reduced distinct redox state. Redox cycling between Cu(I) to Cu(II) catalyses the generation of highly reactive oxygen species (Stohs et al., 1995). Free copper ion (Cu(I)) is formed when copper is in the presence of a reducing agent, such as superoxide or ascorbic acid, which subsequently catalyses the formation of hydroxyl free radicals from hydrogen peroxide via the Haber-Weiss reaction (Gaetke et al., 2003). Hydroxyl free radical is the most powerful oxidizing radical likely to arise in the biological system (Buettner et al., 1993). Hydroxyl free radicals are capable of attacking cellular protein, nucleic acid and lipids and causes damage such as altering protein properties and function, inducing DNA damage and mutation, and altering
lipid membrane structure and leading to interference with membrane function (Riley et al., 1994).

The ability for copper to adopt either oxidized or reduced distinct redox state enables copper to act as an essential cofactor for many vital oxidative and reductase enzymes. For this reason copper is essential and is utilized by approximately 300 enzymes in the human body (Crisponi et al., 2010). Some of the better known enzymes are: cytochrome c oxidase, the terminal enzyme of electron transport and oxidative phosphorylation; superoxide dismutase, an antioxidant enzyme for free radical detoxification; lysyl oxidase, required for collagen and elastin cross-linking; doamine β-monooxygenase, essential for conversion of dopamine to noradrenaline, monophenol mono-oxygenase, required for the synthesis of dopamine; tyrosinase, which catalyses melanin biosynthesis; and peptidyl-glycine α-amidating monooxygenase, an enzyme needed to synthesis of pituitary hormones (Danks et al., 1988, Eipper et al., 1988, Friedman et al., 1965, Lerner et al., 1950, Linder et al., 1996, Marks et al., 2001, Uauy et al., 1998, Uriu-Adams et al., 2005, Wachnik et al., 1988).

Although there are variations between redox states of copper, it is distributed throughout the body and it is consistently found in high levels in organs such as the liver, brain, heart and kidneys; 10% of copper is found in the liver alone. Copper concentrations are found at intermediate levels in the lung, intestine and spleen, and at low levels in the endocrine glands, muscle and bone (Evans et al., 1973, Pena et al., 1999).

1.4.2 Cellular Transport of Copper

To sustain a delicate balance of copper in cells, ensuring that copper is delivered to copper requiring enzymes without causing oxidative stress, mammals have evolved highly conserved pathways to maintain copper homeostasis. These pathways consists of a series of copper binding proteins, chaperones and copper transporters to ensure that copper is delivered to essential copper protein without releasing highly toxic free copper ions.
Copper is absorbed from dietary intake in the small intestine (Linder et al., 1996). The mechanism by which copper is transported across the brush border and the uptake at the apical plasma membrane of the intestinal cells is currently unknown (Crisponi et al., 2010, Sharp et al., 2003). Thereafter newly absorbed copper enters the liver, the organ that controls copper levels. Hepatic copper is then incorporated into ceruloplasmin or endogenous enzymes and to maintain copper homeostasis while excess copper is secreted into the bile for removal (Mercer et al., 2001).

The basic aspects of cellular copper transport are shown in Figure 1.2. Majority of copper in the serum are bound to ceruloplasmin, a multi-copper oxidase that can bind up to seven copper atoms (Harris et al., 2000). Copper absorption in the liver is presumably mediated by ceruloplasmin absorption by the hepatocytes (Irie et al., 1986, Tavassoli et al., 1986a, Tavassoli et al., 1986b). Bound copper is transferred from ceruloplasmin at the cell surface without penetrating the cell (Percival et al., 1990) by a high-affinity cellular copper uptake transporter on the plasma membrane, CTR1, putatively by endocytosis (Pena et al., 1999, Petris et al., 2003). Depending on the target protein, intracellular copper is then loaded on to one of three known pathway specific cytoplasmic chaperones; cytochrome c oxidase 17 (COX17) (Glerum et al., 1996), antioxidant protein 1 (Atox1) (Lin et al., 1997), and copper chaperone for superoxide dismutase (CCS) (Culotta et al., 1997), and copper is subsequently delivered to protein of target. COX17 directs copper to the mitochondria for insertion into the terminal oxidase of the respiratory chain, cytochrome c oxidase, for ATP production (Glerum et al., 1996, Hamza et al., 2002). The second copper chaperone, Atox1, guides copper to the ATP7A/B localized in the trans-Golgi network (Hamza et al., 1999, Huffman et al., 2000, Larin et al., 1999) where it subsequently gets translocated into the lumen of the Golgi (Voskoboinik et al., 1998). ATP7A and ATP7B normally resides in the trans-Golgi membrane where they deliver copper to secreted cupro-protein (Dierick et al., 1997, Hung et al., 1997, Yamaguchi et al., 1996), but when cytoplasmic copper concentration increases, it traffics to the plasma membrane and efflux excess copper to the extracellular milieu (Petris et al., 1996). Cupro-protein includes tyrosinase, lysyl oxidase, ceruloplasmin and blood clotting factors V and VIII (Puig et al., 2002). The third copper chaperone, CCS, is a protein
Copper is taken up by CTR1 and then transferred to pathway specific chaperones; the three currently known are COX, ATOX1, and CCS (shown in green). Each chaperone delivers copper to a specific protein – cytochrome c oxidase in the mitochondria, post-Golgi compartment and superoxide dismutase (SOD) respectively. Once copper exits from the cell it is likely to be transported by ceruloplasmin. The copper pathways are indicated by arrow. Copper is represented in orange.

required to deliver copper to cytoplasmic superoxide dismutase (SOD) where it utilizes the redox properties of copper to protect cells against oxidative damage, this is achieved by catalyzing the disproportionation of superoxide to hydrogen peroxide (Fukai et al., 2011, Rae et al., 1999). Once copper exits from the cell it is likely to be transported by ceruloplasmin, the tightly bound pool that accounts for at least 90% of the total plasma copper in most species (Gubler et al., 1953).

1.4.3 Copper Efflux and Influx Transporters

Copper transporting proteins, ATP7A, ATP7B and CTR1, play an essential role for the delivery of copper to essential cuproenzymes. Copper transporters have evolved along with other components of copper regulatory pathways to ensure that copper is transported without releasing highly cytotoxic free copper ions (Camakaris et al., 1999, Linder et al., 1996,). The P-type ATPases, ATP7A and ATP7B are both copper efflux transporters and recent studies have suggests that they have distinct physiological roles and differ in enzymatic characteristics, trafficking properties, interacting partners, and regulation (Barnes et al., 2005, Linz et al., 2007). However, ATP7A and ATP7B both deliver copper to the trans-Golgi network (Lutsenko et al., 2007) where the biosynthetic incorporation of copper into copper enzymes are thought to take place. Consistent with that idea, the protein localization of ATP7A and ATP7B are found to be within the perinuclear region of the cytoplasm where they deliver copper to secreted cupro-protein (Dierick et al., 1997, Hung et al., 1997, Huster et al., 2003, Roelofs en et al., 2000, Yamaguchi et al., 1996). The trafficking of ATP7A and ATP7B from the cytoplasm to the plasma membrane has been reported when intracellular copper levels are elevated subsequently allowing both ATP7A and ATP7B to act as a copper efflux transporter (Cobbold et al., 2003, Lutsenko et al., 2003, Pase et al., 2004, Petris et al., 1996, Petris et al., 1999).

CTR1 is a plasma membrane protein that functions as a high-affinity cellular copper uptake transporter and is the major pathway for copper entry into the cells (Lee et al., 2002a). Trafficking of CTR1 is an important regulatory mechanism for controlling copper homeostasis (van den Berghe et al., 2010). Elevated copper level has shown
to stimulate the endocytosis (Guo et al., 2004, Petris et al., 2003) and degradation (Ooi et al., 1996, Petris et al., 2003) of the human Ctr1 protein.

1.4.4  Tissue Expression and Localization of Copper Transporters

The expression of copper transporters has been characterized extensively in various tissues and appears to be related to their biological role. Cloning of the ATP7A and ATP7B gene revealed that ATP7A and ATP7B are co-expressed in numerous tissues like the brain, kidney, heart, lung, muscle, pancreas, placenta, bone marrow, intestine, liver and eye. (Bull et al., 1993, Chelly et al., 1993, Kuo et al., 1997, Linz et al., 2007, Mercer et al., 1993, Petrukhin et al., 1994, Ravia et al., 2005, Tanzi et al., 1993, Vulpe et al., 1993, Yamaguchi et al., 1993). However, ATP7A and ATP7B have very high expressions in the intestine and liver respectively (Bull et al., 1993, Chelly et al., 1993, Mercer et al., 1993, Tanzi et al., 1993, Vulpe et al., 1993, Yamaguchi et al., 1993). CTR1 is a ubiquitously expressed protein, with particular high expression reported in the liver (Lee et al., 2000, Zhuo et al., 1997). CTR1 is expressed in tissues such as brain, heart, kidney, mammary gland, intestine, lung, spleen, retina, testes, skeletal muscles (Bauerly et al., 2005, Hsiao et al., 2001, Kuo et al., 2006, Lee et al., 2000, Samsonov et al., 2006, Zhuo et al., 1997). The ubiquitous pattern of CTR1 expression in tissue is likely to be reflective of the essential requirement of copper in most tissues.

1.4.4.1.  Brain

The mRNA expression of ATP7A, ATP7B and CTR1 have been reported in neuronal tissues of human (Hsiao et al., 2001, Roth et al., 2006, Shyamsunda et al., 2005, Yanai 2 et al., 2005), rodents (Choi et al., 2009, Kuo et al., 2006, Platonova et al., 2004) and Drosophila (Burke et al., 2008). However each copper transporter exhibited a cell-type specific pattern of expression and activity in different regions of the brain (Barnes et al., 2005, Platonova et al., 2005). This is likely to reflect the requirements for copper in different brain region to support the functions of diverse cuproenzymes, such as dopamine-β-monooxygenase and peptidylglycine α-
amidating monooxygenase that convert dopamine to norepinephrine and synthesize neuropeptides, respectively.

In the rat, ATP7B has been reported to be continuously expressed in the Purkinje neurons (Barnes et al., 2005), and in the mouse the Atp7b gene was found to be only active in the hypothalamus (Platonova et al., 2005). ATP7A switches expression from the Purkinje neurons to Bergmann glia during development of the rat (Barnes et al., 2005) and the Atp7a gene was active in all brain regions of mouse (Platonova et al., 2005). The CTR1 mRNA in the rat was found to be relatively high in all regions of the brain, particularly the choroid plexus, which was approximately two times higher (Platonova et al., 2005).

1.4.4.2. Kidney

ATP7A and ATP7B were reported to be co-expressed in the epithelial cells of the proximal and distal renal tubules and is thought to act as a copper efflux pump to protect against copper overload in the renal parenchyma (Linz et al., 2007). Both ATP7A and ATP7B were suggested to regulate the copper levels in the filtrates in the mouse glomeruli. Moore et al found the expression of ATP7B in the kidney medulla where it may have a role in the re-absorption of copper (Moore et al., 2003).

CTR1 was expressed in the renal tubular cells of mice, localized to the basolateral side of the renal tubules (Kuo et al., 2006, Lee et al., 2000, Pabla et al., 2009). Pabla et al connected the cisplatin induced nephrotoxicity with the expression of CTR1 in mice localized to the basolateral membrane, the site of cisplatin uptake. It was shown that the deletion of Ctr1 ameliorated tubular cell apoptosis in these mice which could suggest that the transport of cisplatin into the site of damage in the kidney were via CTR1 mediated transport (Pabla et al., 2009).

1.4.4.3. Eye

In human, both ATP7A and ATP7B were found to be expressed in the Golgi of the retinal pigment epithelial cells and in addition ATP7A was found to also be present in the neurosensory retina (Krajacic et al., 2006). CTR1 has been reported to be
expressed in the connective tissues of the eye during mouse development and adulthood (Kuo et al., 2001).

It has been suggested that ATP7A and ATP7B may play an important role in retinal copper homeostasis. Krajacic et al showed that copper stimulates the relocalization of ATP7B. In addition, retinal degeneration is associated with genetic inherent diseases, Menkes and Wilson’s disease, where ATP7A and ATP7B are dysfunctional respectively. Thus, the loss of local retinal activity of ATP7A and ATP7B proteins may contribute to the retinal degeneration that manifest in Menkes and Wilson diseases (Krajacic et al., 2006).

1.4.4.4. Heart

Copper is crucial for normal cardiac tissue functioning because copper necessitates ATP synthesis in the mitochondria, which is required for muscle contraction (Medeiros et al., 1993). Furthermore copper plays a role in oxidative stress protection (Takahashi et al., 2000) and peptide hormone biogenesis, both of which are vital for functioning of the heart, including lysyl oxidase involved in collagen crosslinking (Medeiros et al., 1993). The knockdown of Ctr1 in Drosophila and mouse models demonstrated severe cardiovascular dysfunction resulting in cardiac hypertrophy (Kim et al., 2010). Thus, it is not surprising that the expression of ATP7A, ATP7B and CTR1 in the heart has been reported. The expression of ATP7B was found to be associated in early embryonic development of mouse (Kuo et al., 1997). Kuo et al detected abundant amount of CTR1 in the intercalated disks of the heart using Immunohistochemical analyses (Kuo et al., 2006). The expression of ATP7A was found in the heart using immunoblotting analysis (Kim et al., 2010).

1.4.4.5. Lung

Using RNA in situ hybridization Kuo et al reported the expression of ATP7B during the later gestation period of the mouse (Kuo et al., 1997). Recently ATP7A, ATP7B and CTR1 have shown to be expressed differentially in the primary culture of mouse tracheal epithelial cells (Ibricevic et al., 2010). The expression of ATP7A, ATP7B and
CTR1 were found in non-ciliated cells, ciliated cells and airway epithelial cell, respectively. ATP7B was linked to having a protective role in the detoxification of silver in the lung (Ibricevic et al., 2010). In HepG2 cells, the knockout of ATP7B resulted in a significant increase in cell death when methylated caffeine silver acetate compound, a silver containing toxin, was exposed to the cells. Ibricevic et al proposed that the specific expression of ATP7B in airway epithelial cells and the abovementioned results suggested detoxification of silver via ATP7B trafficking and secretion in the lung.

1.4.4.6. Smooth Muscles

ATP7A were found to be abundantly expressed in vascular smooth muscle cells, vascular endothelial cells, fibroblasts and aorta in mice (Qin et al., 2006). Another independent researcher has also described the expression of ATP7A and CTR1 in vascular smooth muscle cells of rat, mouse and human using RT-PCR detection method (Ashino et al., 2010). It was proposed that ATP7A transports copper to SOD which is essential for the full activation of SOD3 in these smooth muscles and ATP7A function to modulate vascular O$_2^-$ levels via the modulation of SOD3 activity in the vasculature (Qin et al., 2006). ATP7A has also been suggested to play a role in the platelet-derived growth factor induced vascular smooth muscle cells migration in a copper dependent manner (Ashino et al., 2010). Vascular smooth muscle cell migration is important in vascular injury because it is a key component of neointimal formation required for in vivo vascular remodeling in response to vascular injury (Volker et al., 1997).

1.4.4.7. Placenta

ATP7A and ATP7B were found to be differentially localized within the human placenta (Chelly et al., 1993, Hardman et al., 2004, Mercer et al., 1993, Vulpe et al., 1993). ATP7A was present at the basal side of the syncytiotrophoblast layer and also within the fetal vascular endothelial cells, whereas ATP7B was localized at the microvillous membrane of the syncytiotrophoblast (Hardman et al., 2011). Hardman et al suggested that the differential expression of ATP7A and ATP7B provide
definitive evidence for distinct roles that each copper transporter plays in the human placenta; ATP7A transports copper into the fetal circulation for copper requiring cuproenzymes in the placenta while ATP7B aids in the maintenance of copper homeostasis by transporting copper to the maternal circulation (Hardman et al., 2007). It appears that ATP7A and ATP7B are important throughout the whole duration of the pregnancy because the levels of both proteins did not change across pregnancy when measured by Western blot analysis (Hardman et al., 2004).

CTR1 is speculated to be involved in copper transport in human placental cells by regulating copper uptake through the endocytic pathway. In term placental tissue hCTR1 was found to be localized within the syncytiotrophoblast layer and the fetal vascular endothelial cells in the placental villi and toward the basal plasma membrane. CTR1 were not found to co-localize with ATP7A or ATP7B (Hardman et al., 2006).

1.4.4.8. Mammary Gland

It has been suggested that ATP7A plays a regulatory role in copper transport within the mammary gland during lactation which functions to export excess copper out of milk (Camakaris et al., 1995, Kelleher et al., 2006). ATP7A expression was found in the mammary gland of mice and humans in both lactating and nonlactating state and the location of ATP7A protein supports this proposition (Ackland et al., 1999, Camakaris et al., 1995, Grimes et al., 1997, Kelleher et al., 2006). During the nonlactating state ATP7A was expressed in the perinuclear region and relocalized during mid-lactation to the luminal membrane of mammary epithelial (Ackland et al., 1999, Camakaris et al., 1995, Kelleher et al., 2006). Llanos et al demonstrated the expression of ATP7A in the basolateral membrane of mammary epithelial cells within the mammary gland in lactating mice. In addition, they showed that the expression of human ATP7A in the transgenic mice resulted in a significant reduction in copper concentration in the mammary gland of transgenic mice compared to their non-transgenic counterparts suggesting that ATP7A plays a role in removing excess copper from the mammary epithelial cells rather than supplying copper to milk (Llanos 2008). Furthermore, Ackland et al showed that the levels of ATP7A mRNA
expression in human mammary gland breast epithelial increased during the lactation period (Ackland et al., 1999). For these reasons, ATP7A is primarily involved in minimizing copper levels in the mammary glands.

The level of ATP7B expression is comparable between the lactating and nonlactating mice but the localization of ATP7B differed between the nonlactating and lactating state in mammary gland (Michalczyk et al., 2000). ATP7B was found to localize primarily to the luminal region of the cells and presented a punctate staining pattern, but the localization of ATP7B shifts from the trans-Golgi network in the nonlactating state to a vesicular compartment in the lactating period (Kelleher et al., 2006, Michalczyk et al., 2000). Results from animal models with dysfunctional ATP7B have suggested that ATP7B is important in the delivery of copper into milk in the mammary gland. The milk produced by lactating ATP7B-null mice had significantly lower copper concentrations compared to the control mice with functional ATP7B (Buiakova et al., 1999). Moreover, Michaczyk et al showed that mice with an ATP7B mutation that prevents its translocation from the trans-Golgi network to an intracellular vesicular compartment are associated with low copper concentration in milk (Michaczyk et al., 2000).

The role CTR1 plays in mammary gland is not yet known. The expressions of CTR1 in the mammary gland has been observed for various cell lines and were reported to localize at the plasma membrane and within vesicular compartments (Klomp et al., 2002). In the mouse RT-PCR analysis revealed that CTR1 is expressed in the epithelium of mammary ducts (Samsonov et al., 2006). In the rat mammary glands both mRNA and protein expression of CTR1 has been shown (Kelleher et al., 2003, Kuo et al., 2006).

1.4.4.9. Intestine

ATP7A is likely to have a role of copper efflux in the intestine. ATP7A is abundantly expressed in the intestine of humans (Shyamsunda et al., 2005), rodents (Bauerly et al., 2005, Collins et al., 2009, Ravia et al., 2005, Nyasae et al., 2007,) and Drosophila (Burke et al., 2008). During basal copper level ATP7A is localised to the trans-Golgi
network in the perinuclear region of the enterocytes of the intestine (Monty et al., 2005, Nyasae et al., 2007). Under high copper conditions ATP7A vesicles has been reported to relocalise close to the basolateral membrane (Monty et al., 2005, Nyasae et al., 2007). In addition, the induction of ATP7A mRNA in the small intestine was observed when dietary intake of copper was enhanced in older pups with no changes in copper accumulation in the intestine (Bauerly et al., 2005).

ATP7B is localized in the Golgi apparatus of the enterocytes of the small intestine and in the stomach in murine models (Weiss et al., 2008). When Caco-2 cells, an established model for enterocytes of the small intestine, were exposed to copper, ATP7B was shown to translocate from the perinuclear region to a diffused punctuate staining pattern, the copper level dependent distribution pattern of ATP7B is likely to reflect the contribution ATP7B has in intestinal copper excretion and maintenance of cellular copper homeostasis (Weiss et al., 2008).

The expression of CTR1 has been found in the intestines of mice (Kuo et al., 2005), rats (Bauerly et al., 2005) and humans (Lee et al., 2000). Studies suggested that CTR1 is involved in the uptake of copper into the small intestines and the movement of CTR1 in cells is important to manage copper homeostasis (Bauerly et al., 2005, Kuo et al., 2005). Kuo et al studied the protein expression of CTR1 in copper deficient mice and found a higher expression of CTR1 in the duodenum of copper deficient mice (Kuo 2 et al., 2005). The upregulation of CTR1 protein in the intestine supports that CTR1 levels increase to enhance copper uptake when dietary copper level is low.

The importance of changes in the cellular localization of CTR1 from the plasma membrane to the cytoplasm in the regulation of copper homeostasis was demonstrated by Bauerly et al. Bauerly et al found that CTR1 protein level were altered in rats that were given copper supplementation but there were no changes in the mRNA levels of CTR1 suggesting that the function of CTR1 in the regulation of copper homeostasis is also regulated by post-transcriptional events which induces endocytosis of CTR1 (Bauerly et al., 2005).

1.4.4.10. Liver
The expression of ATP7B is particularly high in liver of human (Bull et al., 1993, Roth et al., 2006, Shyamsundar et al., 2005, Yamaguchi et al., 1993, Yanai et al., 2005,) and rat (Bauerly et al., 2005, Schilsky et al., 2000) as the liver is commonly described as a central organ for systemic copper homeostasis. ATP7B is located in the Golgi apparatus in the liver under basal conditions and delivers copper across the membrane of the trans-Golgi network for the incorporation into ceruloplasmin (Bingham et al., 1998, Lutsenko et al., 2007, Tao et al., 2003,). When copper levels are elevated, ATP7B translocates from the secretory compartment to cytosolic vesicular structures to excrete excess copper into the bile (Cater et al., 2006, Guo et al., 2005, Lutsenko et al., 2007, Roelofsen et al., 2000, Tao et al., 2003,). It has been reported that the translocation of ATP7B is essential for the excretion of copper (Cater et al., 2007, Forbe et al., 2000), but the exact mechanism copper translocation in these vesicles to the bile is unknown (Weiss et al., 2008).

ATP7A mRNA is found to be expressed in the liver of cattle (Han et al., 2009) and human (Roth et al., 2006). Hepatic mRNA and protein expression of ATP7A has been reported in the mice of various ages and peaking during the neonatal phase (Lenartowicz et al., 2010).

CTR1 mRNA levels are found to be high in the liver of mice, cattle and humans (Han et al., 2009, Lee et al., 2001, Zhou et al., 1997). In transgenic mice lacking the expressions of CTR1 in liver showed the important role of CTR1 and copper in growth. Mice without CTR1 in the liver suffered growth defects at 2 and 3 month of age because the lack of copper was likely to be required at a high level for rapid growth in younger animals (Kim et al., 2009).

1.4.4.11. Testes

CTR1 were found to be expressed during mouse development and adulthood in the testes (Kuo et al., 2001). The role copper transporter plays in the testes remains largely unknown.
1.4.5 Structures of Copper Transporters

1.4.5.1. ATP7A and ATP7B

ATP7A and ATP7B belong to the P-type ATPases cation-transporting protein family (P1B-ATPases) and uses energy of ATP hydrolysis to transport copper. ATP7A and ATP7B share 50-60% sequence homology and has distinct structural and mechanistic characteristic (Lutsenko et al., 2007). The ATP7A/7B proteins contain six metal-binding domains (MBD) near the N-terminus, a phosphorylation domain, a phosphatase domain, an actuator domain, eight trans-membrane helices and both amino and carboxy termini protruded into the cytosol (Lutsenko et al., 2007) (Figure 1.3).

The copper transporting P-type ATPases contain several specific motifs that are essential for copper transport. The NH2-terminal portion of the Cu-ATPases contains six repetitive sequences containing the sequence motif GMT/HCxxCxxxE. Each of these repeats form a subdomain with a single metal-binding site allowing a total stoichiometry of six copper ions per NH2-terminal domain (Cobine et al., 2000, DiDonato et al., 1997, Jensen et al., 1999, Lutsenko et al., 1997, Wernimont et al., 2004). The two Cys residues CxxC, located in the metal-binding motif, are the only two copper-coordinating ligands (DiDonato et al., 2000, Ralle et al., 1998) and mutations of the cysteines in the MBD5 or MBD6 of ATP7B have shown altered affinity of the intramembrane binding sites for copper (Huster et al., 2003).

The binding and hydrolysis of ATP is mediated through coordinated action of the A-domain (the actuator domain that contains the signature TGEA sequence motif essential for enzymatic function of P-type ATPase) and the ATP-binding domain. The ATP-binding domain contains the TGDN sequence motif that binds ATP and consists of two portions; the P-domain where the catalytic phosphorylation site is located with the invariant aspartic acid in the DKTG motif; and the nucleotide binding-domain with the conserved SEHPL motif (N-domain) (Lutsenko et al., 2007). These sequence motifs are conserved and essential for the ATPase activity and is characteristic for all P-type ATPases. The di-leucine motif is located in the C-terminal...
Figure 1.3: Interpretation of the structure and functional model of P-type ATPases

The ATP7A/7B proteins contain six metal-binding domains near the N-terminus (MBD 1-6), a phosphorylation domain (P-domain), a phosphatase domain (N-domain), an actuator domain (A-domain). The di-leucine motif is located in the C-terminal region (LL). In addition ATP7A/7B has eight trans-membrane helices and both amino and carboxy termini protrudes into the cytosol.

region and is involved in endocytosis (Lutsenko et al., 2007).

1.4.5.2. CTR1

CTR1 is a glycosylated trans-membrane protein consisting of three putative trans-membrane segments (Dancis et al., 1994, Lee et al., 2002, Pena et al., 2000) and forms oligomers with the functional unit of a trimer (Aller et al., 2004, Lee et al., 2002). The C terminus is localized within the cell and the N terminus is located in the extracellular space (Figure 1.4) (Elsses et al., 2002, Klomp et al., 2003, Puig et al., 2002). CTR1 is unlikely to be an ion pump nor a secondary transporter because ATP hydrolysis (Lee et al., 2002) or an ion gradient is not required for copper uptake. Two distinct copper uptake mechanisms has been suggested; the first, uptake of copper takes place by binding of copper on an essential site in the extracellular membrane surface followed by a conformational change which releases copper into the intracellular surface (Klomp et al., 2003). The second mechanism is by far the most supported which suggests the entry of copper through the plasma membrane into cells via the formation of a pore structure by the oligomerizaton of CTR1 (Aller et al., 2004, Dancis et al., 1994, Elsse et al., 2002, Elsse et al., 2005, Klomp et al., 2003, Lee et al., 2002, Pena et al., 2000, Puig et al., 2002, Zhou et al., 2001).

Several stretches of the CTR1 gene are structurally conserved in yeast, plants and mammals and highlights the functional importance of these regions for copper sensing, uptake and oligomerization believed to be important to mediate copper entry into cells. Most of the studies conducted to reveal the important functional motifs were mutagenesis experiments where vital protein residues were altered to observe copper uptake functions or protein folding and oligomerization. CTR1 contains an amino-terminal region rich in methionines arranged as MxxM and MxM motifs in the second transmembrane domain that has been suggested to be involved in copper binding prior to the transport of copper inside the cell (Dancis et al., 1994, Puig et al., 2002). The essentiality of the two conserved methionine residue in the second transmembrane domain of the CTR1 gene for the uptake of copper were demonstrated in mutagenesis experiments where it is required for high affinity transport of copper (Puig 2002). It appears that the third transmembrane domain is
Figure 1.4: Interpretation of the structure and functional model of Ctr1

Left (blue), diagram of a CTR1 monomer shows the extracellular N terminus, three putative transmembrane domains, and an intracellular C terminus. Two conserved amino acid motifs in the entire family of CTR copper uptake proteins, Met-X-X-Met in TM2 and Gly-X-X-Gly (GG4) motif in TM3. The extracellular amino-terminal domain contains two histidine-rich regions H1 and H2 (yellow squares) and two Mets motifs M1 and M2 (red oblongs). The cytoplasmic carboxy-terminal contains a conserved His-Cys-His motif, the putatively though to generate thermodynamic energy to move copper into the cell. Right, the symmetrical homotrimer of Ctr1 is shown where nine transmembrane forms a pore enabling the passage of copper ions across the lipid bilayer (each CTR1 monomer is represented by one colour).

responsible for the assembly of CTR1 into functional trimer that takes up copper into the cells (Aller et al., 2004). The GG4 motif in the third transmembrane is necessary for CTR1 processing and the formation of fully functional trimers.

In addition, a cluster of metal binding residues at the C-terminus has been shown to play a pivotal role in copper transport (Eisses et al., 2005). The carboxy-terminal portion of CTR1 protein is rich in charged amino acids and contains well conserved cysteines and histidines close to the carboxyl terminus. It has been suggested that the Cys-His motif which are located in the cytosol is responsible for the generation of thermodynamic energy that drives copper into the cells while preventing the entry of unwanted ‘free’ copper (van den Berghe et al., 2010).

The N-terminal end of the CTR1 protein contains 4 clusters of methionines and histidines which interact with copper (H1, M1, H2, M2). The M2 regions have shown to be important in copper transport when environmental levels of copper are low (Eisses et al., 2002, Guo et al., 2004, Liang et al., 2009, Puig et al., 2002). Moreover, the copper-stimulated endocytosis of the hCtr1 appears to be dependent on the M2 cluster located in the N-terminal end that is closest to the transmembrane region (Guo et al., 2004).

1.4.6 Genetics and Diseases Involving Copper Transporters

The importance of these transporters can be illustrated by serious human diseases resulting from defective copper transporter. Genetically defective ATP7A transporters give rise to a copper deficient disorder, Menkes disease, a fatal X-linked neurodegenerative disorder where the intestinal ATP7A transporter fails to transport dietary copper for absorption. Due to the lack of copper required for normal cellular functions, characteristics of patients with Menkes disease include poor psychomotor development, failure to thrive, seizures and abnormality in hair structure. Since brain growth and motor neurodevelopment requires copper particularly in the first 12 months of life. Untreated cases of Menkes disease are usually fatal (Mercer et al., 2001).
Wilson disease is associated with devastating neurological deficits caused by mutation of ATP7B, where ATP7B fails to pump excess copper out into the bile from the liver. This leads to pathological accumulation of copper and the clinical manifestation depends on the organs being saturated by copper. Typically liver failure is the first symptom to be seen, as liver is the first site of copper accumulation. Neurologic symptoms occur at a later stage after the saturation of copper in the liver. The deposit of copper in the eye results in a yellow-brown ring appearance in the cornea known as the “Kayser-Fleischer ring”. If the excess copper deposit is not removed from the body in a timely manner, the outcome is usually fatal, and fatalities are expected to occur within 9 months to 3 years of treatment cessation (Das et al., 2006).

In human there is no reported genetic disease associated with CTR1 mutations. This is not surprising given the demonstration of the essentiality of CTR1 for embryonic development in mouse. In the homozygous mutant Ctr1 knockout mouse early embryonic lethality was observed. These mice were shown to die in utero in mid-gestation suggesting a critical role for mCtr1 in copper homeostasis and embryonic development (Kuo et al., 2001, Lee et al., 2001). It is likely that a mutation in CTR1 in human results in a similar embryonic lethal phenotype since CTR1 appears to have an important role in the mammalian embryonic development.

1.4.7 Treatment of Wilsons and Menkes Disease

The pharmacological treatment for Menkes and Wilson’s disease aims is to restore the imbalance of copper in the body. Copper chelating agents are given to Wilson’s disease patients to remove the toxic deposit of copper. These agents include zinc, trientine, ammonium tetrathiomolybdate and D-penicillamine (Das et al., 2006). Copper supplements are given to patients with Menkes disease in the form of copper histidine (Kaler et al., 2008).
1.4.8 Regulation of Copper Transporters

Although the exact mechanism that controls the function of copper transporting ATPase is currently unclear (Lutsenko et al., 2007), current literature suggests that the regulation of copper homeostasis is achieved through posttranslational mechanism where changes in intracellular localization of these protein plays a role (van den Berghe et al., 2010). At basal copper levels the P-type ATPases, ATP7A and ATP7B, are localized within the perinuclear region of the cytoplasm (Dierick et al., 1997, Hung et al., 1997, Huster et al., 2003, Roelofsen et al., 2000, Yamaguchi et al., 1996). The trafficking of ATP7A and ATP7B from the cytoplasm to the plasma membrane has been reported when intracellular copper levels are elevated, subsequently allowing both ATP7A and ATP7B to act as a copper efflux transporter to eliminate excess copper within the cell (Cobbold et al., 2003, Lutsenko et al., 2003, Pase et al., 2004, Petris et al., 1996, Petris et al., 1999, Monty et al., 2005). Thus P-type ATPases transporters have dual function which is dependent on copper levels within the cell and the localization changes accordingly. At basal copper level ATP7A and ATP7B are localized in the cytoplasm and provide copper to the secretary vesicles, when copper level elevates, trafficking to the plasma membrane takes place and they act as an efflux copper pump. The trafficking of ATP7A is reversible, energy-dependent and appears to not require de novo synthesis of new protein because both protein and mRNA transcripts level of ATP7A remain constant (Petris et al., 1996). Silver molecule (Ag(I)) which is chemically similar to copper (Cu(I)) have shown to induce the trafficking of ATP7A to the plasma membrane (Solioz et al., 1995).

Under basal conditions human CTR1 protein is located on the surface of the plasma membrane and internalizes into the cell when copper levels are elevated. Similar to ATP7A and ATP7B, it appears that CTR1 is regulated posttranslationally and trafficking of CTR1 is an important regulatory mechanism for controlling copper homeostasis (van den Berghe et al., 2010). Elevated copper level has shown to stimulate the endocytosis (Guo et al., 2004, Petris et al., 2003) and degradation (Ooi et al., 1996, Petris et al., 2003) of the human CTR1 protein. Upon extracellular
copper level returning to baseline, internalized hCTR1 rapidly returns to the plasma membrane in as little as 30 minutes (Molloy et al., 2009). Thus, CTR1 responds rapidly to elevations of extracellular copper but also returns rapidly to allow for optimal uptake when the danger of excess copper is removed (Molloy et al., 2009). The uptake of copper by CTR1 is time and temperature dependent, saturable, stimulated by low extracellular pH and high potassium concentrations (Lee et al., 2002a). In addition, the transport of copper by CTR1 is specific for reduced copper (Lee et al., 2002a). Similar to ATP7A and ATP7B, silver molecules have shown to be taken up by CTR1 (Bertinato et al., 2010).

1.5. Evidence of Platinum Drug Transport by Copper Transporters

Although the transport mechanism of copper transporters are not well understood and that platinum drugs shares little structural similarity to elemental copper, multiple lines of evidence implicate a role for copper transporters in the transport of platinum drugs. Evidence for the proposition that platinum drugs enter cells and are distributed to various subcellular compartments via copper transporters is discussed below.

1.5.1 Altered Platinum Drug Transport in Genetically Engineered Cells

There is a strong body of evidence implying that copper transporters play a role in controlling the cellular movement of platinum drugs. The first study that implicated the role for copper transporters in the transport of platinum drugs was achieved by altering the expression of copper transporters to observe the effects on the cellular pharmacology of platinum drugs. There is now quite a large body of evidence to support this, derived from multiple cell models originating from different species where the expressions of copper transporters were manipulated through genetic engineering.

1.5.1.1. Yeast

Ishida et al was the first to show the link between copper efflux transporter, CTR1, and platinum drug transport. Using yeast cells Ishida et al demonstrated that the
deletion of the \textit{ycr1} lead to a reduction of cisplatin accumulation compared to the wild-type yeast cells. In addition, they also found a reduction in copper uptake with the co-incubation of cisplatin and the cytotoxicity of cisplatin was reduced when the cells were co-incubated with copper sulphate (Ishida et al., 2002). Since then similar findings were reported; Lin \textit{et al} showed a reduction in both cellular uptake of cisplatin and DNA-platinum adduct formation with the deletion of \textit{ycr1} (Lin \textit{et al.}, 2002). In addition the cells with the deletion of \textit{ycr1} acquired markedly increased resistance to cisplatin, carboplatin and oxaliplatin compared to the wild type yeast cells (Lin \textit{et al.}, 2002).

1.5.1.2. Mouse

Copper transporter mediated platinum drug transport has been investigated using mouse embryonic fibroblasts. Platinum accumulation was found to be reduced in the heterozygous \textit{mCtr1}^{+/−} and homozygous \textit{mCtr1}^{−/−} mouse embryonic fibroblasts by approximately 35% and 70% respectively compared to the wild type (Ishida \textit{et al.}, 2002). In addition, cisplatin resistance were increased by several fold compared to the wild type cells after 2 hours of cisplatin exposure (Ishida \textit{et al.}, 2002). Other research groups reported similar findings with homozygous \textit{mCtr1}^{−/−} mouse embryonic fibroblasts where the cellular accumulation and cytotoxicity of cisplatin were found to be reduced compared to the wild type (Holzer \textit{et al.}, 2006, Ishida \textit{et al.}, 2010, Larson \textit{et al.}, 2009, Samimi \textit{et al.}, 2006).

Larson \textit{et al} also demonstrated the link between copper transporters and platinum drugs by re-expressing \textit{hCTR1} in \textit{mCtr1}^{−/−} mouse embryonic fibroblasts and showed reversal of cell sensitivity to the growth-inhibitory effects of cisplatin to a level similar to the wild type cells. Moreover, with the re-expression of \textit{hCTR1} to the \textit{mCtr1} knockout, the initial influx rate of cisplatin was restored. Finally, they also demonstrated that the \textit{mCtr1} knockout had a more prominent effect on the initial drug influx than the ensuing steady-state level of cisplatin, carboplatin and oxaliplatin (Larson \textit{et al.}, 2009).
However contradicting results has also been reported, Holzer et al reported that although mCtr1 knockout in mouse embryonic fibroblasts reduced cellular platinum uptake significantly, it did not alter cell sensitivity to the growth inhibitory effect of carboplatin and oxaliplatin (Holzer et al., 2006).

1.5.1.3. Human

1.5.1.3.1. CTR1

Different isogenic human carcinoma cell line has been utilized to study the involvement of copper transporters in the transport of platinum drugs. In the human small cell lung cancer line SR2, overexpression of CTR1 significantly increased the uptake of cisplatin, carboplatin and oxaliplatin, and increased the sensitivity of the cells to cisplatin and carboplatin but not oxaliplatin (Song et al., 2004). Two research groups showed an increase in cisplatin cellular uptake in human ovarian carcinoma cells, A2780-hCTR1, when compared to the empty vector-transfected mock cells (Holzer et al., 2004). It has been shown independently that the overexpression of CTR1 enhances drug toxicity in the SR2 and A2780-hCTR1 cell line (Holzer et al., 2004, Song et al., 2004). Recently, More et al investigated the accumulation of platinum in hCTR1-overexpressing HEK293 cell line and found a threefold increase in cisplatin accumulation compared to mock cell. In addition, they reported the inhibition of CTR1-stimulated cytotoxicity; cell platinum uptake and DNA adduct formation in HEK293-hCTR1 by copper sulphate (More et al., 2010).

1.5.1.3.2. ATP7A and ATP7B

Copper efflux transporters, ATP7A and ATP7B, are linked to the resistance of platinum drugs. There is ample evidence to support that ATP7A and ATP7B transports platinum out of cells or into specific sub-cellular compartments. Studies have found that with the overexpression of ATP7B, an 8.9-fold higher resistance to cisplatin were conferred to human epidermoid KB carcinoma cells (Komatsu et al., 2000). Furthermore, the efflux-dependent cellular cisplatin accumulation was reduced by approximately half compared to the mock cells (Komatsu et al., 2000). Katano et al reported that the overexpression of ATP7B in human ovarian carcinoma
cells 2008 and IGROV-1, and head and neck squamous carcinoma cell UMSCC10b resulted in an increase resistance to both cisplatin and carboplatin (Katano et al., 2003). They also demonstrated decreased steady state cellular level of \([^{14}\text{C}]\text{carboplatin}\) and a more rapid platinum efflux with the 2008-ATP7B overexpression cell line compared with the mock transfectants (Katano et al., 2003). Utilizing siRNA-mediated gene silencing to knockdown of ATP7B in parental line of A2780 and its cisplatin-resistant subline, resulted in dramatic increase in the sensitivity to cisplatin and DNA adduct formation (Mangala et al., 2009).

The human fibroblast cell line Me32a is derived from Menkes’ disease patient and lacks the expression of both ATP7A and ATP7B. Me32a sublines stably expressing ATP7B and ATP7A, MeWND and MeMNK respectively, are available and have been employed for the investigation of platinum drug transport by ATP7A and ATP7B (Rabik et al., 2009, Samimi et al., 2004, Samimi et al., 2006). The ATP7B-expressing MeWND cells were significantly more resistant to cisplatin, carboplatin and JM118 when compared to the parental Me32a cell line (Rabik et al., 2009, Samimi et al., 2004, Samimi et al., 2006). However these studies also showed conflicting results which did not support the idea that ATP7B effluxes platinum drugs out of cells. The results from these studies showed that the ATP7B expressing cell lines accumulated more platinum drugs compared to Me32a (Rabik et al., 2009, Samimi et al., 2004, Samimi et al., 2006). Samimi et al and Rabik et al reported inconsistent findings in ATP7B-expressing MeWND cells where oxaliplatin-induced cytotoxicity, platinum uptake and DNA adduct formation were not changed in one study (Rabik et al., 2009) but increased in another (Samimi et al., 2004). It has been suggested that the reduction in the formation of DNA adducts in the ATP7B expressing MeWND cells might be due to the potential effect of ATP7B sequestrating drugs from the cytoplasm into vesicular structures thus preventing them from reaching DNA targets (Samimi et al., 2006).

ATP7A has also been identified to play a role in platinum drug sensitivity and resistance. While platinum drug accumulation were greater for cisplatin, carboplatin and oxaliplatin in the ovarian carcinoma 2008 cells expressing ATP7A, the same study
also reported an increase in resistance to these platinum drugs of cisplatin compared to the mock cell lines (Samimi et al., 2004). A possible explanation is that ATP7A, like ATP7B, also sequesters platinum drugs into cytoplasmic vesicles and prevents cytotoxic effects of platinum drugs in the ovarian carcinoma 2008 cells. In noncancerous cell lines, namely MeMNK and Chinese hamster ovary CHO-K1-ATP7A, the overexpression of ATP7A has shown to increase the resistance to platinum drugs (Owatari et al., 2007, Rabik et al., 2009, Samimi et al., 2004, Samimi et al., 2006). Conversely siRNA knockdown of ATP7A in human gastric adenocarcinoma subline S3 reversed the resistance to both oxaliplatin and cisplatin (Rabik et al., 2009).

1.5.2 Correlation of Expression of Copper Transporters and Platinum Drug Resistance

Indirect evidence to support the idea that platinum drugs are transported by copper transporters is the correlation between the expressions of copper influx and efflux transporters in platinum-resistant cells or tumours. A common characteristic of cells with the acquired platinum drug resistance is reduced drug accumulation (Andrews et al., 1990, Kartalou et al., 2001), and an important mechanism that controls drug accumulation is the transport system. Many studies have reported that platinum drug-resistant cells are associated with a lower expression of copper influx transporter, CTR1, or an overexpression of copper efflux transporter, ATP7A/ATP7B.

The decrease in expression of copper influx transporter, CTR1, has been linked to altered intracellular platinum drug concentration. Song et al investigated the mRNA expression of CTR1 in a panel of cisplatin-resistant carcinoma cell sublines of lung, colon and glioblastoma and found lower platinum uptake compared with its sensitive parental line (Song et al., 2004).

Furthermore, platinum-resistant cells have been reported to have higher expressions of copper efflux transporter, ATP7A and ATP7B. The overexpression of ATP7A in a gastric cancer cell line has been attributed to oxaliplatin resistance (Chen et al., 2007). The impaired uptake and resistance of cisplatin in human prostate carcinoma PC-5 cells were linked to the endogenous overexpression of ATP7B (Komatsu et al.,
Yoshizawa et al reported that the overexpression of ATP7B in oral squamous cell carcinoma cells is linked to acquire platinum drug resistance (Yoshizawa et al., 2007). Lastly, cross-resistant to cisplatin has been found in copper-resistant CuR27 hepatoma cells. Compared with the parental HuH7 cells with a normal ATP-7B expression, the overexpression of ATP7B was linked to a reduction in intracellular platinum accumulation (Safaei et al., 2005).

1.5.3 Evidence of Platinum Drug Transport by CTR1 from In Vivo Animal Models

The in vivo roles of copper influx transporter, CTR1, in platinum drug uptake has been investigated using a tumor xenografts model in mice (Jandial et al., 2009, Larson et al., 2009) and mouse model of human cervical cancer (Ishida et al., 2010). These studies demonstrated the importance of CTR1 in the uptake and antitumour activities of platinum drugs in the whole animal model.

The antitumor efficacy cisplatin was shown to be dependent on CTR1 expression by Larson et al in a mice tumour xenograft model. The \(\text{ctr1}(-/-)\) xenografts failed to respond to the treatment of cisplatin but the growth of the \(\text{ctr1}(-/-)\) xenografts were reduced by a single maximum tolerated dose of cisplatin once CTR1 levels were restored by a lentivirus infection (Larson et al., 2009).

Jandial et al prevented the cisplatin-induced down-regulation of \(h\text{CTR1}\) with a proteasomal inhibitor, bortezomib, and showed that the i.p., administration of bortezomib before i.p. cisplatin increased platinum accumulation in peritoneal tumors by 33% in an in vivo murine model of human 2008 tumor xenografts.

In the study by Ishida et al, tetrathiomolybdate, a copper chelator was shown to enhance the in vivo antitumour activity of cisplatin by increasing platinum–DNA adduct levels in xenograft tumors of HPV16/E2 mice bearing human cervical cancer (Ishida et al., 2010). The results linked the expression of CTR1 to cisplatin accumulation and tumor sensitivity because it has been previously reported that copper chelators prevents CTR1 degradation in vitro (Ishida et al., 2010).
1.5.4 CTR1 Expression and Association to Platinum Drug-induced Toxicity

Recent reports have implicated the link between CTR1 and platinum drug-induced toxicities. A recent report revealed association of copper transporter to cisplatin-induced ototoxicity. The hallmark of ototoxicity includes blebbing and loss of outer and inner hair cells, degeneration of stria vascularis, and loss of spiral ganglion cells. These pathological abnormalities have been correlated with accumulation of platinum in the cochlear and CTR1 was found to be abundantly expressed in the inner ear, the site of primary damage in cisplatin-induced ototoxicity (More et al., 2010). More et al showed that intratympanic administration of copper sulphate, a substrate for CTR1 transporter, prior to intraperitoneal administration of cisplatin in the mice prevented hearing loss at click stimulus and 8, 16 and 32 kHz frequencies. The results from this study suggest that CTR1 is involved in the mechanism of ototoxicity induced by cisplatin via transporting cisplatin into the site of damage (More et al., 2010).

Moreover, Pabla et al demonstrated that the cisplatin-induced nephrotoxicity were ameliorated by the deletion of Ctr1 in the mouse model and that the expression of CTR1 in mice was localized to the basolateral membrane, the site of cisplatin uptake (Pabla et al., 2009). Based on these results Pabla et al concluded that the cisplatin-induced nephrotoxicity may be due to CTR1 mediated transport into the site of damage in the kidney (Pabla et al., 2009).

1.5.5 Associations between the Expression of Copper Transporters and Clinical Platinum-based Chemotherapeutic Outcomes

Several reports from clinical studies have established the association between copper transporter expression and patient outcomes from platinum-based chemotherapy. In these studies, tumour specimens from cancer patients were analyzed for the level of copper transporter expression by the means of RT-PCR (Ishida et al., 2010, Martinez-Balibrea et al., 2009, Nakayama et al., 2002), and immunohistochemical analysis (Miyashita et al., 2003, Nakayama et al., 2004,
Specimens from several types of cancer were examined including ovarian carcinoma (Ishida et al., 2010, Nakayama et al., 2002, Nakayama et al., 2004, Samimi et al., 2003), head and neck oral cavity squamous cell carcinoma (Miyashita et al., 2003) and advanced colorectal cancer (Martinez-Balibrea et al., 2009). The expression of ATP7A (Samimi et al., 2003), ATP7B (Martinez-Balibrea et al., 2009, Miyashita et al., 2003, Nakayama et al., 2002, Nakayama et al., 2004) and CTR1 (Ishida et al., 2010) were investigated.

High tumour expression of platinum efflux transporters was consistently associated with poorer clinical outcomes from platinum-based chemotherapy across several cancer types. It has been reported that a high expression of ATP7B is associated with shorter survival (Miyashita et al., 2003, Nakayama et al., 2002, Nakayama et al., 2004), lower tumour response (Miyashita et al., 2003, Nakayama et al., 2004), and a poor grade of tumour differentiation (Nakayama et al., 2002, Nakayama et al., 2004). Conversely, Martinez-Balibrea et al showed that a lower ATP7B mRNA and protein expression level were associated with a higher tumour response and had a longer time to disease progression (Martinez-Balibrea et al., 2009). Samimi et al reported that a higher ATP7A protein expression in ovarian cancer tumor samples was associated with shorter patient survival (Samimi et al., 2003).

The expression of copper influx transporter, CTR1, to the clinical outcomes of ovarian cancer treated with platinum-based chemotherapy was examined by Ishida et al. It was reported that a higher expression of CTR1 in tumours is associated with a better efficacy of platinum-based therapy (Ishida et al., 2010).

These clinical findings suggest that copper efflux pumps transport platinum drugs out of cells or into specific sub-cellular compartments resulting in a reduction in cell sensitivity to platinum drugs and causing clinical treatment resistance. In contrast copper influx transporter act to influx platinum drugs and is a determinant of tumour sensitivity to platinum drugs.
1.6. Summary and Aim of Thesis

A major dose-limiting toxicity of oxaliplatin drug treatment is neuropathy and the primary site of damage occurs in the cell bodies of the sensory neurons of the dorsal root ganglion (Cavaletti et al., 1998, Cavaletti et al., 2001, Gregg et al., 1992, Holmes et al., 1998, Jamieson et al., 2005, Krarup-Hansen et al., 1999, Luo et al., 1999a, McDonald et al., 2005, McKeage et al., 2001, Ta et al., 2006, Thompson et al., 1984). At present, the mechanisms of toxicity for oxaliplatin induced neuropathy remain unknown. The main pharmacological action of platinum drugs does not adequately explain the selective damage to dorsal root ganglion cells since these neurons are post-mitotic and does not undergo DNA replication. Recent evidence suggests that platinum drugs may bind and competitively inhibit copper transporters but their expression in the dorsal ganglia have never been considered before now. Little is currently known about the expression of copper transporters in the dorsal root ganglion, the cell bodies of primary sensory neurons which become damaged by platinum drugs. We hypothesized that oxaliplatin might induce neuropathy by utilizing copper transporters to move in and out of DRG neurons. With this background, the purpose of this thesis is to study copper transporters expression in the cells that is primarily damaged by platinum drug-induced neuropathy. In addition, to test copper related treatments for the prevention of platinum drug induced toxicity. The goal of this thesis was to provide insights to mechanisms of neurotoxicity induced by oxaliplatin involving copper transporters.

Therefore, the overall aims of this thesis were:

- To investigate the expression of copper transporters in dorsal root ganglion neurons of rats in both healthy control Wistar rats and those treated with oxaliplatin
- To investigate the efficacy of copper modifying compounds and substrates for copper transporters for ameliorating oxaliplatin induced neuropathy in a Wistar rat model
- To investigate the expression of copper transporters in untreated and oxaliplatin treated primary cultures of rat dorsal root ganglion neurons
To hypothesize potential mechanism by which copper transporters are associated with oxaliplatin induce peripheral neuropathy

In order to achieve these overall aims, experiments were carried out to:

- Examine the expression of copper transporters in dorsal root ganglion tissue of untreated and oxaliplatin treated Wistar rats
- Investigate copper transporter expression in the dorsal root ganglion neuronal subpopulation that are damaged by oxaliplatin
- Determine if the co-administration of agents that altered copper levels with oxaliplatin may ameliorate oxaliplatin-induced neurotoxicity in a Wistar rat model
- Study copper transporter related mechanism of oxaliplatin-induced neuropathy using an ex-vivo rat model
Chapter 2

Materials and Methods

2.1. Animals

Animals were housed by the Vernon Jansen Unit at the University of Auckland in a temperature controlled environment on a 12 hour light-dark cycle with access to food and water ad libitum. A maximum of 6 animals were housed in each cage and the animals treated with oxaliplatin were checked regularly for signs of toxicity. Animals showing signs of toxicity were immediately euthanized. The Animal Ethics Committee of the University of Auckland approved all animal procedures (AEC No. R591, R432, R828) and all animals experimental procedures were carried out in compliance with the ethical guidelines.

2.2. Drug Treatments

For oxaliplatin treatment studies, animals were treated with a 1.85 mg/kg dose of oxaliplatin twice weekly for 8 weeks. Aged-matched female Wistar rats that weighted approximately 280 g were used in the beginning of the experiment. Oxaliplatin as injection solution of Eloxatin (50mg/10mL, Donated by Oncology Unit from Auckland City Hospital) was diluted in 5% dextrose (Baxter Healthcare, Old Toongabbie, Australia) and was administered to rats intraperitoneally (i.p.) at an injection volume of 10 ml/kg. To avoid chronopharmacologic variations, injections were performed between 1400 and 1600 hours on Wednesday and Friday. Control animals were treated with the vehicle control solution of 5% dextrose.
2.3. Paraformaldehyde Perfusion and Dissection

For euthanasia at the end of study, an intraperitoneal dose of pentobarbitone was used (0.9 mL of 3 mg/mL; Chemstock Animal Health Ltd., Christchurch, New Zealand). After onset of deep anaesthesia, which is indicated by loss of righting reflexes and pain response, the chest of the rat was cut open and a 16 gauge needle inserted into the left ventricle of the heart. The left and right atria were cut and 60 ml of saline was perfused into the heart via the needle followed by 60-120 ml of 4% paraformaldehyde (PFA; Sigma-Aldrich) in 0.1 M phosphate buffer. Following perfusion, the spine of the animal was exposed by cutting the skin along its back. A laminectomy was performed to uncover the DRG. Lumbar 5 (L5) DRG were carefully dissected from their centrally- and peripherally-directed nerve fibres under a dissection microscope and were stored in 4% PFA.

2.3.1 Dissection for Total RNA Extraction

A separate perfusion protocol was used when extracting RNA to avoid potential PFA interference. Deep anaesthesia was induced as above by an intraperitoneal injection of 0.9 ml of 3 mg/ml pentobarbitone. The animal were cut open to expose lumbar DRG, brain, spinal cord, liver, kidney and small intestine for dissection. The dissected tissues were washed in cold PBS and immediately snap-frozen in liquid nitrogen and stored at -80°C until ready for homogenization.

2.3.2 Dissection for Primary Culture of DRG Neurons

For primary culture of DRG neurons 19 to 21 day old rats were used. Following the onset of deep anesthesia, a large transverse incision was made down the middle of the back skin to expose the spine. The spinal column was removed from the animal and hemisected to uncover the spinal cord and DRG. Lumbar 1 – 6 (L1 – L6) DRG were carefully microdissected from connecting central and peripheral nerve fibres and adherent connective tissues and kept in ice-cold Leibovitz L-15 medium supplemented with 10% horse serum (Invitrogen, Carlsbad, CA, USA), 100 U/mL
penicillin, 0.1 mg/mL streptomycin, and 2 mM glutamine. DRG from 3 – 4 animals were pooled together for subsequent cell isolation procedures.

2.4. Histology

L5 DRG were removed from storage in 4% PFA and were washed in Milli-Q water for 20 minutes, dehydrated in 70%, 95% and 100% ethanol for 1 hour each, and cleared in xylene for 10 minutes. After tissue processing, L5 DRG were stored in liquid paraffin wax for 1 hour, and were then embedded in an embedding mould with paraffin wax. The embedding moulds were left to cool on a cold plate to allow the wax to solidify. On solidification, the wax blocks were removed from the embedding moulds and a microtome (Leica RM 2145, Nussloch, Germany) was used to cut each dorsal root ganglion into 6 µm sections. The sections were placed in a warm water bath and transferred to slides coated with poly L-lysine (Sigma-Aldrich), which were subsequently dried in a 50°C oven for 50 minutes. Approximately 100 sections were collected from each DRG.

For morphometric evaluation, slides containing paraffin sections of L5 DRG were stained with haematoxylin and eosin. In preparation for staining, slides were dewaxed in xylene for 10 minutes, and were rehydrated by undergoing 12-20 dips in 100% ethanol, 95% ethanol and 70% ethanol, prior to being washed in Milli-Q water. The slides were stained with Gill’s haematoxylin for 4 minutes, washed in Milli-Q water before and after a 1 minute wash in Scott’s tap water, and stained with Moore’s eosin for 1 minute. The slides were then dehydrated in 95% and 100% ethanol for 12-20 and 6-10 dips, respectively and for 10-15 dips in a 1:1 ethanol/xylene mixture. Finally, slides were cleared in xylene for at least 10 minutes and coverslipped with DPX mounting medium (Scharlau Chemie S. A.).

2.4.1 Immunohistochemistry

For immunohistochemistry procedures, DRG were sectioned onto frozen slides. Animals were perfused and DRG removed as described in section 2.4. Dissected DRG were post-fixed in 4% PFA for 2-6 hours then cryoprotected in 30% sucrose in PBS
solution overnight or until the tissues sunk. Once sunken, the DRG were immersed in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA), and snap-frozen in liquid nitrogen for 10 seconds. The frozen DRG were then stored at -80°C prior to sectioning. Each dorsal root ganglion was sectioned on a cryostat (Leica CM 3050) at a thickness of 10 µm at -18°C. A total of 4 or 5 sections were transferred on to each polylysine-coated slide. Each section was separated from its adjacent section by at least 60 µm to reduce the likelihood of a DRG neuronal cell body being present in more than 1 section on the same slide. After sectioning, the slides were stored at -80°C prior to immunostaining.

2.5. DRG Morphometry

For each morphometric procedure, DRG sections stained with haematoxylin and eosin were examined under an Axiostar light microscope (Carl Zeiss Vision, Hallbergmoos, Germany). The light microscope was attached to an Axiocam camera (Carl Zeiss Vision), which captured digital images and displayed them on a computer screen. All analyses and measurements of DRG morphometry were performed using AxioVision 3.0 software (Carl Zeiss Software).

2.5.1 Measurement of Cell Body and Nucleolus Area

For defining the morphometry of neurons expressing a protein of interest, the cell body area was measured for approximately 1000 cells that contained a nucleus. The frequency of cell body area for both non- and immuno-reactive neurons was measured and categorized into three size-based groups: small (<750 µm²), medium (750-1750 µm²) and large (>1750 µm²) based on similar studies (Bergman et al., 1998, Price et al., 1985, Zhang et al., 1994).

For assessing oxaliplatin treatment effects, each DRG was cut to yield approximately 100 sections. Ten sections at regular intervals were randomly selected for analysis. In each of these 10 sections, the largest 3-4 cells with visible nucleoli were measured along with their cell body area. From these, the 10 largest cells for each dorsal root ganglion were selected and the average value of their nucleolus size was calculated.
In the primary culture of DRG neurons study reported in chapter 6, the effect of the oxaliplatin or copper compounds on the cell body area size of the neurons was determined by measuring the first approximately 1000 cells that get randomly captured on the microscope containing both non- and immuno-reactive neurons.

2.6. **Immunohistochemistry**

2.6.1 **Single Label Immunohistochemistry**

Twelve-µm DRG cryosections mounted onto poly-L-lysine pre-coated slides were warmed to room temperature for approximately 20 minutes then processed with 0.2% Triton X-100 in PBS. Next, the slides were incubated in 1% hydrogen peroxide in 50% methanol for 10 minutes to prevent endogenous peroxidase activity, followed by 3 successive 5 minute washes in PBS. If excessive background staining was known to occur, the slides were placed in a blocking buffer containing PBS with 3% normal goat serum (Sigma-Aldrich) or 3% normal donkey serum (Sigma-Aldrich) and 20 mg/ml bovine serum albumin (ICPbio Ltd, Auckland, New Zealand), 0.2% Triton X-100 for 1 hour to block non-specific binding. Next, the slides were incubated over-night in a humidity chamber with either the chicken anti ATP7A (1:1000; ab13995 from Abcam, Cambridge, MA), rabbit anti ATP7B antibody (NB100–360, Novus Biologicals), rabbit polyclonal anti-hCTR1 primary antibody (1:500, Novus Biologicals, Littleton, CO, USA) or anti-phosphorylated neurofilament heavy subunit (pNF-H) antibody (1:100, Swant, Bellinzona, Switzerland) diluted in immunobuffer overnight at room temperature. Following several washes in PBS containing 0.2% Triton X-100, slides were incubated with either a biotinylated anti-chicken (1:500 Jackson 703-065-155) or anti-rabbit antibody (1:500, Sigma), an extravidin-peroxidase conjugate (1:500, Sigma) diluted immunobuffer. Three further 5 minute washes in PBS were performed before slides were incubated in a humidity chamber for 3 hours with an extravidin-peroxidase conjugate (1:500; Sigma-Aldrich) diluted in immunobuffer. The tertiary antibody was washed from the slides in 3 rinses in PBS for 5 minutes each, then staining was visualised with 3 - 10 minute incubation of 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich). Following 3 final 5 minute washes in PBS, the slides were dehydrated through 5 minute rinses in 70%, 95% and 100% ethanol,
before being cleared for 5 minutes in 1:1 ethanol/xylene and for 10 minutes in xylene. The slides were then coverslipped with DPX mounting medium and analysed by light microscopy. All steps in this procedure were performed at room temperature. Sections incubated without primary antibodies served as a control.

2.6.2 Fluorescent Double-Labelling Immunohistochemistry

For immunofluorescence staining, after H₂O₂ exposure and non-specific blocking DRG sections were incubated in 200 µl Invitrogen Image-iT FX signal enhancer for 30 min. The abovementioned chicken anti ATP7A (1:1000, Abcam), anti-hCTR1 antibody (1:1000, Novus) or anti-phosphorylated neurofilament heavy subunit (pNF-H) antibody (1:100, Swant, Bellinzona, Switzerland) was applied to the DRG sections for 48 h at 4°C, followed by either Alexa Fluor 594-labeled anti-chicken, Alexa Fluro 594-labeled anti-mouse, Alexa Fluro 488-labeled anti-rabbit IgG (H+L) (1:500, Invitrogen) or DyLight 488-labeled anti-chicken IgG, for 3 h. Then the sections were coverslipped with Vectorshield anti-fade mounting medium (Vector Laboratories, Burlingame, CA) and stored overnight at 4°C to prevent bleaching. Apart from cooling on storage, all steps in this procedure were carried out at room temperature. Reciprocal omission controls were included to ensure there was no cross-bleeding between the channels. Fluorescent images were acquired using a Leica DMR upright fluorescence microscope (Leica Microsystems, Wetzler, Germany) with a cooled colour Nikon digital camera attached, and analyzed using Nikon EclipseNet and ImageJ software (National Institutes of Health, USA).

2.6.3 Imaging and Analysis

Standard non-fluorescent immunolabelling was analysed by light microscopy using AxioVision 3.0 software. Digital images were obtained using an Axiocam digital camera attached to an Axiostar light microscope and analyzed using Axiovision 3.0 software on a PC (Carl Zeiss, Hallbergmoos, Germany). The cross-sectional area was measured for every DRG neuron containing a nucleus or nucleolus that expressed immunostaining. DRG cells were again categorised on the basis of size into small- (<750 µm²), medium- (750-1750 µm²) and large-sized (>1750 µm²) cells. Finally, the
frequency of immunostaining for each immunohistochemical marker was generated by counting every cell and expressing the frequency of immunoreactive neurons as a percentage of the total cell count.

For double-labelling experiments, fluorescent images were acquired using a Leica DMR upright fluorescence microscope equipped with fluorescent rhodamine (red) and FITC (green) filters with excitation wavelength ranges of 534-558 nm and 450-490 nm, respectively (Leica Microsystems, Wetzler, Germany). A cooled colour Nikon digital camera was attached; images were captured and analyzed using Nikon EclipseNet and ImageJ software (National Institutes of Health, USA).

2.7. Western Blot Analysis

Following euthanasia of animals with intraperitoneal injection of pentobarbitone (0.9 mL of 3 mg/mL; Chemstock Animal Health Ltd.), lumbar DRG tissues were dissected and homogenized using a Dounce homogenizer (Glas-Col) for 3 min in a lysis buffer containing 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 0.1% SDS, and a protease inhibitor mixture (Complete Mini Protease Inhibitor Cocktail tablets, Roche Diagnostics, Indianapolis, IN, USA). The homogenate was centrifuged at 500 \( \times g \) for 15 min at 4°C to remove nuclei and large particulate matter and the protein concentration of the resulting supernatant was determined by a bicinchoninic acid (BCA) assay as previously reported (Liu et al., 2008). Protein samples (40 µg) were heated at 95°C for 30 min, resolved in 8% SDS-PAGE, and then transferred to a nitrocellulose membrane (Amersham Pharmacia) using a Transblot SD apparatus (Bio-Rad). Following blocking with 5% milk/bovine serum albumin solution, ATP7A was detected by chemiluminescence using anti-ATP7A antibody (1:1000, no. ab13995: Abcam, Cambridge, UK), horseradish peroxidase (HRP)-conjugated anti-chicken antibody (Sigma-Aldrich) and the ECL Advance Detection reagent (Amersham Biosciences). Beta actin was probed to determine the equal loading using anti-beta actin antibody (Abcam) and a HRP-conjugated anti-rabbit IgG antibody (Amersham).
2.8.  Reverse transcription – Polymerase Chain Reaction (RT-PCR)

2.8.1.  RNA Extraction

Total RNA was isolated from rat tissues using an Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules, CA, USA). The manufacturer’s suggested protocol was followed. Tissues were rapidly collected after dissection and homogenized in PureZol reagent by a Dounce homogenizer (Glas-Col, Terre Haute, IN, USA). Tissues were further homogenised by being passed through a 20-gauge needle and syringe. The homogenised sample was incubated for 5 min followed by centrifuged at 12000g for 10 min. Chloroform was added to the supernatant and shaken for 5 min prior to centrifugation at 12000g for 15 min. Equal volume of 70% ethanol was mixed into the resultant upper aqueous phase and was transferred to a RNA binding column and centrifuged at 12000g for 30 seconds. Low stringency, DNase I (1 unit/µg, Bio-Rad) and high stringency were centrifuged through the column individually before total RNA was eluted in 40–60 µl of elutions solution.

The concentration and purity of the RNA were determined using a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies). The purity of the RNA sample was determined by measuring the absorbance of the sample at the wavelengths of 260 nm (A_{260}) and 280 nm (A_{280}). An A_{260}/A_{280} ratio greater than 1.8 indicated that the RNA samples were pure.

2.8.2.  cDNA Synthesis

Total RNA of each sample (0.25 µg) was reverse-transcribed into cDNA using a SuperScript first strand synthesis kit (Invitrogen, Carlsbad, CA, USA) according to instructions, followed by digestion. Following digestion with DNase I (1 unit/µg, Bio-Rad) to eliminate residual genomic DNA from the RNA sample prior to the onset of RT-PCR, total RNA of each sample (0.25 µg) was reverse-transcribed into cDNA using a SuperScript first strand synthesis kit (Invitrogen) according to instructions. Briefly, EDTA (25mM) was added to the sample and heated to 65°C to inactivate DNase 1. RNA was then denatured at 65°C in the presence of free nucleotides. First strand
cDNA synthesis from the isolated RNA template was primed with oligo(dT<sub>12-18</sub>) in a reaction catalysed by superscript II reverse transcriptase. Ribonuclease H (Invitrogen) was used to digest the cDNA product to remove the RNA templates. A sample without reverse transcriptase was prepared as control for the presence of residual genomic DNA.

### 2.8.3. Amplification of cDNA by PCR

cDNA was amplified by PCR in a reaction mixture containing dNTP, MgCl<sub>2</sub>, Platinum Taq DNA polymerase (Invitrogen) and custom primers, using a GeneAmp 9700 PCR System (Applied Biosystems, Foster City, CA, USA) at 52°C for 40 cycles. Forward and reverse primers for rat Atp7a were: 5’-tag acg gca tgc att gta aat c-3’ and 5’-tgg att tta cac ctg gct tct t-3’ (amplicon of 375 bp); for rat Atp7b were 5’-att cca gga ctg tcc gtt cta a-3’ and 5’-cac ttg ctc ctc tct gag gat t-3’ (amplicon of 396 bp); for rat Ctr1 were: 5’-ttg gct tta aga atg tgg acc t-3’ and 5’-cat aag gat ggt tcc att tgg t-3’ (amplicon of 206 bp); and for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5’-tgc tga gta tgt cgt gga gtc t-3’ and 5’-aca gtc ttc tga gtg gca gta a-3’ (amplicon of 291 bp), as a control for the densitometric analysis.

### 2.8.4. Analysis of Amplified RT-PCR Products

Amplified PCR products analyzed by gel electrophoresis on a 2% agarose gel at 110V using a EC 250-90 power supply (Thermo electron Corporation, San Jose, CA, USA) after staining with ethidium bromide (Invitrogen) and photographed using Gel Doc 2000 System (Bio-Rad). A 1 kb plus DNA ladder (Invitrogen) was ran beside the RT-PCR products for the determination of band size. Each band was quantified by densitometry analysis using Image J software (National Institute of Health, Bethesda, MD, USA).

### 2.9. Real-Time PCR (qPCR)

Multiplex real-time PCR was performed using ABI PRISM 7900HT Sequence Detection Systems and SDS 2.3 software (Applied Biosystems). Primers and probe sets were purchased as TaqMan Gene Expression Assays containing forward and reverse
unlabelled PCR primer pair and a fluorescent reporter dye-labelled TaqMan MGB probe (Table 2.1). Samples were analyzed in triplicate in 10-µl total volume containing 2× TaqMan universal PCR Master Mix, 20× TaqMan FAM-labelled probe for rat \(Atp7a\) gene, 20× TaqMan FAM-labelled probe for rat \(Atp7b\) gene, 20× TaqMan FAM-labelled probe for rat \(Ctr1\) gene, 20× VIC-labelled 18S ribosomal RNA as endogenous control probe, and 25 ng of cDNA. The abundance of mRNA of ATP7A, ATP7B, CTR1 or rRNA was measured as the threshold cycle values (Ct) after each reaction. The relative RNA expression level was calculated using the \(2^{-\Delta Ct}\) method (Livak et al., 2001), where gene of interest expression normalized to 18S rRNA was reported. \(\Delta Ct = (Ct_{ATP7a, ATP7b, CTR1} - Ct_{rRNA})\).

2.10. Metal Analysis in Plasma and Tissues

Plasma, DRG, brain, spinal cord, sciatic nerve, liver, kidney and intestine were removed and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) to determine if oxaliplatin given alone or with copper histidine altered the tissue platinum or copper content. All plasma and tissue content was determined from standards made up in the relevant matrix.

2.10.1. Collection of Blood Plasma Samples for Oxaliplatin Pharmacokinetic Study in Rats

After the last dose of oxaliplatin at the end of the 8 weeks treatment 7 blood samples were taken from the tail vein to prepare plasma. Approximately 100µl of blood was collected at 0, 1, 15, 30 45 60 and 120 minutes after dosing using a 20-gauge needle and were transferred into heparinised tubes to prevent clotting. These samples were then centrifuged at 5000 rpm for 10 minutes and equal volume of cold methanol was added to the resultant plasma to precipitate out proteins remaining in the plasma samples. The samples were then subjected to centrifugation at 5000 rpm for 10 minutes before the collection of supernatant and storage at -80°C prior to ICP-MS analysis.
2.10.2. Collection of Tissue Samples for Measurement of Levels of Platinum, Copper and Other Metals

Ninety-six hours after the last oxaliplatin dose, animals were exsanguinated and L4, L5 and L6 DRG, brain, spinal cord, sciatic nerve, liver, kidney and intestine were dissected out and frozen as described in section 2.3.1. It has been reported previously that using wet tissue weight for metal determination in tissues can produce sizable error due to inconsistency in the wetness of the tissue (Adrian et al., 1979). Thus, a pilot experiment was conducted where the wet and dried tissue weight of different tissue were compared to assess the unit to use for elemental measurements between ng of element per wet or dried tissue. Water content was removed from wet tissues by placing tissues no bigger than 5mm by 5mm into eppendorf tubes followed by drying in a Jouan RC-10 centrifugal evaporator for a minimal time of 1 hr or until the tissue appeared dried (Jouan, St Nazaire, France). The wet tissue weight correlated well with dry tissue weight and all results were reported as ng of element per gram wet tissue. Prior to analysis, tissues were digested overnight by 1 ml of 70% nitric acid and then heated to 90°C for 1hr; it was then diluted to 2ml with Milli-Q Water spiked with 50 ppb thallium and yttrium as internal standard. The platinum and copper content of each samples was analysed using a Varian 820MS ICP-MS.

2.10.3. ICP-MS Analysis

Platinum and copper counts were generated by running each plasma or tissue sample on a Varian 820MS ICP-MS (Agilent Technologies Inc., Santa Clara, CA, USA) at LabPLUS (Auckland, New Zealand). The ICPMS was tuned on a daily basis to maximize sensitivity using a multi-element solution containing platinum, yttrium and thallium (10 ng/mL in 0.7% HNO3 (v/v) (Assurance Grade Multi-Element Standards, Spex CertiPrep, Metuchen, NJ, USA). The ICP-MS operating conditions are listed in Table 2.1. A series of plasma and relevant tissue standards of known platinum and copper concentration were made up in the relevant matrix and were run on the ICP-MS at the time the samples were ran to generate a standard curve of platinum and copper concentration in plasma and other tissues examined. Each samples and
Table 2.1: ICP-MS operating conditions.

**Plasma measurement parameters**

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<th>Parameter</th>
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</thead>
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<td>Nebulizer gas flow rate</td>
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<tr>
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<td>Pump rate</td>
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**Analysis modes**

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<th>Value</th>
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</tr>
<tr>
<td>Replicates/sample</td>
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**Sampling**

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</tr>
<tr>
<td>Replicate time</td>
<td>3.53 – 5.86 sec</td>
</tr>
<tr>
<td>Spray chamber temperature</td>
<td>3°C</td>
</tr>
<tr>
<td>Mass range</td>
<td>194-195</td>
</tr>
</tbody>
</table>
standards were spiked with 50 ppb thallium as internal standard. The platinum and copper content of each sample was calculated from the standard curve using platinum or copper counts generate by ICP-MS and scaling for the dilution factor. These values were then used to plot a time course of plasma platinum and copper content. AUC was calculated as the area under the plasma platinum or copper concentration in plasma from the time points used and $T_{max}$ was the time taken to reach maximal concentration.

2.11. Primary Culture of Isolated Rat DRG Neurons

Culturing of primary DRG neurons was adapted from Delree et al (Delree et al., 1989).

2.11.1. Harvesting DRGs

Animals were euthanized with an intraperitoneal dose of 0.9 ml of 3 mg/ml pentobarbitone. The spinal column was dissected from the animal and fully submerged in L5 medium supplemented with horse serum and penicillin-streptomycin-glutamine (PSG). Lumbar one-L7 DRG was dissected using the microscope under sterile conditions with forceps. A minimum of three rats were used per culture and the DRGs of these rats were pooled together for dissociation.

2.11.2. Dissociation and Plating of DRG

Pooled DRGs were washed with cold PBS then centrifuged for 5 min at 300 g at 7°C. The solutions are discarded and the filtrates are incubated for 40 min at 37°C in freshly made collagenase and dispase in NBA1 solution followed by centrifugation at 300 g for 5 min at 7°C. The solution were then discarded and replaced with 0.25% trypsin in NBA1 solution and incubated for 30 min at 37°C. NBA2 solutions are then added to neutralize the trypsin followed by centrifugation at 300 g for 5 min at 7°C. The DRG pellet were re-suspended in NBA2 and triturated by passing through a Pasteur pipette. DRG cells are then filtered using a BD Falcon 70 µm-/100 µm-cell strainer and then suspended in freshly made isosmotic Percoll solution (density = 1.040 g/mL), followed by centrifugation at 800 g for 20 min at room temperature.
The filtrate was discarded without disturbing the cell pellet and then re-suspend with NBA3 prior to centrifugation at 800 g at 7°C for 5 min. The cells were made visible by dilution with trypan blue and counted using a haemocytometer. Poly-L-ornithine and laminin coated wells are seeded at 5000 cells per well containing NBA3 with 5’-fluoro-2’deoxyuridine (FudR) at a concentration of 40 µM to reduce the proliferation of non-neuronal cells.

2.11.3. Culturing of DRG Neurons

Adult DRG neurons are incubated in a humidified 37°C, 5% CO2 incubator and mediums are changed every other day. Because adult neurons have been axotomized, plated neurons are grown for 3 days before addition of any experimental factors. The cultures were not kept for more than 4 days and the survival of primary DRG neurons in these conditions has been previously demonstrated for up to 4 days (Delree et al., 1989).

2.12. Statistical Analysis

The statistical significance of difference between the means and trends were assessed using linear regression analysis, unpaired t-test, 1-way analysis of variance (ANOVA), where there were more than two groups 1-way ANOVA with Bonferroni’s multiple comparison test or Dunnett’s post-test were performed when appropriate. Prism 5.0 software (GraphPad Software, CA, USA) or Microsoft Excel 2007 was used to perform statistical tests. The test for normality and variance was performed using Prism prior to subjecting data to the above-mentioned statistical tests. Statmate 2 software (GraphPad) was used for the calculation of the power of an experiment that found no significant differences between the mean values of the groups of data. A P-value less than 0.05 were regarded as indicating statistically significant differences. In linear regression analysis, an $r^2$ (co-efficient of determination) value approaching 1 with a P-value less than 0.05 indicated a linear relationship between the 2 parameters investigated.
CHAPTER THREE

DIFFERENTIAL EXPRESSION OF COPPER TRANSPORTERS IN RAT DORSAL ROOT GANGLION

3.1. Introduction

Copper transporting proteins ATP7A, ATP7B and CTR1 play an essential role for the delivery of copper to essential cuproenzymes. Copper transporters have evolved along with other components of copper regulatory pathways to ensure that copper is transported without releasing highly cytotoxic free copper ions (Camakaris et al., 1999, Linder et al., 1996). The P-type ATPases, ATP7A and ATP7B, both transport copper out of cells, or deliver it to the trans-Golgi network (Lutsenko et al., 2007), whereas CTR1 is a plasma membrane protein that functions as a high-affinity cellular copper uptake transporter (Lee et al., 2002).

There is a strong body of evidence which implies that copper transporters play a role in controlling the cellular accumulation and cytotoxicity of platinum drugs, with CTR1 mediating platinum uptake into cells and ATP7A and ATP7B transporting platinum out of cells or into specific sub-cellular compartments. Genetic knockout and down-regulation of CTR1 has demonstrated altered uptake and toxicity of platinum drugs (Holzer et al., 2006, Ishida et al., 2002, Lin et al., 2002, Song et al., 2004). Studies have found that an increase in ATP7B expression leads to more efflux of platinum
drugs (Katano et al., 2003, Komatsu et al., 2000) and enhanced resistance of cells to platinum drugs with the increased expression of ATP7A (Owatari et al., 2007). The correlation of increased expression of ATP7A to a reduction in response to platinum drug treatment indirectly indicates that ATP7A might play a role in the export of platinum drugs (Samimi et al., 2003).

Platinum drugs are associated with peripheral sensory neuropathies that limit their use in clinical cancer chemotherapy (Windebank et al., 1999). Sensory neurons of the dorsal root ganglion (DRG) are the putative primary site of damage for platinum drug induced neuropathy. In patients receiving platinum drugs, it has been shown that platinum selectively accumulates in DRG (Cavaletti et al., 1990, Gregg et al., 1992, Krarup-Hansen et al., 1999). Previously animal studies have shown similar selective accumulation of platinum drugs in the DRG after chronic platinum drug treatment (Cavaletti et al., 1992, Cavaletti et al., 2001, Holmes et al., 1998, Jamieson et al., 2005, Screnci et al., 2000, Toniwa et al., 1986). This damage is observed in the form of damaged sensory neurons (Cavaletti et al., 2001, Holmes et al., 1998, Jamieson et al., 2005, Krarup-Hansen et al., 1999, McKeage et al., 2001, Screnci et al., 1997, Thompson et al., 1984, Tomiwa et al., 1986). Moreover, morphological changes in the form of DRG cell body, nucleus and nucleolus shrinkage have been observed in animal models of platinum induced neuropathy (Cavaletti et al., 2001, Holmes et al., 1998, McKeage et al., 2001, Screnci et al., 2000).

Little is currently known about the expression of copper transporters in the DRG that contains the cell bodies of primary sensory neurons which become damaged by platinum drugs. These neurons may require copper transport as they strongly express cuproenzymes, such as cytochrome C oxidase (Karmy et al., 1991), Cu/Zn superoxide dismutase (Rosenfeld et al., 1997) and peptidylglycine α-amidating monooxygenase (Jeng et al., 1997) and are sensitive to copper deficiency (Kennerson et al., 2010, Zara et al., 2009).

The objective of this study was to study the expression level of ATP7A, ATP7B and CTR1 in DRG tissue from adult rats. In addition, it aimed to study the distribution and localization of copper transporter protein within the DRG tissue of rat in both
healthy control and oxaliplatin treated animals. These studies will provide data that enables the adjusted expression of copper transporters to be related to the neurotoxicity of oxaliplatin and might give insight into the mechanism of neurotoxicity involving copper transporters. Neuronal atrophy was used as the endpoint for measuring the neurotoxicity of oxaliplatin in DRG tissues, as in previous studies (Jamieson et al., 2005, Jamieson et al., 2009, Liu et al., 2009, Muller et al., 1990, Tomiwa et al., 1986).

3.2. Methods

3.2.1. Animals and Drug Treatment

Aged-matched, 10 week old, female Wistar rats that weighed approximately 280 g at the beginning of the experiment were used as described in chapter 2.1. The rats were acclimatised to handling for two weeks prior to the drug treatment. Animals that were in the oxaliplatin treatment group (n=5) were injected intraperitoneally with 1.85 mg/kg of oxaliplatin (Eloxatin solution) twice weekly for 8 weeks and the control groups (n=5) were treated with vehicle control solution of 5% dextrose at a volume of 10 ml/kg. Seventy-two hours after the conclusion of multiple dose treatment animals were euthanased with an intraperitoneal dose of 0.9 ml of 3 mg/ml pentobarbitone (Chemstock Animal Health Ltd).

3.2.2. RT-PCR and Real-Time PCR

3.2.2.1. RT-PCR

Extraction of RNA from lumbar DRG, brain, spinal cord, liver, kidney and small intestine as described in chapter 2.7.1. Briefly RNA was extracted using an Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad). For the oxaliplatin-treated group, tissues were removed from animal 96 hours after last oxaliplatin dosing for RNA extraction.
For cDNA synthesis total RNA of each sample (0.25 µg) was reverse-transcribed into cDNA using a SuperScript first strand synthesis kit (Invitrogen) according to instructions, followed by digestion as described in chapter 2.7.2.

Amplification of cDNA were performed by PCR in a reaction mixture containing dNTP, MgCl₂, Platinum Taq DNA polymerase (Invitrogen) and custom primers, using a GeneAmp 9700 PCR System (Applied Biosystems) at 52°C for 40 cycles. Forward and reverse primers for rat Atp7a, Atp7b and Ctr1 are outlined in chapter 2.7.3.

Amplified PCR products analysed by gel electrophoresis on a 2% agarose gel at 110V using a EC 250-90 power supply (Thermo electron Corporation) after staining with ethidium bromide (Invitrogen) and photographed using Gel Doc 2000 System (Bio-Rad). A 1kb plus DNA ladder (Invitrogen) was ran beside the RT-PCR products for the determination of band size. Each band was quantified by densitometry analysis using ImageJ software (National Institute of Health, Bethesda, MD, USA).

3.2.2.2. Real Time PCR

Multiplex real-time PCR was performed using ABI PRISM 7900HT Sequence Detection Systems and SDS 2.3 software (Applied Biosystems). Primers and probe sets were purchased as TaqMan Gene Expression Assays containing forward and reverse unlabelled PCR primer pair and a fluorescent reporter dye-labelled TaqMan MGB probe. Samples were analyzed in triplicate in 10-µl total volume containing 2× TaqMan universal PCR Master Mix, 20× TaqMan FAM-labelled probe for rat Atp7a gene, 20× TaqMan FAM-labelled probe for rat Atp7b gene, 20× TaqMan FAM-labelled probe for rat Ctr1 gene, 20× VIC-labelled 18S ribosomal RNA as endogenous control probe, and 25 ng of cDNA. The abundance of mRNA of ATP7A, ATP7B, CTR1 or rRNA was measured as the threshold cycle values (Ct) after each reaction. The relative RNA expression level was calculated using the 2^-ΔCt method (Livak et al., 2001), where gene of interest expression normalized to 18S rRNA was reported. 

\[
\triangle C_t = (C_t\text{ of ATP7a, ATP7b, CTR1} - C_t \text{ of rRNA})
\]
3.2.3. Single Labelled Immuno-histochemistry

Twelve-µm DRG cryosections were prepared for single label immuno-histochemistry as described in chapter 2.6.1. Three biochemical markers were used to distinguish DRG sub-populations; Chicken anti ATP7A (1:1000; ab13995 from Abcam), rabbit polyclonal anti-hCTR1 primary antibody (1:500, Novus Biologicals, Littleton, CO, USA) or mouse anti-RT-97 (a clone of phosphorylated NF-H; 1:100; Chemicon International). Secondary antibodies used were biotinylated anti-chicken (1:500 Jackson 703-065-155), anti-rabbit antibody (1:500, Sigma) or anti-mouse antibody (1:500, Sigma), then an extravidin-peroxidase conjugate (1:500, Sigma) prior to visualisation using DAB (Sigma-Aldrich). Sections incubated without primary antibodies served as a control.

3.2.4. Double Labelled Immuno-fluorescence

Immuno-fluorescence staining was performed on the above mentioned primary antibodies as described in section chapter 2.6.2 followed by either Alexa Fluor 594-labeled anti-chicken, Alexa Fluro 594-labeled anti-mouse, Alexa Fluro 488-labeled anti-rabbit IgG (H+L) (1:500, Invitrogen) or DyLight 488-labeled anti-chicken IgG for 3 h.

3.2.5. Western Blot Analysis

Following euthanasia of animals with intraperitoneal injection of pentobarbitone (0.9 mL of 3 mg/mL; Chemstock Animal Health Ltd.), lumbar DRG tissues were dissected and for western blot analysis as described in 2.7.

3.2.6. Size Distribution of ATP7A and CTR1 Expressing Neurons

Cross-sectional cell body area was measured for DRG neurons immuno-reactive towards ATP7A and CTR1 as described in 2.5.1. The number of cells measured per DRG ranged from 962 to 1586. The cell body area measurements were used to generate a histogram of the frequency of the size distribution of DRG neurons that
were immuno-reactive towards ATP7A and CTR1 in control vehicle group or following treatment with oxaliplatin.

3.2.7. Statistical Test

Prism software was employed for the calculation of statistical significance value. Students T-test (unpaired and Two-sided) and ANOVA were applied when appropriate. For multiple group comparison Bonferroni’s multiple comparison post-test was used unless otherwise stated. To ensure that ANOVA assumptions were not violated normality and variance test were perform on data using Prism.

3.3. Results

3.3.1. Studies of DRG from Healthy Animals

3.3.1.1. mRNA Expression

The expression of ATP7A, ATP7B and CTR1 was investigated in the rat DRG and other reference tissues reported to express these genes. ATP7A, ATP7B and CTR1 gene transcript levels were analysed qualitatively using RT-PCR and quantitatively using qPCR.

3.3.1.1.1. ATP7A

When examined by RT-PCR, abundant mRNA expression of ATP7A was found in DRG tissue of all three rats examined, as shown by visible bands at 375 bp (Figure 3.1). Highest mRNA levels of the ATP7A gene was detected in neuronal tissues, such as spinal cord, DRG and brain, compared to non-neuronal tissues. ATP7A protein bands were clearly visible in the neuronal tissue of all three animals examined. Compared to neuronal tissue, the brain, compared to non-neuronal tissues. ATP7A protein bands were clearly visible in the neuronal tissue of all three animals examined. Compared to neuronal tissue, the mRNA expression of ATP7A was found to be lower in non-neuronal tissues measured, in descending order of kidney, liver then intestine. Faint but visible ATP7A bands were seen for all three animals for kidney, the positive reference tissue. Intestine and liver had the faintest bands of all tissues
Figure 3.1: ATP7A gene expression in rat tissues determined by RT-PCR.

Electrophoresis of RT-PCR products in 1% agarose gel with subsequent staining with ethidium bromide. D, dorsal root ganglion; B, brain; SC, Spinal cord; L, liver; K, kidney; I, intestine. ATP7A and GAPDH protein band at 375 and 291 base pair (bp) respectively.
examined. One out of the three animals showed no visible ATP7A band in intestinal tissue. ATP7A mRNA was found to be expressed in DRG tissue of rats by real-time PCR. Highest mRNA levels of the ATP7A gene was detected in the neuronal tissues such as spinal cord followed by brain and DRG. The lowest detection was in the non-neuronal reference tissues in the order of liver, kidney then intestine. The relative RNA expression level of individual animal, range and medium, normalized to 18S rRNA, is presented in Table 3.1. The RT-PCR and qPCR findings corresponded well with each other.

3.3.1.1.2. ATP7B

ATP7B was barely detectable by RT-PCR in the DRG of the 3 rats examined (Figure 3.2). Relative to other tissues the highest expression of ATP7B mRNA was found in the liver. It showed a particular strong band in one animal and was also detectable, although fainter, in the other two rats. ATP7B showed low expression in the other neuronal tissues of brain and spinal cord. ATP7B showed faint expression in kidney and there was no detectable band seen for intestine.

The results for real-time PCR were similar to that of RT-PCR in that ATP7B was barely detectable in DRG. ATP7B mRNA transcripts were detected by qPCR in DRG of only two of six animals. ATP7B mRNA transcripts were detected by qPCR in all six animals in the liver, where expression was the highest. Low expression was found in all other tissues studied. The mRNA of ATP7B was undetectable in 3 animals in kidney, 2 animals in spinal cord and 1 animal in brain (Table 3.1).

3.3.1.1.3. CTR1

Abundant mRNA expression of CTR1 was found in DRG tissue of rats, as shown by visible bands at 206 bp in all 3 rats examined (Figure 3.3). CTR1 mRNA levels were found to be high across all tissues examined by RT-PCR; all tissues examined showed similar staining intensity with ethidium bromide.

CTR1 mRNA was found to be expressed in DRG tissue of rats by real-time PCR. In contrast to ATP7A and ATP7B, CTR1 mRNA transcripts were detectable by qPCR in all
Table 3.1: Copper transporter gene expression in rat tissue determined by quantitative PCR.

<table>
<thead>
<tr>
<th>Copper transporter mRNA relative to 18S ($2^{-\Delta Ct} \times 10^4$)</th>
<th>Rat no.</th>
<th>DRG</th>
<th>Brain</th>
<th>SC</th>
<th>Liver</th>
<th>Kidney</th>
<th>Intestine</th>
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<tr>
<td><strong>ATP7A</strong></td>
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<tr>
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<td>84.5</td>
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<tr>
<td><strong>Range</strong></td>
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<td>7-84</td>
<td>5-289</td>
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<td>2-84</td>
<td>0-43</td>
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<td><strong>ATP7B</strong></td>
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<td><strong>Range</strong></td>
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<td>11-275</td>
<td>6-243</td>
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</table>

Values represent the median and range of determinations in six animals.
Figure 3.2: ATP7B gene expression in rat tissues determined by RT-PCR.

Electrophoresis of RT-PCR products in 2% agarose gel with subsequent staining with ethidium bromide. D, dorsal root ganglion; B, brain; SC, Spinal cord; L, liver; K, kidney; I, intestine. ATP7B and GAPDH protein band at 396 and 291 base pair (bp) respectively.
Figure 3.3: CTR1 gene expression in rat tissues determined by RT-PCR.

Electrophoresis of RT-PCR products in 2% agarose gel with subsequent staining with ethidium bromide. D, dorsal root ganglion; B, brain; SC, Spinal cord; L, liver; K, kidney; I, intestine. CTR1 and GAPDH protein band at 206 and 291 base pair (bp) respectively.
tissues, and in all animals, at levels higher than ATP7A and ATP7B. CTR1 mRNA levels were found to be highest in non-neuronal tissue and lowest in DRG (Table 3.1). The RT-PCR and qPCR findings both showed abundant CTR1 mRNA expression in all the tissues examined.

3.3.1.2. Protein Expression

Immuno-histochemistry was used to study the localization of copper transporter proteins within the rat DRG. The location of ATP7A protein in relation to CTR1 protein was further investigated using double labelling immuno-fluorescence.

3.3.1.2.1. ATP7B

No specific immuno-reactivity for ATP7B was found in rat DRG tissue in comparison to a negative control (Figure 3.4), even though the primary antibody (NB100–360, Novus Biologicals) is reported to detect ATP7B in rat liver (Goss et al., 2008).

3.3.1.2.2. ATP7A

ATP7A had a specific pattern of distribution within rat DRG tissue, with intense cytoplasmic staining of the cell bodies of smaller DRG neurons. ATP7A immuno-histochemistry of rat DRG tissue visualized by ABC-peroxidase revealed that this efflux copper transporter was most strongly expressed within smaller-sized DRG neurons that showed intense immuno-reactivity in a rather punctuate pattern localised to the cytoplasm of their neuronal cell bodies. Other DRG neurons showed lighter and more diffuse cytoplasmic immunostaining for ATP7A with occasional examples of a granular pattern of immuno-reactivity detected inside the plasma membrane. No immunostaining for ATP7A was apparent in the satellite cells, nerve fibres or other non-neuronal tissue elements of the rat DRG (Figure 3.5 A-D). The specificity of anti-ATP7A primary antibody was confirmed by Western blotting showing a 170 kDa protein band on the ATP7A immunoblots of DRG tissue homogenates from rats aged 4, 12 and 20 weeks (Figure 3.5 F). The lack of immunostaining of DRG tissue in the absence of the primary antibody in negative controls is shown in Figure 3.5 E.
Figure 3.4: ATP7B immuno-histochemistry of rat DRG tissue.

Representative sections of DRG tissue of control animal visualized by ABC peroxidise showing no immuno-reactivity of ATP7B in DRG neurons (A). Negative control for ABC peroxidise immuno-histochemistry without primary antibody (B). Scale bar, 20 µm.
Figure 3.5: ATP7A immuno-histochemistry of rat DRG tissue.

(A, C) Representative sections of DRG tissue of control animal visualized by ABC peroxidise showed intense punctuate vesicular pattern of ATP7A immuno-reactivity throughout the cytoplasm of cell body, mostly within smaller-sized DRG neurons (solid arrows). ATP7A had a more diffuse cytoplasmic localization in addition to a granular component detected around the plasma membrane (broken arrow) Other neurons showed lighter diffuse cytoplasmic label of ATP7A (d) without staining of cell nucleus (n). B and D are inserts (broken lines) of A and C at a higher magnification respectively. Negative control for ABC peroxidise immuno-histochemistry without primary antibody (E). Scale bar, 20 µm.

Figure 3.5 F: – ATP7A protein expression in rat DRG tissue.

Detection of ATP7A protein by Western blot analysis in DRG of rats aged 4 weeks (lane 1), 12 weeks (lane 2) and 20 weeks (lane 3). Beta actin was probed as a loading control. Note: Western blotting study of ATP7A was kindly done by Dr Johnson Liu.
3.3.1.2.3. **CTR1**

CTR1 immuno-histochemistry of rat DRG tissue showed a pattern of immunostaining that differed from ATP7A. CTR1 immuno-reactivity visualised by ABC peroxidase showed plasma membrane and vesicular cytoplasmic staining of DRG neurons (Figure 3.6). CTR1 immuno-reactivity is mostly associated with neurons with large-sized cell bodies. There was lighter background staining of other neuronal cell bodies and no staining of nerve fibres, satellite glial cells or other tissue elements.

3.3.1.2.4. **Co-localization Studies**

Double-label immuno-fluorescence of ATP7A and CTR1 confirmed their primary localization to neuronal cell bodies, distinct patterns of immuno-reactivity and non-overlapping distribution within rat DRG tissue that was consistent to the immuno-histochemical staining. ATP7A was most strongly expressed within the cell bodies of smaller-sized DRG neurons. CTR1 showed plasma membrane and vesicular cytoplasmic staining of DRG neurons within large-sized cell bodies. No immunostaining for ATP7A or CTR1 was apparent in the satellite cells, nerve fibres, strong DAPI nuclear staining or other non-neuronal tissue elements of the rat DRG. The expression of ATP7A and CTR1 appear to have little overlapping, as shown by lack of co-localization (Figure 3.7). In addition, ATP7A was co-labelled with a protein known to be co-expressed with CTR1, pNF-H. Figure 3.8 showed that the cytoplasmic localization of ATP7A in smaller DRG neurons did not overlap with the intense pNF-H immuno-reactivity of nerve fibres and larger neuronal cell bodies. This information further confirmed that ATP7A and CTR1 are expressed in different sub-populations of neurons.

3.3.1.3. **Morphometry Analysis**

3.3.1.3.1. **ATP7A**

For this analysis, ATP7A immuno-reactive neurons were defined as those with intense cytoplasmic staining. ATP7A immuno-reactive neurons accounted for about one third (35.1 ± 2.9%) of the overall population of DRG neurons in control animals.
Figure 3.6: CTR1 immuno-histochemistry of rat DRG tissue.

(A, C) Representative sections of DRG tissue of control animal visualized by ABC peroxidise showing immuno-reactivity of the plasma membrane (broken arrow) and vesicular cytoplasmic element (solid arrow) of large neurons with lighter diffuse immunostaining of the cytoplasm of other neurons (d) without nucleus (n) or nerve fibre (f) staining. B and D are inserts (broken lines) of A and C at a higher magnification respectively. Negative control for ABC peroxidise immuno-histochemistry without primary antibody (E). Scale bar, 20 µm.
Figure 3.7: Immuno-fluorescent staining for ATP7A (A, E), CTR1 (B, F), DAPI (C, G) in lumbar DRG of female adult Wistar rats. Arrows (→) indicates small neurons with strong immuno-reactivity for ATP7a. Broken arrow (←) indicates neurons with high immuno-reactivity. DAPI-stained satellite cells are shown with upward arrow (↑). There appears to be no co-localization of ATP7a and CTR1 (D, H). Scale bars, 20 µm.
Figure 3.8: Immuno-fluorescent staining for ATP7a (A, E), pNF-H (B, F) and DAPI (C, G) in lumbar DRG of female adult Wistar rats. Arrows (→) indicates small neurons with strong immuno-reactivity for ATP7a. Solid arrow (←) indicates neurons with high immuno-reactivity to pNF-H. DAPI-stained satellite cells are shown with upward arrow (↑). There appears to be no co-localization of ATP7a and pNF-H (H). Scale bars, 20 µm.
The neurons were classified into three size groups; small (<750 µm²), medium (750-1750 µm²), and large (>1750 µm²). Most of the ATP7A immuno-reactive neurons had cell bodies measuring less than 750 µm² (64.2 ± 6.9%) and only a small proportion of the ATP7A immuno-reactive neurons measured greater than 1750 µm². The percentage of neurons between 750 to 1750 µm² that showed immuno-reactivity towards ATP7A was 29.1 ± 4.7%. The mean cell body area of ATP7A immuno-reactive neurons was 767.1 ± 87.6 µm².

CTR1

CTR1 immuno-reactive neurons were defined as those with plasma membrane or vesicular cytoplasmic staining. About one tenth of neurons were immuno-reactive for CTR1 10.9 ± 1.8%. Very few of the CTR1 immuno-reactive neurons had cell bodies measuring less than 750 µm² (2.0 ± 1.3%), most of the CTR1 immuno-reactive neurons were greater than 1750 µm² (58.2 ± 16.1%). The percentage of neurons between 750 to 1750 µm² showing immuno-reactivity towards CTR1 was 40.0 ± 16.0%. The mean cell body area of CTR1-immuno-reactive neurons was 1936 ± 278 µm².

Morphometric analysis of control animal DRG tissue showed that ATP7A and CTR1 were expressed by different neuronal subpopulations with differing size profiles (Figure 3.9). Most of the ATP7A immuno-reactive neurons had cell bodies measuring less than 750 µm² but very few of the CTR1 immuno-reactive neurons were this size (Table 3.2, P<0.01; 1 way ANOVA). The percentage of ATP7A and CTR1 immuno-reactive neurons in the large size group were also significantly different (Table 3.2, P<0.01; 1 way ANOVA). Most of the CTR1 immuno-reactive neurons had cell bodies measuring greater than 1750 µm² and only a small proportion of the ATP7A immuno-reactive neurons were this size. The percentage of neurons between the size of 750 to 1750 µm² that showed immuno-reactivity towards ATP7A and CTR1 was numerically different but did not reach statistical significant (Table 3.2, P=0.63; 1 way ANOVA). ATP7A immuno-reactive neurons accounted for about one third of the overall population of DRG neurons in control animals whereas only about one tenth of neurons were immuno-reactive for CTR1 (Table 3.2, P<0.01; 1 way ANOVA).
Figure 3.9: Cell body size profiles of ATP7A (A) and CTR1 immuno-reactive (C) neurons in DRG tissue from control untreated animals (B).

Size profiles of ATP7A immuno-reactive and CTR1 immuno-reactive neurons show little overlap. B and D are the total neuronal population size profile of ATP7A and CTR1 respectively, and show no differences between the two populations. Values represent the mean and standard deviation of five animals.
Table 3.2: Expression profile of copper transporters in DRG.

<table>
<thead>
<tr>
<th></th>
<th>Mean cell body area (µm²)</th>
<th>Expression frequency (%)</th>
<th>Small cells &lt;750 µm² (%)</th>
<th>Medium cells 750-1750 µm² (%)</th>
<th>Large Cells &gt;1750 µm² (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP7A</td>
<td>767 ± 88</td>
<td>35.1 ± 2.9</td>
<td>64.2 ± 6.9</td>
<td>29.1 ± 4.7</td>
<td>6.7 ± 2.5</td>
</tr>
<tr>
<td>CTR1</td>
<td>1936 ± 278*</td>
<td>10.9 ± 1.8*</td>
<td>2.0 ± 1.3*</td>
<td>39.9 ± 15.9</td>
<td>58.2 ± 16.1*</td>
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</tbody>
</table>

Data presented as mean ± standard deviation of 5 animals. Significantly different from ATP7A indicate by * at $P <0.01$. 
mean cell body area of ATP7A immuno-reactive neurons was significantly smaller than that of the CTR1 immuno-reactive neurons (Table 3.2, \( P < 0.01 \); 1 way ANOVA).

### 3.3.2. Studies of DRG from oxaliplatin-treated animals

Animals were treated intraperitoneally with oxaliplatin (1.85 mg/kg) twice weekly for 8 weeks to observe chronic oxaliplatin treatment effect. DRG were removed at the end of the study period to investigate the effect of oxaliplatin on cell body size profile of ATP7A and CTR1 immuno-reactive neurons.

#### 3.3.2.1. Morphometric Analysis

##### 3.3.2.1.1. ATP7A

The size profile of ATP7A was examined in rats treated chronically with oxaliplatin. The size distribution frequency of ATP7A immuno-reactive neurons appears to be unchanged after oxaliplatin treatment (Figure 3.10 A). Representative photomicrographs of each treatment group are shown in Figure 3.11. The mean cell body area of neurons that showed strong immuno-reactivity towards ATP7A for the control group and oxaliplatin treated group were 767.1 ± 87.6 µm² and 641.1 ± 38.6 µm² respectively (Figure 3.12 A, Table 3.3). The percentage of strongly ATP7A immuno-reactive neurons that were <750 µm² and between 750-1750 µm² for the control group and oxaliplatin treated group were 64.2 ± 6.9% and 70.5 ± 4.0%, and 29.1 ± 4.7 and 27.1 ± 3.7% respectively. The percentage of neurons over 1750 µm² that showed immuno-reactivity towards ATP7A was 6.8 ± 2.5% and 2.5 ± 1.3% for control group and oxaliplatin group respectively (Figure 3.12 A, Table 3.3 A). After chronic oxaliplatin treatment the expression frequency of ATP7A immuno-reactive neurons remains similar to that of the control untreated group (35.1 ± 2.9% in control verses 35.1 ± 1.8% in oxaliplatin-treated, Table 3.3).

##### 3.3.2.1.2. CTR1

The cell size profile of neurons that had immuno-reactivity towards CTR1 appears to have shifted to the left after oxaliplatin treatment indicating neuronal atrophy.
**Figure 3.10: Cell body size profiles of ATP7A (A) and CTR1 (B) immuno-reactive neurons in DRG tissue from control and oxaliplatin-treated rats.**

Size profile of ATP7A immuno-reactive neurons in the control and oxaliplatin-treated rats did not appear to show any differences. In contrast oxaliplatin caused a leftward shift of the size profile of CTR1 immuno-reactive neurons compared to control.
**Figure 3.11: ATP7A immuno-histochemistry of rat DRG tissue.**

Representative sections of DRG tissue of control animal (A, B, C) and oxaliplatin-treated animal (D, E, F) visualized by ABC peroxidise showing intense punctuate vesicular pattern of ATP7A immuno-reactivity throughout the cytoplasm of cell body mostly within smaller-sized DRG neurons (B and E). C and F are inserts (broken lines) of B and E, respectively, at a higher magnification. Oxaliplatin treatment did not change the ATP7A immuno-reactive profile of neurons. Negative controls for ABC peroxidise immuno-histochemistry without primary antibody (A and D). Scale bar, 20 µm.
Figure 3.12: Morphometry of ATP7A (A) and CTR1 (B) immuno-reactive neurons in DRG tissue from control and oxaliplatin-treated rats.

Significant difference from control is represented with * at $P<0.01$, one way ANOVA with Bonferroni multiple comparison post-test. NS, not significant. Bars represent mean and standard deviation.
Table 3.3: Size profiles of ATP7A immuno-reactive (A) and CTR1 immuno-reactive (B) neurons after oxaliplatin treatment.

<table>
<thead>
<tr>
<th>A</th>
<th>Mean cell body area (µm²)</th>
<th>Small cells &lt;750 µm² (%)</th>
<th>Medium cells 750-1750 µm² (%)</th>
<th>Large Cells &gt;1750 µm² (%)</th>
<th>Frequency of IR neurons (%)</th>
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<tr>
<td>Control</td>
<td>767 ± 88</td>
<td>64.2 ± 6.9</td>
<td>29.1 ± 4.7</td>
<td>6.7 ± 2.5</td>
<td>35.1 ± 2.9</td>
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<td>Oxaliplatin</td>
<td>641 ± 39</td>
<td>70.5 ± 4.0</td>
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<table>
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<tr>
<th>B</th>
<th>Mean cell body area (µm²)</th>
<th>Small cells &lt;750 µm² (%)</th>
<th>Medium cells 750-1750 µm² (%)</th>
<th>Large Cells &gt;1750 µm² (%)</th>
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<td>2.0 ± 1.3</td>
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<tr>
<td>Oxaliplatin</td>
<td>1461 ± 64.1*</td>
<td>5.2 ± 2.0</td>
<td>66.4 ± 5.5*</td>
<td>28.5 ± 5.5*</td>
<td>11.3 ± 3.5</td>
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</table>

Data presented as mean ± standard deviation of >4 animals. Significantly different from control indicate by * at P<0.01.
Figure 3.10 B). Figure 3.13 shows representative photomicrographs of the control and oxaliplatin treatment group. Oxaliplatin treatment of animals caused reduction in their mean cell body areas from $1936 \pm 278 \, \mu m^2$ compared to the control group of $1461 \pm 64 \, \mu m^2$ (Figure 3.12, Table 3.3 B, $P<0.01$; 1 way ANOVA). After oxaliplatin treatment there were significant changes in the percentage of small and large neurons (Figure 3.12 B, Table 3.3 B, $P<0.01$; 1 way ANOVA). The percentage of small and large sizes neurons changed from $2.0 \pm 13$ to $5.2 \pm 2.0\%$ and $58.2 \pm 16.1\%$ to $28.5 \pm 5.4\%$ respectively. The percentage of medium size neurons increased in the oxaliplatin group from $39.9 \pm 15.9\%$ to $66.4 \pm 5.5\%$. There was no difference in the frequency of neurons that had strong CTR1 immunoreactivity in the animals of the control group ($10.9 \pm 2.1\%$) compared to the oxaliplatin treated-group ($11.3 \pm 3.5\%$) (Table 3.3).

3.3.2.2. mRNA Expression

In the control group of animals, the starting mRNA extraction per mg of wet DRG tissue was higher compared to the oxaliplatin-treated group (control; $0.46 \pm 0.14$ verses oxaliplatin-treated; $0.28 \pm 0.07$ ng/ml, $P<0.05$; Students T-test). However, the level of ATP7A and CTR1 mRNA expression in DRG tissue was measured by qPCR in control and oxaliplatin-treated animal using equal amount of starting mRNA. No significant changes were observed for the cycle threshold value of ATP7A and 18S in control and oxaliplatin treated group ($P=0.44$ and $P=0.42$ respectively; Students T-test, Table 3.4). Similarly, following chronic oxaliplatin drug treatment there were no significant changes observed in cycle threshold for mRNA expression of CTR1 and 18S in the rat DRG tissue ($P=0.29$ and $P=0.39$ respectively; Students T-test, Table 3.4).

The relative expression of ATP7A was compared to 18S rRNA and normalised to control group and no significant changes were observed. In the control group and oxaliplatin-treated group the $2^{(\Delta\Delta Ct)}$ was $1.7 \pm 1.2$ and $1.9 \pm 0.8$ respectively (Figure 3.14 A). Following chronic oxaliplatin drug treatment there were no significant changes observed in CTR1 mRNA expression in the DRG neurons. The $2^{(\Delta\Delta Ct)}$ for the control and oxaliplatin was $1.2 \pm 1.0$ and $1.2 \pm 0.5$, respectively (Figure 3.14 B).
Figure 3.13: CTR1 immuno-histochemistry of rat DRG tissue.

Representative sections of DRG tissue of control animal (A, B, C) and oxaliplatin-treated animal (D, E, F) visualized by ABC peroxidise. Intense neuronal membrane immuno-reactivity was found for CTR1 (B and E). Oxaliplatin treatment caused a reduction in DRG neurons that are immuno-reactive for CTR1 (F) compared to control (C). (C and F are inserts (broken lines) at a higher magnification from (B and E, respectively). Negative controls for ABC peroxidise immuno-histochemistry without primary antibody (A and D). Scale bar, 20 µm.
Table 3.4: mRNA expressions of ATP7A (A) and CTR1 (B) in untreated and oxaliplatin-treated animals. C, initial yield from mRNA extraction.

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Significantly different from control indicate by * at P<0.05.
Figure 3.14: mRNA expression of ATP7A (A) and CTR1 (B) in control untreated and oxaliplatin-treated animals. Relative expression compared to control group and normalized to 18S (mean with standard deviation).
3.4. Discussion

In the experiments described in this chapter the expression of ATP7A, ATP7B and CTR1 in DRG tissue from healthy untreated and oxaliplatin-treated adult rats was investigated. This study was the first description of the expression of copper-transporting P-type ATPases in DRG tissue from rats or any other animal species. In addition, the changes in expression of copper transporters after chronic oxaliplatin treatment may shed light on the involvement of copper transporters in the transport and accumulation of oxaliplatin in DRG tissues.

The studies in this chapter showed that ATP7A and CTR1, but not ATP7B, were expressed in rat DRG tissue. ATP7A and CTR1 were both found to be expressed at the gene and protein level in the DRG tissue of rats. The ATP7B mRNA was barely detectable in the rat DRG when studied by RT-PCR and qPCR but the expression in the reported positive reference tissue, liver, was confirmed. There was no specific immuno-reactivity for ATP7B protein found in DRG tissue using immunohistochemistry studies. ATP7A and CTR1 protein were detected by immunohistochemistry and western blot. Using immunohistochemistry techniques ATP7A and CTR1 was found to localize within the cell bodies of sensory neurons with little or no immunostaining of nerve fibres, satellite cells or other tissue elements. The protein expression of ATP7A in rat DRG was further validated using western blotting. Unsuccessful attempts were made to verify the specificity of the CTR1 antibody by the means of western blotting, although the antibody was specified by the supplier not to be suitable for western blotting. One rationale for the expression of ATP7A and CTR1 in these post-mitotic DRG neurons is that copper transporting proteins are required to maintain tight copper balance as failure to do so may result in irreversible death of neurons. The finding of ATP7A and CTR1 expression in DRG neurons is consistent with the number of reports of its expression in other mammalian neuronal tissues (Barnes et al., 2005a, Bauerly et al., 2005b, Niciu et al., 2006, Platonova et al., 2005, Samsonov et al., 2006).
Although the expression of ATP7A and CTR1 were both confined to the DRG neuronal cell body, their pattern of expression differed considerably. ATP7A immuno-reactive neurons were characterised by their small cell bodies and intense punctuate cytoplasmic immunostaining and accounted for about one third of the overall total population of DRG neurons. In contrast, strongly CTR1 immuno-reactive neurons were characterised by their large cell bodies, intense plasma membrane and infrequent vesicular cytoplasmic immunostaining and was found in about one tenth of the overall population of DRG neurons. The cell body size-profiles of ATP7A determined by morphometric analysis differed markedly from that of CTR1 immuno-reactive neurons. Studies by immuno-fluorescence double labelling inveterate that distinct subsets of sensory neurons intensely expressed either ATP7A or CTR1, but not both. Neuronal immuno-reactivity for ATP7A did not co-localise with CTR1, or with pNF-H, a protein that has previously shown to co-localize with CTR1 (Liu J et al., 2009). These findings show that ATP7A and CTR1 have neuron subtype-specific and largely non-overlapping distribution in adult rat DRG tissue suggesting that these copper transporters have distinct roles in supporting the functions of primary sensory neurons. One rationale for this differential pattern of expression of copper transporters within DRG tissue might be due to different subsets of peripheral sensory neurons having distinct requirements for copper delivery to specific cuproenzymes. The reason for differential expression of copper transporter in DRG remains speculative. However the activity of copper transporter has been shown to considerably differ in different region of the rat brain (Platonova et al., 2005).

The studies in the current chapter showed that oxaliplatin treatment of adult rats caused atrophy of the CTR1 immuno-reactive subpopulation of DRG neurons without changing the size profile of the ATP7A immuno-reactive subpopulation of DRG neurons. In other cell types, the cellular accumulation and cytotoxicity of platinum drugs is controlled, at least in part, by copper transporters, with CTR1 transporting platinum drugs into cells (Holzer et al., 2004b, Ishida et al., 2002, Lin et al., 2002), and ATP7A and ATP7B transporting platinum drugs out of cells or into specific subcellular compartments (Kalayda et al., 2008, Katano et al., 2003, Safaei et al., 2008, Samimi et al., 2004a, Samimi et al., 2004b). Atrophy of CTR1 immuno-reactive
neurons but not ATP7A immunoreactive neurons, induced by oxaliplatin, leads us to hypothesize that ATP7A expressing DRG neurons are less sensitive to oxaliplatin neurotoxicity because the high levels of ATP7A facilitate the cellular efflux of oxaliplatin, reducing its availability for reactions with DNA or other key neurotoxicity targets. In contrast, DRG neurons expressing high levels of CTR1 would be expected to take up more oxaliplatin leading to toxic effects in this neuronal subtype. Thus it can suggest that the neuronal subtype-specific and largely non-overlapping distribution of ATP7A and CTR1 within DRG tissue may influence the neurotoxicity of oxaliplatin by controlling its cellular accumulation and sub-cellular distribution within primary sensory neurons.

In the current study, the gene expression and the protein localization of CTR1 and ATP7A remained unchanged in DRG tissues after chronic oxaliplatin treatment. This finding is not in accordance with copper transporters being involved in the transport of oxaliplatin in rat DRG. However, the sampling time in this study of 96 hours after the last oxaliplatin dose may be too late for any changes in protein localization to be detected. It has been found that after cessation of oxaliplatin exposure there are rapid restoration of ATP7A and CTR1 protein to the perinuclear region and plasma membrane respectively. The resynthesis and trafficking of hCTR1 back to its normal cellular membrane locations in as little as 30 minutes upon cessation to platinum drugs exposure has been reported previously (Holzer et al., 2006, Molloy et al., 2009). Similarly, ATP7A have shown to return to perinuclear region from the cell periphery within hours after removal of cisplatin (Kalayda et al., 2008). The tissue utilized for the current immuno-histochemical studies were obtained from animals 96 hours after the last oxaliplatin dose, where the levels of circulating oxaliplatin would be minimal since it would have exceed four half-lives of oxaliplatin. Thus it can be speculated that the static protein localization of ATP7A and CTR1 found after oxaliplatin treatment may be related to the end sampling time point.

After oxaliplatin treatment there was a reduction of mRNA level in DRG tissues that was not specific to only copper transporters. The starting mRNA yield of the DRG tissue in the control group was significantly higher than that of the oxaliplatin treated group. This is in accordance with literature report of the ability of platinum
drugs to inhibit RNA synthesis (Jung et al., 2006). However, minimal changes in the relative mRNA levels of ATP7A and CTR1 after chronic oxaliplatin treatment was observed with qPCR studies. There was less than 1 cycle difference in the qPCR run between the control and oxaliplatin group in all instances reflecting less than 2 fold changes in the mRNA expression level. This change is of small magnitude and questionable biological significance. In addition the differences in mRNA expression levels were not statically significant so there is a high likelihood that these changes are false positive. The unchanged transcriptional activity of ATP7A and CTR1 after oxaliplatin treatment does not indicate that lack of involvement of ATP7A and CTR1 in the transport of oxaliplatin in the DRG tissue in this current study because posttranscriptional events are responsible for the regulation of CTR1 function (Bauerly 2005 et al., Petris et al., 2003). It has been shown in previous studies that the relocation of ATP7A does not require new protein synthesis (Petris et al., 1996) and therefore the mRNA levels are not likely to change after exposure to its substrate.

In conclusion/summary, the findings from this study suggest that ATP7A and CTR1 might play a role in the transport of oxaliplatin in DRG tissues of rats and could influence the neurotoxicity of oxaliplatin in these subsets of sensory neurons. However this hypothesis is made based on the assumption that neurons with transporters that putatively transport platinum drugs into the cells are damaged, whereas neurons with transporters that putatively transport platinum drugs out of the cells are spared but causality is not inferred. To address this possibility, substrates of copper transporters can be tested in an in vivo model of neuropathy. Theoretically these compounds will compete with the transport of oxaliplatin at the copper transporter level, while also having the ability to stimulate the endocytosis and degradation of CTR1, and this can be achieved by giving copper supplements. Under the same principle, copper chelators can be used to enhance the ATP7A function of exporting platinum drug to alleviate the effect of neuropathy. Therapeutic compounds currently being used for the treatment of Menkes and Wilson’s disease in attempt to reverse the state of copper deficiency and copper overload in patients, respectively, are good candidates for in vivo studies because
safety has been established. In such a study, copper supplements or chelators can be given along with oxaliplatin to observe the effect on neuropathy. This theory will be tested in the next chapter.
CHAPTER FOUR

PILOT STUDY OF COPPER TREATMENT
FOR PREVENTING OXALIPLATIN
NEUROTOXICITY

4.1. Introduction

Copper (Cu) is an essential element for the survival of mammals but it can also be potentially toxic to life. Cu ions can exist in both an oxidized, cupric (Cu\(^{2+}\)), or reduced, cuprous (Cu\(^+\)) state (Linder et al., 1996) and can drive the generation of highly reactive oxygen species, so excess level of copper is harmful to cells (Li et al., 1993, Li et al., 1994). To sustain a delicate balance of copper in cells, mammals have evolved highly conserved pathways to maintain copper homeostasis. These pathways consist of series of copper binding proteins, including copper transporters CTR1, ATP7A and ATP7B. These transporters ensure that copper is delivered to essential copper proteins without releasing highly toxic free copper ions.

The importance of copper transporters to health and well-being is illustrated by serious human diseases resulting from defective copper transporters. Genetically defective ATP7A transporters give rise to Menkes disease, a copper deficient and fatal X-linked neurodegenerative disorder where the intestinal ATP7A transporter fails to transport dietary copper for absorption. Patients with Menkes disease lacks copper required for normal cellular functions resulting in characteristics such as poor
psychomotor development, failure to thrive, seizures and abnormality in hair structure. In untreated cases of Menkes disease fatalities is usually seen in the first 12 months of life since copper is essential during this period for brain growth and motor neurodevelopment (Mercer et al., 2001). Wilson disease is associated with devastating neurological deficits caused by mutation of ATP7B, where ATP7B fails to pump excess copper out into the bile from the liver. This leads to pathological accumulation of copper and the clinical manifestation depending on the organs being saturated by copper. Typically liver failure is the first symptom to be seen because liver is the first site of copper accumulation. Neurologic symptoms develop at a later stage and commonly present itself as tremor, dystonia and dysarthria. In addition, deposits of copper are frequently seen in the eyes of patients with Wilson’s disease resulting in a yellow-brown ring appearance in the cornea known as the “Kayser-Fleischer ring”. If the excess copper deposit is not removed from the body in a timely manner the outcome are usually fatal, with fatalities expected to occur within 9 months to 3 years of treatment cessation (Das et al., 2006).

The pharmacological treatment used for Menkes and Wilson’s disease aims is to restore the imbalance of copper in the body. Copper supplements are given to patients with Menkes disease in the form of copper histidine (Kaler et al., 1998). Due to the lack of intestinal absorption of copper by defective ATP7A transporters, copper histidine is usually given parenterally or subcutaneously. Early intervention with copper histidine has shown to enhance survival (Kaler et al., 2008), reduce seizures (Kaler et al., 2010) and improves neuro-developmental outcomes (Christodoulou et al., 1998, Kaler et al., 2008, Tang et al., 2008, Tumer et al., 1996). Patients with Wilson’s disease are treated with copper chelating agents such as trientine, ammonium tetrathiomolybdate and D-penicillamine to remove toxic deposits of copper (Das et al., 2006). Zinc has also been given to inhibit the intestinal absorption of copper by inducing metallothionein, which has high affinity for copper and enhances the excretion of copper in the feces (Pfeiffer et al., 2011). The abovementioned compounds are practical and effective agents to reduce copper levels in the body and have successfully ameliorated Wilson’s disease related
neurologic dysfunction and reversed the presentation of Kayser-Fleischer ring (Brewer et al., 1991, Brewer et al., 2006, Lorincz et al., 2006).

Evidence presented in the previous chapter showed that subpopulations of dorsal root ganglia neurons strongly express either copper influx or efflux transporters and neurons expressing CTR1, the copper influx transporter, were selectively damaged by platinum drugs. Recent literature suggests that copper transporter may be implicated in platinum drug transport in other cell types. Altered expressions of copper transporter have shown to correspondingly affect the accumulation of platinum drugs in yeast (Ishida et al., 2002, Lin et al., 2002), mammalian cell lines (Holzer et al., 2004, Holzer et al., 2006, Ishida et al., 2002) and human cell lines (Chen et al., 2007, Komatsu et al., 2000, Matsumoto et al., 2007, Song et al., 2004). Cells with resistant to copper have shown parallel resistant to platinum drugs and vice versa (Katano et al., 2002, Safaei et al., 2001, Safaei et al., 2004). An increase in copper efflux transporter ATP7A has been correlated with poor survival in ovarian cancer patients (Samimi et al., 2003). There appear to be an apparent association between the transport and efficacy of platinum drugs to the corresponding altered levels of copper transporters.

This opens up the possibility of modulating the expression of copper transporters in order to alleviate platinum drug induced toxicity. Theoretically, the levels of copper transporter can be manipulated by exposing the cells to substrates of copper transporter which consequently leads to trafficking and degradation of transporters (Guo et al., 2004, Holzer et al., 2004, Holzer et al., 2006, Petris et al., 1996, Petris et al., 2003). Thus, copper supplements may lower the expression of CTR1 to reduce platinum drug uptake whereas copper chelators may increase the expression of ATP7A resulting in enhanced efflux of platinum drugs out of the cells. A reduction in platinum drug accumulation in DRG neurons, the cells that selectively take up platinum drugs, may improve platinum drug induced neuropathy. There are many compounds that can be used to modify levels of copper in the cells. The agents used for the treatment of patients with Wilson’s or Menkes disease are good candidates because the safety of the compounds has been established. These copper level
modifying compounds include copper histidine, copper sulfate, trientine, ammonium tetrathiomolybdate, and Zinc.

Although the safety has been established, the dose had to be carefully chosen because of the potential of adverse effects that can confound study results when these copper modifying compounds are given in high doses. Excessive zinc and D-penicillamine intake induces gastrointestinal discomfort (Grasedyck et al., 1988, Vallee et al., 1993), and excess of copper in the form of copper histidine or copper sulfate can cause death and liver failure (Gaetke et al., 2003). The dose selection of the copper modifying compounds used in the current studies were chosen based on previous reported studies that have successfully elevated or reduced copper levels in the rat without toxicity observed. For example, the dose of zinc, trientine, ammonium tetrathiomolybdate, D-penicillamine and copper sulfate was referenced from studies by Walter et al, Yanagisawa et al, Ogra et al, Domingo et al and Naganuma et al respectively (Domingo et al., 1998, Naganuma et al., 1985, Ogra et al., 2000, Walter et al., 2002, Yanagisawa et al., 1998). In the case of copper sulfate, where no safe dose data was found in the literature, allometric scaling was used to convert the human dose used in Menkes’ patients to the dose for rats. Allometric scaling is a helpful approach for the selection of a suitable dose for first-time administration where no previous data is available on the safety (Boxenbaum et al., 1995, Riviere et al., 1997, Mahmood et al., 1999).

Chronic oxaliplatin treatment in rat provides a strong experimental approach to studying the dose limiting neuropathy induced by oxaliplatin and has been utilized by many researchers (Cavaletti et al., 2001, Homles et al., 1998, Jamieson et al., 2005, Screnci et al., 1997). The pattern of neurotoxicity seen in human appear to accurately reflect the rat model demonstrating concordant changes in electrophysiological, morphological and behavioral endpoints of peripheral nerve damage. For example, the major site of platinum accumulation has been reported in the DRG tissue of both humans (Gregg 1992) and rats (Cavaletti et al., 2001, Holmes et al., 1998, Luo et al., 1999, Screnci et al., 1997, Screnci et al., 2000, Ta et al., 2006). Moreover, the reduction in sensory nerve conduction velocity is evident in humans
(Hamers et al., 1991, Mollman et al., 1990) and rats (McKeage et al., 1994). Thus, the Wistar rat model appears to be a well characterized model which enables valid evaluation of oxaliplatin induced neuropathy.

With this background, the purpose of the studies presented in this chapter is to investigate the potential efficacy of selected copper modifying agents in the prevention of oxaliplatin induced neuropathy using the established Wistar rat model of neuropathy. A series of copper modifying agents were tested with the aim of finding the most effective compound against oxaliplatin induced neuropathy. Hereafter, a dose-response study was conducted with the most promising agent to find the optimal response for the prevention of oxaliplatin induced neuropathy. Due to the fact that there are many compounds being screened in this study it was impractical to select multiple assessment endpoint of neuropathy. In previous studies of oxaliplatin neuropathy, atrophy of DRG neuronal cell body and cell nucleolus was apparent (Cavaletti et al., 1991, Cavaletti et al., 1993, Jamison et al., 2005, Tomiwa et al., 1986), and this pathological endpoint in the form of morphological changes in DRG neurons were used in the experiments described in this chapter to assess the efficacy of copper modifying agent on the prevention of oxaliplatin induced neuropathy. Due to resources constraint behavior or electrophysiological endpoints were not used for the assessment of oxaliplatin induced neuropathy.

4.2. Experimental Design

4.2.1 Animals and Drug Treatment

Aged-matched, 10 weeks old, female Wistar rats that weighed approximately 280 g were used in the beginning of the experiment as described in 2.1. Animals were treated with a 1.85 mg/kg dose of oxaliplatin twice weekly for 8 weeks. Oxaliplatin as injection solution of Eloxatin (50mg/10mL, Donated by Oncology Unit at Auckland City Hospital) was diluted in 5% dextrose (Baxter Healthcare, Old Toongabbie, Australia) and was administered to rats intraperitoneally at an injection volume of 10 ml/kg. To avoid chronopharmacologic variations, injections were performed between 1400 and 1600 hours on Wednesday and Friday. Control animals were
treated with the vehicle control solution of 5% dextrose. In addition to oxaliplatin, each treatment animals were treated by oral gavage 5 days a week for duration of 8 weeks with one of the copper chelating agents or copper supplementary agents diluted in deionized water at various dose. These copper agents included copper histidine (CH), copper sulfate, ammonium tetrathiomolybdate (TTM), trientine and zinc sulfate. Control animals were treated by oral gavage with water as control at a dosing volume of 1 ml/kg. Using StatMate 2.0 software (GraphPad), a samples size of 3 – 6 animals was determined to be adequate from previous studies (Jamieson 2009, Liu 2009) to detect changes in cell body and nucleolus size induced by oxaliplatin with statistical power compared to control. Table 4.1 shows the list of copper modifying agents, doses used and number of animals in the studies presented in this chapter.

4.2.2 Body Weight Changes and General Toxicity

All animals in the study were weighted five times a week and signs of toxicity such as lack of movement, ruffled fur and posture indicative of discomfort or pain were noted for tolerability of the drugs.

4.2.3 DRG Morphometry and Stereology

At the end of the 8 week study, all animals were euthanased and their DRG collected for morphometry assessments, 24 hours after the last oxaliplatin dose as described in 2.5. The cross-sectional cell body area and nucleolus area of neurons containing discernible nucleoli were measured for approximately 40 of the largest neurons out of approximately 1000 DRG cells. The mean of the largest 10 cells for each individual animal was then calculated as previously (Cavletti et al., 1992, Cavletti et al., 1998, Holmes et al., 1998, McKeage et al., 2001, Muller et al., 1990, Tomiwa et al., 1986).

4.2.4 Statistical Test

Linear regression was used to assess if the rate of body weight increase between treatment groups were different. Prism was used to check if the overall body weight versus time slopes were different between groups. The statistical significance of
Table 4.1: Copper treatment groups and doses

<table>
<thead>
<tr>
<th>Copper Compound</th>
<th>n</th>
<th>Dose (mg/kg)</th>
<th>Dose Volume (ml/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper Histidine</td>
<td>3</td>
<td>0.02</td>
<td>1</td>
</tr>
<tr>
<td>Copper Histidine</td>
<td>4</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>Copper Histidine</td>
<td>4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Copper Histidine</td>
<td>3</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>Copper Sulfate</td>
<td>4</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>TTM</td>
<td>4</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>TTM</td>
<td>3</td>
<td>30</td>
<td>3</td>
</tr>
<tr>
<td>TTM</td>
<td>1</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>Trientine</td>
<td>4</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>Zinc Sulfate</td>
<td>4</td>
<td>15</td>
<td>1</td>
</tr>
</tbody>
</table>

In addition to receiving copper compounds all animals were treated with a 1.85 mg/kg dose of oxaliplatin twice weekly for 8 weeks at an injection volume of 10 ml/kg. TTM; ammonium tetrathiomolybdate.
differences between means was assessed using 1-way analysis of variance (ANOVA) with the application of post-test when appropriate. For the comparison of starting weight the Bonferroni’s multiple comparison post-test was used, in all other cases the 1-way ANOVA with Dunnett’s multiple comparison post-test comparing all groups against control was employed. The assessment of relationship between different experimental parameters was performed using linear regression. These statistical procedures were undertaken using Prism 5.0 software (GraphPad Software, CA, USA). Statmate 2.0 software (GraphPad) was used to calculate the power of an experiment that found no significant difference between the mean values of 2 groups of data. For each statistical test, a $P$-value less than 0.05 were regarded as indicating a statistically significant difference. Ammonium tetrathiomolybdate at 30 mg/kg had one animal in the group due to premature death prior to study ending and was omitted in all ANOVA statistical test performed.

4.3. Results

4.3.1 Body Weight and General Toxicity

All groups of animals received oxaliplatin at 1.85 mg/kg twice weekly for 8 weeks except for the control group which received drug vehicle alone. In addition all animals received their respective copper treatment aside from the control and oxaliplatin alone group which received water vehicle. There were two treatment-related deaths prior to the end of the experiment; both animals received ammonium tetrathiomolybdate (30 mg/kg and 100 mg/kg). Another animal from the ammonium tetrathiomolybdate (30 mg/kg) died from accidental dosing to the lungs. Post mortem was not performed on any of the rat. Aside from that, all rats had a healthy appearance and no apparent toxicity such as ruffled fur or hinged posture was observed. Upon dissection, ascites were found in at least one animal from all oxaliplatin treated groups.

The starting weight of rats in this experiment varied from 260 – 299 grams and was not statistically different between treatment groups (Table 4.2). At the end of the
Table 4.2: Summary body weight data of the average in each treatment groups

<table>
<thead>
<tr>
<th>Copper Agent Dose (mg/kg)</th>
<th>n</th>
<th>Baseline Body Weight (g)</th>
<th>SD</th>
<th>Final Body Weight (g)</th>
<th>SD</th>
<th>Absolute Weight Gained (g)</th>
<th>SD</th>
<th>% Weight Increase</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>6</td>
<td>273</td>
<td>21</td>
<td>326</td>
<td>48</td>
<td>52</td>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td>Oxaliplatin alone*</td>
<td>-</td>
<td>6</td>
<td>287</td>
<td>32</td>
<td>313</td>
<td>28</td>
<td>24</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td>Copper Histidine*</td>
<td>0.02</td>
<td>3</td>
<td>260</td>
<td>16</td>
<td>286</td>
<td>24</td>
<td>26</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Copper Histidine*</td>
<td>0.2</td>
<td>4</td>
<td>283</td>
<td>26</td>
<td>310</td>
<td>34</td>
<td>27</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Copper Histidine*</td>
<td>2</td>
<td>4</td>
<td>252</td>
<td>16</td>
<td>289</td>
<td>24</td>
<td>37</td>
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</tr>
<tr>
<td>Copper Histidine*</td>
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<td>267</td>
<td>11</td>
<td>294</td>
<td>6</td>
<td>26</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Copper Sulfate*</td>
<td>0.2</td>
<td>4</td>
<td>298</td>
<td>19</td>
<td>309</td>
<td>31</td>
<td>11</td>
<td>22</td>
<td>4</td>
</tr>
<tr>
<td>TTM*</td>
<td>10</td>
<td>4</td>
<td>299</td>
<td>10</td>
<td>319</td>
<td>22</td>
<td>20</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>TTM*</td>
<td>30</td>
<td>1</td>
<td>274</td>
<td>-</td>
<td>304</td>
<td>-</td>
<td>30</td>
<td>-</td>
<td>11</td>
</tr>
<tr>
<td>Trientine*</td>
<td>50</td>
<td>4</td>
<td>285</td>
<td>15</td>
<td>318</td>
<td>34</td>
<td>33</td>
<td>22</td>
<td>11</td>
</tr>
<tr>
<td>Zinc sulfate*</td>
<td>15</td>
<td>4</td>
<td>298</td>
<td>19</td>
<td>322</td>
<td>20</td>
<td>30</td>
<td>41</td>
<td>10</td>
</tr>
</tbody>
</table>

*Animals were treated intraperitoneally with a 1.85 mg/kg dose of oxaliplatin twice weekly for 8 weeks at an injection volume of 10 ml/kg.

TTM; ammonium tetrathiomolybdate.
treatment period, all rats in the study gained weight regardless of the treatment groups they belonged to, with the control untreated group gaining the most weight (18.7 ± 10.4% of starting weight; Table 4.3). Percentage of weight gained from the start of the treatment in groups receiving oxaliplatin in descending order are as follow; copper histidine (2 mg/kg) 14.8 ± 4.6%, trientine 11.4 ± 7.3%, ammonium tetrathiomolybdate (30 mg/kg) 10.9, zinc sulfate 10.0 ± 14.0%, copper histidine (20 mg/kg) 9.9 ± 2.8%, copper histidine (0.02 mg/kg) 9.9 ± 4.6%, copper histidine (0.2 mg/kg) 9.5 ± 3.4%, oxaliplatin 9.1 ± 7.8%, ammonium tetrathiomolybdate (10 mg/kg) 3.9 ± 4.2%, then lastly copper sulfate 3.7 ± 7.5%. Ammonium tetrathiomolybdate (10 mg/kg) and copper sulphate treatment group was the only two groups with a lower weight gain compared to oxaliplatin. Although there were numerical differences in the percentage of weight gained from the control group compared to other treatment groups they were not of statistical significances. However the rate of weight gained is significantly different between treatment groups with control gaining at the fastest rate (P<0.01, Linear regression). Figure 4.1 shows the weight progression of each individual animal in different treatment group during the treatment period of the study. In the control group there was an average of 0.3258% of weight gain each day compared to oxaliplatin alone at 0.1537% of weight gain per day. Three other treatment groups that received both oxaliplatin and copper modifying compound had a greater percentage of weight gain than oxaliplatin treated alone; copper histidine (0.02 mg/kg, 2 mg/kg) and ammonium tetrathiomolybdate (30 mg/kg) had an average weight gain of 0.2029, 0.2380 and 0.2450% per day respectively.

4.3.2 Morphometry of Dorsal Root Ganglion

4.3.2.1. Neuronal Cell Body Size

The cell body size of DRG neurons for the oxaliplatin alone group was significantly smaller than the control group (control; 2620 ± 599 versus oxaliplatin alone; 1853 ± 165 µm², Table 4.4, P<0.01; 1-way ANOVA). All other treatment groups that received oxaliplatin in addition to copper modifying compounds had neuronal cell body areas that were smaller than the control group but larger than the oxaliplatin alone group.
Table 4.3: Percentage of weight gained from the start of the experiment

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Oxaliplatin Alone*</th>
<th>CH 0.02 mg/kg*</th>
<th>CH 0.2 mg/kg*</th>
<th>CH 2 mg/kg*</th>
<th>CH 20 mg/kg*</th>
<th>Copper Sulfate*</th>
<th>TTM 10 mg/kg*</th>
<th>TTM 30 mg/kg*</th>
<th>Trientine*</th>
<th>Zinc Sulfate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat 1</td>
<td>32.9</td>
<td>7.1</td>
<td>13.8</td>
<td>7.1</td>
<td>8.8</td>
<td>6.8</td>
<td>-7.2</td>
<td>1.6</td>
<td>10.9</td>
<td>4.4</td>
<td>9.9</td>
</tr>
<tr>
<td>Rat 2</td>
<td>17.6</td>
<td>-1.5</td>
<td>11.2</td>
<td>6.5</td>
<td>16.1</td>
<td>12.1</td>
<td>6.7</td>
<td>4.9</td>
<td>11.0</td>
<td>11.0</td>
<td>-2.2</td>
</tr>
<tr>
<td>Rat 3</td>
<td>2.9</td>
<td>7.8</td>
<td>4.8</td>
<td>10.4</td>
<td>19.9</td>
<td>10.8</td>
<td>5.8</td>
<td>9.2</td>
<td>21.5</td>
<td>29.7</td>
<td></td>
</tr>
<tr>
<td>Rat 4</td>
<td>24.9</td>
<td>8.2</td>
<td>13.9</td>
<td>14.3</td>
<td>9.6</td>
<td>-0.3</td>
<td>8.7</td>
<td>2.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat 5</td>
<td>21.6</td>
<td>10.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat 6</td>
<td>12.1</td>
<td>22.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>18.7</strong></td>
<td><strong>9.1</strong></td>
<td><strong>9.9</strong></td>
<td><strong>9.5</strong></td>
<td><strong>14.8</strong></td>
<td><strong>9.9</strong></td>
<td><strong>3.7</strong></td>
<td><strong>3.9</strong></td>
<td><strong>10.9</strong></td>
<td><strong>11.4</strong></td>
<td><strong>10.0</strong></td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td><strong>10.4</strong></td>
<td><strong>7.8</strong></td>
<td><strong>4.6</strong></td>
<td><strong>3.4</strong></td>
<td><strong>4.6</strong></td>
<td><strong>2.8</strong></td>
<td><strong>7.5</strong></td>
<td><strong>4.2</strong></td>
<td><strong>7.3</strong></td>
<td><strong>14.0</strong></td>
<td></td>
</tr>
<tr>
<td>Weight gain reduction compared to control (%)</td>
<td>9.6</td>
<td>8.8</td>
<td>9.2</td>
<td>3.9</td>
<td>8.8</td>
<td>15.0</td>
<td>14.8</td>
<td>7.8</td>
<td>7.3</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>Increase in weight gain compared to oxaliplatin (%)</td>
<td>9</td>
<td>4</td>
<td>63</td>
<td>9</td>
<td>-59</td>
<td>-57</td>
<td>20</td>
<td>25</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Animals were treated intraperitoneally with a 1.85 mg/kg dose of oxaliplatin twice weekly for 8 weeks at an injection volume of 10 ml/kg. CH; copper histidine, TTM; ammonium tetrathiomolybdate.
Figure 4.1: Body weight measurements of each animal in different treatment group during the 8 weeks treatment period of the study

(A) control, (B) oxaliplatin, (C) copper histidine 0.02 mg/kg, (D) copper histidine 0.2 mg/kg, (E) copper histidine 2 mg/kg, (F) copper histidine 20 mg/kg, (G) copper sulfate, (H) ammonium tetrathiomolybdate 10 mg/kg, (I) ammonium tetrathiomolybdate 30 mg/kg, (J) trientine and (K) zinc sulphate. Aside from the control group all animals were treated intraperitoneally with a 1.85 mg/kg dose of oxaliplatin twice weekly for 8 weeks at an injection volume of 10 ml/kg. Each individual point on graphs represents a single weight measurement while each colour is designated to a different animal. Black line indicates linear regression line. The rate of weight gained calculated by linear regression for each treatment group is placed on the right bottom corner of each graph. (L) is a table showing the slope and the 95% confidence interval of the weight regression for each treatment group. Control group showed the fastest rate of weight gain during the 8 weeks treatment period. CH; copper histidine, TTM; ammonium tetrathiomolybdate.
Table 4.4: DRG neuronal cell body area by treatment group

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Oxaliplatin alone*</th>
<th>CH 0.02 mg/kg*</th>
<th>CH 0.2 mg/kg*</th>
<th>CH 2 mg/kg*</th>
<th>CH 20 mg/kg*</th>
<th>Copper Sulfate*</th>
<th>TTM 10 mg/kg*</th>
<th>TTM 30 mg/kg*</th>
<th>Trientine*</th>
<th>Zinc Sulfate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat 1</td>
<td>2995</td>
<td>2011</td>
<td>2034</td>
<td>2312</td>
<td>2061</td>
<td>2298</td>
<td>2050</td>
<td>2385</td>
<td>2219</td>
<td>1962</td>
<td>1854</td>
</tr>
<tr>
<td>Rat 2</td>
<td>3110</td>
<td>1898</td>
<td>1799</td>
<td>2240</td>
<td>2766</td>
<td>2037</td>
<td>1894</td>
<td>1852</td>
<td>1751</td>
<td>1751</td>
<td>2234</td>
</tr>
<tr>
<td>Rat 3</td>
<td>2322</td>
<td>1949</td>
<td>1927</td>
<td>2191</td>
<td>2065</td>
<td>2270</td>
<td>2211</td>
<td>2026</td>
<td>1982</td>
<td>1982</td>
<td>2091</td>
</tr>
<tr>
<td>Rat 4</td>
<td>1757</td>
<td>1881</td>
<td>1715</td>
<td>2327</td>
<td>2024</td>
<td>2378</td>
<td>1751</td>
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<td>1751</td>
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<td>2007</td>
</tr>
<tr>
<td>Rat 5</td>
<td>2250</td>
<td>1843</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Rat 6</td>
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<td>1538</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Mean</td>
<td>2620</td>
<td>1853**</td>
<td>1920†</td>
<td>2115</td>
<td>2305</td>
<td>2202</td>
<td>2045†</td>
<td>2160</td>
<td>2219</td>
<td>1861††</td>
<td>2046†</td>
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<td>271</td>
<td>332</td>
<td>143</td>
<td>130</td>
<td>266</td>
<td>128</td>
<td>128</td>
<td>159</td>
</tr>
<tr>
<td>% of reduction compared to control</td>
<td>29</td>
<td>27</td>
<td>19</td>
<td>12</td>
<td>16</td>
<td>22</td>
<td>18</td>
<td>15</td>
<td>29</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>% of protection from oxaliplatin-induced shrinkage</td>
<td>9</td>
<td>34</td>
<td>59</td>
<td>45</td>
<td>25</td>
<td>40</td>
<td>48</td>
<td>1</td>
<td>25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Animals were treated intraperitoneally with a 1.85 mg/kg dose of oxaliplatin twice weekly for 8 weeks at an injection volume of 10 ml/kg. Significant differences from control at †, \(P<0.05\) and ††, \(P<0.01\) (1-way ANOVA). Note: TTM 30 mg/kg was omitted from this statistical test, CH; copper histidine, TTM; ammonium tetrathiomolybdate, aside from the percentage values all values expressed as in \(\mu m^2\).
Among the groups treated with oxaliplatin and copper modifying compounds, copper histidine (0.2, 2 and 20 mg/kg) and the ammonium tetrathiomolybdate group (10 mg/kg) had neuronal cell body sizes that were not significantly different from the control (Figure 4.2, 1-way ANOVA). The neuronal cell body sizes of the treatment groups that were given oxaliplatin and copper modifying compounds in descending order of size were as follows; copper histidine 2 mg/kg 2305 ± 332 µm², ammonium tetrathiomolybdate 30 mg/kg 2219 µm², copper histidine 20 mg/kg 2202 ± 144 µm², ammonium tetrathiomolybdate 10 mg/kg 2160 ± 266 µm², copper histidine 0.2 mg/kg 2115 ± 271 µm², zinc sulfate 2046 ± 159 µm², copper sulfate 2045 ± 130 µm², copper histidine 0.02 mg/kg 1920 ± 118 µm² and trientine 1861 ± 128 µm². When given along with oxaliplatin, copper histidine at 2 mg/kg appeared to have provided the best protection against oxaliplatin-induced neuronal-shrinkage compared to all other treatment groups, and was not significantly different from that value of the control group.

4.3.2.2. Nucleolus Size

The control group had a larger nucleolus area compared to the oxaliplatin alone group of 22.1 ± 5.3 µm² and 12.0 ± 0.8 µm² respectively (P <0.0001; 1-way ANOVA). Of all groups receiving oxaliplatin, the oxaliplatin alone group had the smallest nucleolus size than all groups also receiving copper compounds aside from the copper histidine 0.02 mg/kg group, with a nucleolus size of 11.4 ± 1.0 µm² (Table 4.5, Figure 4.3). The addition of copper histidine at 2 mg/kg to oxaliplatin provided the best protection against oxaliplatin-induced nucleolus shrinkage of 50% (Table 4.5, cell nucleolus area; 17.1 ± 6.2 µm²) and this difference was not significantly different from control (1-way ANOVA). The cell nucleolus size of all other treatment groups that were given oxaliplatin and copper modifying compounds were significantly smaller than control (Figure 4.3). The cell nucleolus area of the treatment groups in descending order are as follow; copper histidine 2 mg/kg 17.1 ± 6.2 µm², ammonium tetrathiomolybdate 30 mg/kg 15.9 µm², ammonium tetrathiomolybdate 10 mg/kg 14.5 ± 2.4 µm², copper histidine 0.2 mg/kg 14.2 ± 1.2 µm², copper histidine 20 mg/kg
Figure 4.2: Mean cell body area of DRG neurons for each animal by treatment group

Aside from control, all animals were treated intraperitoneally with a 1.85 mg/kg dose of oxaliplatin twice weekly for 8 weeks. Reductions in cell body area are seen in all groups treated with oxaliplatin. Solid black and dotted red lines represents the upper and lower 95% confidence interval for the control group and oxaliplatin only treated group respectively. Copper histidine at 2 and 20 mg/kg providing the most protection against oxaliplatin induced cell body shrinkage, with all values sitting above the upper 95% confidence interval of the oxaliplatin only treated group. Copper histidine; CH, ammonium tetrathiomolybdate; TTM.
Table 4.5: Cell nucleolus size of treatment groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Oxaliplatin Alone*</th>
<th>CH 0.02 mg/kg*</th>
<th>CH 0.2 mg/kg*</th>
<th>CH 2 mg/kg*</th>
<th>CH 20 mg/kg*</th>
<th>Copper Sulfate*</th>
<th>TTM 10 mg/kg*</th>
<th>TTM 30 mg/kg*</th>
<th>Trientine*</th>
<th>Zinc Sulfate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat 1</td>
<td>21.6</td>
<td>11.0</td>
<td>11.9</td>
<td>15.5</td>
<td>18.3</td>
<td>13.0</td>
<td>13.8</td>
<td>14.9</td>
<td>15.9</td>
<td>14.3</td>
<td>12.3</td>
</tr>
<tr>
<td>Rat 2</td>
<td>26.3</td>
<td>11.3</td>
<td>12.1</td>
<td>13.6</td>
<td>25.4</td>
<td>15.5</td>
<td>15.1</td>
<td>13.7</td>
<td>14.1</td>
<td>14.1</td>
<td>14.3</td>
</tr>
<tr>
<td>Rat 3</td>
<td>20.8</td>
<td>12.8</td>
<td>10.3</td>
<td>14.9</td>
<td>11.4</td>
<td>12.9</td>
<td>14.0</td>
<td>11.7</td>
<td>11.8</td>
<td>13.2</td>
<td>12.2</td>
</tr>
<tr>
<td>Rat 4</td>
<td>20.6</td>
<td>12.9</td>
<td>12.9</td>
<td>13.2</td>
<td>13.0</td>
<td>17.5</td>
<td>12.7</td>
<td>14.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat 5</td>
<td>13.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat 6</td>
<td>29.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>22.0</td>
<td><strong>12.0</strong>*</td>
<td><strong>11.4</strong>*</td>
<td><strong>14.2</strong>*</td>
<td><strong>17.1</strong>*</td>
<td><strong>13.8</strong>*</td>
<td><strong>14.0</strong>*</td>
<td><strong>14.5</strong>*</td>
<td><strong>15.9</strong>*</td>
<td><strong>13.2</strong>*</td>
<td><strong>13.2</strong>*</td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td>5.3</td>
<td>0.8</td>
<td>1.0</td>
<td>1.2</td>
<td>6.2</td>
<td>1.5</td>
<td>0.8</td>
<td>2.4</td>
<td>1.2</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>% of reduction compared to control</td>
<td>45</td>
<td>48</td>
<td>36</td>
<td>23</td>
<td>37</td>
<td>37</td>
<td>34</td>
<td>28</td>
<td>40</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>% of protection from oxaliplatin-induced shrinkage</td>
<td>-6</td>
<td>22</td>
<td>50</td>
<td>17</td>
<td>19</td>
<td>24</td>
<td>39</td>
<td>12</td>
<td>12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Animals were treated intraperitoneally with a 1.85 mg/kg dose of oxaliplatin twice weekly for 8 weeks at an injection volume of 10 ml/kg. Significantly different from control at †††, *P*<0.001 (1-way ANOVA). Note: TTM 30 mg/kg was omitted from this statistical test, CH; copper histidine, TTM; ammonium tetrathiomolybdate, aside from the percentage values all values expressed as in µm².
Figure 4.3: Cell nucleolus area of DRG in un-treated, oxaliplatin alone, and various copper modifying compounds

Aside from control, all animals were treated intraperitoneally with a 1.85 mg/kg dose of oxaliplatin twice weekly for 8 weeks. All oxaliplatin treated groups showed a significantly reduction in nucleolus area. Solid black and dotted red lines represents the upper and lower 95% confidence interval for the control group and oxaliplatin only treated group respectively. There were 3 groups with cell nucleolus body area of all animals treated sitting above the upper 95% confidence interval of the oxaliplatin only treated group; copper histidine at 0.2 and 20 mg/kg and copper sulfate. Copper histidine; CH, ammonium tetrathiomolybdate; TTM.
13.8 ± 1.5 µm², copper sulfate 14.0 ± 0.9 µm², zinc sulfate 13.2 ± 1.1 µm², trientine 13.2 ± 1.2 µm² and copper histidine 0.02 mg/kg 11.4 ± 1.0 µm².

4.3.3 Relationships between Bodyweight and Experimental Parameters

To determine if the changes in DRG morphometry were related to the altered bodyweight of oxaliplatin-treated animals, linear regression was used to analyze the relationship between these parameters. The r² value of the linear regression of body weight against DRG cell body area and DRG nucleolus area were 0.03458 and 0.07383. Therefore, body weight could not significantly explain the variation observed between the control and treatment groups for the reduction in cell body area and cell nucleolus area that were significantly altered by oxaliplatin and copper modifying compounds.

4.4. Discussion

In this study, five different compounds were tested for their efficacy against oxaliplatin induced neuropathy including copper supplements and chelators. The hypothesis was that modifying the expression of copper transporters would result in a reduction of platinum drug accumulation in the DRG neurons and resulting neurotoxicity. This initial pilot experiment provided insights into the tolerability of the test compounds in combination with oxaliplatin, and allowed an initial evaluation of their potential for ameliorating oxaliplatin induced neuropathy. The current pilot study also aided the selection of which copper modifying compound had the best potential for further study. However there are weaknesses in the study. Firstly, the statistical power of this study were low because there were a total of 11 treatment groups each with only 3 to 6 animals. In addition, the assessment of oxaliplatin induced neuropathy was limited to established pathological endpoints (Cavaletti et al, 1991, 1992; Tomiwa et al., 1986, Holmes 1998, Jamieson 2005) without behavioral or electrophysiological endpoints.

Oxaliplatin altered the body weights when given with or without copper modifying compounds. The actual body weight changes were similar in both groups treated
with oxaliplatin. At the end of the treatment period there was a 19% of weight gained in control group compared to 9% from the oxaliplatin alone group. The reduction in weight gain were expected and a common observation by other researchers after oxaliplatin treatment (Cavaletti et al., 2001, Holmes et al., 1998, Jamieson et al., 2005). Although there was no significant difference between the percentage and absolute weight gained at the end of the treatment period between any of the treatment groups, the rate of weight gained was highest for the control group and there was a significant difference between the rates of weight gain between groups. Aside from copper histidine at 2mg/kg, where the percentage of weight gained was 15%, the percentage of weight gained in the groups treated with copper modifying compounds was similar to the oxaliplatin alone treated group and varied from 4-10%. Despite the fact that the weight gained differences was only numerically different and not statistically significant these data were still experimentally relevant and important. The reasons for a lack of statistical significance could be due to small experimental animal number and a variable response leading to high standard deviation. The weight data from this study provided information that of all compounds tested, copper histidine at 2mg/kg were the best tolerated by animals.

Oxaliplatin treated rats showed morphological changes in DRG neurons that were indicative of neuropathy. A prominent neuronal cell body and cell nucleolus shrinkage was observed in rats treated with oxaliplatin that is consistent with the previously reported (Cavaletti et al., 2001, Holmes et al., 1998, Jamieson et al., 2005, McKeage et al., 2001). The addition of copper modifying compounds did not ameliorate morphological changes of DRG tissue induced by oxaliplatin. However, of all compounds tested copper histidine at 2 mg/kg provided the best protection against oxaliplatin induced neuronal cell body atrophy (59%) and cell nucleolus shrinkage (50%).

Based on the weight and morphological results copper histidine at 2 mg/kg was chosen as the compounds that had the most potential in providing protection against oxaliplatin induced neuropathy. In the next chapter a more comprehensive
and in-depth study were conducted using copper histidine at 2 mg/kg for the alleviation of oxaliplatin induced neuropathy. The study expanded to examine morphological, physiological and pharmacokinetic endpoints with the aim to provide a more in depth analysis to oxaliplatin induced neuropathy with sufficient statistical power.
Table 4.6: Summary table of copper compounds tested for the protection against oxaliplatin-induced neuropathy

<table>
<thead>
<tr>
<th>Cu Agent Dose (mg/kg)</th>
<th>n</th>
<th>Weight Increased (%)</th>
<th>Cell Body Size (µM²)</th>
<th>Cell Body Size Reduction from Control (µM²) (%)</th>
<th>Nucleolus size (µM²)</th>
<th>Nucleolus Size Reduction from Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control -</td>
<td>6</td>
<td>19 ± 10</td>
<td>2620 ± 599</td>
<td>-</td>
<td>22.0 ± 5.3</td>
<td>-</td>
</tr>
<tr>
<td>Oxaliplatin*</td>
<td>6</td>
<td>9 ± 8</td>
<td>1853 ± 165</td>
<td>767</td>
<td>12.0 ± 0.8</td>
<td>10.0</td>
</tr>
<tr>
<td>Cu Histidine* 0.02</td>
<td>3</td>
<td>10 ± 5</td>
<td>1920 ± 118</td>
<td>700</td>
<td>11.4 ± 1.0</td>
<td>10.6</td>
</tr>
<tr>
<td>Cu Histidine* 0.2</td>
<td>4</td>
<td>10 ± 3</td>
<td>2115 ± 271</td>
<td>505</td>
<td>14.2 ± 1.2</td>
<td>7.8</td>
</tr>
<tr>
<td>Cu Histidine* 2</td>
<td>4</td>
<td>15 ± 5</td>
<td>2305 ± 332</td>
<td>315</td>
<td>17.1 ± 6.2</td>
<td>4.9</td>
</tr>
<tr>
<td>Cu Histidine* 20</td>
<td>3</td>
<td>10 ± 3</td>
<td>2202 ± 143</td>
<td>418</td>
<td>13.8 ± 1.5</td>
<td>8.3</td>
</tr>
<tr>
<td>Cu Sulfate* 0.2</td>
<td>4</td>
<td>4 ± 8</td>
<td>2045 ± 130</td>
<td>575</td>
<td>14.0 ± 0.8</td>
<td>8.1</td>
</tr>
<tr>
<td>TTM* 10</td>
<td>4</td>
<td>4 ± 4</td>
<td>2160 ± 266</td>
<td>460</td>
<td>14.4 ± 2.4</td>
<td>7.6</td>
</tr>
<tr>
<td>TTM* 30</td>
<td>1</td>
<td>11</td>
<td>2219</td>
<td>401</td>
<td>15.9</td>
<td>6.1</td>
</tr>
<tr>
<td>Trientine* 50</td>
<td>4</td>
<td>11 ± 7</td>
<td>1861 ± 128</td>
<td>759</td>
<td>13.2 ± 1.2</td>
<td>8.8</td>
</tr>
<tr>
<td>Zinc Sulfate* 15</td>
<td>4</td>
<td>10 ± 14</td>
<td>2046 ± 159</td>
<td>574</td>
<td>13.2 ± 1.1</td>
<td>8.8</td>
</tr>
</tbody>
</table>

*Animals were treated intraperitoneally with a 1.85 mg/kg dose of oxaliplatin twice weekly for 8 weeks at an injection volume of 10 ml/kg. Ammonium tetrathiomolybdate; TTM.
CHAPTER FIVE

EFFICACY OF COPPER HISTIDINE, A CTR1 SUBSTRATE, IN A RAT MODEL OF OXALIPLATIN-INDUCED NEUROTOXICITY

5.1 Introduction

The results from chapter 3 and 4 lead to the hypothesis that copper transporters are involved in the transport of oxaliplatin and play a role in mediating oxaliplatin-induced neurotoxicity in the Wistar rat. There are several basis to support this proposition. Firstly, copper transporters appear to mediate oxaliplatin uptake in DRG tissue. In chapter 3 it was found that the sub-population of neurons expressing CTR1 underwent atrophy after oxaliplatin treatment whereas ATP7A immunoreactive neurons were spared. This finding suggested that neurons expressing copper influx transporter, CTR1, are transporting oxaliplatin into the cells making them more susceptible to neurotoxicity compared to neurons expressing copper efflux transporter, ATP7A, which might be able to transport oxaliplatin out of the cells. Secondly, the neurotoxicity induced by oxaliplatin appeared to be mediated through copper transporters. In chapter 4, a trend in the reduction of neurotoxicity was shown when oxaliplatin was co-administrated with a substrate of CTR1. This suggested that CTR1 may be involved in the mediation of oxaliplatin-induced neurotoxicity in the Wistar rat via the transport of oxaliplatin into the DRG neurons and inhibiting that may ameliorate oxaliplatin-induced neuropathy.
The objective of this chapter was to test this hypothesis. In order to do so the efficacy of copper histidine at 2 mg/kg were tested in addition to oxaliplatin treatment in a Wistar rat model of oxaliplatin-induced neuropathy. In the previous chapter, copper histidine at the dose of 2 mg/kg provided numerical protection against oxaliplatin induced cell body shrinkage, cell nucleolus shrinkage and body weight loss when co-administered with oxaliplatin. The aims of the current chapter were to reproduce these results with sufficient statistical power and to add more endpoints with the goal to provide a more comprehensive assessment to test this hypothesis.

To assess the severity of oxaliplatin-induced neurotoxicity, morphometry endpoints including DRG cell body and cell nucleolus area were utilized and compared between the untreated controls, oxaliplatin alone and a combination of oxaliplatin plus copper histidine treatment groups. In addition, the cell body area of the neuronal sub-population expressing phosphorylated heavy neurofilament subunit (pNFH) was measured and used as a neurotoxicity endpoint because these neurons were damaged by oxaliplatin treatment and underwent atrophy in previous studies (Jamieson et al., 2009).

To investigate if the addition of copper histidine to oxaliplatin reduced the uptake of oxaliplatin, platinum levels were measured in DRG of the oxaliplatin alone and the combinational treatment groups using an elemental specific detector, inductively coupled plasma mass spectrometry (ICP-MS). In addition, the effects of copper histidine on the levels of other relevant metals including copper and iron were also examined in liver, kidney, heart, intestine and other neuronal tissues such as the sciatic nerve, brain and spinal cord at the end of the experiment. Iron levels were also of interest because the absorption and excretion of copper have been shown to be inversely affected by iron levels in rats (Bremner et al., 1981, Yu et al., 1994).

To reveal potential interactions of oxaliplatin with the addition of copper histidine, the platinum levels in plasma were compared between groups treated with oxaliplatin alone and in combination with copper histidine. It was important to evaluate the plasma levels of oxaliplatin when copper histidine was co-administered
because it is essential for a neuro-protective agent to not change the plasma level and alter the efficacy of oxaliplatin when administered together. Although the metabolism of oxaliplatin is non-enzymatic, but previous neuro-protective treatment of calcium and magnesium significantly lowered the response rate in patients given oxaliplatin (Hochster et al., 2007) highlighting the importance of investigating pharmacokinetic interactions.

Lastly, the mRNA levels of copper transporters were investigated in the copper histidine in addition to oxaliplatin treatment group and compared to the oxaliplatin alone treatment group to investigate if the addition of copper histidine altered copper transporter expressions in DRG.

A model of chronic oxaliplatin treatment in the rat was used to study the oxaliplatin-induced neurotoxicity as previously (Cavaletti et al., 2001, Homles et al., 1998, Jamieson et al., 2005, Screnci et al., 1997). The pharmacokinetic of platinum drugs have shown to be similar in rodents and human by many research groups (Gregg et al., 1992, Holmes et al., 1998, Screnci et al., 1998). In addition, the pattern of neurotoxicity seen in humans appear to accurately reflect the rat model demonstrating concordant changes in electrophysiological, morphological and behavioral endpoints of peripheral nerve damage previously (Cavaletti et al., 2001, Homles et al., 1998, Jamieson et al., 2005, Screnci et al., 1997).

5.2 Experimental Design

5.2.1 Animals and Drug Treatment

Aged-matched, 10 weeks old, female Wistar rats that weighed approximately 280 g in the beginning of the experiment were used as described in 2.1. Using StatMate 2.0 software (GraphPad), a samples size of 10 - 11 animals per group was determined to be adequate from previous pilot studies conducted in chapter 4 to detect changes in cell body (1000 µm² ± 600 µm²) and nucleolus size (10 µm² ± 6 µm²) induced by oxaliplatin with the addition of copper histidine compared to oxaliplatin alone with a significance level of 0.05 and a power of 90%. Additional rats were used for the study
of platinum, copper and iron measurements for the comparison of oxaliplatin alone versus oxaliplatin plus copper histidine treatment \( n = 4 \). Figure 5.1 shows treatment group details and experimental design. A total of 51 animals were used for the studies described in this chapter. There were 18, 17 and 16 animals allocated to the control untreated, oxaliplatin treatment only and oxaliplatin plus copper histidine group respectively.

Animals allocated to the oxaliplatin alone or oxaliplatin plus copper histidine groups were treated with a 1.85 mg/kg dose of oxaliplatin twice weekly for 8 weeks. Oxaliplatin as injection solution of Eloxatin (50mg/10mL, Donated by Oncology Unit at Auckland City Hospital) was diluted in 5% dextrose (Baxter Healthcare, Old Toongabbie, Australia) and was administered to rats intraperitoneally at an injection volume of 10 ml/kg. To avoid chronopharmacologic variations, injections were performed between 1400 and 1600 hours on Wednesday and Friday. Control animals were treated with the vehicle control solution of 5% dextrose at the same time, dosing volume and frequency as groups receiving oxaliplatin.

In addition to oxaliplatin or dextrose, each animal received by oral gavage copper histidine or control vehicle 5 days a week for a duration of 8 weeks. The control and oxaliplatin group both received water as control vehicle at a dosing volume of 1 ml/kg. The group receiving oxaliplatin plus copper histidine received by oral gavage copper histidine at 2mg/kg diluted in deionized water at a dosing volume of 1 ml/kg.

5.2.2 Body Weight Changes and General Toxicity

All animals in the study were weighed five times a week and signs of toxicity such as lack of movement, ruffled fur and posture indicative of discomfort or pain were noted.

5.2.3 DRG Morphometry

At the end of the 8 week treatment period and 24 hours after the last oxaliplatin dose, animals were euthanased and their DRG collected for morphometry
### Treatments (for 8 weeks)

<table>
<thead>
<tr>
<th>Treatments (for 8 weeks)</th>
<th>Treatment groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>N</td>
<td>18</td>
</tr>
<tr>
<td>Oxaliplatin (i.p.)</td>
<td>Vehicle only</td>
</tr>
<tr>
<td>Copper Histidine (p.o.)</td>
<td>Vehicle only</td>
</tr>
</tbody>
</table>

**Figure 5.1: Experimental groups, timeline and design**

(A) Experimental groups and treatments. (B) Schematic of experimental design and timeline.
assessments as described in 2.5 (n = 11 for control, n = 10 each for both the oxaliplatin alone and oxaliplatin plus copper histidine groups). Cross-sectional cell body areas and nucleolus areas of DRG neurons containing discernible nucleoli were measured for approximately 40 of the largest neurons out of approximately 1000 DRG neurons to total. The mean cell body and nucleolus areas of the largest 10 neurons for each individual animal, was then calculated as previously (Cavletti et al., 1992, Cavletti et al., 1998, Holmes et al., 1998, McKeage et al., 2001, Muller et al., 1990, Tomiwa et al., 1986).

5.2.4 Single Label Immuno-histochemistry

Immuno-histochemistry was used to stain sub-populations of DRG neurons with immunoreactivity for ATP7A, CTR1 and pNF-H for the control, oxaliplatin alone and oxaliplatin plus copper histidine treatment animals. After the dissection of DRG from rats, DRG cryosections (12 µm thick) were prepared for single label immuno-histochemistry as described in 2.6.1. Three primary antibodies were used to immunostain DRG sub-populations; A chicken anti ATP7A (1:1000; ab13995 from Abcam), rabbit polyclonal anti-hCTR1 primary antibody (1:500, Novus Biologicals, Littleton, CO, USA), or mouse anti-RT-97 (a clone of phosphorylated NF-H; 1:100; Chemicon International, Temecula, CA, USA). Secondary antibodies used were biotinylated anti-chicken (1:500 Jackson 703-065-155), anti-rabbit antibody (1:500, Sigma), or anti-mouse antibody (1:500, Sigma), followed by an extravidin-peroxidase conjugate (1:500, Sigma) prior to extravidin-peroxidase conjugation (1:500; Sigma-Aldrich) and visualisation using DAB (Sigma-Aldrich). Sections incubated without primary antibodies were served as negative controls.

5.2.5 Platinum, Copper and Iron Tissue Measurements

At the end of the 8 weeks oxaliplatin treatment, platinum, copper and iron levels in plasma, DRG, brain, spinal cord, sciatic nerve, liver, kidney and intestine were measured using ICP-MS in 8 animals (n = 4 for each group treated with oxaliplatin alone and oxaliplatin plus copper histidine). Tissues were removed from animals 96
hours after last oxaliplatin dosing. All results were reported as ng of element per gram of wet tissue. The tissues were stored at -80°C prior to overnight digestion by 1 ml of 70% nitric acid. It was then heated to 90°C for 1 hr and diluted to 2 ml with Milli-Q Water spiked with 50 ppb thallium and yttrium as internal standard.

For plasma preparation, approximately 200 µl of blood was collected via the tail vein at 0, 0.25, 0.5, 0.75, 1 and 2 hr relative to the timing of the oxaliplatin i.p. injection at time 0 hr and copper histidine oral dosing at -4 hr. Blood was centrifuged at 5000 rpm for 10 minutes and equal volume of cold methanol was added to the resultant plasma to precipitate out proteins remaining in the plasma samples prior to centrifugation at 5000 rpm for 10 minutes. The supernatants were then collected and stored at -80°C prior to platinum, iron and copper analysed using a Varian 820MS ICP-MS as described in 2.9.3.

### 5.2.6 Real-Time PCR (qPCR)

Multiplex real-time PCR was performed using ABI PRISM 7900HT Sequence Detection Systems and SDS 2.3 software (Applied Biosystems). Primers and probe sets were purchased as TaqMan Gene Expression Assays containing forward and reverse unlabelled PCR primer pair and a fluorescent reporter dye-labeled TaqMan MGB probe. Samples were analyzed as described in 2.9. The abundance of mRNA of ATP7A, ATP7B, CTR1 or rRNA was measured as the threshold cycle values (Ct) after each reaction. The relative RNA expression level was calculated using the $2^{-\Delta Ct}$ method (Livak 2001), where the expressions of the gene of interest were normalized to 18S rRNA. $\Delta Ct = (Ct_{ATP7a, ATP7b, CTR1} - Ct_{rRNA})$.

### 5.2.7 Statistical Tests

The statistical significance of differences between means was assessed using 1-way analysis of variance (ANOVA) with the application of post test when appropriate. For the comparison of starting weight the Bonferroni’s multiple comparison post-test was used for comparison of all treatment groups against each other to ensure that the starting weight was not different. In all other cases the Dunnett’s multiple
comparison post-test comparing all groups against control was employed to assess the difference from the oxaliplatin alone group and oxaliplatin plus copper histidine group. The test for normality and variance was performed using Prism prior to subjecting data to ANOVA. The assessment of relationship between different experimental parameters was performed using linear regression. These statistical procedures were undertaken using Prism 5.0 software (GraphPad Software, CA, USA). Statmate 2.0 software (GraphPad) was used to calculate the power of an experiment that found no significant difference between the mean values of 2 groups of data. For each statistical test, a $P$-value less than 0.05 were regarded as indicating a statistically significant difference.

5.3 Results

5.3.1 In-life Animal Study

5.3.1.1 Body Weight and General Toxicity

The weight of each animal was measured daily for five days each week during the 8-week treatment period. There were no differences in the starting weights between each of the treatment groups (control; 270.6 ± 29.9 g, oxaliplatin alone; 267.8 ± 23.9 g, oxaliplatin plus copper histidine; 269.7 ± 31.5 g, $P=0.9540$; 1-way ANOVA). All treatment groups showed weight gain throughout the treatment period. Figure 5.2 A, B and C shows the weight progression of each individual animal in the different treatment groups during the course of the study. At the end of the experiment, the amount of body weight gained for control, oxaliplatin alone and oxaliplatin plus copper histidine groups were 41.9 ± 16.4, 32.2 ± 15.4 and 31.5 ± 14.5 g respectively. The body weight of the animals at the end of the experiment in the oxaliplatin alone and with the addition of copper histidine were not statistically different from the control group ($P=0.5049$; 1-way ANOVA). However, linear regression analysis showed that the rate of body weight increase was significant different between treatment groups (Figure 5.2 D, $P=0.0109$; Linear regression comparing the slopes and intercept of percentage of starting weight versus days). Animals in the control group gained
<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Slope</th>
<th>95% CI of Slope</th>
<th>$r^2$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.3333 ± 0.0062</td>
<td>0.3212 to 0.3455</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>Oxaliplatin Alone</td>
<td>0.2337 ± 0.0063</td>
<td>0.2214 to 0.2460</td>
<td>0.91</td>
<td>0.0197</td>
</tr>
<tr>
<td>Oxaliplatin plus Copper Histidine</td>
<td>0.1897 ± 0.0064</td>
<td>0.1772 to 0.2022</td>
<td>0.94</td>
<td>0.0058</td>
</tr>
</tbody>
</table>

Figure 5.2: Body weight of animals in control, oxaliplatin alone and oxaliplatin plus copper histidine treatment groups during the 8 weeks treatment period of the study

Each individual point on (A) control, (B) oxaliplatin alone, (C) oxaliplatin plus copper histidine represents a single weight measurement while each colour is designated to a different animal on each graph. Black line indicates linear regression line. (D) Linear regression analysis of weight from each treatment group. Control group showed the fastest rate of weight gain during the 8 weeks treatment period followed by oxaliplatin alone then oxaliplatin plus copper histidine. P value assesses if the slopes are different from control. The slope of weight gained in the oxaliplatin alone is not different from oxaliplatin plus copper histidine group ($P=0.6669$).
weight at a rate of $0.3333 \pm 0.0062\%$ per day compared to the oxaliplatin alone group ($0.2337 \pm 0.0063\%$) and the group treated with oxaliplatin plus copper histidine ($0.1897 \pm 0.0064\%$). A summary of the body weight data by treatment group is presented in Table 5.1.

5.3.1.2 Mortality

Four animals died prior to the end of the experiment; three of which were treatment-related and one was related to anaesthetic overdose and occurred during a nerve conduction velocity study. Of the three animals that had treatment related death while receiving oxaliplatin, two animals belonged to the oxaliplatin alone group and one animal received oxaliplatin plus copper histidine. Aside from that, all rats had a healthy appearance and no apparent toxicity was observed. Upon dissection, ascites were found in 13 out of 17 rats (76%) receiving only oxaliplatin, and 7 out of 16 rats (44%) receiving oxaliplatin plus copper histidine. The volumes of these ascites varied but could not be taken into account for final weight measurements.

5.3.2 DRG Neuronal Morphometry

Morphometry parameters of DRG neurons were used as an endpoint for assessing oxaliplatin neurotoxicity. The addition of copper histidine to oxaliplatin did not provide protection against oxaliplatin-induced changes in DRG morphometry.

5.3.2.1 Cell Body Size

Both groups treated with oxaliplatin, either alone or with copper histidine, had significantly smaller cell body areas than the control group (Figure 5.3, $P<0.0001$; 1-way ANOVA). The cell body size of the control group, oxaliplatin alone and oxaliplatin plus copper histidine group were $3071 \pm 199$, $2038 \pm 213$ and $2194 \pm 213 \mu m^2$ respectively. Oxaliplatin plus copper histidine group had a larger cell body size compared to the oxaliplatin alone group, but that difference was not statistical significant. Treatment with oxaliplatin alone or oxaliplatin plus copper histidine
Table 5.1: Individual weight parameters of animals in each treatment group

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Mean 271 312 42 15 268 300 32 12 270 300 31 12
SD 30 40 16 6 24 30 15 6 31 34 15 6
95% CI 256 292 34 13 256 285 24 9 254 282 23 8
n 18 17 16
A + sign indicates that the animal died prior to the end of the experiment. An asterisk indicates that ascites were found in the animal at the end of the experiments. There were no statistical significant differences between the oxaliplatin alone or oxaliplatin plus copper histidine group to the control group in starting weight or end of study weight ($P=0.9540$ and $P=0.5049$ respectively, 1-way ANOVA).
Figure 5.3: Representative photomicrographs of DRG neurons after treatment with the control vehicle (A, D), oxaliplatin alone (B, E) or oxaliplatin plus copper histidine (C, F). Cell body and cell nucleolus areas of DRG neurons in animals treated with oxaliplatin (with or without copper histidine) (G).

Oxaliplatin treatments (with or without copper histidine) induced significant neuronal cell body and cell nucleolus shrinkage ($P<0.0001$, 1 way ANOVA with Bonferroni’s multiple comparison post-test). Bars represent mean and standard deviation of 11 - 10 animals. Scale bar = 20 µm.
induced cell body-shrinkage of DRG neurons by 33.6 and 28.6% compared to control respectively (Table 5.2).

5.3.2.2. Cell Nucleolus Size

The control group had larger nucleolus size compared to the oxaliplatin alone group and oxaliplatin plus copper histidine group, with mean DRG nucleolus size of 24.8 ± 2.9, 15.7 ± 2.9 and 14.8 ± 2.4 µm² respectively (Figure 5.3 and Table 5.2, P<0.0001; 1-way ANOVA). Compared to the control group, the oxaliplatin alone and oxaliplatin plus copper histidine group showed cell nucleolus-shrinkage of DRG neurons by 33.6 and 40.6% respectively (Figure 5.3). There was no difference in cell nucleolus sizes between the oxaliplatin alone group and oxaliplatin plus copper histidine group.

5.3.2.3. Neurons Expressing Phosphorylated NF-H

The effect of copper histidine given in addition to oxaliplatin on the expression of nerve injury marker phosphorylated heavy neurofilament subunit (pNF-H) was investigated in lumber 5 DRG. The cell body size and nucleolus size were recorded for each immuno-reactive and immuno-negative neuronal population for each protein marker. The neurons were subsequently categorised based on cell body size and the percentage which falls within every 200 µm² interval was used to generate size frequency histograms. In addition, the neuronal population were categorised into small (<750 µm²), medium (750-1750 µm²), and large sized neurons (>1750 µm²) to observe any changes in the portion of neurons within this size after treatment. The addition of copper histidine to oxaliplatin did not change the morphometry of neurons expressing protein marker of peripheral neuropathy.

Compared to control, both oxaliplatin treated groups, with or without copper histidine, had smaller neurons that were immuno-reactive towards RT-97, which stains phosphorylated NF-H (RT-97). Representative photomicrographs of each treatment groups are shown in Figure 5.4. The atrophy of RT-97 immuno-reactive neurons in the oxaliplatin alone and oxaliplatin plus copper histidine group is shown in the size frequency histogram where there was an apparent leftward-shift in the
Table 5.2: Cell body size and cell nucleolus size of DRG neurons in treatment groups

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<th>Oxaliplatin plus Copper Histidine</th>
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Mean 3071 24.8 2038*** 15.7*** 2193*** 14.8***
SD 199 2.93 213 2.85 247 2.39
n 11 11 10 10 10 10
95% CI 2937-3205 22.9-26.8 1886-2191 13.7-17.8 2017-2370 13.1-16.5

% changes compared to control 33.6 33.6 28.6 40.6
% of protection compared to oxaliplatin alone 15.0 -10.8

All values expressed are in µm². *** indicates that it is significantly different from control (P<0.0001), 1 way ANOVA with Bonferroni’s multiple comparison post-test.
**Figure 5.4**: Representative sections of DRG tissue of control (A, B, C), oxaliplatin alone (D, E, F) and oxaliplatin plus copper histidine treated animals (G, H, I) visualized by ABC peroxidise.

Intense RT97 immuno-reactivity was found throughout neuronal cell body (B, E and H) and nerve fibres (f). Oxaliplatin treatment alone or with copper histidine caused a reduction in DRG neurons that were immuno-reactive for RT97 (F and I respectively) compared to control (C). C, F and I are inserts (broken lines) at a higher magnification from B, E and H, respectively. Negative controls for ABC peroxidise immunohistochemistry without primary antibody (A, D and G). Scale bar, 20 µm.
oxaliplatin alone and mean cell body area between the oxaliplatin alone group and the oxaliplatin plus copper histidine treated group.

Similar to previous report by Jamieson et al. (Jamieson et al., 2009), the loss of RT-97 immuno-reactivity was observed in the oxaliplatin treated animals. The frequency of oxaliplatin plus copper histidine group was lower when compared to the control group (Figure 5.5 A). The mean cell body area of neurons that were immuno-reactive towards RT-97 was reduced in oxaliplatin alone (706 ± 118 µm²) and in oxaliplatin plus copper histidine treated animals (768 ± 30.0 µm²) compared to that in the control group (Figure 5.5 B, 1131 ± 71 µm²; P<0.01; 1-way ANOVA). There were no differences in RT-97 immuno-reactive neurons dropped from 20.9 ± 1.1% in the control group to 9.1 ± 1.0 and 8.9 ± 0.5% in the oxaliplatin alone and oxaliplatin plus copper histidine treated animals, respectively (Figure 5.5 B, P<0.001; 1-way ANOVA). The staining frequency of RT-97 immuno-reactive neurons did not differ between the two oxaliplatin treated groups, given with or without copper histidine.

The percentage of strongly RT-97 immuno-reactive neurons that had cell body area that were <750 µm² for the control group was 24.0 ± 3.8% and increased in the oxaliplatin alone and oxaliplatin plus copper histidine treated group to 64.0 ± 12.4% and 58.5 ± 4.1%, respectively (Figure 5.5 B, P<0.01; 1-way ANOVA). The percentage of RT-97 immuno-reactive neurons that were between 750-1750 µm² reduced after oxaliplatin treatment from 63.8 ± 9.6% in the control group to 33.9 ± 11.9 and 37.8 ± 3.3% in the oxaliplatin alone and oxaliplatin plus copper histidine group, respectively (Figure 5.5 B, P<0.05; 1-way ANOVA). There was a reduction in the percentage of RT-97 immuno-reactive neurons that had cell body area >1750 µm² between the control (12.2 ± 7.0%) and both oxaliplatin treated group (oxaliplatin alone; 2.1 ± 0.8%, oxaliplatin plus copper histidine; 3.7 ± 0.7%) but it was not of statistical significance. The percentage of small, medium and large-size neurons did not differ in the oxaliplatin alone and the oxaliplatin plus copper histidine group. Individual RT-97 immuno-reactive neurons values of each group are presented in Table 5.3.
Figure 5.5: Cell body size frequency graph of RT-97 immuno-reactive (IR) neurons in DRG tissue of control, oxaliplatin alone and oxaliplatin plus copper histidine treated animals (A). Bar graph of cell body area, staining frequency, percentage of small, medium and large-size neurons that were immuno-reactive for RT-97 in the control, oxaliplatin alone and oxaliplatin plus copper histidine group.

Treatment of oxaliplatin alone and with copper histidine caused a left-ward shift of the size profile of RT-97 immuno-reactive neurons compared to control. The addition of copper histidine did not prevent the atrophy of reduction in staining frequency of RT-97 immuno-reactive neurons after oxaliplatin treatment. Significantly different from control at $P<0.05$, $P<0.01$ and $P<0.001$ are represented by *, **, *** respectively, 1 way ANOVA with Bonferroni’s multiple comparison post-test.
Table 5.3: Morphometry of pNF-H immuno-reactive subpopulation of DRG neurons

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<td>35.1</td>
<td>3.2</td>
</tr>
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<td></td>
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<td>738</td>
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<td>62.0</td>
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<tr>
<td>Mean</td>
<td></td>
<td>768**</td>
<td>8.9***</td>
<td>58.5**</td>
<td>37.8*</td>
<td>3.7</td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td>30.0</td>
<td>0.5</td>
<td>4.0</td>
<td>3.3</td>
<td>0.7</td>
</tr>
<tr>
<td>95% CI</td>
<td></td>
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<td>7.7-10.1</td>
<td>48.5-68.6</td>
<td>29.5-46.1</td>
<td>1.8-5.5</td>
</tr>
<tr>
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<td></td>
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<td>-1.8</td>
<td>-8.5</td>
<td>+1.8</td>
<td>+77.4</td>
</tr>
</tbody>
</table>

*, ** and *** indicates significant difference from control (P<0.05, P<0.005, P<0.001, respectively; 1 way ANOVA with Bonferroni’s multiple comparison post-test).
5.3.2.4. Copper Transporter Expression in DRG Neurons

5.3.2.4.1 Neurons with Strong ATP7A Immuno-reactivity

The staining pattern of ATP7A was similar between the control, oxaliplatin alone and oxaliplatin plus copper histidine group. ATP7A were found to be expressed strongly within smaller-sized DRG neurons with a light and diffused cytoplasmic immuno-staining for ATP7A (Figure 5.6). There was a significant decrease in the frequency of neurons that had strong ATP7A immuno-reactivity in the animals of the control group (35.1 ± 2.9%) compared to the oxaliplatin plus copper histidine treated group (29.1 ± 1.7%) (Figure 5.7 and Table 5.4, \( P=0.0245 \); 1-way ANOVA). Thirty-three point six ± 2.3% of neurons had strong ATP7A immuno-reactivity from the oxaliplatin alone group and was not statistically different from the control or oxaliplatin plus copper histidine group. Individual ATP7A immuno-reactive neurons values of each group are presented in Table 5.4. There were no significant changes in the size of ATP7A immunoreactive after treatment with oxaliplatin alone or oxaliplatin plus copper histidine compared to the control group.

5.3.2.4.2 Neurons with Strong CTR1 Immuno-reactivity

There was a reduction in mean cell body areas of the CTR1 immuno-reactive neurons for both groups treated with oxaliplatin compared to the control group (control; 1936 ± 278, oxaliplatin alone; 1461 ± 64, oxaliplatin plus copper histidine; 1419 ± 195 µm², Figure 5.8, \( P<0.01 \); 1-way ANOVA). The atrophy of CTR1 immuno-reactive neurons after oxaliplatin treatment is also evident by a clear leftward shift in their size distribution profile in the oxaliplatin alone and oxaliplatin plus copper histidine groups compared with the control group (Figure 5.9 A). After oxaliplatin treatment, there was a reduction in the percentage of large neurons measuring greater than 1750 µm² from 58.2 ± 16.1% in the control group to 28.5 ± 5.4% in the oxaliplatin alone group (\( P<0.01 \); 1-way ANOVA). Consequently, there was an increase in the percentage of medium sized neurons measuring between 750 to 1750 µm² from 39.9 ± 15.9% in the control group to 66.4 ± 5.5% in the oxaliplatin alone group (Figure 5.9 B and Table 5.5, \( P<0.01 \); 1-way ANOVA).
Figure 5.6: Representative sections of DRG tissue of control animals (A, B, C), oxaliplatin alone treated animals (D, E, F) and oxaliplatin plus copper histidine treated animals (G, H, I) visualized by ABC peroxidise.

Intense punctuate vesicular pattern of ATP7A immuno-reactivity were found throughout the cytoplasm of cell body mostly within smaller-sized DRG neurons. C, F and I are inserts (broken lines) of B, E and H, respectively, at a higher magnification. Oxaliplatin alone or given with copper histidine did not alter the staining pattern of ATP7A. Negative controls for ABC peroxidise immunohistochemistry without primary antibody (A, D and G). Scale bar, 20 µm.
Figure 5.7: (A) Cell size frequency profile of ATP7A immuno-reactive neurons in the control, oxaliplatin alone and oxaliplatin plus copper histidine. (B) Morphometry profile of ATP7A-immuno-reactive neuron in the control, oxaliplatin alone and oxaliplatin plus copper histidine treated groups.

The treatment of oxaliplatin alone or with copper histidine did not appear to have changed the cell size expression profile and cell body size of ATP7A immuno-reactive neurons in DRG tissue. Oxaliplatin plus copper histidine induced a reduction in staining frequency of ATP7A immuno-reactive neurons ($P<0.05$, 1 way ANOVA with Bonferroni’s multiple comparison post-test). Bars represent mean and standard deviation.
Table 5.4: Morphometry of ATP7A immuno-reactive subpopulation of DRG neurons

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rat no</th>
<th>Mean Cell Body Area (µm²)</th>
<th>Frequency of IR neurons (%)</th>
<th>% Small Cells (&lt;750µm²)</th>
<th>% Medium Cells (750-1750µm²)</th>
<th>% Large Cells (&gt;1750µm²)</th>
</tr>
</thead>
<tbody>
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<td>1.9</td>
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<tr>
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* indicates significant differences from control (P<0.05; 1 way ANOVA with Bonferroni’s multiple comparison post-test).
Figure 5.8: Representative sections of DRG tissue of control animals (A, B, C), oxaliplatin alone animals (D, E, F) and oxaliplatin plus copper histidine treated animals (G, H, I) visualized by ABC peroxidise.

Intense neuronal membrane immuno-reactivity was found for CTR1 (B, E and H). Oxaliplatin treatments (with or without copper histidine) induced atrophy in CTR1 immuno-reactive neurons of rat DRG tissue. C, F and I are inserts (broken lines) at a higher magnification from B, E and H, respectively. Negative controls for ABC peroxidise immunohistochemistry without primary antibody (A, D and G). Scale bar, 20 µm.
Figure 5.9: Cell body size frequency graph of CTR1 immuno-reactive neurons in DRG tissue in oxaliplatin treated animals with and without copper histidine (A). Bar graph of cell body area, staining frequency, percentage of small, medium and large-size neurons that were immuno-reactive for CTR1 in oxaliplatin treated group with and without copper histidine.

Oxaliplatin caused a left-ward shift of the size profile of CTR1 immuno-reactive neurons compared to control. The addition of copper histidine did not prevent the atrophy of CTR1 immuno-reactive neurons after oxaliplatin treatment. Significantly different from control at $P<0.05$ and $P<0.01$ are represented by * and ** respectively, 1 way ANOVA with Bonferroni’s multiple comparison post-test.
Table 5.5: Morphometry of the CTR1 immuno-reactive subpopulation of DRG neurons

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rat no</th>
<th>Mean Cell Body Area (µm²)</th>
<th>Frequency of IR neurons (%)</th>
<th>% Small Cells (&lt;750µm²)</th>
<th>% Medium Cells (750-1750µm²)</th>
<th>% Large Cells (&gt;1750µm²)</th>
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<td>+5.2</td>
<td>-17.6</td>
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* and ** indicates significant differences from control (P<0.05, P<0.01; 1 way ANOVA with Bonferroni’s multiple comparison post-test).
The addition of copper histidine to oxaliplatin did not significantly alter the mean cell body area, frequency of immuno-reactive neurons, percentage of small, medium or large-sized neurons compared to oxaliplatin alone group (Figure 5.9 B and Table 5.5). In the oxaliplatin plus copper histidine group, the cell body size distribution profile, mean cell body area (1419 ± 195 µm²), percentage of small (6.8 ± 5.7%), medium (69.8 ± 9.1%) and large (23.5 ± 12.2%) CTR1 immuno-reactive neurons were similar to that of the oxaliplatin alone group (Figure 5.9 B and Table 5.5).

The frequency of CTR1 immuno-reactive neurons, expressed as a percentage of all DRH neurons, were not significantly different between groups after oxaliplatin treatment compared to the control group (P=0.9655; 1-way ANOVA). The percentage of CTR1 immuno-reactive neurons in the control, oxaliplatin alone and the oxaliplatin plus copper histidine treated groups were 10.9 ± 1.8, 11.3 ± 3.5 and 10.9 ± 1.9% respectively (Figure 5.9 B and Table 5.5).

5.3.3 Tissue and Plasma Platinum, Copper and Iron Levels

To determine if the addition of copper histidine to oxaliplatin altered the levels of platinum in tissue and plasma, samples were analysed using ICP-MS. The effects of copper histidine on copper levels in plasma and tissues were also studied by comparing copper levels between animals treated alone with oxaliplatin and with the co-administration of copper histidine. Furthermore, since iron has been reported to affect copper levels in rats (Yu et al., 1993, Klevay et al., 2001), the levels of iron were also monitored to ensure that any variation found wasn’t attributed by a change in iron levels.

For the tissue study of platinum, copper and iron levels, DRG, sciatic nerve, brain, spinal cord, liver, kidney, heart and intestine were collected at the end of the eight week dosing period in oxaliplatin alone and oxaliplatin plus copper histidine treated rats. The levels of element measured were then converted to ng per wet weight of tissue. In addition, plasma pharmacokinetic profile of platinum, copper and iron were examined in both treatment groups immediately post oxaliplatin dosing for up to 120 minutes. There
were similar tissue and plasma levels of platinum, copper and iron in both oxaliplatin treated group (with or without copper histidine).

5.3.3.1  Platinum

5.3.3.1.1  Tissue Platinum Levels

The addition of copper histidine to oxaliplatin treatment had no significant effect on the levels of platinum in DRG and other neuronal tissues. In DRG, platinum levels were 1035 ± 99.6 ng/gram of tissue in the oxaliplatin alone group and 1044 ± 22.4 ng/gram of tissue in the oxaliplatin plus copper histidine group ($P=0.8725$; Prism Two-tailed unpaired T-test). Likewise, there were other differences in the platinum levels of liver, kidney, heart and intestine between the oxaliplatin alone and oxaliplatin plus copper histidine groups (Figure 5.10).

5.3.3.1.2  Plasma Platinum Levels

The plasma platinum level profile was similar in the oxaliplatin alone and the oxaliplatin plus copper histidine groups. The amount of unbound platinum was measured at time point 0, 15, 30, 45, 60 and 120 minutes after oxaliplatin dosing for the oxaliplatin alone and oxaliplatin plus copper histidine groups. The platinum plasma level time-course profile of both treatment groups appeared similar (Figure 5.11). The area under the curve was 9.5 ± 3.3 and 11.4 ± 2.1 µg.hr/ml for the oxaliplatin alone and the oxaliplatin plus copper histidine group respectively ($P=0.3918$; Prism two-tailed unpaired T-test). The time to maximal platinum concentration was 20.7 ± 11.6 and 19.3 ± 9.29 minutes for the oxaliplatin alone and oxaliplatin plus copper histidine group respectively ($P=0.7548$; Prism Two-tailed unpaired T-test). Maximal platinum concentration achieved in the oxaliplatin alone group and oxaliplatin plus copper histidine group were 127 ± 38.7 and 170 ± 48.0 ng/ml ($P=0.2642$; Prism Two-tailed unpaired T-test).

5.3.3.2  Copper

5.3.3.2.1  Tissue Copper Levels

The addition of copper histidine to oxaliplatin did not significantly change the tissue
**Figure 5.10:** Tissue platinum levels of oxaliplatin alone and oxaliplatin with copper histidine group 96 hours after last oxaliplatin dose (A). Platinum levels (ng/gram wet tissue) of individual animal treated with oxaliplatin alone (oxaliplatin) and oxaliplatin plus copper histidine (CH) in neuronal tissues (B) and other tissues (C). Bars represent mean and standard deviation.

The addition of copper histidine to oxaliplatin did not significantly alter the tissue levels of platinum.
The addition of copper histidine to the treatment of oxaliplatin (1.8 mg/kg) twice weekly for eight weeks did not significantly change the plasma platinum levels, area under the curve, $C_{\text{max}}$ and $T_{\text{max}}$. 

Figure 5.11: (A) Platinum plasma concentration-time curves post oxaliplatin dose (mean and standard deviation). (B) Area under the curve, $C_{\text{max}}$ and $T_{\text{max}}$ in oxaliplatin alone and the oxaliplatin plus copper histidine group. Individual values are presented in (C).
copper level in any of the tissues examined (Figure 5.12). The copper level in DRG for the oxaliplatin alone and oxaliplatin plus copper histidine groups were 16.2 ± 2.95 and 16.4 ± 2.18 µg/g of tissue, respectively ($P=0.9383$; Prism Two-tailed unpaired T-test). In liver, the copper level was 2.6 ± 0.2 and 2.5 ± 0.1 µg/g of tissue for the oxaliplatin group and oxaliplatin plus copper histidine group respectively ($P=0.7328$; Prism Two-tailed unpaired T-test). The copper levels in sciatic nerve, brain, spinal cord, kidney, heart and intestine were similar in both treatment groups.

5.3.3.2.2. **Plasma Copper Levels**

Blood was taken from animals at time 0, 15, 30, 45, 60 and 120 minutes after oxaliplatin and at least 24 hr after copper histidine dose. The oxaliplatin alone group and the oxaliplatin plus copper histidine group had a similar pharmacokinetic profile and no significant differences were found between the treatment groups (Figure 5.13). The AUC and $T_{max}$ for the oxaliplatin alone group were 27.3 ± 6.7 µg.hr/ml and 23.3 ± 29.9 minutes respectively. In the oxaliplatin plus copper histidine group, the AUC was 28.7 ± 4.0 µg.hr/ml and $T_{max}$ was 28.7 ± 4.0 minutes respectively. There were no significant differences in the AUC or $T_{max}$ between the two groups ($P=0.7424$ and $P=0.2097$ respectively; Prism Two-tailed unpaired T-test). The maximal concentration of copper measured were 394 ± 133 and 328 ± 48.3 ng/ml for oxaliplatin alone group and oxaliplatin plus copper histidine group respectively ($P=0.3930$; Prism Two-tailed unpaired T-test).

5.3.3.3 Iron

5.3.3.3.1. **Tissue Iron Levels**

The iron levels in DRG of the oxaliplatin alone and oxaliplatin plus copper histidine group were 446 ± 42.8 and 430 ± 26.9 µg/g tissue respectively ($P=0.5747$; Prism Two-tailed unpaired T-test). Both group had similar iron measurements in sciatic nerve, brain, spinal cord, liver, kidney and intestine (Figure 5.14). The iron level in heart tissue for oxaliplatin alone and oxaliplatin plus copper histidine groups were 37.9
Figure 5.12: Copper tissues levels in oxaliplatin alone and oxaliplatin plus copper histidine group 96 hours after last copper histidine and oxaliplatin dose (1.8 mg/kg) (A) (mean and standard deviation). Copper level (ng/gram wet tissue) of individual animal treated with oxaliplatin alone (oxaliplatin) and oxaliplatin plus copper histidine (CH) in neuronal tissues (B) and other tissues (C).

Baseline of copper indicated by * in (A). Tissue copper levels are similar in both groups.
Figure 5.13: (A) Plasma copper concentration-time curves post oxaliplatin dose. (B) Area under the curve, $C_{\text{max}}$ and $T_{\text{max}}$ in the oxaliplatin alone group and the oxaliplatin plus copper histidine group (mean and standard deviation). Individual values are presented in (C).

The addition of copper histidine to the treatment of oxaliplatin (1.8 mg/kg) twice weekly for eight weeks did not significantly change the plasma copper levels, area under the curve, $C_{\text{max}}$ and $T_{\text{max}}$. 
Figure 5.14: Iron tissue levels in animals treated with oxaliplatin with or without copper histidine (A) (mean and standard deviation). Iron level (ng/gram wet tissue) of individual animal treated with oxaliplatin alone (oxaliplatin) and oxaliplatin plus copper histidine (CH) in neuronal tissues (B) and other tissues (C). Baseline of iron indicated by * in (A). Plasma and tissue levels of iron were not significantly altered with the addition of copper histidine to oxaliplatin except for heart tissue (P=0.0481, Prism Two-tailed unpaired student’s t-test).
±0.6 and 34.5 ± 2.2 µg/g tissue respectively, which was just statistically different between these two groups ($P=0.0481$; Prism Two-tailed unpaired T-test).

### 5.3.3.3.2. Plasma Iron Levels

The plasma levels of iron of both groups treated with oxaliplatin were similar (Figure 5.15). The AUC for plasma iron for the oxaliplatin alone and oxaliplatin plus copper histidine groups were 183 ± 38.2 and 192 ± 7.3 µg.hr/ml respectively (Figure 5.15; $P=0.8463$, Prism Two-tailed Unpaired T-test). The $T_{\text{max}}$ and $C_{\text{max}}$ for the oxaliplatin alone and oxaliplatin plus copper histidine groups were 48.0 ± 21.7, 34.3 ± 25.6 min and 2671 ± 71, 2752 ± 662 ng/ml respectively ($P=0.4790$ and $P=0.9324$ respectively; Prism Two-tailed unpaired T-test).

### 5.3.4 mRNA Expression of ATP7A and CTR1 in Dorsal Root Ganglia of Rats

Real time PCR was utilised to investigate changes in ATP7A and CTR1 mRNA expression in DRG after treatment with oxaliplatin alone or oxaliplatin plus copper histidine compared to a control group. The amount of mRNA extracted per mg of DRG tissue was higher in the control group of animals compared to both the oxaliplatin alone and the oxaliplatin plus copper histidine groups (control; 0.46 ± 0.14, oxaliplatin alone; 0.28 ± 0.07, oxaliplatin plus copper histidine; 0.26 ± 0.12 ng/ml, Table 5.6 C, $P=0.0156$; 1-way ANOVA with Dunnett’s multiple comparison post-test comparing all groups against control).

Quantification of mRNA expression levels of ATP7A and CTR1 in DRG tissue were performed using equal starting concentrations of mRNA, and determined by the cycle threshold value at which the reaction reached a fluorescent intensity above background set in the exponential phase of the amplification. In addition, the relative amount of mRNA of the target gene of interest was normalized to a housekeeping gene, 18S. No significant changes were observed for the cycle threshold value of ATP7A and 18S in control, oxaliplatin alone group and the oxaliplatin plus copper histidine group (Table 5.6 A, $P=0.2516$ and $P=0.1132$ respectively; 1-way ANOVA). Similarly, there were no significant changes observed in
Figure 5.15: Iron plasma levels: (A) Plasma concentration-time curves post oxaliplatin dose (mean and standard deviation). (B) Bar graph of area under the curve, $C_{\text{max}}$ and $T_{\text{max}}$ from animals given with and without copper histidine. Individual values are presented in (C).

Plasma iron levels, area under the curve, $C_{\text{max}}$ and $T_{\text{max}}$ are similar in the oxaliplatin alone group and the oxaliplatin plus copper histidine group.
Table 5.6: Initial yield from mRNA extraction (A), mRNA expression of ATP7A (B) and CTR1 (C) in DRG tissue from animals after treatment in the control, oxaliplatin-alone and oxaliplatin plus copper histidine groups.

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Significantly different from control (*, P<0.05, 1-way ANOVA, Dunnets’s post-test comparing against control).
cycle threshold for mRNA expression of CTR1 in the rat DRG tissue between the control, oxaliplatin alone and the oxaliplatin plus copper histidine group (Table 5.6 B, \(P=0.0818\); 1-way ANOVA). There was a significant increase in cycle threshold for 18S mRNA expression in the oxaliplatin plus copper histidine group compared to the control (31.0 ± 1.9; control versus 33.7 ± 1.3; oxaliplatin plus copper histidine Table 5.6 B, \(P=0.0239\); 1-way ANOVA with Dunnett’s multiple comparison post-test comparing all groups against control).

Figure 5.16 shows the relative expression of ATP7A and CTR1 compared to the control group and normalized with 18S as internal standard. The ATP7A \(2^{\Delta\Delta Ct}\) were 1.67 ± 1.23, 1.91 ± 0.78 and 2.31 ± 1.31 for the control, oxaliplatin alone and oxaliplatin plus copper histidine group respectively (\(P=0.6010\); 1-way ANOVA). The CTR1 \(2^{\Delta\Delta Ct}\) were 1.24 ± 0.97, 1.17 ± 0.52 and 3.34 ± 3.89 for the control, oxaliplatin alone and oxaliplatin plus copper histidine group respectively (\(P=0.1952\); 1-way ANOVA). Although there was no statistical significance the relative expression between any of the groups, a trend of lower ATP7A and CTR1 mRNA expression normalized to 18S were seen in the oxaliplatin alone and oxaliplatin plus copper histidine group.

### 5.3.5 Relationships between Bodyweight and DRG Morphometry Parameters

To determine if the changes in DRG morphometry caused by oxaliplatin treatment were related to the altered bodyweight of oxaliplatin treated animals, linear regression was used to analysis the relationship between cell body area and cell nucleolus area to weight of animal. The \(r^2\) value of the linear regression of body weight against DRG cell body area and DRG nucleolus area were 0.04426 and 0.0090 respectively. None of these \(r^2\) values reached statistical significance.

### 5.4 Discussion

The experiments in this chapter aimed to determine if the neurotoxicity of oxaliplatin in DGR neurons could be inhibited or reduced with the co-administration
Figure 5.16: Relative mRNA expression of ATP7A (A) and CTR1 (B) in untreated, oxaliplatin-alone and copper histidine treated animals normalized to 18S (mean and standard deviation).

Treatment of oxaliplatin alone or with the addition of copper histidine at 2 mg/kg did not alter the mRNA expression of ATP7A or CTR1 in rat DRG tissue.
of a known CTR1 substrate, thereby reducing the neurotoxicity in DRG. Several approaches were taken to examine this proposition; the neurotoxicity of rats treated with copper histidine in addition to oxaliplatin was compared to the oxaliplatin alone treated group using morphometry data of overall neuronal cell body area, nucleolus area and sub-population of neurons expressing pNF-H that were known to be damaged by oxaliplatin. In addition, the platinum levels in DRG were compared between these two treatment groups. The protein and mRNA expression of CTR1 and ATP7A were also studied to examine whether the addition of copper histidine to oxaliplatin treatment altered the expression of the primary copper transporters in DRG.

The results obtained from this chapter suggested that the addition of copper histidine at 2 mg/kg to oxaliplatin treatment in the Wistar rat model did not alter the neurotoxicity induced by oxaliplatin in DRG neurons or other tissues examined. The measured levels of platinum in DRG and in other tissues examined were similar in both oxaliplatin treated groups despite the addition of copper histidine. Possibly as a result the neurotoxicity in these two groups was comparable. There were no statistically significant differences in neuronal cell body or nucleolus area between the oxaliplatin alone and oxaliplatin plus copper histidine groups. In accordance with previous findings by this lab the reported oxaliplatin-induced atrophy in neurons with immuno-reactivity towards pNF-H and CTR1 was also evident in the current study (Jamieson et al., 2009, Liu et al., 2009). However, co-administration of copper histidine with oxaliplatin did not provide any statistically significant protection against this oxaliplatin-induced cell body shrinkage in neuronal subpopulations with immunoreactivity for pNF-H or CTR1. In addition, the reported reduction in staining frequency of p-NF-H immuno-reactive neurons after oxaliplatin treatment (Jamieson et al., 2009) were also seen in this current study and were similar between both oxaliplatin alone and the oxaliplatin plus copper histidine treated group.

One explanation for the lack of protection on oxaliplatin-induced neurotoxicity in DRG could be that the current study failed to increase the levels of copper in DRG tissues of the animals treated with oral gavaged copper histidine. The liver, intestine
and plasma levels of copper were found to be similar in the oxaliplatin alone and oxaliplatin plus copper histidine group. The unexpected results of the lack of differences in copper level measured in liver, intestine and plasma leads to the conclusion that the daily oral dosing of 2 mg/kg copper histidine described in this study did not achieve an increase in systemic copper levels. The intestine is the site of copper absorption and the liver is the organ responsible for copper homeostasis where excess copper is thought to be stored and prepared to be excreted in the bile (Evans et al., 1973). Thus, an increase in copper level in the animal should be reflected in increased levels of copper in both intestine and the liver. In previous studies this was evident; Bauerly et al dosed infant rats with 25 µg of copper per day for a total of 20 days and observed an approximate two-fold increase in liver and intestine tissue copper levels (Bauerly et al., 2005). In addition, the plasma concentration of copper was significantly increased in these rats. The daily dose and the cumulative dose of copper given to rats in the current study were approximately 20 and 40 times, respectively, higher than the dose Bauerly et al administered. Moreover, a common method of drug delivery via oral gavage was employed by Bauerly et al and this current study. In another study where an identical dosing schedule is shared with the current study (5 days a week for 8 weeks) copper sulphate were given orally to adult rats at 500 mg/kg and an increase in liver copper concentration was also found (Zhang et al., 2000). However, it must be noted that a much higher copper dose (250 times higher) was used by Zhang et al.

Reasons other than the oral route of administration were more likely to have contributed to the failure of elevating systemic copper levels in the rat. In previous studies copper was shown to be absorbed well when given orally. Orally administered $^{64}$Cu is rapidly absorbed and peak absorptions occur at 30 minutes (Marceau et al., 1970), and 30% of the dose is absorbed within 8 hours in rats (Marceau N 1970) and human (Sternlie et al., 1967). Other possible factors that could affect copper levels were also investigated. Iron levels can affect the absorption and excretion of copper (Bremner et al, 1981, Yu et al., 1994) but the iron levels were shown to be similar in both oxaliplatin alone and oxaliplatin plus copper histidine treated group which eliminates the potential of varying iron levels in
affecting copper levels. Thus the dose, route of administration and other possible confounding factor may not explain the reason why this study failed to raise copper level in the rat.

Another observation that supported the lack of changes in copper levels of DRG is that the protein localization of CTR1 or ATP7A in DRG neurons was not altered by the addition of copper histidine. The qualitative localization of CTR1 or ATP7A in rat DRG tissue was similar in the oxaliplatin alone and the oxaliplatin plus copper histidine groups when examined by immuno-histochemistry. This is not the results expected if DRG copper levels were increased because the movement of CTR1 protein induced by its substrate has been reported by several research groups previously. It was observed that substrate exposure to CTR1 induced a reduction of CTR1 protein on the plasma membrane (Guo et al., 2004, Larson et al., 2010, Petris et al., 2003, Molloy et al., 2009).

The lack of changes in DRG copper level was supported with the results that the mRNA expressions of CTR1 and ATP7A in rat DRG tissue were not significantly different between the copper histidine in addition to oxaliplatin and the oxaliplatin alone group. The mRNA expression levels of copper transporters were likely to be altered in rat DRG exposed to increased levels of copper. In previous studies utilizing other tissues that putatively transports copper using ATP7A, the mRNA level of ATP7A went up significantly after the rats were successfully exposed to more copper (Bauerly et al., 2005). However, minimal changes were observed in the relative mRNA levels of ATP7A and CTR1 between the control, oxaliplatin alone group and oxaliplatin plus copper histidine treatment group when studied with qPCR. In the current study there was less than 1 cycle difference in the qPCR run reflecting less than 2 fold changes in the mRNA expression level. This change is of small magnitude and questionable biological significance.

Oxaliplatin treatment appeared to have reduced the steady state global expression of genes. The amount of mRNA extracted from DRG was reduced significantly in both groups treated with oxaliplatin in both the copper transporter and the housekeeping 18S ribosomal RNA gene. Moreover, oxaliplatin treatment reduced the mRNA
expression level in DRG tissues that was not specific to only copper transporters. There was a lower trend in the relative mRNA expression levels of ATP7A, CTR1 and 18S in both group treated with oxaliplatin compared to the control untreated group. This is in accordance with literature report of the ability of platinum drugs to inhibit RNA synthesis (Jung et al., 2006).

Oxaliplatin treatment seems to have selectively increased copper and iron levels in DRG, sciatic nerve and heart. Although the enhanced copper and iron level in these tissues after oxaliplatin treatment is only by positive association, the increases observed were not trivial as there was almost a tenfold difference. To substantiate if oxaliplatin treatment truly enhanced copper and iron levels in the DRG, sciatic nerve and heart further studies are required. However, the copper and iron baseline level found in the current study was almost equivalent to that reported in the literature. Cempel et al reported that the iron levels of healthy untreated Wistar male rats were around 100 µg of iron per gram of wet tissue (µg/g) in liver and kidney and approximately 20 µg/g in the brain, which this study also obtained (Cempel et al., 2004). Furthermore, the copper baseline level found in this study were similar to those reported in healthy untreated Wistar male rats by Cempel et al; around 5µg/g in liver and kidney and approximately 2 µg/g in brain (Cempel et al., 2002). In a recent study by Ademuyiwa et al, they reported that the copper levels in the heart of the male albino Sprague-Dawley rat was 2.8 µg/g, in the current study 2.2 µg/g of copper was found as the baseline copper level in the heart (Ademuyiwa et al., 2010). Thus, it appears that the copper and iron baseline levels estimation using linear regression were adequate and the levels reported here were true reflections.

The relevance of the observed increase in copper and iron level by oxaliplatin treatment, if true, is unknown. But it can be speculated that the increased levels of copper and iron may contribute to the neurotoxicity induced by oxaliplatin. The accumulation of copper is a suspected etiology in several neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease (Tiffany-Castiglioni et al., 2011, Waggoner et al., 1999). The accumulation of iron is associated with Friedreich’s ataxia, an autosomal recessive disease that affects the peripheral
nervous systems. The neurological features of Friedreich’s ataxia are atrophy of DRG neurons and sensory neuropathy (Koeppen et al., 2011, Simon et al., 2004). Iron accumulation has been described in the cardiac tissues and neurons of animal model and in patients with Friedreich’s ataxia (Bayot et al., 2011, Koeppen, et al., 2003, Koeppen, et al., 2011a, Koeppen, et al., 2011b, Rahman et al., 2011). Excess copper and iron level in the DRG, sciatic nerve and heart could contribute to tissue damage by generation of reactive oxygen species as both are very redox-active in tissues (Halliwell et al., 1990, Stohs et al., 1995). Further studies are required to investigate if an increase in copper and iron level contributes to neurotoxicity of oxaliplatin.

The platinum level reported in the current study was similar to previous studies looking at platinum levels after oxaliplatin treatments. Platinum levels were studied after eight weeks of oxaliplatin chronic treatment and sampled at 96 hours post oxaliplatin dose. Similar to Scrinci et al’s finding a high level of platinum was found to accumulate in DRG and sciatic nerve, double that of the brain and spinal cord (Scrinci et al., 1997). The kidney showed the highest platinum level of all tissues measured and Pestieau et al has also reported high levels of platinum in kidneys after intraperitoneal oxaliplatin administration in male Sprague-Dawley rats (Pestieau et al., 2001). The high platinum level in kidneys coincides with the route of oxaliplatin elimination which is largely via renal excretion. While the accumulation of platinum in the DRG and sciatic nerve corresponds to the selective peripheral neuropathy symptoms of oxaliplatin.

The evidence presented in chapter 5 suggested that the oral dosing of 2 mg/kg copper histidine in addition to the eight week chronic oxaliplatin treatment did not provide an increase in copper level that was of biological significance. In the studies described in this chapter copper histidine at 2 mg/kg failed to show any benefit against oxaliplatin induced neuropathy in the Wistar rat in vivo model.
CHAPTER SIX
COPPER TRANSPORTER EXPRESSION AND OXALIPLATIN TOXICITY IN PRIMARY CULTURES OF RAT DORSAL ROOT GANGLION NEURONS

6.1. Introduction

The studies described in this chapter utilized an ex vivo tissue culture system to investigate the role of copper transporter in oxaliplatin-induced neuropathy. The possible role CTR1 plays in oxaliplatin-induced neuropathy via facilitating the uptake of oxaliplatin into the DRG remained unanswered using the in vivo Wistar rat model. The attempt to inhibit oxaliplatin uptake with copper histidine, a CTR1 substrate was not achieved in the in vivo Wistar rat model of oxaliplatin-induced neuropathy because copper levels were not shown to be modified in the DRG tissue. This could be due to a number of reasons such as ADME or copper homeostasis of the whole animal. However using the ex vivo system, the exposure time and concentration of copper to DRG neurons can be tightly controlled. Therefore, an ex vivo primary cultures of rat DRG neurons provided a possible way to test the hypothesis that copper transporters may be involved in oxaliplatin-induced neuropathy by modulating the uptake of oxaliplatin into DRG. In the past many researchers have successfully utilized primary rat DRG cultured neurons for the investigation of
platinum drug induced neuropathy (Gill et al., 1998, Jong et al., 2011, McDonald et al., 2005, Ta et al., 2006).

There is substantial evidence that the tissue culture model is useful for studying the cellular and molecular events leading to neuropathy in patients and that it is comparable to the in vivo model of peripheral neuropathy. For example Gill et al used primary DRG neuronal cultures to examine the role of cell cycle regulatory elements in cisplatin-induced neurotoxicity and showed that cisplatin caused similar morphologic changes of apoptotic cell death in primary sensory neurons both in vitro and in vivo (Gill et al., 1998). Ta et al also found biochemical evidence of apoptosis in cultures of DRG and in vivo morphological evidence of apoptosis suggesting that neuronal death induced by oxaliplatin was due to apoptosis both in vitro and in vivo (Ta et al., 2006). McDonald et al observed that after platinum drug exposure, platinum preferentially accumulated in ex vivo cultured DRG neurons and in vivo DRG tissue over other neuronal cell lines and tissues respectively (McDonald et al., 2005). The pattern and severity of neurotoxicity induced by the different platinum drugs were similar between the in vitro rat DRG culture and the in vivo model (Holmes et al., 1998, Luo et al., 1999). In addition, experimental investigations of primary cultures of DRG neurons were relatively simple to perform compared to in vivo models and the use of animal numbers was reduced.

Primary cultures of rat DRG neurons are live neurons isolated from micro-surgically dissected lumbar DRG neurons. These cells were prepared fresh and maintained in a nutrient rich medium that kept them alive for the duration of the study for each experiment carried out in this chapter. Primary cultures from dorsal root ganglia contained both neuronal and non-neuronal cells. The non-neuronal cells consisted of fibroblast and Schwann cells that formed a support on which these neurons can live on. These dissociated postnatal DRG sensory neurons offered the possibility to study mature and completely developed neurons surrounded by cellular substrates that normally exist in the in vivo environment.

The studies in the current chapter aimed to address several questions; 1) whether copper transporters were also expressed in the cultured DRG neurons in rats, 2)
whether a similar differential pattern of copper transporter expression is seen in the cultured DRG neurons in rats as the rat DRG tissues, 3) whether copper transporter expressing primary cultured DRG neurons respond to oxaliplatin in a similar manner to the rat in vivo model of peripheral neuropathy. Effects of oxaliplatin on copper transporter in primary DRG neurons were investigated in this chapter using a reliable and established ex vivo model (Delree et al., 1989).

6.2. Experimental Design

6.2.1. Animals

Nineteen to 21 day old female Wistar rats were used for the dissection of DRG and as a source of DRG neurons at the beginning of the experiment, as described in 2.3.2.

6.2.2. Primary Culture of Rat DRG Neurons

Culturing of primary DRG neurons from animals described in 6.2.1 was adapted from Delree et al (Delree et al., 1989). Harvesting, dissociation, plating and culture of DRG were performed as described in 2.10.

6.2.3. Oxaliplatin Time Course Study in Primary Culture of DRG Neurons

To define the time course of the effect of oxaliplatin on primary DRG neurons in culture, the primary DRG cultures were treated with 10 µM of oxaliplatin for 0, 1, 3, 8 and 24 hr. Following oxaliplatin treatment, cells were washed with phosphate both buffered saline at 37°C prior to immunofluorescence staining.

6.2.4. Oxaliplatin Concentration Study in Primary Culture of DRG Neurons

To define the concentration-dependence effect of oxaliplatin on primary DRG neurons in culture, they were treated with oxaliplatin at 0, 1, 10 and 100 µM for 3 hr. Following oxaliplatin treatment, cells were washed with phosphate buffered saline at 37°C prior to immunofluorescence staining.
6.2.5. **Fluorescent Single-Label Immunohistochemistry**

The chambers containing cells were washed with phosphate buffered saline, fixed with paraformaldehyde (4%) for 15 min at room temperature, washed permeabilized with 0.1% Triton X-100 and incubated at 4°C with conjugated antibodies overnight. The immunofluorescence staining was performed with three antibodies; a chicken anti ATP7A (1:1000; ab13995 from Abcam), rabbit polyclonal anti-hCTR1 primary antibody (1:500, Novus Biologicals, Littleton, CO, USA), or mouse anti-MAP2 followed by either Alexa Fluor 594-labeled anti-chicken, Alexa Fluro 594-labeled anti-mouse, Alexa Fluro 488-labeled anti-rabbit IgG (H+L) (1:500, Invitrogen) for 3 h as described in section 2.6.2. After incubation, cells were washed and coverslipped with Vectashield mounting medium with 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Sections incubated without primary antibodies were served as a control.

6.2.6. **Fluorescent Double-Label Immunohistochemistry**

Immunofluorescence staining was performed on the above mentioned primary antibodies as described in section 2.6.2. followed by either Alexa Fluor 594-labeled anti-chicken, Alexa Fluro 594-labeled anti-mouse, Alexa Fluro 488-labeled anti-rabbit IgG (H+L) (1:500, Invitrogen) for 3 h.

6.2.7. **DRG Morphometry**

The cross-sectional cell body area of approximately 1000 DRG cells was measured for each experimental condition. This neuronal population were then further divided into CTR1 or ATP7A immuno-reactive and non immuno-reactive neurons. Strongly CTR1-expressing DRG neurons were defined as those having intense plasma membrane and/or punctuate cytoplasmic immuno-reactivity to CTR1. Those negative for strong CTR1 expression had only diffuse cell body immuno-reactivity without plasma membrane or punctuate cytoplasmic reactivity. Strongly ATP7A-expressing neurons were defined as those having intense diffuse or punctuate cytoplasmic staining. Those negative for strong ATP7A expression had no or low-
intensity diffuse or punctuate cytoplasmic staining without plasma membrane immunoreactivity.

6.2.8. Statistical Analysis

Data were reported from at least three different cultures. The cell body area of the total neuronal CTR1 and ATP7A immuno-reactive population were not normally distributed but skewed toward the smaller cell body area. Therefore median was examined as well as the mean to obtain a more accurate reflection of the average value of the current data sample collected in this chapter. Unless stated otherwise, median values used were the median cell body area of total neuronal population for that particular culture. Contingency tables were used to analysis the proportions of neurons with cross-sectional cell body areas greater to or less than the median value of the control group/culture/well. Chi-square tests were generated from these contingency tables. Prism software was employed for the calculation of statistical significance value. Dunnett’s multiple comparison post-test comparing against CTR1 immuno-reactive neurons at 0 hr was utilized in addition to ANOVA and applied when appropriate unless otherwise stated.

6.3. Results

This chapter investigated the expression of copper transporters in untreated and oxaliplatin treated primary culture of DRG neurons. Data were obtained from three or more different cultures of DRG each dissected from a minimum of three rats that were pooled together for dissociation for culture. DRG neurons were cultured for up to 4 days after dissection and the cultures appeared viable during this time as previously (Delree et al., 1989). At the end of the each culture, neurons were processed by immunohistochemistry staining with anti-CTR1 or -ATP7A primary antibodies. Photomicrographs were taken and used for cross-sectional cell body area measurements of CTR1 immuno-reactive, ATP7A immuno-reactive and their respective negative neuronal sub-populations in each culture. These data were plotted as frequency-size histograms and the proportions of each neuronal
subpopulation with cell body area greater than or less than the median of the total population was calculated.

6.3.1. Copper Transporter Expression in Primary Cultures of Untreated Rat DRG Neurons

6.3.1.1 Morphology

6.3.1.1.1 Phase Contrast Morphology

When viewed under phase contrast microscope, cultured neurons of various sizes with highly refringent soma and well developed neuritic trees were seen. These characteristics are indicative of healthy neuronal morphology and viable neurons (Ransom et al., 1977). There were several types of cells found in the culture; neurons and non-neuronal cells. Phase-bright dorsal root ganglions were supported by phase-dark non-neuronal cells (fibroblasts and Schwann cells) that grew over the entire surface. Neuronal cell bodies were seen interspersed between the fibres with axon visibly extending out from the neuronal cell bodies (Figure 6.1 A).

Figure 6.1 B shows a phase contrast image of primary culture of DRG neurons overlaid with staining for neurofilament heavy subunit (NFH) and DAPI. NFH, a major component of neuronal cytoskeleton, was found to be expressed within all neuronal cell body. DAPI stained the nucleus of all cell including both neuronal and non-neuronal cells.

6.3.1.1.2 CTR1

Immunostaining of CTR1 was restricted to sub population of neuronal cell bodies without being expressed in the non-neuronal supporting cells. It showed strong membrane and occasional cytoplasmic localisation (Figure 6.1 C and D)

6.3.1.1.3 ATP7A

ATP7A showed strong immuno-reactivity in the perinuclear and cytoplasm of some
Figure 6.1: Microphotographs of primary DRG cultures

(A); Phase contrast image showing the appearance of a primary culture of rat DRG neurons grown on laminin. Phase-bright DRG neurons (thick arrows) were present along with phase-dark non-neuronal cells (fibroblasts; closed arrows). Nerve fibers are shown extending out from the neuronal cell bodies (open arrow).

(B); Phase contrast image of a primary culture of rat DRG neurons (thick black arrows; large neurons, thick red arrows; small neurons) overlaid with DAPI labeling of nuclei (blue; orange thin arrows) and NFH staining of DRG neuronal cell bodies and nerve fibers (open black arrow).

(C) and (D); Immunofluorescent staining of CTR1 (green) and DAPI (blue). Distinct staining patterns were seen in CTR1 immuno-reactive neurons. Prominent CTR1 staining was seen in the plasma membrane and cytoplasm (punctuate staining) of mostly large DRG neurons without apparent staining in non-neuronal supporting cells. Arrow points to large neurons with strong CTR1 staining.

(E) and (F); Immunofluorescent staining of ATP7A (red) and DAPI (blue). ATP7A cytoplasmic staining was seen in the cell bodies of DRG neurons that appeared smaller than the CTR1 staining neurons and without staining of non-neuronal supporting cells. Arrow points to neurons with strong ATP7A immuno-reactivity.

(G) and (H); Double Immunofluorescent labelling of ATP7A (red) and CTR1 (green) with DAPI nuclear staining (blue). Cytoplasmic staining of ATP7A and CTR1 in the cell bodies of DRG neurons showed little co-localization of CTR1 and ATP7A within the same neuron cell bodies. Scale bar = 20 µm.
neurons in a punctuate fashion. Its expression was limited to the cell body of some neurons and not in the non-neuronal supporting cells (Figure 6.1 E and F).

6.3.1.4. **Double Labeling of CTR1 and ATP7A**

Double-immunolabeling of CTR1 and ATP7A in primary culture of DRG neurons revealed that there was minimal overlap in the expression of these two copper transporters (Figure 6.1 G and H).

6.3.1.2 **Morphometry**

The cell body areas of between 972 – 1146 neurons were scored for each culture which included CTR1 immuno-reactive and CTR1 negative or ATP7A immuno-reactive and ATP7A negative neurons. Three cultures were analysed to define morphometry parameters for each neuronal subpopulation.

6.3.1.2.1. **CTR1 Immuno-reactive Neurons**

The cell body size frequency distribution of CTR1 immuno-reactive neurons is graphically presented in Figure 6.2. The median cell body cross sectional area for CTR1-immunoreactive neurons from three cultures combined was 319 µm². A bigger percentage of CTR1 immuno-reactive neurons had cell body size areas larger than the median value of the total neuronal population (76 ± 8%, Figure 6.2). The percentage of CTR immuno-reactive neurons smaller than the median was 24 ± 8%. The results from the three experiments were consistent.

The number of immuno-reactive neurons for CTR1 as a percentage of the total neuronal population was 23.6 ± 1.7%. Similar trends were observed across all three experiments and the expression frequency for culture 1, 2 and 3 was 23.6, 21.9 and 25.2% respectively.

6.3.1.2.2. **ATP7A Immuno-reactive Neurons**

The cell body size frequency distribution of ATP7A immuno-reactive neurons is graphically presented in Figure 6.3. The median cell body area of ATP7A immuno-
Figure 6.2: Cell body size frequency histograms of CTR1 immuno-reactive neurons in primary cultures of DRG from healthy animals

(A) to (D) cell body size frequency histograms of CTR1-IR (green bars) and CTR1-negative neurons (black bars) from experiment 1 (A), experiment 2 (B), experiment 3 (C) and combined data from experiment 1 to 3 (D). Dotted black vertical lines and values show the median cross-sectional cell body area for the total neuronal population for each experiment. The percentage value shown on each graph represents the proportion of CTR1-IR neurons that had cell body cross-sectional areas greater than the median value of the total population. Most of the CTR1 neurons (76%) were larger than the average DRG neuron.

(E) to (H) Contingency tables for experiment 1 (E), experiment 2 (F), experiment 3 (G) and combined data from experiment 1 to 3 (H) showing the number and percentages of CTR1-IR and total neurons with cross-sectional cell body areas less than or greater than the median value for the total neuronal population. P values are from Chi square tests.
**Table E**

<table>
<thead>
<tr>
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<th>No. of neurons (%)</th>
<th>No. of neurons (%)</th>
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<tbody>
<tr>
<td>Total</td>
<td>549 (52)</td>
<td>513 (48)</td>
</tr>
<tr>
<td>CTR1-IR</td>
<td>58 (23)</td>
<td>193 (77)</td>
</tr>
<tr>
<td>Magnitude of difference</td>
<td>-29%</td>
<td>+29%</td>
</tr>
</tbody>
</table>

P < 0.0001

**Table F**

<table>
<thead>
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<th>No. of neurons (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>502 (52)</td>
<td>470 (48)</td>
</tr>
<tr>
<td>CTR1-IR</td>
<td>70 (33)</td>
<td>143 (67)</td>
</tr>
<tr>
<td>Magnitude of difference</td>
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<td>+19%</td>
</tr>
</tbody>
</table>

P < 0.0001

**Table G**

<table>
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<th>No. of neurons (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>554 (52)</td>
<td>505 (48)</td>
</tr>
<tr>
<td>CTR1-IR</td>
<td>46 (17)</td>
<td>221 (83)</td>
</tr>
<tr>
<td>Magnitude of difference</td>
<td>-35%</td>
<td>+35%</td>
</tr>
</tbody>
</table>

P < 0.0001

**Table H**

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<th>No. of neurons (%)</th>
<th>No. of neurons (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>1605 (52)</td>
<td>1488 (48)</td>
</tr>
<tr>
<td>CTR1-IR</td>
<td>174 (24)</td>
<td>557 (76)</td>
</tr>
<tr>
<td>Magnitude of difference</td>
<td>-28%</td>
<td>+28%</td>
</tr>
</tbody>
</table>

P < 0.0001
Figure 6.3: Cell body size frequency histograms of ATP7A immuno-reactive neurons in primary cultures of DRG from healthy animals

(A) to (D) cell body size frequency histograms of ATP7A-IR (red bars) and ATP7A-negative neurons (black bars) from experiment 1 (A), experiment 2 (B), experiment 3 (C) and combined data from experiment 1 to 3 (D). Dotted black vertical lines and values show the median cross-sectional cell body area value for the total neuronal population for each experiment. The percentage value shown on each graph represents the proportion of ATP7A-IR neurons that had cell body cross-sectional areas greater than the median value of the total population. Approximately half of the ATP7A neurons (51%) were larger than the average DRG neuron.

(E) to (H) Contingency tables for experiment 1 (E), experiment 2 (F), experiment 3 (G) and combined data from experiment 1 to 3 (H) showing the number and percentages of ATP7A-IR and total neurons with cross-sectional cell body areas less than or greater than the median value for the total neuronal population. $P$ values are from Chi square tests.
<table>
<thead>
<tr>
<th></th>
<th>No. of neurons (%) ≤ median</th>
<th>No. of neurons (%) &gt; median</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>559 (55)</td>
<td>451 (45)</td>
</tr>
<tr>
<td>ATP7A-IR</td>
<td>248 (51)</td>
<td>235 (49)</td>
</tr>
<tr>
<td>Magnitude of difference</td>
<td>-4%</td>
<td>+4%</td>
</tr>
<tr>
<td>P</td>
<td>0.1467</td>
<td></td>
</tr>
</tbody>
</table>

| **F** |                            |                             |
| Total | 621 (54)                   | 525 (46)                   |
| ATP7A-IR | 294 (61)             | 187 (39)                   |
| Magnitude of difference | -7%                        | +7%                         |
| P     | <0.0001                    |                             |

| **G** |                            |                             |
| Total | 537 (54)                   | 463 (46)                   |
| ATP7A-IR | 157 (34)             | 301 (66)                   |
| Magnitude of difference | -20%                       | +20%                        |
| P     | <0.0001                    |                             |

| **H** |                            |                             |
| Total | 1717 (54)                  | 1439 (46)                  |
| ATP7A-IR | 699 (49)             | 723 (51)                   |
| Magnitude of difference | -5%                        | +5%                         |
| P     | 0.0010                     |                             |
reactive neurons for three cultures combined was 354 µm² and the results from all three cultures were similar and consistent. ATP7A immuno-reactive neurons were distributed across all cell size and were evenly spread with approximately half the ATP7A immuno-reactive neurons spit at the median (49 ± 14% below median, 51 ± 14% above median, Figure 6.3).

The staining frequency for ATP7A for each culture was 47.8, 42.0 and 44.0% (45.2 ± 3.0%). The mean cell body area for the three cultures combined for ATP7A immuno-reactive neurons was 410 ± 47.0 µm².

### 6.3.1.2.3. Comparison of CTR1 and ATP7A Immuno-reactive Neurons

CTR1 and ATP7A immuno-reactive neurons appeared to have largely overlapping size profile (Figure 6.4 A). However, the portion of CTR1 and ATP7A immuno-reactive neurons above the median was significantly different, suggesting that CTR1 and ATP7A were expressed in different neurons with CTR1 have a slightly bigger cell body area (Figure 6.4 B; \( P < 0.0009 \), Prism two-tailed Chi-square test).

The percentage of neurons that had immuno-reactivity towards the two copper influx and efflux transporter were significantly different. ATP7A had 21.6% more staining than CTR1 (Table 6.1; \( P = 0.0004 \), Student’s T-test).

The mean cell body area for the three cultures combined for CTR1 and ATP7A immuno-reactive neurons were 428 ± 17.2 and 410 ± 47.0 µm² respectively and were not significantly different (Table 6.1, \( P = 0.5670 \); Student’s T-test).

### 6.3.2. Effect of Oxaliplatin on Copper Transporter Expressing Neurons In Vitro

#### 6.3.2.1. CTR1- Immuno-reactive Neurons

To observe the time dependence of oxaliplatin on CTR1 immuno-reactive neurons, primary cultures of DRG neurons were exposed to 10 µM of oxaliplatin for 0, 1, 3, 8 and 24 hr. To observe the concentration dependence of effects of oxaliplatin on
Figure 6.4: Comparison of cell body cross-sectional area frequency histograms of CTR1-IR and ATP7A-IR neurons cultured from the DRG of healthy rats. CTR1- and ATP7A-IR neurons had overlapping but non-identical size profiles.

(A) Frequency histogram of the cross-sectional cell body areas of CTR1 (green bars) and ATP7A (red bars) immuno-reactive neurons using data pooled from experiments 1 to 3. The dotted black vertical lines and value represents the median cross-sectional area value for the combined CTR1 and ATP7A neuronal populations. (B) Contingency table for pooled data from experiment 1 to 3 showing the number and percentages (%) of CTR1-IR and ATP7A neurons with cross-sectional cell body areas equal to or less than and greater than the median value of both populations. The $P$ value is from a Chi square test.
Table 6.1: Morphometry of CTR1 and ATP7A immuno-reactive neurons cultured from the DRG of healthy rats. CTR1 immuno-reactive neurons were fewer in number than ATP7A immuno-reactive neurons.

<table>
<thead>
<tr>
<th></th>
<th>Neuronal number</th>
<th>Cell body area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n IR</td>
<td>n total</td>
</tr>
<tr>
<td><strong>CTR1-IR neurons</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture 1</td>
<td>251</td>
<td>1046</td>
</tr>
<tr>
<td>Culture 2</td>
<td>213</td>
<td>972</td>
</tr>
<tr>
<td>Culture 3</td>
<td>267</td>
<td>1059</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>244</td>
<td>1026</td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td>28</td>
<td>47</td>
</tr>
<tr>
<td><strong>95% CI</strong></td>
<td>189 - 299</td>
<td>934 - 1118</td>
</tr>
<tr>
<td><strong>ATP7A-IR neurons</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture 1</td>
<td>483</td>
<td>1010</td>
</tr>
<tr>
<td>Culture 2</td>
<td>481</td>
<td>1151</td>
</tr>
<tr>
<td>Culture 3</td>
<td>458</td>
<td>1040</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>474</td>
<td>1067</td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td>14</td>
<td>74</td>
</tr>
<tr>
<td><strong>95% CI</strong></td>
<td>447 - 501</td>
<td>922 - 1212</td>
</tr>
</tbody>
</table>

| **P value**    | 0.0002         | 0.4609               | 0.0004 | 0.5670 |        |            |        |

P values for differences between the mean values for CTR1 and ATP7A immuno-reactive neurons (Student's T-test).
CTR1 immunoreactive neurons, primary cultures of DRG were treated with 0, 1, 10, 30 and 100 µM of oxaliplatin for 3 hr.

6.3.2.1.1. Morphology

Oxaliplatin treatment induced atrophy in CTR1-IR neurons as apparent by reduction in cell body area of neurons (Figure 6.5). The localization of CTR1 protein appeared to change as oxaliplatin exposure time increased as seen by a lost in membrane staining and gained in intensity of cytoplasmic staining of CTR1 after oxaliplatin treatment.

6.3.2.1.2. Morphometry

6.3.2.1.2.1. Total CTR1 Immuno-reactive Neuronal Population

6.3.2.1.2.1.1. Time Course

Oxaliplatin appeared to have altered the size distribution of CTR1 immuno-reactive neurons in a time dependent manner by inducing a leftward shift on the cell body size frequency histogram which is indicative of neuronal atrophy (Figure 6.6 A). The portion of CTR1 immuno-reactive neurons with cell body area larger than the median of the CTR1 immuno-reactive neurons from two cultures reduced with a longer time treatment of oxaliplatin (Figure 6.6 B). Chi square analysis showed that the time dependent reduction was of statistical significance (Figure 6.6 C). No changes in the expression frequency were observed for CTR1 immuno-reactive neurons during this time course (Figure 6.6 D; \( P = 0.8002 \); 1 way ANOVA). The mean expression frequency of total CTR1 immuno-reactive neurons at 0 hr of oxaliplatin treatment was 33.0 ± 6.1%. The mean expression frequency was 35.1% at 1 hr, 30.3 ± 12.0% at 3 hr, 27.7 ± 8.2% at 8 hr and 25.5 ± 2.4% at 24 hr.

6.3.2.1.2.1.2. Concentration Response

The cell body size frequency histogram of CTR1 immuno-reactive neurons showed an oxaliplatin concentration dependent leftward shift from 0 to 100 µM (Figure 6.6 E). The percentage of CTR1 immuno-reactive neurons with cell body area larger than
Figure 6.5: Altered CTR1 staining in cultured DRG neurons after exposure to oxaliplatin at 10 µM for 24 hr.

Compared to control (A), oxaliplatin-treated DRG neurons (B) appeared smaller and to have lost membrane staining and gained intensity of cytoplasmic staining of CTR1. Scale bar = 20 µM.
CTR1-IR Neurons (total)

Time Course

- 0 hr
- 1 hr
- 3 hr
- 8 hr
- 24 hr

Conc. Dependent

- 0 µM
- 1 µM
- 10 µM
- 30 µM
- 100 µM

Below lower quartile
Lower quartile – Median
Median – upper quartile
Above upper quartile

B

% of neurons

% of neurons

Time of Oxaliplatin Treatment (hr)

Frequency of staining (%)

Frequency of staining (%)

Time of Oxaliplatin Treatment (hr)

Concentration of Oxaliplatin Treatment (µM)

Concentration of Oxaliplatin Treatment (µM)

D

<table>
<thead>
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<th>Time (hr)</th>
<th>No. of neurons (%) &lt; median</th>
<th>No. of neurons (%) &gt; median</th>
</tr>
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<tbody>
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<td>0</td>
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<td>310 (46)</td>
</tr>
<tr>
<td>1</td>
<td>345 (79)</td>
<td>93 (21)</td>
</tr>
<tr>
<td>3</td>
<td>364 (79)</td>
<td>96 (21)</td>
</tr>
<tr>
<td>8</td>
<td>345 (76)</td>
<td>111 (24)</td>
</tr>
<tr>
<td>24</td>
<td>370 (79)</td>
<td>99 (21)</td>
</tr>
</tbody>
</table>

P < 0.0001

H

<table>
<thead>
<tr>
<th>Conc. (µM)</th>
<th>No. of neurons (%) &lt; median</th>
<th>No. of neurons (%) &gt; median</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>241 (57)</td>
<td>185 (43)</td>
</tr>
<tr>
<td>1</td>
<td>257 (74)</td>
<td>89 (26)</td>
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<tr>
<td>10</td>
<td>292 (76)</td>
<td>93 (24)</td>
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<tr>
<td>30</td>
<td>198 (74)</td>
<td>71 (26)</td>
</tr>
<tr>
<td>100</td>
<td>230 (78)</td>
<td>66 (22)</td>
</tr>
</tbody>
</table>

P < 0.0001
Figure 6.6: Time course (A-D) and concentration dependence (E-H) of the effect of oxaliplatin exposure on the morphometry of the total population of CTR1-IR neurons

(A) Cell body size frequency histogram of CTR1 immuno-reactive neurons (total) exposed to oxaliplatin (10 µM) for various times.

(B) Bar graphs showing the portion of neurons with cell body area below lower quartile, between lower quartile and median, between median and upper quartile and above upper quartile in the control group with immuno-reactivity for CTR1 exposed to oxaliplatin (10 µM) for various times.

(C) Contingency table showing the numbers and proportion of CTR1 immuno-reactive neurons > or ≤ to the median value of the control group of CTR1 immuno-reactive neuronal population in each treatment group exposed to oxaliplatin (10 µM) for various times.

(D) The staining frequency of CTR1 immuno-reactive neurons as a percentage of the total population of neurons exposed to oxaliplatin (10 µM) for various times.

(E) Cell body size frequency histogram of CTR1 immuno-reactive neurons (total) exposed to oxaliplatin (3 hr) at various concentrations.

(F) Bar graphs showing the portion of neurons with cell body area below lower quartile, between lower quartile and median, between median and upper quartile and above upper quartile in the control group with immuno-reactivity for CTR1 exposed to oxaliplatin (3 hr) at various concentrations.

(G) Contingency table showing the numbers and proportion of CTR1 immuno-reactive neurons > or ≤ to the median value of the control group of CTR1 immuno-reactive neuronal population in each treatment group exposed to oxaliplatin (3 hr) at various concentrations.
The staining frequency of CTR1 immuno-reactive neurons as a percentage of the total population of neurons exposed to oxaliplatin (3 hr) at various concentrations.

Oxaliplatin induced a leftwards shift in the size profile of CTR1 immuno-reactive neurons without affecting their frequency of staining for CTR1. These changes were dependent on both time and concentration.
the median of the CTR1 immuno-reactive neurons from two cultures reduced significantly as oxaliplatin concentration increased (Figure 6.6 F and G; \( P < 0.0001 \); Chi Square Analysis). The expression frequencies for CTR1 immuno-reactive neurons were not statically different with an increase in concentration (Figure 6.6 H; \( P = 0.5357 \); 1 way ANOVA). The frequency of total CTR1 immuno-reactive neurons in the control group, 1, 10, 30 and 100 µM were 24.0 ± 5.5% , 20.3 ± 6.6, 21.0 ± 4.6, 19.7 ± 1.9 and 18.3 ± 2.9% respectively.

6.3.2.1.2.2. CTR1 Immuno-reactive Neurons (Plasma Membrane Staining)

6.3.2.1.2.2.1. Time Course
Oxaliplatin altered the size distribution of CTR1 immuno-reactive neurons with prominent plasma membrane staining by shifting these neurons to the left on the size distribution graph (Figure 6.7 A). The percentage of CTR1 immuno-reactive neurons with prominent plasma membrane staining that had cell body area larger than the median of the CTR1 membrane neurons from two cultures reduced significantly as oxaliplatin time increased (Figure 6.7 B and C; \( P < 0.0001 \); Chi Square Analysis). Oxaliplatin induced a time dependant reduction in staining frequency in neurons with prominent CTR1 plasma membrane staining (Figure 6.7 D). At 0 hr 26.0 ± 4.2% of neurons showed strong CTR1 plasma membrane staining. After 1, 3, 8 and 24 hr of oxaliplatin treatment the percentage of neurons that had strong CTR1 plasma membrane staining dropped to 21.5, 16.8 ± 5.8, 12.1 ± 7.3 and 7.2 ± 1.6% respectively. The frequency of CTR1 membrane staining was significantly lower at 24 hr compared to 0 hr (\( P = 0.0272 \); 1-way ANOVA).

6.3.2.1.2.2.2. Concentration Response
CTR1 immuno-reactive neurons with prominent plasma membrane staining shifted to the left on the cell body size frequency graph after oxaliplatin treatment from 0 to 100 µM (Figure 6.7 E). The percentage of CTR1 immuno-reactive neurons with prominent plasma membrane staining that had cell body area larger than the median of the CTR1 membrane neurons from two cultures reduced significantly as
**CTR1-IR Neurons (with membrane staining)**

**Time Course**

A. 

- Cell body cross-sectional area (μm²)
- % of neurons
- Time: 0 hr, 1 hr, 3 hr, 8 hr, 24 hr

**Conc. Dependent**

E. 

- Cell body cross-sectional area (μm²)
- % of neurons
- Concentration: 0 μM, 1 μM, 10 μM, 30 μM, 100 μM

**B**

- Time of Oxaliplatin Treatment (hr)
- % of neurons

**C**

- Frequency of staining (%)
- Time of Oxaliplatin treatment (hr)

**D**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>No. of neurons (% &lt; median)</th>
<th>No. of neurons (% &gt; median)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>277 (53)</td>
<td>249 (47)</td>
</tr>
<tr>
<td>1</td>
<td>199 (91)</td>
<td>20 (9)</td>
</tr>
<tr>
<td>3</td>
<td>202 (79)</td>
<td>53 (21)</td>
</tr>
<tr>
<td>8</td>
<td>157 (75)</td>
<td>52 (25)</td>
</tr>
<tr>
<td>24</td>
<td>107 (80)</td>
<td>26 (20)</td>
</tr>
</tbody>
</table>

\( P < 0.0001 \)

**E**

- Concentration of Oxaliplatin Treatment (μM)
- % of neurons

**F**

- Frequency of staining (%)
- Concentration of Oxaliplatin treatment (μM)

**G**

<table>
<thead>
<tr>
<th>Conc. (μM)</th>
<th>No. of neurons (% &lt; median)</th>
<th>No. of neurons (% &gt; median)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>166 (56)</td>
<td>128 (44)</td>
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<td>62 (24)</td>
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<td>10</td>
<td>109 (69)</td>
<td>50 (31)</td>
</tr>
<tr>
<td>30</td>
<td>69 (68)</td>
<td>23 (32)</td>
</tr>
<tr>
<td>100</td>
<td>117 (83)</td>
<td>24 (17)</td>
</tr>
</tbody>
</table>

\( P < 0.0001 \)
Figure 6.7: Time course (A-D) and concentration dependence (E-H) of the effect of oxaliplatin exposure on the morphometry of a sub-population of CTR1-IR neurons with plasma membrane staining

(A) Cell body size frequency histogram of CTR1 immuno-reactive neurons with plasma membrane staining exposed to oxaliplatin (10 µM) for various times.

(B) Bar graphs showing the portion of neurons with cell body area below lower quartile, between lower quartile and median, between median and upper quartile and above upper quartile in the control group with CTR1 plasma membrane immuno-reactivity exposed to oxaliplatin (10 µM) for various times.

(C) Contingency table showing the numbers and proportion of CTR1 immuno-reactive neurons with plasma membrane staining > or ≤ to the median value of the control group of CTR1 immuno-reactive neuronal population in each treatment group exposed to oxaliplatin (10 µM) for various times.

(D) The staining frequency of CTR1 immuno-reactive neurons with plasma membrane staining as a percentage of the total population of neurons exposed to oxaliplatin (10 µM) for various times.

(E) Cell body size frequency histogram of CTR1 immuno-reactive neurons with plasma membrane staining exposed to oxaliplatin (3 hr) at various concentrations.

(F) Bar graphs showing the portion of neurons with cell body area below lower quartile, between lower quartile and median, between median and upper quartile and above upper quartile in the control group with CTR1 plasma membrane immuno-reactivity exposed to oxaliplatin (3 hr) at various concentrations.

(G) Contingency table showing the numbers and proportion of CTR1 immuno-reactive neurons with plasma membrane staining > or ≤ to the median value of the control group of CTR1 immuno-reactive neuronal population in each treatment group exposed to oxaliplatin (3 hr) at various concentrations.
(H) The staining frequency of CTR1 immuno-reactive neurons as a percentage of the total population of neurons with plasma membrane staining exposed to oxaliplatin (3 hr) at various concentrations.

Oxaliplatin induced a leftwards shift in the size profile of CTR1 immuno-reactive neurons with plasma membrane staining and reduced their frequency of staining. These changes were dependent on both time and concentration.
oxaliplatin concentration increased (Figure 6.7 F and G; \( P < 0.0001 \); Chi Square Analysis). The expression frequencies of CTR1 immuno-reactive neurons with prominent plasma membrane staining in the control group were 16.6 ± 3.8% and decreased in a concentration dependant manner with oxaliplatin treatment at 0.3, 1, 3, 10, 30 and 100 µM to 9.8 ± 1.3, 14.8 ± 7.0, 10.0 ± 2.8, 8.6 ± 5.0, 7.4 ± 1.5 and 8.5 ± 3.6% respectively. The reduction in staining frequency plateau at 30 µM, however it was not of statistical significances (\( P = 0.3068 \); 1 way ANOVA).

6.3.2.1.2.3. CTR1 Immuno-reactive Neurons (Without Plasma Membrane Staining)

6.3.2.1.2.3.1. Time Course

CTR1 immuno-reactive neuronal population without membrane staining shifted to the left on the cell body size frequency histogram after treatment of oxaliplatin with increasing time which was indicative of neuronal atrophy (Figure 6.8 A). Oxaliplatin induced a significant time dependent reduction in the percentage of CTR1 cytoplasmic neurons that had cell body area larger than the median of the CTR1 cytoplasmic neurons in the two cultures combined. (Figure 6.8 B and C, \( P < 0.0001 \); Chi Square Analysis). The mean expression frequency of CTR1 immuno-reactive neurons without membrane staining increased with time and was highest at 24 hr. In the control group 7.0 ± 1.9% of neurons showed CTR1 immuno-reactivity without membrane staining. As the oxaliplatin exposure time increased to 1, 3, 8 and 24 hr, the staining frequency rouse gradually to 13.6, 13.5 ± 6.1, 15.6 ± 0.9 and 18.3 ± 0.8% respectively. The expression frequency of neurons without CTR1 plasma membrane staining was significantly different at 0 hr when compared to 24 hr (Figure 6.8 D, \( P < 0.05 \); 1-way ANOVA).

6.3.2.1.2.3.2. Concentration Dependent

The cell body size frequency histogram of CTR1 immuno-reactive neuronal population without membrane staining showed an oxaliplatin concentration dependent leftward shift with an increasing concentration of 0 to 100 µM (Figure 6.8 E). As the concentration of oxaliplatin increased there was a significant rise in the
Figure 6.8 Time course (A-D) and concentration dependence (E-H) of the effect of oxaliplatin exposure on the morphometry of a sub-population of CTR1-IR neurons without plasma membrane staining

(A) Cell body size frequency histogram of CTR1 immuno-reactive neurons without plasma membrane staining exposed to oxaliplatin (10 µM) for various times.

(B) Bar graphs showing the portion of neurons with cell body area below lower quartile, between lower quartile and median, between median and upper quartile and above upper quartile in the control group without CTR1 plasma membrane immuno-reactivity exposed to oxaliplatin (10 µM) for various times.

(C) Contingency table showing the numbers and proportion of CTR1 immuno-reactive neurons without plasma membrane staining > or ≤ to the median value of the control group of CTR1 immuno-reactive neuronal population in each treatment group exposed to oxaliplatin (10 µM) for various times.

(D) The staining frequency of CTR1 immuno-reactive neurons without plasma membrane staining as a percentage of the total population of neurons exposed to oxaliplatin (10 µM) for various times.

(E) Cell body size frequency histogram of CTR1 immuno-reactive neurons without plasma membrane staining exposed to oxaliplatin (3 hr) at various concentrations.

(F) Bar graphs showing the portion of neurons with cell body area below lower quartile, between lower quartile and median, between median and upper quartile and above upper quartile in the control group without CTR1 plasma membrane immuno-reactivity exposed to oxaliplatin (3 hr) at various concentrations.

(G) Contingency table showing the numbers and proportion of CTR1 immuno-reactive neurons > or ≤ to the median value of the control group of CTR1 immuno-reactive neuronal population without plasma membrane staining in each treatment group exposed to oxaliplatin (3 hr) at various concentrations.
(H) The staining frequency of CTR1 immuno-reactive neurons without plasma membrane staining as a percentage of the total population of neurons exposed to oxaliplatin (3 hr) at various concentrations.

Oxaliplatin decreased the size of CTR1-IR neurons without plasma membrane staining but increased the staining frequency in a time and concentration dependent manner.
percentage of CTR1 cytoplasmic neurons that had cell body area larger than the median of the CTR1 cytoplasmic neurons in the two cultures combined (Figure 6.8 F and G; $P<0.0001$; Chi Square Analysis). CTR1 immuno-reactive neurons without membrane staining increased in staining frequency with increasing oxaliplatin concentration and reached a plateau at 10 µM (Figure 6.8 H, $P<0.01$; 1 way ANOVA). The staining frequency for CTR1 cytoplasmic neurons at 0, 0.3, 1, 3, 10, 30 and 100 µM were 7.4 ± 1.7, 5.3 ± 0.2, 5.5 ± 0.4, 9.4 ± 1.2, 12.4 ± 0.4, 12.3 ± 0.3 and 9.8 ± 0.7% respectively.

### 6.3.3. Comparison of Oxaliplatin Toxicity on CTR1 and ATP7A Immuno-reactive Neurons

To compare the effect of oxaliplatin on CTR1 and ATP7A immuno-reactive neurons, primary cultures of DRG neurons were exposed to 10 µM of oxaliplatin for 3 hr. The cultured DRG neurons were then fixed and incubated with anti-CTR1 or ATP7A antibodies after the exposure of oxaliplatin. The cross-sectional cell body area of between 460 and 804 neurons that had immuno-reactivity for CTR1 or ATP7A were measured. These oxaliplatin treatment conditions were selected based on the experiments conducted in 6.3.3 and 6.3.4 where optimal oxaliplatin exposure time and concentrations were determined based on a maximal observable effect in the form of cell body shrinkage and a reduction in staining frequency induced by oxaliplatin. Two cultures were used to generate results presented in this section and the data are presented as the mean of the two cultures. Primary DRG neurons expressing ATP7A seemed to be affected by oxaliplatin treatment at 10 µM for 3 hr at a lesser degree compared to CTR1 immuno-reactive neurons.

#### 6.3.3.1 Qualitative Changes or Morphology

The localization of CTR1 protein appeared to change as oxaliplatin exposure time increased (Figure 6.9). Oxaliplatin induced a loss of the CTR1 membrane staining frequency. Unlike CTR1, the pattern of ATP7A protein localization in DRG neurons did not appear to be affected by oxaliplatin treatment (Figure 6.9). The pattern of
Figure 6.9: CTR1 staining in cultured DRG neurons was altered by oxaliplatin treatment at 10 µM without any apparent change in ATP7A immunostaining.

Top and bottom panels show images of untreated and oxaliplatin treated cultured DRG neurons respectively. Oxaliplatin treatment (10 µM) at 3 hr and 24 hr diminished CTR1 membrane staining compared to control untreated neurons without reducing its cytoplasmic staining. Oxaliplatin did not appear to affect the pattern of ATP7A protein localization in DRG neurons, where cytoplasmic staining are seen in both untreated and oxaliplatin treated neurons. Scale bar = 20 µM.
ATP7A protein expression was similar after oxaliplatin treatment and the localization remained in the cytoplasm of neuronal cell bodies.

6.3.3.2 Morphometry

Oxaliplatin treatment at 10 µM for 3 hr induced a leftward shift in the size distribution of CTR1 immuno-reactive neurons which was indicative of neuronal atrophy (Figure 6.10 A). In contrast, the size distribution of ATP7A immuno-reactive neurons appeared relatively unchanged by oxaliplatin (Figure 6.10 E). After oxaliplatin treatment the percentage of CTR1 immuno-reactive neurons that were smaller than the median value of the untreated control group significantly increased from 54 to 80% (Figure 6.10 B and C, \( P < 0.0001 \); Two-tailed Chi-square test). After oxaliplatin treatment the percentage of ATP7A immuno-reactive neurons that were smaller than the median value of the untreated control group increased numerically from 53% to 65% but it was not of statistically significance (Figure 6.10 F and G, \( P = 0.0602 \); Two-tailed Chi-square test).

The frequency of staining remained unchanged after oxaliplatin treatment in both CTR1 and ATP7A immunoreactive neuronal sub-populations. The percentage of neurons with immuno-reactivity towards CTR1 was 33.0 ± 6.1% in control and 30.3 ± 12% in the oxaliplatin treated group (Figure 6.10 D, \( P = 0.8035 \); Student’s T-test). The expression frequency of ATP7A in the control and oxaliplatin treatment group was 46.9 ± 1.0% and 46.2 ± 1.5% respectively (Figure 6.10 H, \( P = 0.6299 \); Student’s T-test).

6.4 Discussion

In the experiments described in this chapter, the expression of ATP7A and CTR1 in both healthy untreated and oxaliplatin treated primary cultures of rat DRG neurons were investigated. The DRG primary culture neurons model utilized here provided a useful model to study the role of copper transporters in oxaliplatin induced neuropathy. The time and concentration dependent changes of oxaliplatin induced cellular changes of copper transporter were investigated in the primary cultures of rat DRG neurons. Cultured rat DRG neurons presented as large cells with highly refringent soma and well developed neuritic trees. Both neuronal and non-neuronal
Figure 6.10: Comparative effect of oxaliplatin (10 µM for 3 hr) on the morphometry of CTR1-IR and ATP7A-IR neurons

(A) and (E) Cell body size frequency histograms. The vertical dotted line represents the median cell body area of the immuno-reactive neurons in the respective control group. Oxaliplatin induced a more prominent left shift of CTR1 immuno-reactive neurons compared to ATP7A immuno-reactive neurons.

(B) and (F) are bar graphs showing the portions of neurons > or ≤ to the median cell body area of the control group for CTR1 and ATP7A.

(C) and (G) are tables showing the frequency of neurons > or ≤ to the median cell body area of respective immuno-reactive neuronal population of the control group, compared to the oxaliplatin treated group. Prism unpaired Two-tailed Chi-square test.

(D) and (H) shows the frequency of staining for CTR1 (D) and ATP7A (H) immuno-reactive neurons after oxaliplatin treatment at 10 µM for 3 hr.

Oxaliplatin reduced the staining of CTR1 immuno-reactive neurons without affecting the number of ATP7A immuno-reactive neurons.
supporting fibroblasts and Schwann cells were found in the culture. Neuronal cell bodies were seen interspersed between with axon visibly extending out from the neuronal cell bodies.

In untreated cultures, CTR1 and ATP7A protein were found to be expressed in distinct sub-populations of neurons cultured from rat DRG with overlapping size profile population. The expression of CTR1 and ATP7A were both restricted to neuronal cell bodies without immuno-staining of nerve fibres, satellite cells or other tissue elements. However the pattern of expression of CTR1 differed from ATP7A. CTR1 was expressed in the plasma membrane and cytoplasmic cell body and ATP7A was expressed only in the cytoplasm of neurons. CTR1 and ATP7A were found to be expressed in largely overlapping but distinct sub-population of neurons with minimal co-localization when examined by immunofluorescence double labelling. The expression frequency of CTR1 immuno-reactive and ATP7A immuno-reactive neurons were also notably different. The expression frequency of ATP7A was approximately double of that of CTR1 (45% versus 24%). The size frequency profile and mean cell body area of CTR1 and ATP7A were similar and appear to have overlapping size profile. However, the portion of CTR1 and ATP7A immuno-reactive neurons above the median were significantly different when analysed using statistical test suggesting that although they appear to be comparable in cell body area but CTR1 and ATP7A were not expressed in the same neuronal sub-population.

Oxaliplatin induced atrophy in CTR1 immuno-reactive neurons in primary cultures of rat DRG. The mean cell body area of total CTR1 immuno-reactive neurons was significantly reduced after oxaliplatin treatment at different concentration. A similar trend in neuronal cell body shrinkage was observed in the time point experiments but it was not of statistical significance. In addition oxaliplatin significantly altered the cell size frequency distribution of CTR1 immuno-reactive neurons inducing a leftward shift in this neuronal sub-population which was indicative of neuronal atrophy.

Oxaliplatin induced a time dependent reduction in numbers of neurons with strong CTR1 plasma membrane staining and an increase in numbers of CTR1 immuno-
reactive neurons lacking plasma membrane staining, without altering the total number of neurons expressing CTR1. The oxaliplatin induced reduction in membrane staining of CTR1 in CTR1 immuno-reactive neurons was also dependent upon the concentration of oxaliplatin. The drop in plasma membrane staining frequency was inversely proportional to the increase in neurons immuno-reactive for CTR1 without plasma membrane staining, thus the overall frequency of expression in total CTR1 immuno-reactive neurons remained unchanged as time and concentration of oxaliplatin increased. The time and concentration dependent effect of oxaliplatin on CTR1 plasma membrane neuronal expression frequency could suggest that oxaliplatin is a substrate of CTR1 in primary culture of rat DRG.

The results from this chapter suggest that oxaliplatin exposure to cultured DRG neurons induces trafficking of CTR1 protein in the plasma membrane to the cytoplasmic. Although the movement of CTR1 protein was not investigated but the overall expression frequency of CTR1 immuno-reactive neurons remained similar at all time points suggesting that the loss in CTR1 plasma membrane staining resulted in an increase in CTR1 immuno-reactive neurons without plasma membrane staining. A rational hypothesis is that the trafficking of CTR1 in primary cultures of DRG neurons was induced by oxaliplatin treatment in a time dependent manner. This is not surprising because the movement of CTR1 protein induced by its substrate has been reported by several research groups previously. It was observed that substrate exposure to CTR1 caused a reduction of CTR1 protein seen on the plasma membrane (Guo et al., 2004, Larson et al., 2010, Petris et al., 2003, Molloy et al., 2009).

CTR1 and ATP7A immuno-reactive neurons differed in their response to oxaliplatin. Both CTR1 and ATP7A immuno-reactive neuronal population underwent atrophy after oxaliplatin treatment but the degree of atrophy was more severe for CTR1 immuno-reactive neurons. Oxaliplatin induced a significant drop in the portion of CTR1 immuno-reactive neurons that had cell body area above the median of the culture in the control group but the reduction in ATP7A immuno-reactive neurons were not of significance. In addition, oxaliplatin affected the staining pattern of CTR1 immuno-reactive neurons causing a reduction in membrane staining frequency and
an increase in cytoplasmic staining frequency as mentioned above. In contrast, the cytoplasmic staining pattern of ATP7A immuno-reactive neurons appeared not to be affected by oxaliplatin. Lastly, oxaliplatin induced an obvious shift in the size distribution frequency of CTR1 immuno-reactive neurons compared to ATP7A immuno-reactive neurons.

In conclusion, CTR1 and ATP7A were found to be expressed in distinct sub-population of cultured rat DRG neurons. Oxaliplatin caused atrophy in the neurons expressing CTR1 and a loss of membrane staining frequency without altering the number of neurons. Neurons expressing ATP7A was not affected by oxaliplatin treatment.
CHAPTER SEVEN

GENERAL DISCUSSION

7.1. Implications of Specific Localization of ATP7A and CTR1 Protein in Rat DRG Tissue and Primary Culture of DRG Neurons

In the current study ATP7A was found to be expressed in the cell cytoplasm while CTR1 had strong immunoreactivity in the plasma membrane and occasional diffuse reactivity in the cytoplasm of rat DRG neurons. The protein localization of ATP7A and CTR1 in DRG neurons is consistent with their reported protein localization and the role these copper transporters play in other cell types. For example, ATP7A is putatively located in the trans-Golgi network and functions to sequester excess copper into vesicles (Petris et al., 1996). CTR1 is reported to be expressed in the plasma membrane to influx copper into the cells (Klomp et al., 2002). The immunostaining pattern of CTR1 found in this study corresponded well with the recent reports of CTR1 expression in DRG from our lab (Liu et al., 2009). In addition, the protein expression patterns of ATP7A and CTR1 in DRG tissue is consistent with the findings in primary culture of DRG neurons.

To our knowledge this is the first time that the protein localization of copper efflux transporting protein is reported in DRG of rats. The physiological significance of the differential expression of copper transporters by DRG neurons is unclear and requires further studies. However, it can be speculated that ATP7A and CTR1 may be required by distinct sub-types of DRG neurons to deliver copper to specific cuproenzymes vital for the synthesis of neuropeptides and ATP. ATP7A, for example, delivers copper to peptidylglycine alpha-amidating monooxygenase in cell types other than DRG neurons (El Meskini et al.,
2003, Hansel et al., 2001, Stevenson et al., 2003), but this cuproenzyme activity is required by DRG neurons for the synthesis of substance P (Erion et al., 1994, Jeng et al., 1997, Wong et al., 1994). Like ATP7A, substance P is primarily expressed by small DRG neurons (Hokfelt et al., 1975, Jamieson et al., 2005, McCarthy et al., 1989), and the size of substance P-expressing DRG neurons was not altered by oxaliplatin treatment (Jamieson et al., 2005). This suggests the existence of a subset of sensory neurons that co-express ATP7A, peptidyl alpha-amidating monooxygenase and substance P to support neuronal functions requiring neuropeptide synthesis. In other cell types, the level of expression of CTR1 corresponds closely with the activity of cytochrome C oxidase (Kim et al., 2009, Lee et al., 2001, Lee et al., 2002), which is a cuproenzyme involved in oxidative phosphorylation for ATP synthesis. Like CTR1, cytochrome C oxidase is expressed intensely by large-sized DRG neurons (Karmy et al., 1991), which may have reduced capacity for glycolysis compared to small DRG neurons (Gardiner et al., 2007), consistent with their strong need for CTR1 to meet their high demands of copper for delivery to cytochrome C oxidase and subsequent ATP synthesis via oxidative phosphorylation. In this way, the neuronal subtype-specific and largely non-overlapping distribution of ATP7A and CTR1 in DRG tissue may relate to specific cuproenzyme requirements by distinct subsets of primary sensory neurons.

The presence of copper transporting protein must be of importance in DRG neurons and are likely to be evolutionarily conserved similar to the structural features (Puig et al., 2002) and functions (Hua et al., 2010) that are conserved between species. It appears that copper is essential for the cellular function of DRG neurons. In untreated control animals, the copper levels measured in the DRG were at a level similar to that of the heart, a tissue that were reported to have a high need for copper for the generation of a high demand of ATP required for normal cardiac tissue functioning (Medeiros et al., 1993). This idea that DRG utilizes high copper is also supported by clinical reports of long-term copper deficiency being linked to the development of peripheral sensory neuropathy (Khaleeli et al., 2010, Zara et al., 2009). Thus the expression of copper transporting proteins may aid to maintain copper balance and deliver copper for basic cellular activities in the DRG neurons.

7.2. Possible Roles of CTR1 and ATP7A in Oxaliplatin-Induced Neuropathy
The finding in the current study suggested that copper transporters are possible determinants of oxaliplatin-induced neurotoxicity in DRG by modulating the movement of oxaliplatin into the neurons. We reported that copper transporters appeared to be involved in the transport of oxaliplatin into DRG neurons and that neurons expressing copper influx transporters are more vulnerable to the toxicity of oxaliplatin than neurons expressing copper efflux transporters.

The results from both the *in vivo* Wistar rat model and the *in vitro* primary cultures of rat DRG neurons showed that oxaliplatin induced changes in CTR1-IR neurons to a greater degree than ATP7A-IR neurons. These findings suggested that neurons expressing copper influx transporter are transporting oxaliplatin into DRG neurons and making them more vulnerable to the toxicity of platinum drugs, whereas neurons expressing copper efflux transporter are sequestering oxaliplatin in to cytoplasmic vesicles thereby sparing them from harm.

Other observations from the *in vitro* studies also supports that copper transporters are involved in the transport of oxaliplatin into DRG neurons. It was shown that oxaliplatin induced changes in the protein localization of the CTR1 in primary cultures of DRG neurons. This coincides with previous studies where alterations in the distribution and sub-cellular localization of CTR1 were documented after exposure to platinum drugs along with the evidence of platinum drug uptake into these cells by CTR1 (Holzer et al., 2004a, Holzer et al., 2004b). For example, Holzer et al showed that upon cisplatin exposure, hCTR1 internalized into the cytoplasm from the plasma membrane (Holzer et al., 2004). In parallel experiments they reported that the cell lines overexpressing of CTR1 accumulated approximately 50% more cisplatin than the respective normal cell line (Holzer et al., 2004). The finding of CTR1 protein relocation stimulated by its substrate is consistent with the common conception that the expressions of transporters are usually regulated by their substrate. An example of that will be the regulation of CTR1 by exposure to copper (Dancis et al., 1994) and the putative platinum drug substrate, cisplatin (Holzer et al., 2004a, Holzer et al., 2004b).

Although copper transporters may play a role in the transport of oxaliplatin in DRG neurons, the degree of involvement in oxaliplatin-induced neurotoxicity remains unknown and requires further studies. Copper transporters appeared not to be the only transporters that
mediate the uptake of platinum drugs into cells because complete abolishment of platinum drug uptake with deletion of CTR1 was not seen (Holzer et al., 2004). Thus, the part copper transporter plays and the contribution it has in oxaliplatin-induced neurotoxicity via the uptake of oxaliplatin into the DRG neurons require further clarification.

7.3. Possible Reasons of Atrophy in CTR1-IR Neurons

It is a common conception that oxaliplatin causes peripheral neuropathy by platinum accumulation within the DRG leading to atrophy or loss of peripheral sensory neurons (Cavaletti et al., 1992, Cavaletti et al., 1998, Cavaletti et al., 2001, Daugaard et al., 1987, Gregg et al., 1992, Holmes et al., 1998, Jamieson et al., 2005, Krarup-Hansen et al., 1999, Krarup-Hansen et al., 2007, Roelof et al., 1984, McKeage et al., 2001, Screnci et al., 1997, Screnci et al., 2000, Thompson et al., 1984, Tomiwa et al., 1986). Atrophy of DRG neurons would be expected to lead to altered sensory nerve conduction velocities that characterise oxaliplatin-induced peripheral neuropathy because DRG cell body size, axonal calibre and nerve conduction velocity are strongly correlated (Harper et al., 1985, Kishi et al., 2002, Lee et al., 1986). Although DRG neuronal shrinkage is a common observation in neuropathy the mechanism underlying atrophy of neurons remains unknown.

A possible cause of peripheral sensory neuronal atrophy may be due to energy deprivation in sub-populations of DRG neurons leading to autophagy. A hallmark of cells undergoing autophagy is atrophy; where structural proteins and organelles of a cell are destroyed, with a parallel reduction in the size and functional capacity of the cell (Lockshin et al., 2007, Melli et al., 2008, Mizushima et al., 2008). This is an adaptive response as it allows the cell to survive in adverse conditions by reducing its metabolic overheads and to provide fuel required to maintain an active metabolism and ATP production (Mizushima et al., 2008, Scarlatti et al., 2009). A reduction in energy required by the cells for proper cellular functioning could be the grounds behind neuropathy associated with diabetes and platinum drugs. In diabetic induced neuropathy, hyperglycemia is a feature where the cells are starved of energy (Dobretsov et al., 2007). In the current study it is possible that the neuronal sub-population that underwent atrophy were impaired in the generation of ATP. The results in the current study showed that neurons expressing copper influx transporters underwent oxaliplatin-induced atrophy. The copper levels in these neuronal sub-
populations may be reduced due to the competitive uptake of oxaliplatin into the cells via CTR1, or the degradation/relocation of CTR1 stimulated by oxaliplatin exposure. The downstream effect of a reduction in cellular copper is that oxidative phosphorylation is impaired in the mitochondria resulting in decreased ATP generation. Consistent with this idea, autophagy has been reported in primary neurons as a result of nutrient deprivation (Young et al., 2009). In addition, autophagy has been observed in DRG of patients with Friedreich Ataxia; a neurodegenerative disease with neuropathic symptoms (Simon et al., 2004).

It is possible that the competitive uptake of oxaliplatin into the neurons by CTR1 caused the reduction of cellular copper being delivered to the mitochondria for ATP synthesis, subsequently leading to energy deprivation that resulted in autophagy. Indeed, there are growing evidence that mitochondria dysfunction is connected to a number of neurodegenerative diseases highlighting the importance of mitochondrial function in maintaining healthy neurons (Chen et al., 2009, Filosto et al., 2011). The production of ATP is generated by oxidative phosphorylation and driven by the mitochondrial membrane potential through the electron transport chain. Transport of copper to the mitochondria and maintenance of their membrane potential are closely linked phenomena. Therefore, interference with the production of energy can potentially affect mitochondrial transport along axons, leading to cytoskeleton dysfunction and derangement (Melli et al., 2008). Evidence that aligns with this theory included the reduction in frequency and cell body area of DRG neurons with immunoreactivity towards phosphorylated neurofilament (pNF-H), the main cytoskeleton element that supports the axon cytoplasm in neuronal cells, in oxaliplatin-induced neuropathy (Jamieson et al., 2009). There was another study that demonstrated the importance of energy production in cisplatin-induced neuropathy; Melli et al showed the prevention of mitochondrial energetic failure with alpha-lipoic acid (aLA) treatment, showing a reduction in the neurotoxicity induced by cisplatin in primary cultures of DRG neurons (Melli et al., 2008).

Thus, there appears to be indirect evidence in the literature to support the theory that energy deprivation in neurons may lead to autophagy that manifest in neuronal atrophy. It
may be possible that the cause of this energy deprivation is due to the disruption of copper homeostasis in CTR1 expressing neurons via the competitive transport of oxaliplatin.

7.4. Possible Reason for Lack of Efficacy in Copper Histidine for the Amelioration of Oxaliplatin-Induce Neuropathy

Several reasons could contribute to the lack of protection of copper on oxaliplatin-induced neurotoxicity. Firstly, effective tissue copper concentrations were not achieved in DRG in vivo. It is possible that the copper homeostasis in the whole animal prevented an increase in systemic copper level but further studies are required to clarify this likelihood. In a small pilot study, copper histidine at the dose of 2 mg/kg showed a numerical protection against oxaliplatin induced cell body and nucleolus shrinkage when given in addition to oxaliplatin. However, the apparent efficacy of copper histidine against oxaliplatin induced cell body and nucleolus shrinkage was not reproducible in a subsequent study with a bigger number of animals. The plasma and tissue findings of animals given copper histidine in addition to oxaliplatin suggested that, despite oral dosing of copper histidine at 2 mg/kg for 5 days a week over an eight weeks study period it failed to increase the levels of systemic copper and in DRG tissues. It is unlikely to be attributed to the route of copper administration or inadequate dose. Copper has been shown to be absorbed well when given orally in previous studies. Orally administered $^{64}$Cu was rapidly absorbed and peak absorptions occurs at 30 minutes (Marceau et al., 1970), and 30% of the dose is absorbed by 8 hours in rats (Marceau et al., 1970) and human (Sternlie et al., 1967). Furthermore, the current study dosed rat with a higher daily and cumulative dose of copper with similar duration of time in study compared to Bauerly et al, whom observed a two-fold increase in liver and intestine tissue copper levels that was not replicated by this study (Bauerly et al., 2005). The dose of 2 mg/kg was chosen due to concerns with copper poisoning in rats treated with oxaliplatin. Acute overdose of copper resulted in anorexia, lethargy and weakness (Semple et al., 1960, Winge et al., 1990) and were taken into consideration with the addition of oxaliplatin since animals usually experience a 10% reduction in weight gain (Jamieson et al., 2005, Screnci et al., 1997).

Secondly, the absorption of copper from the diet can be affected by the presence of other dietary components such as zinc, calcium and iron (Arredondo et al., 2006). This is an
unlikely reason why the levels of copper were not enhanced in rats given copper histidine oral dosing because animals were fed standard rat chow with no additional zinc, calcium or iron. However because the levels of zinc and iron were only measured in the oxaliplatin treated animals it cannot be compared to the control animals to assess if the administration of oxaliplatin has altered the levels of zinc or iron. To the best of our knowledge there is no data in the literature about the systemic induction of zinc, iron or calcium levels by oxaliplatin.

There does not appear to be a clear reason for the inability to enhance systemic copper levels in Wistar rats treated with oral dosing of copper histidine at 2 mg/kg. Further experiments can be conducted using primary cultures of rat DRG neurons to decipher if an increase in copper level in DRG can ameliorate oxaliplatin-induced neuropathy because the levels of copper exposure can be controlled. This will be discussed under section 7.7.1.1.

7.5. Applicability of Primary Culture for the Investigation of Platinum Drug-Induced Neuropathy

The DRG primary culture system functioned as a useful model to examine the events underlying oxaliplatin induced neuropathy involving copper transporters. For example, the changes in CTR1 protein localization were not captured in the in vivo studies of DRG after oxaliplatin treatment presumably due to the late DRG sampling time. The trafficking of copper transporter induced by oxaliplatin was only seen in primary culture and not in DRG tissues of rats. A likely reason for this finding may be due to the rapid restoration of CTR1 to the plasma membrane after removal of substrate exposure. Previous studies have shown that the resynthesis and trafficking of hCTR1 back to its normal cellular membrane locations occurs in as little as 30 minutes upon cessation of platinum drug exposure (Holzer et al., 2006, Molloy et al., 2009). Similarly, ATP7A has shown to return to the perinuclear region from the cell periphery within hours after removal of cisplatin (Kalayda et al., 2008). The tissues utilized for the current immunohistochemical studies were obtained from animals 96 hours after the last oxaliplatin dose, where the levels of circulating oxaliplatin would be minimal since it would have exceed four half lives of oxaliplatin. ATP7A and CTR1 protein are likely to have returned to the normal location and any changes to the trafficking of transporter to the plasma membrane or cytoplasm of ATP7A or CTR1, respectively, were not
detected. Therefore, in line with other researcher’s view, it was found here that primary cultures of DRG neurons represented a tightly controlled system to study the basics of cellular responses to external disturbances to the nerve system homeostasis including trafficking of protein (Melli et al., 2009).

The copper transporter expression in primary cultures of DRG appeared to be reflective of the DRG tissue. Primary culture DRG neurons showed similar copper transporters expression characteristics to rat DRG tissue. As mentioned above, CTR1 and ATP7 protein are found to be expressed in primary DRG neurons in a similar pattern to rat tissue. In addition, the size profile of CTR1 and ATP7A expression bear similarities in both the rat DRG tissue and primary cultures of DRG neurons; CTR1 was found to be expressed in bigger neurons and ATP7A were found in smaller neurons. Lastly, CTR1 and ATP7A were found to be expressed in largely non-overlapping and different sub-population of neurons in both rat tissue and primary cultures of DRG neurons.

The *in vivo* DRG tissue and *in vitro* primary cultures of DRG neurons expressing copper transporters responded similarly to oxaliplatin treatment. Neurons expressing CTR1 underwent atrophy in both the *in vivo* and *in vitro* model of oxaliplatin-induced neuropathy, whereas ATP7A immuno-reactive neurons were less affected. The DRG primary culture system displayed similar responses to oxaliplatin insults compared to the *in vivo* Wistar rat model.

It must be noted however, that the expression frequencies of CTR1 and ATP7A in primary culture were both proportionally higher than that of the *in vivo* rat tissue; the expression of both CTR1 and ATP7A were respectively 13 and 10% higher than in the primary culture compared to primary culture. The differences in staining frequency may be due to the more direct exposure and reduced fixation time of CTR1 and ATP7A protein in the primary culture compared to the tissue resulting in an enhanced binding of antibodies to their target protein.

In general, the *in vitro* primary cultures of DRG neurons appear to be a valuable model for studying the events involving copper transporters in oxaliplatin induced neuropathy. It not
only resembles the rat in vivo model but also enables questions that are difficult to be addressed in the complex whole animal system to be isolated and answered.

7.6. Limitations of Studies

The specificity of the CTR1 antibody towards CTR1 was not sufficiently validated because the CTR1 antibody used was not suitable for western blotting. In addition, the morphometric analyses of immunochemically defined subpopulations of cells in tissue sections and primary cultures of DRG neurons were inherently subjective as qualitative interpretation of cell positivity and arbitrary definitions of positive and negative cells were required. For these reasons there were limitations in interpreting the data for immunohistochemistry. Nonetheless, the neurons that were deemed to be CTR1 immunoreactive showed expected staining pattern and suggested that the antibody labelled the expected structures such as plasma membrane staining of CTR1. Furthermore, the mRNA expression of CTR1 in the DRG neurons was confirmed using both RT-PCR and qPCR. The finding of CTR1 expression in DRG neurons is consistent with the number of reports of its expression in other mammalian neuronal tissue (Bauerly et al., 2005, Platonova et al., 2005, Samsonov et al., 2006). In addition, the specificity of the CTR1 antibody was validated by pre-absorption assay using immunizing peptide in the A2780 human carcinoma cell line by Holzer et al (Holzer et al 2006).

The ATP7B antibody utilized for immunohistochemical studies was not validated. This weakens the validity of the finding that ATP7B protein is not expressed in DRG. However the low ATP7B RNA level found by RT-PCR and real time PCR in DRG confirms that the lack of protein expression of ATP7B in DRG. Moreover, Goss et al have shown the protein expression of ATP7B in rat liver using this ATP7B antibody (Goss et al 2008).

This study would have benefited with an additional functional assessment endpoint of neuropathy such as sensory nerve conduction velocity measurements. DRG cell body size, axonal calibre and nerve conduction velocity are strongly correlated so the atrophy of DRG neurons would be expected to lead to altered sensory nerve conduction velocities that characterise oxaliplatin-induced peripheral neuropathy (Harper et al., 1985, Kishi et al., 2002, Lee et al., 1986). The functional assessment of peripheral neuropathy by sensory
nerve conduction velocity measurement was a desirable additional endpoint to assess the severity of neuropathy. There were attempts made to evaluate sensory nerve conduction velocity measurements in the current study, however, this was unsuccessful. The effects of oxaliplatin on the sensory nerve conduction velocity of the Wistar rat were relatively small compared to the high background variation. Therefore a large number of animals were required to elicit statistical significant findings as reported by others (Screnci 1999). In previous in vivo studies a minimal of 20 animals in each treatment groups were used to detect a significant change in sensory nerve conduction velocity in Wistar rats after oxaliplatin treatment (Jamieson 2003 et al., Jamieson et al., 2005).

7.7. Future Directions

7.7.1 The In Vitro Uptake of Oxaliplatin by CTR1 in DRG Neurons

To decipher the role CTR1 plays in the uptake of oxaliplatin into the DRG neurons several in vitro approaches can be taken and some are discussed below.

7.7.1.1. Inhibition of Oxaliplatin Uptake by Copper via CTR1

To substantiate the role CTR1 plays in the accumulation of oxaliplatin in cultured rat DRG neurons the effects of a CTR1 inhibitor, concentration, temperature and competing substrate on the uptake of oxaliplatin can be investigated. If the transport of oxaliplatin can be suppressed by inhibition of the functional uptake of CTR1 it is likely to result in a reduction in cellular accumulation and cytotoxicity induced by oxaliplatin. Silver have shown to be an inhibitor of CTR1 mediated copper transport temperature. In addition, the transport of copper by CTR1 has been demonstrated to be stimulated by ascorbate because it reduces copper from Cu(II) to Cu(I) which is a more preferable CTR1 substrate (Lee et al., 2002a, Lee et al., 2002b). By using validated experimental conditions these compounds can be tested to observe if the accumulation of oxaliplatin can be altered.

To assess the uptake of oxaliplatin in cultured rat DRG neurons, platinum content in DRG neurons and cytotoxicity of oxaliplatin can be measured. The accumulation of oxaliplatin can be measured by platinum uptake content in the DRG neurons using an element specific detector, ICP-MS (Jamieson et al., 2003, Screnci et al., 1997, Screnci et al., 2000). The
cytotoxicity of oxaliplatin induced in DRG neurons can also be assessed with a cytotoxic assay to estimate uptake of oxaliplatin into DRG neurons such as the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Jong et al., 2011).

7.7.1.2. Over-Expression of CTR1 in Cell Lines

Using an overexpressing cell line of CTR1 one can verify the role it plays in rat DRG neurons uptake of oxaliplatin. For example, the generation of rCtr1 overexpressing and mock-transfected isogenic cell lines on a HEK293 background can be used to verify the biological role CTR1 has on oxaliplatin uptake. The platinum accumulation and cytotoxicity of oxaliplatin can be compared with the mock-transfected control cells. Previously the overexpression of hCTR1 in the HEK293 cell line was generated by Rabik et al (Rabik et al., 2009). Holzer et al showed an increase in cisplatin uptake in forced overexpression of hCTR1 in human ovarian carcinoma cells (Holzer et al., 2003). Similarly, Song et al overexpressed hCTR1 in a non-small cell lung carcinoma cell line and reported an increase uptake of cisplatin (Song et al., 2004). However, the results from overexpression studies should complement other research and interpreted with caution, as it has been shown that the endogenously and exogenously expressed hCTR1 appeared to process cisplatin differently (Holzer et al., 2004).

7.7.1.3. Knockdown of CTR1 in Cell Lines

Knockdown studies were employed successfully by many researchers to study the role copper transporters plays in mediating the transport of platinum drugs. Both Ishida et al and Lin et al significantly reduced the accumulation of platinum drugs by deleting the yCtr1 gene in the yeast (Ishida et al., 2002, Lin et al., 2002). Holzer et al used Ctr1 knockdown model in murine embryonic fibroblasts and showed a reduction on uptake of cisplatin, carboplatin and oxaliplatin (Holzer et al., 2006). Larson et al showed that in the wild-type Ctr1+/+ mouse embryonic fibroblast cell line accumulated more cisplatin, carboplatin and oxaliplatin compared to the Ctr1−/− knockout cell line (Larson et al., 2009). Thus the generation of an isogenic pair of Ctr1+/+ and Ctr1−/− cell lines in rat can reveal if the loss of CTR1 function accompanies a reduction in oxaliplatin uptake.
7.7.2 Mechanisms of CTR1 Membrane Trafficking

There is currently little understanding on the mechanism and regulation that underpin the trafficking of CTR1 from the plasma membrane to the cytoplasm. It is commonly thought that the regulation of CTR1 is post-translational (van den Berghe et al., 2010) and the exposure to copper and platinum drugs induced constitutive cycling (Klomp et al., 2002), degradation (Petris et al., 2003) and endocytosis of CTR1 (Ooi et al., 1996).

It is possible that enzymes involved in the trafficking events and mechanisms in other cell types and trafficking of other types of transporters may also be involved in the regulation of CTR1 membrane relocation. For example, phosphatidylinositol 3-kinase (PI 3-kinase) is a known membrane traffic regulator and plays a crucial early role by activating signalling pathways (De Camilli et al., 1996). PI 3-kinase has been involved in the regulation of the pain-transducing ion channel TRPV1, potentially involved in its trafficking to the plasma membrane in DRG neurons (Stein et al., 2006) and cell surface regulation of dopamine transporters in primary mesencephalic neurons (Hoover et al., 2007).

Thus, the involvement of PI 3-kinase in copper or platinum drug induced membrane loss of CTR1 seems plausible and can be investigated. To test if the regulation of CTR1 membrane movement involves phosphatidylinositol 3-kinase (PI 3), inhibition of PI 3 activity in primary culture can be assessed. Specific inhibitors of PI 3-kinase are available such as LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] which inhibits an ATP binding site specific for PI 3-kinase and abolishes its activity in vitro and in vivo (Vlaho et al., 1994, Zhang et al., 2011). Another PI 3-kinase antagonist that is commonly used to block various signal transduction by PI 3-kinase is Wortmannin (Saito et al., 2009). Many researchers have successfully shown the involvement of PI 3-kinase in signalling pathway that regulate the trafficking of transporters using a PI 3-kinase inhibitor or antagonist (Saito et al., 2009, Vlaho et al., 1994, Zhang et al., 2011).

Regulation of trafficking of CTR1 from the membrane to the cytoplasm may play an important role in the neurotoxicity of oxaliplatin in DRG neurons if the mechanism of toxicity is via the disrupted uptake of copper into the cells. Thus, the study of the regulatory mechanism of CTR1 trafficking may help shed light on the induction of oxaliplatin induced
neuropathy. Utilizing a PI 3-kinase inhibitor or antagonist one can investigate the role of PI 3-kinase in the trafficking of CTR1 from the membrane to cytoplasm and investigate. To determine the contribution of PI 3-kinase to the relocation of CTR1 transporters the presence or absence of a PI 3-kinase inhibitor or antagonist at various concentrations can be tested on primary DRG cultures of rat with the co-incubation of oxaliplatin. Examination of CTR1 protein relocation can be achieved using immunohistochemistry techniques demonstrated in the current study.

7.7.3 The Role of Autophagy in Oxaliplatin-Induced DRG Neuronal Atrophy

To test if autophagy is taking place in the DRG neurons treated with oxaliplatin one can assay for the activation of autophagy within these DRG neurons. One reliable marker is the measurement of the conversion of unconjugated microtubule-associated light chain 3 (LC3-I) to phosphatidylethanolamine-conjugated LC3 (LC3-II). This can be achieved via monitoring the change in subcellular distribution of autophagic vesicles, which are expected to move from a diffused to a punctuate appearance (Young et al., 2009). In addition, the shift of molecular weight from LC3-I to LC3-II can be detected using Western blot analysis (Kabeya et al., 2000).

Furthermore, one can test if the inhibition of autophagy in neurons exposed to oxaliplatin can reduce neuronal atrophy. One pharmacological inhibitor of autophagy activation is class III PI 3-kinase inhibitor 3-methyladenine (3-MA), which inhibits the lipid kinases complex required for the initial formation of the autophagosome isolation membrane (Petiot et al., 2000, Seglen et al., 1982). The oxaliplatin-induced atrophy should be inhibited if autophagy plays a role in the atrophy in the presence of 3-MA.

7.8 Conclusions

In conclusion, the research described in this thesis attempted to understand the mechanisms by which oxaliplatin causes damage to the nervous system. To the best of our knowledge, it was shown here for the first time that copper efflux transporters were expressed in primary sensory neurons \textit{in vitro} and \textit{in vivo} in Wistar female rats. In addition, these DRG neurons exhibited a specific pattern of expression of copper transporters that responded differently to oxaliplatin-induced neuropathy. Neurons expressing copper influx
transporters, CTR1, were more susceptible to oxaliplatin-induced neurotoxicity compared to neurons expressing copper efflux transporter ATP7A. Copper treatment tested for the attempts to ameliorate oxaliplatin-induced neuropathy did not reduce the neurotoxicity of oxaliplatin to DRG neurons in vivo. In primary cultures of rat DRG neurons oxaliplatin caused atrophy in the neurons expressing CTR1 and a loss of membrane staining frequency without altering the number of neurons. Neurons expressing ATP7A was not affected by oxaliplatin treatment.

Why did oxaliplatin, designed to kill rapidly dividing cancer cells, injure nonproliferative, differentiated neurons. We speculate that it may be due to the modulation of oxaliplatin uptake or the disruption of copper homeostasis in these DRG neurons. Further studies are required to elucidate the involvement of copper transporters in oxaliplatin-induced neuropathy in DRG neurons.
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Appendix

Publications Resulting in Part or in Full from this Thesis

Journal Articles:


Abstract:


Differential expression of ATP7A, ATP7B and CTR1 in adult rat dorsal root ganglion tissue

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Abstract

Background: ATP7A, ATP7B and CTR1 are metal transporting proteins that control the cellular disposition of copper and platinum drugs, but their expression in dorsal root ganglion (DRG) tissue and their role in platinum-induced neurotoxicity are unknown. To investigate the DRG expression of ATP7A, ATP7B and CTR1, lumbar DRG and reference tissues were collected for real time quantitative PCR, RT-PCR, immunohistochemistry and Western blot analysis from healthy control adult rats or from animals treated with intraperitoneal oxaliplatin (1.85 mg/kg) or drug vehicle twice weekly for 8 weeks.

Results: In DRG tissue from healthy control animals, ATP7A mRNA was clearly detectable at levels similar to those found in the brain and spinal cord, and intense ATP7A immunoreactivity was localised to the cytoplasm of cell bodies of smaller DRG neurons without staining of satellite cells, nerve fibres or co-localisation with phosphorylated heavy neurofilament subunit (pNF-H). High levels of CTR1 mRNA were detected in all tissues from healthy control animals, and strong CTR1 immunoreactivity was associated with plasma membranes and vesicular cytoplasmic structures of the cell bodies of larger-sized DRG neurons without co-localization with ATP7A. DRG neurons with strong expression of ATP7A or CTR1 had distinct cell body size profiles with minimal overlap between them. Oxaliplatin treatment did not alter the size profile of strongly ATP7A-immunoreactive neurons but significantly reduced the size profile of strongly CTR1-immunoreactive neurons. ATP7B mRNA was barely detectable, and no specific immunoreactivity for ATP7B was found, in DRG tissue from healthy control animals.

Conclusions: In conclusion, adult rat DRG tissue exhibits a specific pattern of expression of copper transporters with distinct subsets of peripheral sensory neurons intensely expressing either ATP7A or CTR1, but not both or ATP7B. The neuron subtype-specific and largely non-overlapping distribution of ATP7A and CTR1 within rat DRG tissue may be required to support the potentially differing cuproenzyme requirements of distinct subsets of sensory neurons, and could influence the transport and neurotoxicity of oxaliplatin.
These neurons may require copper transport as they strongly express cuproenzymes, such as cytochrome C oxidase [8], Cu/Zn superoxide dismutase [9] and peptidylglycine α-amidating monooxygenase [10], and are sensitive to copper deficiency [11,12]. In other cell types, copper transporters have been shown to have a role in controlling the cellular accumulation and cytotoxicity of platinum drugs, with CTR1 mediating platinum uptake into cells [13-15], and ATP7A and ATP7B transporting platinum out of cells or into specific subcellular compartments [16-20]. Platinum-based drugs, such as cisplatin and oxaliplatin, accumulate in DRG tissue [21-26], damage sensory neurons [21,22,24-33], and induce peripheral sensory neuropathies that limit their use in clinical cancer chemotherapy [34]. In the current study, we investigated the expression of ATP7A, ATP7B and CTR1 in DRG tissue from adult rats, either healthy control animals or those treated with oxaliplatin or its drug vehicle. Neuronal atrophy was used as the endpoint for measuring the neurotoxicity of oxaliplatin in DRG tissues, as in previous studies [26,29,35-37]. We aimed to determine patterns of expression and localization of ATP7A and ATP7B within DRG tissue, in an extension to our recent study of CTR1 [35], and to relate the expression of these copper transporters to the neurotoxicity of oxaliplatin.

Results
Copper transporter gene expression in DRG and other tissues

The expression of copper transporter genes in rat DRG tissue was determined by RT-PCR and qPCR in comparison to reference tissues (brain, spinal cord, liver, kidney and intestine). The RT-PCR (Figure 1) and qPCR (Table 1) findings corresponded well with each other. In all of the tissues, CTR1 had the highest mRNA levels, followed by ATP7A and then ATP7B had the least, except in liver where ATP7B levels were higher than ATP7A.

In DRG tissue, ATP7B mRNA was barely detectable with only faint or no bands visible on RT-PCR gels but in liver tissue bands were more clearly visible (Figure 1). ATP7B mRNA transcripts in DRG tissue were detected by qPCR in only two of six animals compared to all six animals for liver (Table 1). In DRG tissue, ATP7A mRNA was more readily detectable than ATP7B, with clearly visible bands on RT-PCR gels (Figure 1) and qPCR-detectable transcripts in all six animals (Table 1). ATP7A mRNA levels in DRG were similar to brain and spinal cord levels but higher than those in the non-neuronal reference tissues.

High levels of CTR1 mRNA were found in DRG, as in other tissues. RT-PCR gels showed clearly visible bands for CTR1 in all tissues (Figure 1). CTR1 mRNA transcripts was detectable by qPCR in all tissues, and in all animals, at levels higher than ATP7A and ATP7B (Table 1).

Copper transporter protein expression in DRG tissue

ATP7A had a specific pattern of distribution within rat DRG tissue, with intense cytoplasmic staining of the cell bodies of smaller DRG neurons as revealed by immunohistochemistry. The specificity of anti-ATP7A primary antibody was confirmed by Western blotting showing a protein band with the size 170 kDa on ATP7A immunoblots of DRG tissue homogenates from rats aged 4, 12 and 20 weeks (Figure 2A). Negative controls that excluded the primary antibody lacked specific immunoreactivity (Figure 2B2a, inset). ATP7A immunohistochemistry of DRG tissue visualized by ABC-peroxidase revealed that this copper efflux transporter was most strongly expressed within smaller-sized DRG neurons that showed intense immunoreactivity in a punctuate pattern localised to the cytoplasm of their neuronal cell bodies (Figure 2B,a and 2b). Other DRG neurons showed lighter and more diffuse cytoplasmic immunostaining for ATP7A, with occasional granular staining of the plasma membrane. No ATP7A immunoreactivity was apparent in the satellite cells, nerve fibres, or other non-neuronal tissue elements of the rat DRG. Furthermore, fluorescent immunohistochemistry showed that ATP7A immunoreactivity was mainly associated with smaller DRG neurons that did not overlap with DAPI-stained non-neuronal cells or with the pNF-H-immunoreactive larger neurons and nerve fibres (Figure 2B,c).
No specific immunoreactivity for ATP7B was found in rat DRG tissue when compared with a negative control (data not shown), even though the primary antibody (NB100-360, Novus Biologicals) detects ATP7B in rat liver [38].

CTR1 immunohistochemistry of rat DRG tissue showed a pattern of immunostaining that differed from ATP7A. The specificity of the Novus antibody for CTR1 immunohistchemistry has been previously determined by preabsorption assay with immunizing peptide using a hCTR1 A2780 human ovarian carcinoma cell line [39]. This antibody, however, is unsuitable for use in Western blot analysis. Strong CTR1 immunoreactivity was associated with the plasma membrane and cytoplasmic vesicular structures of larger-sized DRG neurons, whereas only light staining appeared in the remaining neurons (Figure 3A,B), as previously described [35]. Double label fluorescent immunohistochemistry provided further evidence of CTR1 and ATP7A primary localisation to neuronal cell bodies, and their distinct patterns of immunoreactivity and non-overlapping distribution within rat DRG tissue (Figure 3C).

**Morphometry of ATP7A-positive and CTR1-positive neurons in DRG tissue from control and oxaliplatin-treated rats**

Morphometric analysis of control animal DRG tissue showed that ATP7A and CTR1 were expressed by vesicular structures of larger-sized DRG neurons, whereas only light staining appeared in the remaining neurons (Figure 3A,B), as previously described [35]. Double label fluorescent immunohistochemistry provided further evidence of CTR1 and ATP7A primary localisation to neuronal cell bodies, and their distinct patterns of immunoreactivity and non-overlapping distribution within rat DRG tissue (Figure 3C).

### Table 1 Copper transporter gene expression in rat tissues determined by quantitative PCR

<table>
<thead>
<tr>
<th>Copper transporter mRNA levels (2^{-ΔΔCT} × 10^4) in indicated tissue</th>
<th>Dorsal Root Ganglion</th>
<th>Brain</th>
<th>Spinal Cord</th>
<th>Liver</th>
<th>Kidney</th>
<th>Intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP7B</td>
<td>0^a (0-1)</td>
<td>1^a (0-1)</td>
<td>1^b (0-1)</td>
<td>2 (1-6)</td>
<td>0^b (0-1)</td>
<td>0^b (0-1)</td>
</tr>
<tr>
<td>ATP7A</td>
<td>11 (7-44)</td>
<td>11 (7-84)</td>
<td>19 (5-289)</td>
<td>2^c (0-9)</td>
<td>2 (2-84)</td>
<td>5^c (0-43)</td>
</tr>
<tr>
<td>CTR1</td>
<td>12 (4-323)</td>
<td>19 (4-38)</td>
<td>30 (2-81)</td>
<td>73 (11-275)</td>
<td>13 (6-243)</td>
<td>43 (7-353)</td>
</tr>
</tbody>
</table>

Values represent the median and range (in parenthesis) of determinations in six animals. Symbols indicate when gene expression was undetectable in one^a, two^b, three^c or four^d of six animals.
different neuronal subpopulations with differing size profiles (Figure 4; Table 2). For this analysis, strongly ATP7A-expressing DRG neurons were defined as those having intense diffuse or punctuate cytoplasmic staining and/or plasma membrane immunoreactivity to ATP7A. Those negative for strong ATP7A expression had no or low-intensity diffuse or punctuate cytoplasmic staining without plasma membrane immunoreactivity. Strongly CTR1-expressing DRG neurons were defined as those having intense plasma membrane and/or punctuate cytoplasmic immunoreactivity to CTR1. Those negative for strong CTR1 expression had only diffuse cell body immunoreactivity without plasma membrane or punctuate cytoplasmic immunoreactivity. DRG neurons with strong expression of ATP7A accounted for 35.1 ± 2.9% of the overall population of DRG neurons in control animals, whereas those with strong expression of CTR1 accounted for 10.9 ± 1.8% (P < 0.001). About 64.2 ± 6.9% of the strongly ATP7A-expressing neurons had cell bodies measuring <750 μm², but only 2.0 ± 1.3% of strongly CTR1-expressing neurons were of this size (P < 0.001). About 58.2 ± 16.1% of strongly CTR1-expressing neurons had cell bodies measuring >1750 μm², but only 6.7 ± 2.5% of strongly ATP7A-expressing neurons were of this size (P < 0.001). The mean cell body area of strongly ATP7A-expressing neurons (767.1 ± 87.6 μm²) was significantly smaller than that of the strongly CTR1-expressing neurons (1936 ± 278 μm²; P < 0.001).

Oxaliplatin treatment did not significantly change the size profile of strongly ATP7A-expressing neurons, immunoreactivity pattern of ATP7A or CTR1, or the staining frequencies of ATP7A or CTR1-positive cells (Figure 4A,B). In contrast, oxaliplatin treatment of animals caused atrophy of strongly CTR1-expressing DRG neurons, which showed a clear left-ward shift in their size distribution profile, reduction in their mean cell body areas from 1936 ± 278 μm² to 1461 ± 64 μm² (P < 0.01), a decrease in the percentage of large neurons measuring greater than 1750 μm² from 58.2 ± 16.1% to 28.5 ± 5.4% (P < 0.01), and an increase in the percentage of medium sized neurons measuring between 750 to 1750 μm² from 39.9 ± 15.9% to 66.4 ± 5.5% (P < 0.01) (Table 2).

**Discussion**

This is the first description of the expression of copper-transporting P-type ATPases in DRG tissue from rats or any other animal species. Adult rat DRG tissue exhibited a specific pattern of expression of copper transporters with distinct subsets of sensory neurons intensely expressing either ATP7A or CTR1, but not both or ATP7B. Copper transporter mRNA levels in DRG were highest for CTR1, followed by ATP7A and barely detectable for ATP7B. ATP7A protein was detected in DRG tissue homogenates by Western blotting. ATP7A and CTR1 were detectable in DRG tissue by immunohistochemistry and were localised to the cell bodies of sensory neurons with little or no immunostaining of nerve fibres, satellite cells or other tissue elements, and without specific immunoreactivity for ATP7B. Neuronal immunoreactivity for ATP7A did not co-localise with CTR1, pNF-H or DAPI-stained satellite cells in double-label fluorescent immunohistochemistry studies, and their cell body size-profiles determined by morphometric analysis differed markedly from that of CTR1-immunoreactive neurons. Morphometric analyses of immunohistochemically defined subpopulations of cells in tissue sections is inherently subjective as qualitative...
interpretation of cell positivity and arbitrary definitions of positive and negative cells are required. However, strongly ATP7A-immunoreactive neurons accounted for about one third of the overall total population of DRG neurons, and were characterised by their small cell bodies and intense punctuate cytoplasmic immunostaining. In contrast, strongly CTR1-immunoreactive neurons accounted for about one tenth of the overall population of DRG neurons and were characterised by their large cell bodies, and intense plasma membrane and vesicular cytoplasmic immunostaining, as we recently described [35]. Together, these findings show that ATP7A and CTR1 have neuron subtype-specific and largely non-overlapping distribution in adult rat DRG tissue suggesting that these copper transporters have distinct roles in supporting the functions of primary sensory neurons.

The physiological significance of differential expression of copper transporters by DRG neurons is unclear and requires further study. However, ATP7A and CTR1 may be required by distinct sub-types of DRG neurons to deliver copper to specific cuproenzymes vital for the synthesis of neuropeptides and ATP. ATP7A, for example, delivers copper to peptidylglycine alpha-amidating monooxygenase in cell types other than DRG neurons [40-42], but this cuproenzyme activity is required by DRG neurons for the synthesis of substance P [10,43,44]. Like ATP7A, substance P is primarily expressed by small DRG neurons [26,45,46], and the size of substance P-expressing DRG neurons is not altered by oxaliplatin treatment [26]. This suggests the existence of a subset of sensory neurons that co-express ATP7A, peptidyl alpha-amidating monooxygenase and substance P to support neuronal functions requiring neuropeptide synthesis. In other cell types, the level of expression of CTR1 corresponds closely with the activity of cytochrome C oxidase [4,47,48], which is a cuproenzyme involved in oxidative phosphorylation ATP synthesis. Like CTR1, cytochrome C oxidase is expressed intensely by large-sized DRG neurons [8], which may have reduced capacity for glycolysis compared to small

Table 2 Morphometry of subpopulations of DRG neurons with strong immunoreactivity (IR) for ATP7A and CTR1 in tissues from control and oxaliplatin-treated animals

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Copper transporter</th>
<th>Frequency of IR neurons (%)</th>
<th>Small-sized cells (&lt;750 µm²) (%)</th>
<th>Medium-sized cells (750-1750 µm²) (%)</th>
<th>Large-sized cells (&gt;1750 µm²) (%)</th>
<th>Mean cell body area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ATP7A</td>
<td>35.1 ± 2.9</td>
<td>64.2 ± 6.9</td>
<td>29.1 ± 4.7</td>
<td>67 ± 2.5</td>
<td>767 ± 88</td>
</tr>
<tr>
<td></td>
<td>CTR1</td>
<td>10.9 ± 1.8†</td>
<td>20.0 ± 1.3†</td>
<td>39.9 ± 15.9</td>
<td>582 ± 16.1†</td>
<td>1936 ± 278†</td>
</tr>
<tr>
<td>Oxaliplatin treatment</td>
<td>ATP7A</td>
<td>33.6 ± 2.3</td>
<td>70.5 ± 4.0</td>
<td>27.1 ± 3.7</td>
<td>24 ± 1.3</td>
<td>641 ± 39</td>
</tr>
<tr>
<td></td>
<td>CTR1</td>
<td>11.3 ± 3.5</td>
<td>5.2 ± 2.0</td>
<td>66.4 ± 5.5*</td>
<td>28.5 ± 5.5*</td>
<td>1461 ± 64*</td>
</tr>
</tbody>
</table>

Values represent the mean ± standard deviation of determinations in 4 to 6 animals per group. † P <0.01 for comparison with ATP7a control group; * P <0.01 for comparison with CTR1 control group.
DRG neurons [49], consistent with their strong need for CTR1 to meet their high demands for copper delivery to cytochrome C oxidase and ATP synthesis via oxidative phosphorylation. In this way, the neuronal subtype-specific and largely non-overlapping distribution of ATP7A and CTR1 in DRG tissue may relate to specific cuproenzyme requirements by distinct subsets of primary sensory neurons.

Platinum antitumour drugs, such as oxaliplatin, are known for causing peripheral neuropathy by undefined mechanisms that might involve platinum accumulation within the DRG leading to atrophy or loss of peripheral sensory neurons [21-33,50-52]. Atrophy of DRG neurons would be expected to lead to altered sensory nerve conduction velocities that characterise oxaliplatin-induced peripheral neuropathy because DRG cell body size, axonal calibre and nerve conduction velocity are strongly correlated [53-55]. Furthermore, our previous work has suggested that oxaliplatin may induce atrophy of specific subpopulations of DRG neurons by causing the loss of phosphorylated neurofilament heavy subunit, which is a cyto-skeletal protein that determines the calibre of large myelinated DRG neurons and their axons [37]. In other cell types, the cellular accumulation and cytotoxicity of platinum drugs is controlled, at least in part, by copper transporters, with CTR1 transporting platinum drugs into cells [13-15], and ATP7A and ATP7B transporting platinum drugs out of cells or into specific subcellular compartments [16-20]. In the current study, we showed that oxaliplatin treatment of adult rats caused atrophy of the CTR1-immunoreactive subpopulation of DRG neurons without changing the size profile of the ATP7A-immunoreactive subpopulation of DRG neurons. It is possible that ATP7A expressing DRG neurons are less sensitive to oxaliplatin neurotoxicity because the high levels of ATP7A facilitate the cellular efflux of oxaliplatin reducing its availability for reactions with DNA or other key neurotoxicity targets. In contrast, DRG neurons expressing high levels of CTR1 would be expected to take up more oxaliplatin leading to toxic effects in this neuronal subtype. Thus we suggest the neuronal subtype-specific and largely non-overlapping distribution of ATP7A and CTR1 within DRG tissue influence the neurotoxicity of oxaliplatin by controlling its cellular accumulation and subcellular distribution within primary sensory neurons. If this is so, then oxaliplatin treatment could be expected to alter the expression, distribution and subcellular localisation of ATP7A and CTR1 as in other cell types [15,20,39,56,57] but no evidence for such a change was found in DRG tissue in the current study. The role of CTR1 and ATP7A in oxaliplatin neurotoxicity remains hypothetical but could be tested further in studies comparing the accumulation, subcellular distribution and neurotoxicity of oxaliplatin in CTR1-and ATP7A-expressing neuronal cells in vitro making use of a CTR1 inhibitor to block the neuronal uptake and neurotoxicity of oxaliplatin.

Conclusions
In conclusion, adult rat DRG tissue exhibits a specific pattern of expression of copper transporters with distinct subsets of peripheral sensory neurons intensely expressing either ATP7A or CTR1, but not both or ATP7B. The neuron subtype-specific and largely non-overlapping distribution of ATP7A and CTR1 within rat DRG tissue may be required to support the differing cuproenzyme requirements of distinct subsets of sensory neurons, and could influence the transport and neurotoxicity of oxaliplatin.

Methods

Animals and drug treatment
Age-matched, 12-week-old female Wistar rats were housed in a self-contained unit maintained at 22 ± 2°C, and set to 12 h dark-light cycles with access to food and water ad libitum. Twelve healthy untreated animals were used for Cu transporter expression study by immunoblotting, immunohistochemistry and PCR respectively. In addition, for treatment study, two groups of animals received intraperitoneal injections of either oxaliplatin (Eloxatin; Sanofi-Aventis, Bridgewater, NJ, USA) at a dose of 1.85 mg/kg (n = 13) or dextrose (n = 12), as vehicle control, twice weekly for 8 weeks between 1300 and 1500 h. All animal procedures were approved by the institutional Animal Ethics Committee (AEC No. R591).

Western blot analysis
Following euthanasia of animals with intraperitoneal injection of pentobarbital (90 mg/kg body weight, Chemstock Animal Health, Christchurch, New Zealand), lumbar DRG tissues were dissected and homogenized using a Dounce homogenizer (Glas-Col, Terre Haute, IN, USA) for 3 min in a lysis buffer containing 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 0.1% SDS, and a protease inhibitor mixture (Complete Mini Protease Inhibitor Cocktail tablets; Roche Diagnostics, Indianapolis, IN, USA). The homogenate was centrifuged for 15 min at 500 × g at 4°C to remove nuclei and large particulate matter, and the protein concentration of the resulting supernatant was determined by a bicinchoninic acid (BCA) assay as previously reported [58]. Protein samples (40 μg) were heated at 95°C for 30 min, resolved in 8% SDS-PAGE, and then transferred to a nitrocellulose membrane (Amersham Pharmacia, Tokyo, Japan) using a Transblot SD apparatus (Bio-Rad, Hercules, CA, USA). Following blocking with 5% milk/bovine serum albumin
solution, ATP7A was detected by chemiluminescence using anti-ATP7A antibody (1:1000, no. ab13995: Abcam, Cambridge, UK), horseradish peroxidase (HRP)-conjugated anti-chicken antibody (Sigma-Aldrich, St. Louis, MO, USA), and the ECL Advance Detection reagent (Amersham Biosciences, Buckinghamshire, UK). Beta actin was probed to determine the equal loading using anti-beta actin antibody (Abcam) and a HRP-conjugated anti-rabbit IgG antibody (Amersham).

Reverse transcriptase-PCR
Animals designated for Atp7a, Atp7b and Ctr1 RT-PCR analysis were euthanized with pentobarbitone as above described. The lumbar DRG, brain, spinal cord, liver, kidney and small intestine tissues were collected and homogenized in PureZol reagent for total RNA isolation using an Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad). After digestion with DNase I (1 unit/μg, Bio-Rad), total RNA of each sample (0.25 μg) was reverse-transcribed into cDNA using a SuperScript first strand synthesis kit (Invitrogen, Carlsbad, CA, USA) according to instructions, followed by digestion with Ribonuclease H (Invitrogen) to remove the RNA templates. cDNA was amplified by PCR in a reaction mixture containing dNTP, MgCl2, Platinum Taq DNA polymerase (Invitrogen) and custom primers, using a GeneAmp 9700 PCR System (Applied Biosystems, Foster City, USA) at 52°C for 40 cycles. Forward and reverse primers for rat Atp7a were: 5′-tag acg gca tgt att gta att c-3′ and 5′-tgg att tta cac ctg gct tct t-3′; for rat Atp7b were 5′-att cca gga tgt tcc gta a-3′ and 5′-cac tgg ctc tct ggg gat t-3′ (amplicon of 396 bp); for rat Ctr1 were: 5′-ttg gtc gga att tgt acg gga aat c-3′ and 5′-cat aag atc tgg tct ccg t-3′ (amplicon of 375 bp); and for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5′-tgc tga gta tgt ctt gga gtc tcc t-3′ and 5′-aca gtc ttc tga gca gta a-3′ (amplicon of 291 bp), as a control. PCR products were electrophoresed in 2% agarose gel, stained with ethidium bromide and photographed using Gel Doc 2000 System (Bio-Rad).

Real-time PCR
cDNA was synthesized from total RNA of lumbar DRG, brain, spinal cord, liver, kidney and small intestine tissues of healthy rats as above described, and used for multiplex real-time PCR using ABI PRISM 7900HT Sequence Detection Systems and SDS 2.3 software (Applied Biosystems). Primers and probe sets were obtained as TaqMan Gene Expression Assays containing forward and reverse unlabelled PCR primer pair and a fluorescent reporter dye-labelled TaqMan MGB probe (Invitrogen). Samples were analyzed in triplicate in a 10-μl total volume containing 25 ng of cDNA of each tissue, TaqMan universal PCR Master Mix, TaqMan FAM-labelled probes for rat Atp7a gene, Atp7b gene or Ctr1 gene, respectively, and VIC-labelled 18 S ribosomal RNA as endogenous control probe.

The abundance of mRNA of ATP7A, ATP7B, CTR1 or rRNA was measured as the threshold cycle values (Ct) after each reaction. Fluorescence values were plotted against cycle numbers in SigmaPlot 10.0 using sigmoidal 3 parameter fitting and 50% of the maximum fluorescence was taken as the Ct according to Liu et al’s method [59]. The relative RNA expression level was calculated using the 2^{-ΔΔCt} method [60], where gene of interest expression normalized to 18 S rRNA and ΔCt = (Ct_{ATP7a or ATP7b or CTR1} - Ct_{rRNA}).

DAB and fluorescent immunohistochemistry of DRG
Animals were euthanized with pentobarbitone and perfused with phosphate buffered saline followed by 4% paraformaldehyde solution. Lumbar 5 DRG was dissected, post-fixed in the perfusion fixative for 2 h, cryoprotected in 30% sucrose overnight and embedded in Tissue-Tek (Sakura Finetechnical, Tokyo, Japan). Cryosections (12 μm) were thaw-mounted onto poly-L-lysine-coated Superfrost plus slides, rinsed, permeabilized in 0.2% Triton X-100, incubated with 1% hydrogen peroxide/methanol mixture (1:1), and blocked in 3% normal goat or donkey serum (Sigma-Aldrich) and 2% BSA (ICPbio Ltd, New Zealand). The slides were incubated with a chicken anti-ATP7A (1:1000; Abcam), a rabbit anti-ATP7B antibody (NB100-360, Novus Biologicals, Littleton, CO, USA) or a rabbit polyclonal anti-hCTR1 antibody (1:500, Novus Biologicals, Littleton, CO, USA), respectively, at room temperature overnight. Following rinses, the slides were incubated subsequently with a biotinylated secondary anti-chicken antibody (1:500, Jackson ImmunoResearch laboratories, PA, USA) or anti-rabbit antibody (1:500, Sigma-Aldrich) for 30 min, followed by an extravidin-peroxidase conjugate (1:500, Sigma-Aldrich) for 30 min. The peroxidase reaction was catalyzed using 3,3′-diaminobenzidine tetrahydrochloride (DAB) (AppliChem, Darmstadt, Germany) and hydrogen peroxide as substrates. The sections were dehydrated by gradient alcohols, cleared in xylene and coverslipped with DPX mounting medium. The negative control sections were processed by excluding the primary antibodies. Digital images were obtained using an Axiocam digital camera attached to an Axiostar light microscope and analyzed using Axiovision 3.0 software on a PC (Carl Zeiss, Hallbergmoos, Germany). For fluorescent double labelling, after blocking, incubation with 200 μl of Invitrogen Image-IT FX signal enhancer for 30 min and washes, DRG sections were incubated with the anti-ATP7A antibody (1:1000, Abcam), anti-hCTR1 antibody (1:1000, Novus) or anti-phosphorylated neurofilament heavy subunit (pNF-H) antibody (1:100, Swant,
Bellinzona, Switzerland), respectively, at 4°C for 48 h, followed by subsequently Alexa Fluor 594-labeled anti-chicken or anti-mouse IgG, Alexa Fluor 488-labeled anti-rabbit IgG (1:500, Invitrogen), or DyLight 488-labeled anti-chicken IgG, at room temperature for 3 h. The sections were cover-slipped with Vectorshield anti-fade mounting medium (Vector Laboratories, Burlingame, CA, USA). Reciprocal omission controls were included to ensure there was no cross-bleeding between the channels. Digital images were acquired using an Eclipse Ti fluorescence microscope with a cooled colour digital camera attached (Nikon, Japan), and analyzed using Nikon EclipseNet and ImageJ software (National Institutes of Health, USA).

Morphometry

The size profiles of copper transporter-expressing DRG neurons were determined by measuring the staining frequency, mean cell body size and size distribution. Strongly ATP7A-expressing DRG neurons were defined as those having intense diffuse or punctuate cytoplasmic staining and/or plasma membrane immunoreactivity to ATP7A. Those negative for strong ATP7A expression had no or low-intensity diffuse or punctuate cytoplasmic staining without plasma membrane immunoreactivity. Strongly CTR1-expressing DRG neurons were defined as those having intense plasma membrane and/or punctuate cytoplasmic immunoreactivity to CTR1. Those negative for strong CTR1 expression had only diffuse cell body immunoreactivity without plasma membrane or punctuate cytoplasmic immunoreactivity. Between 1,041 and 1,586 neurons from every seventh tissue section were analyzed per DRG per animal for ATP7A and CTR1, respectively. The ATP7A- or CTR1-positive neurons were further arbitrarily categorized into three size-based groups: small (<750 μm²), medium (750-1750 μm²) and large (>1750 μm²), according to previous studies [26,61]. To determine the neurotoxicity of oxaliplatin, these morphometric parameters of ATP7A-positive and CTR1-positive subpopulations of DRG neurons were compared between the drug-treated and the control animal groups.

Statistics

The differences in mean cell body size and staining frequency between different groups were assessed by one-way ANOVA with Bonferroni multiple comparison post test using Prism 5.01 software (GraphPad, San Diego, CA, USA), with a P value of <0.05 indicating statistical significance.

Acknowledgements

This work was supported by a research grant of Cancer Society of New Zealand. V. Ip was a recipient of a PhD scholarship from the Cancer Society of New Zealand.

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Authors’ contributions

Vt and JI carried out the experimental work. All authors contributed to the research plan, data interpretation and preparation of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 17 June 2010 Accepted: 13 September 2010
Published: 13 September 2010

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doi:10.1186/1744-8069-6-53

Cite this article as: Ip et al. Differential expression of ATP7A, ATP7B and CTR1 in adult rat dorsal root ganglion tissue. Molecular Pain 2010 6:53.
**Oxaliplatin-induced loss of phosphorylated heavy neurofilament subunit neuronal immunoreactivity in rat DRG tissue**

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* Corresponding author    †Equal contributors

**Abstract**

**Background:** Oxaliplatin and related chemotherapeutic drugs cause painful chronic peripheral neuropathies in cancer patients. We investigated changes in neuronal size profiles and neurofilament immunoreactivity in L5 dorsal root ganglion (DRG) tissue of adult female Wistar rats after multiple-dose treatment with oxaliplatin, cisplatin, carboplatin or paclitaxel.

**Results:** After treatment with oxaliplatin, phosphorylated neurofilament heavy subunit (pNF-H) immunoreactivity was reduced in neuronal cell bodies, but unchanged in nerve fibres, of the L5 DRG. Morphometric analysis confirmed significant changes in the number (-75%; *P* < 0.0002) and size (-45%; *P* < 0.0001) of pNF-H-immunoreactive neurons after oxaliplatin treatment. pNF-H-immunoreactive neurons had overlapping size profiles and co-localisation with neurons displaying cell body immunoreactivity for parvalbumin, non-phospho-specific neurofilament medium subunit (NF-M) and non-phospho-specific neurofilament heavy subunit (NF-H), in control DRG. However, there were no significant changes in the numbers of neurons with immunoreactivity for parvalbumin (4.6%, *P* = 0.82), NF-M (-1%, *P* = 0.96) or NF-H (0%; *P* = 0.93) after oxaliplatin treatment, although the sizes of parvalbumin (-29%, *P* = 0.047), NF-M (-11%, *P* = 0.038) and NF-H (-28%; *P* = 0.0033) immunoreactive neurons were reduced. In an independent comparison of different chemotherapeutic agents, the number of pNF-H-immunoreactive neurons was significantly altered by oxaliplatin (-77.2%; *P* < 0.0001) and cisplatin (-35.2%; *P* = 0.03) but not by carboplatin or paclitaxel, and their mean cell body area was significantly changed by oxaliplatin (-31.1%; *P* = 0.008) but not by cisplatin, carboplatin or paclitaxel.

**Conclusion:** This study has demonstrated a specific pattern of loss of pNF-H immunoreactivity in rat DRG tissue that corresponds with the relative neurotoxicity of oxaliplatin, cisplatin and carboplatin. Loss of pNF-H may be mechanistically linked to oxaliplatin-induced neuronal atrophy, and serves as a readily measureable endpoint of its neurotoxicity in the rat model.
Background

Oxaliplatin is a platinum-based chemotherapeutic agent approved for the treatment of colorectal cancer [1]. Although particularly effective for treating colorectal cancer, oxaliplatin causes neurotoxicity in a high percentage of patients [2] that is dose-limiting and can only be prevented by reducing or stopping the drug. Oxaliplatin causes acute and chronic forms of neurotoxicity in the clinic. Acute oxaliplatin neurotoxicity presents with neuro-sensory symptoms that develop during or soon after each drug infusion then recover within a few days or weeks [2,3]. These symptoms are exacerbated by cold exposure and associated with electrophysiological signs of peripheral nerve hyperexcitability [4]. With repeated treatment, oxaliplatin causes a chronic sensory neuropathy with distal paraesthesiae and dysesthesiae, loss of tendon reflexes, vibration sense and proprioception, and sensory ataxia in severe cases [2,3]. The chronic neurotoxicity of oxaliplatin is cumulative and less reversible than its acute syndrome.

There have been previous studies of oxaliplatin-induced neurotoxicity in rodent models. Single doses of oxaliplatin have been reported to acutely disturb nucleolar morphology in DRG neurons [5] and alter behavioural responses indicating sensory alldynia and hyperalgesia [6,7]. Chronic oxaliplatin treatment causes reduced sensory nerve conduction in the tail or hind-limb of treated rodents [8,9], altered sensory responses [10,11] and changes in the size profiles of DRG neurons [8,9,12] suggestive of neuronal atrophy or the loss of DRG neurons. The doses of oxaliplatin employed in these previous rodent studies have varied widely but were often lower than those used clinically, when expressed as per unit of body surface area or considered on the basis of relative susceptibilities to oxaliplatin-induced neurotoxicity, as in recent studies [8,15]. The RT97 primary antibody employed in these studies recognises phosphorylated KSP repeats in the tail domain of phosphorylated neurofilament heavy subunit (pNF-H) [16]. The epitopes of the RT97 antibody are strongly expressed in rat DRG tissue within the cell bodies of subpopulations of large DRG neurons and large-diameter myelinated nerve fibres [17].

Phosphorylated neurofilaments are major cytoskeletal proteins of large myelinated sensory neurons [18]. Disturbance of neurofilament phosphorylation has been implicated in a wide range of neurodegenerative diseases [19] but its role in oxaliplatin-induced neurotoxicity is unknown.

In this paper, we report that neuronal pNF-H expression, as determined by RT97 immunohistochemistry of rat DRG tissue, was significantly reduced after oxaliplatin treatment. This loss of pNF-H immunoreactivity was shown to correspond with the relative neurotoxicity of oxaliplatin, cisplatin and carboplatin, but was not associated with the loss of DRG cells, generalised reduction of neuronal marker or neurofilament expression, or with paclitaxel-induced neurotoxicity.

Results

Oxaliplatin-induces loss of neuronal pNF-H immunoreactivity

After the treatment of rats with oxaliplatin, pNF-H immunoreactivity was reduced in neuronal cell bodies, but appeared unchanged in nerve fibres, of the L5 DRG (Fig 1 and 2). pNF-H immunohistochemistry was carried out using the RT97 primary antibody on cryosections of L5 DRG from animals treated with oxaliplatin or 5% glucose (vehicle control). In control DRG (Figure 1A and 2A), pNF-H immunoreactivity was associated with ganglionic nerve fibres and large neuronal cell bodies, with cytoplasmic staining and nuclei sparing, consistent with a previous report [17]. After treatment with oxaliplatin twice weekly for 8 weeks at a maximum tolerated dose (1.85 mg/kg/dose), pNF-H immunoreactivity of nerve fibres appeared to be relatively unchanged but that of the DRG cell bodies was greatly reduced (Figure 1B and 2B). Cell body size frequency histograms of pNF-H immunoreactive neurons were markedly altered by oxaliplatin treatment (Figure 1C, D).

Morphometric analysis confirmed that oxaliplatin treatment was associated with statistically significant reductions in the number and size of pNF-H immunoreactive neurons (Table 1). Defined as DRG neurons with cell body staining greater than background staining of a negative control, pNF-H immunoreactive neurons accounted for approximately 20% of the overall population of DRG neurons in control animals versus about 5% in animals treated with oxaliplatin (~75%; P < 0.0002). In addition, the size of pNF-H-immunoreactive neurons was altered by oxaliplatin treatment as evident from significant changes in mean cell body size (~45%, P < 0.0001), and in the percentages of neurons with large (>1750 μm²) (~95%; P < 0.001), medium (750-1750 μm²) (~41%; P < 0.0005) or small cell bodies (<750 μm²) (~247%; P < 0.0001).
Neuronal parvalbumin and non-phospho-specific neurofilament immunoreactivity persists following oxaliplatin treatment

Next we examined the effect of oxaliplatin on the expression of parvalbumin, a marker of large DRG neurons. As previously reported [8,20,21], parvalbumin immunoreactivity in control DRG (Fig 2C) was localized to the cell bodies of a subset of neurons, whose size profile (Table 1) was similar to the neuronal subpopulation defined by cell body immunoreactivity for pNF-H. Double immunofluorescence labeling confirmed extensive co-localization of parvalbumin and pNF-H immunoreactivity in the neuronal cell bodies of control DRG (Figure 2E). After oxaliplatin treatment, parvalbumin immunoreactivity persisted
in their mean cell body area (-29%; *P* = 0.0033), and in the percentages of large (>1750 μm²) (-76%; *P* = 0.0003) or small neurons (<750 μm²) (+52%; *P* < 0.05).

Comparative effect of chemotherapeutic drugs on neuronal pNF-H immunoreactivity

Finally we compared the effect of different chemotherapeutic agents on neuronal pNF-H immunoreactivity in rat DRG tissue. Groups of animals were treated for 8 weeks with multiple-doses of oxaliplatin (1.85 mg/kg/dose twice per week), cisplatin (1 mg/kg/dose twice per week) or carboplatin (8 mg/kg/dose twice per week), or for 9 weeks with multiple-doses of paclitaxel (12.5 mg/kg/dose once per week), or their respective drug vehicles as matching control groups. These doses of oxaliplatin [8,23,24], cisplatin [24], carboplatin [24] and paclitaxel [25] were previously shown to alter sensory nerve conduction and DRG neuronal morphometric parameters in this rat model in keeping with the induction of a peripheral neuropathy. There was no mortality during the treatment period but the amount of body weight gained during the experiment was less in the treatment groups (range: 5 to 17% of baseline) than in the control groups (range: 20 to 27% of baseline). The effect of oxaliplatin on the number and size of pNF-H-immunoreactive neurons was confirmed in this independent experiment. After treatment, the number of pNF-H-immunoreactive neurons was significantly altered by oxaliplatin (-77.2%; *P* < 0.0001) and cisplatin (-35.2%; *P* = 0.003) but not by carboplatin or paclitaxel (Figure 4A). The mean cell body area of pNF-H-positive neurons was significantly changed by oxaliplatin.
but not by cisplatin, carboplatin or paclitaxel (Figure 4B).

**Discussion**

In this paper we report, for the first time, an effect of oxaliplatin on neurofilament expression in rat DRG tissue as determined by immunohistochemistry using the RT97 primary anti-neurofilament antibody. The epitopes of the RT97 antibody are phosphorylated KSP repeats within the tail domain of phosphorylated neurofilament heavy subunit [16], which are expressed in a specific pattern in DRG tissue of healthy adult rats [17]. In DRG these epitopes are expressed within the cell bodies of a subpopulation of large neurons, presumably during early post-translational modification of neurofilament subunits, and in their axons, where neurofilament subunits become hyper-phosphorylated during their polymerisation into stabilised polymeric complexes [26]. Aberrant neurofilament phosphorylation, as indicated by altered tissue immunoreactivity for phospho-specific anti-neurofilament primary antibodies, such as the RT97 antibody, has been associated with other disorders of DRG neurons [27,28], but a role in oxaliplatin neurotoxicity has not been considered.

We demonstrated that chronic oxaliplatin treatment was associated with a specific pattern of loss of pNF-H immunoreactivity in rat DRG tissue in the current study. The loss of pNF-H immunoreactivity was evident visually from qualitative changes in the intensity of neuronal cell body immunostaining in DRG sections and by statistically significant reductions in the numbers and size of pNF-H-immunoreactive DRG neurons in oxaliplatin-treated animals, confirmed in two independent experiments. Strong pNF-H immunoreactivity appeared to remain in DRG nerve fibres after oxaliplatin treatment indicating that its loss was specific for the neuronal cell bodies, and that the treatment caused no nonspecific masking of pNF-H epitopes, under these experimental conditions. In control DRG, the neuronal immunoreactivity for pNF-H overlapped or colocalised with parvalbumin, non-phospho-specific-NF-M and non-phospho-specific-NF-H, but the number of DRG neurons displaying immunoreactivity for these primary antibodies was not changed by oxaliplatin. Therefore, the loss of neuronal pNF-H immunoreactivity was not associated with the loss of DRG cells or any generalised reduction of neuronal marker or neurofilament expression induced by oxaliplatin.

The present study also demonstrated that the extent of loss of pNF-H immunoreactivity corresponded to the relative neurotoxicity of oxaliplatin, cisplatin and carboplatin. When these platinum agents were ranked according to their effect on the number of pNF-H-immunoreactive DRG neurons, oxaliplatin had the greatest effect, followed by cisplatin and then carboplatin had the least. Their ranking corresponded with the relative cumulative dose-potencies of oxaliplatin, cisplatin and carboplatin for

<table>
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<tr>
<th>Table 1: Effect of oxaliplatin on morphometry of L5 DRG neurons with immunoreactivity for pNF-H, parvalbumin, NF-M or NF-H.</th>
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<tbody>
<tr>
<td><strong>Frequency of Immunoreactive Cells (%)</strong></td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>pNF-H</strong></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Oxaliplatin</td>
</tr>
<tr>
<td>Percent change</td>
</tr>
<tr>
<td>P</td>
</tr>
<tr>
<td><strong>Parvalbumin</strong></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Oxaliplatin</td>
</tr>
<tr>
<td>Percent change</td>
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<tr>
<td>P</td>
</tr>
<tr>
<td><strong>NF-M</strong></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Oxaliplatin</td>
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<tr>
<td>Percent change</td>
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<tr>
<td>P</td>
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<td><strong>NF-H</strong></td>
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<td>Control</td>
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<tr>
<td>Oxaliplatin</td>
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<td>Percent change</td>
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Animals were treated with oxaliplatin or drug vehicle alone (control group) twice per week for 8 weeks. Values represent the mean and standard error of the mean for 4 to 5 animals/group.

(-31.1%; P = 0.008) but not by cisplatin, carboplatin or paclitaxel (Figure 4B).
reducing sensory nerve conduction velocity in rats, which occurs after cumulative doses of 15, 46.7 and 302 μmol/kg, respectively [24]. In addition, this ranking corresponded with the proportion of patients developing peripheral neurotoxicity of any severity grade after treatment with these platinum drugs, which is reported to occur in ~90% [29], ~50% [30] and ~6% [31] of patients treated with oxaliplatin, cisplatin and carboplatin, respectively.

These findings link the loss of pNF-H with the neurotoxicity of oxaliplatin, although its exact role in this toxicity remains to be elucidated. Phosphorylated neurofilaments have important physiological roles in maintaining axonal calibre and fast conduction velocity of large myelinated nerve fibres [18,26], and their loss causes neuronal and axonal atrophy, and reduced sensory nerve conduction velocity, of DRG neurons [32-34]. Therefore, the loss of neuronal pNF-H expression demonstrated in the current study may be causally linked to the decreased size profiles of DRG neurons and reduced sensory nerve conduction velocity, which are induced by chronic oxaliplatin treatment in rodent models [8,9,12].

The molecular mechanisms responsible for loss of pNF-H immunoreactivity induced by oxaliplatin are unclear and require further study. Defects in early neurofilament phosphorylation could account for the loss of RT97 cell body staining without changes in its nerve fibre immunoreactivity or altered immunoreactivity of non-phospho-specific antineurofilament primary antibodies. The main pharmacological mechanism of platinum-based drugs is the formation of platinum-DNA adducts that inhibit DNA replication and transcription [35]. After exposure to platinum drugs, DNA-platinum adducts have been detected in DRG neurons [36,37] and their level is correlated with the severity of neurotoxicity [38,39]. Therefore, the loss of RT97 immunoreactivity could occur due to inhibited transcription of neurofilament kinase genes. However, confirmation of a mechanism involving defective neurofilament phosphorylation or inhibited transcription would be technically difficult in DRG tissue because of the confounding effects of persisting RT97 immunoreactivity of the ganglionic nerve fibres and non-specific inhibition of DNA transcription by platinum drugs [40]. Whatever the mechanism, it was evident from this study that pNF-H is a specific marker of DRG neuronal subpopulations particularly susceptible to damage from chronic oxaliplatin exposure, and changes in numbers of pNF-H immunoreactive neurons are readily measurable endpoints of oxaliplatin neurotoxicity in the rat. Similarly, the current study confirmed our previous observations [8] of parvalbumin being a specific marker of DRG neurons susceptible to oxaliplatin toxicity and significant changes in size profiles of parvalbumin immunoreactive neurons during this neurotoxicity. Detecting oxaliplatin-induced neurotoxicity in the rat model using pNF-H or parvalbumin immunohistochemistry is statistically more powerful and utilises fewer animals than nerve conduction studies. However, unlike immunochemical endpoints, nerve conduction measurements can be repeated at different times in the same animal and provide functional information.

Paclitaxel causes peripheral neurotoxicity in a high proportion of treated patients [41] and reductions in sensory nerve conduction velocity in the rat [25,42-44], but had no effect on the number or size of pNF-H immunoreactive neurons in this study. The mechanism of paclitaxel neurotoxicity may involve microtubule binding and disturbance of microtubule polymerisation with resulting axonal damage [43,45] and secondary reactive changes in DRG cell bodies [25,46,47]. In contrast, the mechanism of oxaliplatin neurotoxicity may involve a loss of phosphorylated neurofilaments at the level of DRG cell bodies with secondary changes in axonal calibre and conduction velocity. In this way, disturbance of major neuronal cytoskeletal proteins, such as microtubules and neurofilaments, may be a common mechanistic theme whereby...
different anticancer drugs from various classes damage the peripheral nervous system.

**Conclusion**
In conclusion, this study has demonstrated a specific pattern of loss of pNF-H immunoreactivity in rat DRG tissue that corresponds with the relative neurotoxicity of oxaliplatin, cisplatin and carboplatin. Loss of pNF-H may be mechanistically linked to oxaliplatin-induced neuronal atrophy and serves as readily measureable endpoints of oxaliplatin neurotoxicity in the rat model.

**Methods**

**Animals and Drugs**
Age-matched 10-week old female Wistar rats were used for experiments that weighed approximately 270 g at the commencement of the study. All animals were housed in a temperature and humidity-controlled environment with uninhibited access to food and water. Oxaliplatin (Sigma-Aldrich, St. Louis, MO, USA and Sanofi-Synthelabo NZ Ltd, Auckland, NZ) and carboplatin (Mayne Pharma, Vic, Australia) were diluted for injection in 5% dextrose (Baxter Healthcare, Old Toongabbie, Australia) for intraperitoneal injection at 15 ml/kg. Cisplatin (Sigma) was diluted in 0.9% sodium chloride (Baxter Healthcare) for intraperitoneal injection at 15 ml/kg. Paclitaxel (Phytogen Life Sciences Inc., Delta, BC, Canada) was solubilised in a 1:1 solution of Cremophor EL (Sigma-Aldrich) and ethanol to make a stock solution of 6 mg/ml, then further diluted with 0.9% NaCl (Baxter Healthcare) for administration by intraperitoneal injection at an injection volume of 12.5 ml/kg. Animals were treated twice per week either with oxaliplatin (1.85 mg/kg), carboplatin (8 mg/kg) or their control drug vehicle of 5% dextrose, or with cisplatin (1 mg/kg) or its control vehicle of 0.9% sodium chloride, an injection volume of 15 ml/kg for 8 weeks. Paclitaxel-treated animals received 12.5 mg/kg of drug once weekly for a total of 9 weeks, and control animals were treated with the Cremophor EL/ethanol/0.9% NaCl solution at the same dosing frequency and injection volume. To prevent time-dependent variation in pharmacokinetics and pharmacodynamics all injections were performed between 1 and 3 p.m. The Animal Ethics Committee of the University of Auckland approved all animal procedures.

**Single-label Immunohistochemistry**
One week after the conclusion of treatment, terminal anaesthesia was induced by administering 0.9 ml of 3 mg/ml pentobarbitone (Chemstock Animal Health Ltd, Christchurch, New Zealand). Subsequently, transcardiac perfusion with 60 ml of 0.9% NaCl (Baxter Healthcare) followed by 60 ml of 4% paraformaldehyde in 0.1 M phosphate buffer was carried out. L5 DRGs were carefully dissected from each animal, post-fixed in 4% paraformaldehyde for 2-6 hours and cryoprotected in a 30% sucrose solution until the tissues sunk. Following cryoprotection, the DRG were placed in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA), snap frozen in liquid nitrogen and stored at -80°C. Each dorsal root ganglion was sectioned on a cryostat (Leica CM 3050) at a thickness of 10 μm onto polylysine-coated slides that were then stored at -80°C. For immunostaining, frozen tissue slides were warmed to room temperature, washed in PBS containing 0.2% Triton X-100 and incubated in 1% H2O2 in 50% methanol for 10 minutes. To prevent non-specific
binding, the slides were blocked for 1 hour in PBS containing 0.2% Triton X-100 with 3% normal goat serum (Sigma-Aldrich) and 20 mg/ml bovine serum albumin (Sigma-Aldrich). Next, the slides were incubated overnight in a humidity chamber with either the mouse monoclonal antibody to the phospho-specific NF-H subunit (RT-97 clone; 1:100; CBL212, Chemicon International, Temecula, CA, USA), rabbit polyclonal anti-parvalbumin (PVA3) primary antibody (1:1000; P. Emson, Cambridge, UK), rabbit polyclonal non-phospho-specific antineurofilament 200 IgG fraction (1:1000; Sigma N4142) or mouse monoclonal non-phospho-specific antineurofilament medium subunit (1:1000; Sigma N5264). The slides were rewashed and incubated with either an anti-mouse or anti-rabbit biotinylated secondary antibody (1:1500; Sigma-Aldrich) for 2.5 hours. After further washes, the slides were incubated for 3 hours in an extravidin-peroxidase conjugate (1:500; Sigma-Aldrich). Staining was visualised with 0.5 mg/ml 3,3’-diaminobenzidine tetrahydrochloride (AppliChem, Darmstadt, Germany) and 0.01% H2O2 in 0.4 M phosphate buffer for 10 minutes. Finally, the slides were washed, dehydrated through a series of alcohols, cleared in xylene and coverslipped.

The DRG sections were analysed by light microscopy with digital images generated by an Axiocam camera (Carl Zeiss Vision, Hallbergmoos, Germany) and quantitative analysis performed using AxioVision 3.0 (Carl Zeiss Software) software. The cross-sectional area was measured for each immunoreactive neuron and the frequency of expression was generated by counting every cell and expressing the count of immunoreactive neurons as a percentage of the total cell count. Immunoreactive DRG neurons were also categorised on the basis of size into small (cross-sectional area <750 μm2), medium (750-1750 μm2) and large (>1750 μm2) sized cells.

Fluorescent Double Labelling Immunohistochemistry

Frozen DRG slides were defrosted, washed, incubated in H2O2 with methanol and blocked as described previously for single-label immunohistochemistry. Each slide was incubated overnight in a humidity chamber in both mouse anti-pNF-H (1:100) and rabbit anti-parvalbumin (1:1000) primary antibodies. The slides were then washed and incubated in the dark for 4 hours in both anti-rabbit cy3 (1:200; Jackson Laboratories, West Grove, PA, USA) and biotinylated anti-mouse secondary antibodies (1:200, Sigma). The slides were rewashed and incubated in the dark for 3 hours in FITC tertiary antibody (1:200; Sigma-Aldrich). Finally, the slides were washed, cover slipped with Citifluor (Agar Scientific, Essex, UK) and stored overnight at 4°C to prevent bleaching. Fluorescent analysis was performed with a Zeiss Axioplan 2 epifluorescence microscope (Carl Zeiss Microscopy) equipped with fluorescent rhodamine and FITC filters with excitation wavelength ranges of 534-558 nm and 450-490 nm, respectively. Monochrome images were captured by a Dage video camera (Newivicom, Wiesbaden, Germany) and were converted to pseudo-coloured images by Meta morph 6.1 software (Universal Imaging Corporation, Downingtown, PA, USA).

Statistics

The statistical significance of differences in means between treatment and control groups were assessed using unpaired t-tests and analysis of variance (ANOVA). P values < 0.05 indicated statistical significance.

Abbreviations


Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

SJ carried out the oxaliplatin and paclitaxel studies and drafted the manuscript. JS carried out the comparison of oxaliplatin and carboplatin. NJ carried out the cisplatin studies. VI provided technical support. BC participated in the design of the study and its coordination. JL and MM conceived of the study, and participated in its design and coordination and drafted the final manuscript. All authors read and approved the final manuscript.

Acknowledgements

This study was supported by grant funding from the Cancer Society of New Zealand.

References

schedules of oxaliplatin treatment on the peripheral nervous system of the rat. 


Neuronal expression of copper transporter 1 in rat dorsal root ganglia: association with platinum neurotoxicity

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Received: 8 January 2009 / Accepted: 26 April 2009 / Published online: 24 May 2009 © Springer-Verlag 2009

Abstract

Purpose We report the neuronal expression of copper transporter 1 (CTR1) in rat dorsal root ganglia (DRG) and its association with the neurotoxicity of platinum-based drugs.

Methods CTR1 expression was studied by immunohistochemistry and RT-PCR. The toxicity of platinum drugs to CTR1-positive and CTR1-negative neurons was compared in DRG from animals treated with maximum tolerated doses of oxaliplatin (1.85 mg/kg), cisplatin (1 mg/kg) or carboplatin (8 mg/kg) twice weekly for 8 weeks.

Results Abundant CTR1 mRNA was detected in DRG tissue. CTR1 immunoreactivity was associated with plasma membranes and cytoplasmic vesicular structures of a subpopulation (13.6 ± 3.1%) of mainly large-sized (mean cell body area, 1,787 ± 127 μm²) DRG neurons. After treatment with platinum drugs, the cell bodies of these CTR1-positive neurons became atrophied, with oxaliplatin causing the greatest percentage reduction in the mean cell body area relative to controls (42%; P < 0.05), followed by cisplatin (18%; P < 0.05) and carboplatin causing the least reduction (3.2%; P = NS). CTR1-negative neurons, with no immunoreactivity or only diffuse cytoplasmic staining, showed less treatment-induced cell body atrophy than CTR1-positive neurons.

Conclusions CTR1 is preferentially expressed by a subset of DRG neurons that are particularly vulnerable to the toxicity of platinum drugs. These findings, together with its neuronal membrane localization, are suggestive of CTR1-related mechanisms of platinum drug neuronal uptake and neurotoxicity.

Keywords Chemotherapy-induced neurotoxicity · Copper transporter · Platinum drug · Dorsal root ganglion · Sensory neuron · Uptake

Introduction

Chemotherapy-induced peripheral neuropathy is a common and dose-limiting adverse effect of several anticancer drugs. It may limit the delivery of cancer therapy, compromise patient quality of life and persist long beyond the completion of treatment [1]. Chemotherapy-induced peripheral neuropathy occurs in association with particular classes of anticancer drugs, such as platinum and antimitotubule agents, but individual agents of the same class display differing neurotoxicity profiles. Platinum-based drugs, for example, induce a chronic sensory neuropathy with distal paresthesia and dysesthesiae, loss of deep tendon reflexes, vibration sense and proprioception, and sensory ataxia, coming on or worsening with repeated treatments [2, 3]. However, cisplatin, oxaliplatin and carboplatin differ in their acute neurotoxicity and ototoxicity [4, 5], and in the proportion of patients developing peripheral neuropathy of any severity grade after treatment, which is reported to occur in ~50% [6], ~90% [7] and ~6% [8] of treated patients, respectively. Currently, no
approved treatments are available for preventing or limiting chemotherapy-induced peripheral neuropathy, or managing its symptoms, despite a wide range of neuroprotective therapies having been tested clinically [9]. The mechanism of platinum neurotoxicity remains incompletely understood although it may involve platinum accumulation within the dorsal root ganglia (DRG) leading to atrophy or loss of peripheral sensory neurons. Clinical and electrophysiological features of the sensory neuropathy and the sparing of motor function point to damage occurring at the level of the cell body of sensory neurons within the DRG [10–13]. Histopathological studies have shown altered size profiles of DRG neurons after platinum treatment of patients [11, 14] and in rodent models [15–21], consistent with induction of neuronal atrophy or selective loss of large DRG neurons. High levels of platinum accumulate in the DRG compared to peripheral nerves, spinal cord and brain, following exposure to platinum-based drugs in patients [11, 14, 22] and animal models [15, 21, 23–25], but the differing neurotoxicity profiles of cisplatin, carboplatin and oxaliplatin are not simply explained by differences in DRG platinum concentration [16, 23, 25]. Platinum-based drugs exert their antitumour activity by formation of platinum-DNA adducts, particularly 1,2-intrastrand cross-links between N7 residues of adjacent guanine bases [26]. The cellular mechanism of their neurotoxicity is less clear but platinum-DNA adducts have been detected in DRG neurons [27, 28] and their level correlates with the severity of platinum neurotoxicity [29, 30].

Copper transporter 1 (CTR1) is the major high-affinity copper uptake transporter in mammals and an integral membrane protein containing three transmembrane domains with methionine- and histidine-rich metal binding sites [31, 32]. Evidence implicating CTR1 in the transport of platinum drugs includes demonstration of altered uptake and toxicity of platinum drugs associated with genetic knockout, transfection or copper-induced down-regulation of CTR1 in Saccharomyces cerevisiae [33, 34], rodent [33, 35] and human cells [35, 36]. In humans and rodents, CTR1 is expressed by selected normal tissues and cell types [37–39], but it has not been detected before in primary sensory neurons or DRG tissue. However, DRG neurons are known to strongly express several cuproenzymes, such as cytochrome C oxidase [40] and Cu/Zn superoxide dismutase [41], which could indicate significant requirements for copper acquired via CTR1. To investigate the DRG neuronal expression of CTR1, we studied tissues from healthy adult rats by immunohistochemistry and real-time PCR. We then determined the relationship between neuronal CTR1 expression and the neurotoxicity of platinum drugs in animals treated with oxaliplatin, cisplatin and carboplatin.

Materials and methods

Animals and drug treatment

Age-matched, 12-week-old female Wistar rats were housed in a self-contained unit maintained at 22 ± 2°C, and set to 12 h dark–light cycles with food and water ad libitum. Twelve healthy animals were used in immunohistochemistry and PCR studies. In addition, for treatment study, 27 rats were randomly divided into five experimental groups (n = 5 or 6) to receive intraperitoneal injections of oxaliplatin (1.85 mg/kg, Sanofi Aventis), cisplatin (1 mg/kg, Sigma), carboplatin (8 mg/kg, Mayne Pharma), dextrose and physiologic saline (Baxter) as vehicle controls, respectively, twice weekly for 8 weeks, at an injection volume of 15 ml/kg given between 1300 and 1500 h. Animals were weighed twice weekly and inspected daily for signs of drug toxicity. All animal procedures were approved by the institutional Animal Ethics Committee (AEC No. R591) and complied with the UKCCCR guidelines throughout.

Reverse-transcriptase PCR and real-time PCR

Healthy animals designated for CTR1 PCR analysis were euthanized by intraperitoneal injection of pentobarbitone (90 mg/kg body weight, Chemstock Animal Health, Christchurch, New Zealand). Tissues from lumbar DRG, cerebral cortex, spinal cord, liver, kidney and duodenum were collected and homogenized in PureZol reagent by a Dounce homogenizer (Glas-Col, Terre Haute, IN, USA) for total RNA isolation using an Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules, CA, USA). Following digestion with DNase I (1 unit/µg, Bio-Rad), total RNA (0.25 µg) of each sample was reverse-transcribed into cDNA using a SuperScript first strand synthesis kit (Invitrogen, Carlsbad, CA, USA) according to instructions, followed by digestion with Ribonuclease H (Invitrogen). cDNA was amplified by PCR in a reaction mixture containing dNTP, MgCl2, Platinum Taq DNA polymerase (Invitrogen) and custom primers, using a GeneAmp 9700 PCR System (Applied Biosystems, Foster City, CA, USA) at 52°C for 40 cycles. Forward and reverse primers for CTR1 (Genebank accession number NM_133600) were: 5′-ttg get tta aga atg tgg acc t-3′ and 5′-cat aag gat ggt tcc att tgg t-3′; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (accession number NM_017008): 5′-tgc tga ttt tgg gga gtc ttc tgc tga gta gta gaa a-3′, as a control. PCR products were electrophoresed in 2% agarose gel, stained with ethidium bromide and photographed using Gel Doc 2000 System (Bio-Rad). Subsequently, quantitative real-time PCR was performed to compare the relative CTR1 mRNA level in DRG to other tissues using

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ABI PRISM 7900HT Sequence Detection Systems (Applied Biosystems). Primers and probe sets were purchased as TaqMan Gene Expression Assays containing forward and reverse unlabeled PCR primer pair and a fluorescent reporter dye-labeled TaqMan MGB probe. Samples containing 25 ng cDNA of each tissue, TaqMan universal PCR Master Mix, FAM-labeled probe for rat CTR1 gene and VIC-labeled probe for 18S ribosomal RNA as endogenous control, were analyzed in triplicate in 10-μl total volume. A total of six rats were used in this study. The abundance of mRNA of CTR1 or rRNA was measured as the threshold cycle values (Ct) after each reaction. The relative RNA expression level of each sample was calculated, with DRG as the arbitrary calibrator for comparison, using the 2−ΔΔCt method [42], where ΔΔCt = (Ct,CTR1 − Ct,rRNA)target tissue − (Ct,CTR1 − Ct,rRNA)DRG.

Immunohistochemistry of dorsal root ganglia

Under terminal anesthesia, transcardiac perfusion was performed with 120 ml each of saline and 4% phosphate buffered paraformaldehyde. Lumbar 5 (L5) DRG were dissected out, post-fixed in the perfusion fixative for 2 h, cryoprotected in 30% sucrose and embedded with TissueTek OCT compound (Sakura Finetechical, Tokyo, Japan). DRG cryosections of 10-μm mounted onto poly-L-lysine pre-coated slides were processed with 0.2% Triton X-100 in PBS, 1% hydrogen peroxide in 50% methanol, and then the blocking buffer containing 3% normal goat serum and 20 mg/ml bovine serum albumin (ICPbio Ltd, Auckland, New Zealand). A rabbit polyclonal anti-hCTR1 primary antibody (1:500, Novus Biologicals, Littleton, CO, USA) was applied to the DRG sections overnight, followed by incubations with a biotinylated anti-rabbit antibody (1:500, Novus Biologicals, Littleton, CO, USA) and an extravidin-peroxidase conjugate (1:500, Vector Laboratories, Burlingame, CA). Reciprocal omission controls were included to ensure there was no cross-bleeding between the channels. Digital images were acquired using a Leica DMR fluorescence microscope (Leica Microsystems, Wetzler, Germany) with a cooled color Nikon digital camera attached, and analyzed using Nikon EclipseNet and ImageJ software (National Institutes of Health, USA).

Measurement of platinum neurotoxicity by DRG neuronal morphometry

The neurotoxicity of platinum drugs in animals was determined by measuring the mean cell body size and size distribution profile of subpopulations of DRG neurons. CTR1-positive neurons were defined according to the presence of intense plasma membrane and/or punctate cytoplasmic immunoreactivity to CTR1. CTR1-negative neurons were defined as those showing no immunostaining or only light diffuse cytoplasmic immunoreactivity, compared to a negative control. Between 1,847 and 2,635 neurons were analyzed per DRG per animal from evenly spaced tissue sections. The CTR1-positive and CTR1-negative neurons were categorized into three size-based groups: small (<750 μm²), medium (750–1,750 μm²) and large (>1,750 μm²), according to previous studies [18, 43].

Statistics

The differences in mean cell body size, staining frequency and mRNA levels between different groups were assessed by two-tailed t test and one-way analysis of variance (ANOVA) with Dunnett’s post test using Prism 5.01 software (GraphPad, San Diego, CA), with a P value of <0.05 indicating statistical significance.

Results

Neuronal expression and membrane localization of CTR1 in healthy adult rat DRG

Immunohistochemical analysis was carried out using an affinity-purified polyclonal anti-hCTR1 antibody on rat L5 DRG cryosections. The specificity of the antibody had been previously characterized using a hCTR1-transfected human
ovarian carcinoma A2780 cell line and preabsorption assay with immunizing peptide [37]. Cross-reactivity of the antihuman antibody with murine protein was expected based on 100% sequence homology of the C-terminus in both species [38]. As shown in Fig. 1, there was specific CTR1 immunoreactivity associated with the DRG neuronal cell bodies, but not with their nerve fibers, satellite glial cells or other tissue elements. CTR1 immunohistochemical staining was localized to the plasma membranes of large neuronal cell bodies and/or cytoplasmic vesicular structures in a punctate pattern in a portion of DRG neurons of different sizes. In addition, diffuse lighter cytoplasmic staining with nuclear sparing was present in many of the other DRG neuronal cell bodies.

Double label fluorescence immunohistochemistry of CTR1 and phosphorylated neurofilament heavy subunit (pNF-H), a marker for large DRG neurons and their nerve fibers [44], was carried out with DAPI counterstaining (Fig. 2). The result revealed extensive colocalization of CTR1 with pNF-H cell body staining but not with pNF-H nerve fiber staining or with densely DAPI-stained nuclei of satellite glial cells. This was consistent with the single label immunohistochemistry findings described above showing CTR1 immunoreactivity associated with a subpopulation of mainly large DRG neuronal cell bodies, but not with their nerve fibers, satellite glial cells or other tissue elements of the DRG.

Morphometric analysis of CTR1-positive neurons, defined as those having intense plasma membrane and/or punctate cytoplasmic staining, revealed a subpopulation of DRG neurons with a distinct size profile (Fig. 3). CTR1-positive neurons accounted for 13.6 ± 3.1% of the overall population of neurons of L5 DRG of healthy animals (n = 6). The mean cell body size of CTR1-positive neurons was 1,787 ± 127 μm² compared to 746 ± 69 μm² for the overall population (P < 0.05). A greater proportion of CTR1-positive neurons (54.0 ± 12.1%) had large cell bodies (>1,750 μm²) compared to the overall population (7.0 ± 2.5%) (P < 0.05).

We next performed RT-PCR with total RNA purified from different tissues of healthy rats to determine gene expression of CTR1. As shown in Fig. 4, uniformly strong electrophoretic bands were observed in all tissues analyzed, indicative of the abundant expression of CTR1 in lumbar

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**Fig. 1** Neuronal CTR1 immunoreactivity in rat L5 DRG tissue associated with cell bodies (n), plasma membranes (→), punctate cytoplasmic vesicular structures (left arrowhead) and diffuse cytoplasmic staining (↑) without staining of nerve fibers (f) or other tissue elements. a negative control, b and c (enlarged frame in b) DAB IHC, d fluorescence IHC with higher magnification insert.
These results were further confirmed by quantitative real-time PCR. On average, the relative level of CTR1 mRNA in lumbar DRG appeared similar to brain, spinal cord, kidney, liver and small intestine (0.4- to 3.4-fold, \( P \geq 0.05 \), ANOVA).

Fig. 2 CTR1 co-localization with phosphorylated neurofilament heavy subunit (pNF-H) cell body staining (→) but not with its nerve fiber staining (↑) or with densely DAPI-stained satellite cells (→). a CTR1, b pNF-H, c DAPI, d merged. Scale bars 50 μm

Atrophy of CTR1-positive DRG neurons caused by platinum drug treatment

We examined the association between neuronal expression of CTR1 and the neurotoxicity of a series of clinical platinum drugs in the rat. Use was made of a previously established rat model of chemotherapy-induced peripheral neuropathy and known maximum tolerated doses of

Fig. 3 Cell body size frequency histograms for CTR1-positive DRG neurons (solid line) relative to the total DRG neuronal population (broken line). Each bin represents the mean value of six animals

Fig. 4 CTR1 mRNA expression in lumbar DRG and other tissues. Representative gel electrophoretic bands of RT-PCR products for CTR1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene in indicated tissues
oxaliplatin (1.85 mg/kg), cisplatin (1 mg/kg) and carboplatin (8 mg/kg), with a twice per week for 8 weeks dosing schedule in groups of rats [18, 23]. Peripheral neurotoxicity becomes detectable in this model towards the end of 8 weeks treatment. There was no mortality during the treatment period, but the amount of body weight gained was significantly less in each of the treatment groups compared to their respective control groups (Fig. 5a). Mean percentage change in body weight from baseline during repeated treatment with cisplatin (106 ± 5.5%), carboplatin (105 ± 5.2%) and oxaliplatin (108 ± 2.3%) were similar in each of the treatment groups but less than the dextrose (127 ± 4.8%) or saline (120 ± 3.8%) control groups, suggestive of the different doses of platinum drugs having significant but approximately equivalent general toxicity. One week following completion of all treatment, L5 DRGs were collected, processed immunohistochemically for CTR1 and analyzed morphometrically to determine the frequency of neurons expressing CTR1, and to establish size profiles of CTR1-positive and CTR1-negative neuronal subpopulations in the treatment and control groups. The qualitative pattern of neuronal CTR1 expression in L5 DRG was unchanged by platinum drug treatment. The frequency of CTR1-positive neurons was similar in the dextrose control (12.0 ± 1.2%), saline control (12.5 ± 1.8%), oxaliplatin (12.3 ± 1.8%), cisplatin (12.9 ± 1.1) and carboplatin (13 ± 1.5%) groups (P > 0.05, ANOVA).

Treatment of rats with platinum drugs caused atrophy of the cell bodies of CTR1-positive DRG neurons, but the magnitude of their effect varied between the three agents with oxaliplatin causing the greatest toxicity, followed by cisplatin, and carboplatin causing the least. Oxaliplatin was associated with statistically significant reductions in mean cell body size of CTR1-positive neurons (42%, P < 0.05, Fig. 5b) and the percentage of CTR1-positive neurons measuring >1,750 μm² (86%, P < 0.05, Fig. 5c). Oxaliplatin treatment was accompanied by a major leftward deviation of the cell body size frequency histogram for the CTR1-positive neuronal subpopulation (Fig. 6a). In contrast, carboplatin did not cause significant reduction of mean cell body size (3.2%, P = NS, Fig. 5b), the percentage of large neurons (>1,750 μm²) (35%, P = NS, Fig. 5c) or deviation of the cell body size frequency histogram (Fig. 6a) for the CTR1-positive neuronal subpopulation. The toxicity of cisplatin was intermediate to that of oxaliplatin and carboplatin with statistically significantly reductions in mean cell body area of CTR1-positive neurons (18%, P < 0.05, Fig. 5b) and the percentage of CTR1-positive neurons measuring >1,750 μm² (35%, P < 0.05, Fig. 5c), but these reductions were numerically less than those caused by oxaliplatin. In addition, cisplatin caused a smaller leftward deviation in the cell body size frequency

![Fig. 5](image-url) Effect of oxaliplatin (Oxali.), carboplatin (Carb.) and cisplatin (Cispl.) treatment of animals compared with the vehicle control groups (dextrose, saline). a Absolute change in body weight calculated from the body weight of week 8 minus body weight at baseline. b Mean cell body size of CTR1-positive DRG neurons. c Percentage of CTR1-positive DRG neurons measuring >1,750 μm². d Mean cell body size of CTR1-negative DRG neurons. Values are expressed as mean ± standard deviation (n = 5 or 6 animals). NS not significant.
Fig. 6  Comparative effect of oxaliplatin, carboplatin (a, c) and cisplatin (b, d) on cell body size frequency histograms of CTR1-positive (a, b) and CTR1-negative DRG neurons (c, d).

histogram for the CTR1-positive neuronal subpopulation (Fig. 6b) compared to oxaliplatin.

CTR1-negative DRG neurons were less susceptible to platinum toxicity

The CTR1-negative subpopulation of DRG neurons were altered less by the treatment of rats with platinum drugs than the CTR1-positive subpopulation of neurons. CTR1-negative neurons were defined as those showing no immunostaining or only diffuse cytoplasmic immunoreactivity compared to a negative control. Oxaliplatin significantly reduced the mean cell body area of both subpopulations but the effect on CTR1-positive neurons (42%, \( P < 0.05 \), Fig. 5b) was numerically greater than that on CTR1-negative neurons (19%, \( P < 0.05 \); Fig. 5d). Cisplatin significantly reduced the mean cell body area of CTR1-positive neurons versus control (18%, \( P < 0.05 \), Fig. 5b), but the reduction in the mean cell body area of CTR1-negative neurons was not significant versus control (3.1%, \( P = \text{NS} \), Fig. 5d). Furthermore, the cell body size frequency histograms for CTR1-negative neurons (Fig. 6c, d) appeared unchanged or less altered by platinum drugs compared to the clearer leftward deviations in the size profile of CTR1-positive neurons caused by oxaliplatin (Fig. 6a) and cisplatin (Fig. 6b).

Discussion

This study demonstrated the neuronal expression and plasma membrane localization of the copper influx transporter CTR1 in rat DRG. CTR1-immunoreactivity was associated with the cell bodies of DRG neurons but not with their nerve fibers or other tissue elements of the DRG. It was localized to the neuronal surface, with a plasma membrane pattern of immunoreactivity along with staining of cytoplasmic vesicular structures. This pattern of immunoreactivity was seen in a subpopulation of large DRG neurons that accounted for less than 15% of total DRG neurons. These results were further confirmed by the RT-PCR and real-time PCR analysis that indicated abundant CTR1 mRNA level in DRG, comparative to reference tissues. These findings are suggestive of CTR1 having an important but currently uncharacterised physiologic role in subpopulations of primary sensory neurons of the DRG. The localization of CTR1 in the plasma membrane is consistent with it having a role in the uptake of copper into DRG neurons, as in other cell types and tissues [32, 45]. Many neurons categorized as CTR1-negative showed diffuse cytoplasmic immunostaining greater than the negative control but at lower levels than in the CTR-positive neurons. The differential expression of CTR1 may be related to differences in requirements for copper between these two cell types.
Several observations made in the current study suggested significant links between the neuronal expression of CTR1 and the neurotoxicity of platinum-based drugs. For instance, CTR1 expression was demonstrated in a subpopulation of larger-sized DRG neurons that underwent atrophy in response to cisplatin and oxaliplatin treatment, without any change in their number or in the pattern of CTR1 expression. In contrast, the CTR1-negative subpopulation of DRG neurons was smaller, and treatment with oxaliplatin or cisplatin caused less neuronal atrophy, than in the CTR1-positive subpopulation of neurons. Previous studies have suggested that large-sized DRG neurons are more vulnerable to damage from platinum drug treatment than small-sized DRG neurons [18, 19], and that neuronal atrophy could be the morphological basis for the peripheral neurotoxicity that complicates their clinical use [11, 14–20].

In an extension to this finding, we compared the amount of cell body atrophy of CTR1-expressing DRG neurons induced by the treatment of rats with equitoxic doses of cisplatin, oxaliplatin and carboplatin. This quantitative analysis revealed a ranking of these platinum agents according to their effect on the size profiles of CTR1-immunoreactive DRG neurons with oxaliplatin having the greatest effect, followed by cisplatin and then carboplatin. This ranking corresponds with the relative cumulative dose-potencies of oxaliplatin, cisplatin and carboplatin for reducing sensory nerve conduction velocity in rats, which occurs after cumulative doses of 15, 46.7 and 302 μmol/kg, respectively [23]. In addition, this ranking corresponded with the proportion of patients developing peripheral neurotoxicity of any severity grade after treatment with these platinum drugs, which is reported to occur in ~50% [6], ~90% [7] and ~6% [8] of patients treated with cisplatin, oxaliplatin and carboplatin, respectively.

Taken together, these observations implicate CTR1 in the neurotoxicity of platinum-based drugs and suggest a mechanism involving uptake of platinum drugs by CTR1-expressing neurons, leading to neuronal accumulation of platinum and neurotoxicity. A CTR1-based mechanism of neurotoxicity could potentially explain the preferential accumulation of platinum drugs in DRG relative to other tissues [11, 14, 15, 21, 22, 24, 25], and the selective toxicity of platinum drugs to subpopulations of DRG neurons [18, 19]. In addition, differing affinities of platinum drugs for CTR1-mediated uptake [34–36] could contribute to the differing neurotoxicity profiles of individual agents. Alternatively, the mechanism of neurotoxicity could involve disturbance of copper metabolism in CTR1-expressing neurons by platinum drugs, for example via competition for copper uptake [33, 34] or down-regulation of CTR1 protein [46, 47]. The fact that disturbance of copper metabolism has been linked to other neurodegenerative diseases, such as amyotrophic lateral sclerosis [48], strengthens this hypothesis regarding the role of CTR1 in the neurotoxicity of platinum-based drugs. However, until its role is clarified, the possibility remains that it may have no specific mechanistic role in the drug neurotoxicity other than being a marker of the neurons that become damaged by platinum drugs. Higher expression of CTR1 in neurons vulnerable to platinum neurotoxicity may reflect their higher metabolic activity and susceptibility to oxidative damage. In addition, other transporters could be involved in the neurotoxicity, for example those already linked to the transport of platinum-based drugs such as copper transporting P-type ATPases (ATP7A and ATP7B) [49–51], organic cation transporters [52, 53], multidrug resistance proteins [54] and the glutathione S-conjugate efflux pump [55], whose DRG expression is unknown.

In conclusion, CTR1-expressing DRG neurons are particularly vulnerable to the toxicity of platinum drugs, and the extent of their atrophy corresponds with the relative neurotoxicity of oxaliplatin, cisplatin and carboplatin. These findings, together with the neuronal expression and membrane localization of CTR1 in a subset of DRG neurons, are supporting evidence for CTR1-related mechanisms of platinum drug neuronal uptake and neurotoxicity.

Acknowledgments The work was supported by a research grant of Cancer Society of New Zealand.

Conflict of interest statement None.

References

Platinum-specific detection and quantification of oxaliplatin and Pt(R,R-diaminocyclohexane)Cl$_2$ in the blood plasma of colorectal cancer patients†

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Received 1st November 2007, Accepted 22nd February 2008 
First published as an Advance Article on the web 13th March 2008 
DOI: 10.1039/b716925f

Oxaliplatin is a medically-important platinum-based drug for treating advanced colorectal cancer, but its clinical pharmacokinetics and biotransformation are not well understood. We report the development of a reliable sample preparation procedure and a specific HPLC-ICP-MS assay for oxaliplatin and its putative active biotransformation product Pt(R,R-diaminocyclohexane)Cl$_2$ [Pt(DACH)Cl$_2$], and their application to the analysis of the plasma of patients undergoing a standard 2 h infusion of oxaliplatin. HPLC conditions were identified for separating intact oxaliplatin and Pt(DACH)Cl$_2$ that were compatible with on-line detection by ICP-MS. Plasma samples were processed immediately after collection by methanol deproteinization, then stored under conditions in which the analytes of interest were stable. The linearity of calibration curves ($R^2$ = 0.9974), intra- and inter-assay accuracy (101–107%), and precision (3.30–7.12%), drug recovery (95–108%), and short- and long-term stability were adequate to quantify oxaliplatin. Clinical application of the assay showed that intact oxaliplatin was the major active platinum species in the plasma of colorectal cancer patients given oxaliplatin. Pt(DACH)Cl$_2$ was undetectable in patient samples despite the HPLC-ICP-MS assay having a limit of detection of 5 nM (1.9 ppb) for this platinum species.

Introduction

Oxaliplatin is a medically-important platinum-based drug for the treatment of advanced colorectal cancer in combination with fluoropyrimidines. Currently, there is a need for improved sample processing and bioanalytical techniques, for investigating the fate of oxaliplatin and its metabolites in the human body. To date, most pharmacokinetic studies of oxaliplatin have measured the total platinum content of blood fractions by FAAS or ICP-MS, but total platinum measurements may represent both pharmacologically active and inactive platinum species including parent oxaliplatin, active intermediates such as Pt(R,R-diaminocyclohexane)Cl$_2$ [Pt(DACH)Cl$_2$] and inactive nucleophile adducts. In addition, oxaliplatin is unstable in whole blood ($t_{1/2}$ 45 min) and plasma ($t_{1/2}$ 1.5 h), and binds to plasma proteins even during storage at −20 °C. Therefore, analyte stability during sample processing, storage and analysis requires careful consideration.

To date, limited studies of the clinical pharmacokinetics of intact oxaliplatin and its biotransformation have been reported and yielded conflicting results. For example, one study reported several degradation products but no oxaliplatin in the plasma ultrafiltrate at the end of an oxaliplatin infusion, but others detected the parent drug and one or more degradation products, such as Pt(DACH)Cl$_2$. Recently, there has been increasing interest in the use of HPLC coupled with ICP-MS to quantify platinum drugs and their biotransformation products in clinical samples. In this article, we report the development of a sample preparation procedure and HPLC-ICP-MS assay for oxaliplatin and Pt(DACH)Cl$_2$, and their application to the analysis of plasma in patients undergoing a standard 2 h infusion of oxaliplatin. Such analyses could be informative about the pharmacokinetics of intact oxaliplatin and its biotransformation, and assist optimizing chemotherapy for colorectal cancer patients.

Experimental

Materials and chemicals

Oxaliplatin (12.58 mM) was obtained from the Department of Oncology at Auckland City Hospital, Auckland, New Zealand. Pt(DACH)Cl$_2$ was synthesized using a published method. Methanol of chromatographic grade, sodium dodecyl sulfate (SDS) without chloride, triflic acid and nitric acid of analytical grade were purchased from Sigma, St Louis, MO, USA, unless otherwise indicated. Argon of instrument grade was supplied by BOC Gases, Auckland, New Zealand. Milli Q water (Millipore, Bedford, MA, USA) was used throughout the study. Solutions of platinum and thallium (1 µg ml$^{-1}$ in 1% nitric acid or 1% hydrochloric...
acids) were obtained from Spex CertiPrep, Metuchen, NJ, USA. Drug-free blank human plasma was acquired from Auckland Regional Blood Services, Auckland, New Zealand.

Instrumentation

To detect oxaliplatin and Pt(DACH)Cl₂ in plasma samples, an HPLC coupled with an ICP-MS instrument was employed in the study. The HPLC was a HP1100 system, including a binary pump, a rheodyne injector with sample loops (Hewlett Packard, Wilmington, DE, USA), a μBondapak C₁₈ column (Waters, Massachusetts, USA) and a guard column (Phenomenex, Torrance, CA, USA). The mobile phase was spiked with 2 ng mL⁻¹ thallium as the internal standard. The resulting eluate was introduced directly via 0.25 mm diameter PEEK tubing into a HP4500 ICP-MS system (Hewlett Packard, Yokogawa, Japan), which comprised a Scott double-pass spray chamber, a Babington nebulizer, a quartz torch, nickel sampler and skimmer cones, a quadrupole mass analyzer, and an electron multiplier detector (ETP Pty Ltd, Ermington, NSW, Australia). The spray chamber was cooled by a CFT series recirculating chiller (Neslab, Portsmouth, NH, USA). The platinum isotopes with m/z 194, 195 and thallium with m/z 205 were monitored by ICP-MS. Optimization of signal/noise of the system was carried out for each run using 10 ng mL⁻¹ tuning solution of platinum or the mixture of lithium, yttrium, cerium and thallium, by adjusting the following parameters: carrier/blend gas flow rate, extract/einzel/omega ion lenses, AMU/Axis gain/offset and plate/pole bias. Data acquisition and processing were performed using HP4500 ICP-MS ChemStation A.02.00 and chromatographic analysis software C.01.00 (Agilent Technologies, Avondale, PA, USA). Platinum peak areas were normalized by dividing by the thallium counts at the same elution time.

Deproteinization

Methanol deproteinized plasma (MDP) was prepared by adding two volumes of ice-cold methanol to blank or patient plasma samples in a 2 ml Eppendorf tube. The mixture was briefly mixed then centrifuged at 5000 × g, 4 °C for 30 min (Biofuge Stratos, Kendro Laboratory Products, Osterode, Germany). The supernatant was collected as the MDP and transferred to another tube and stored at −80 °C until analysis.

Calibration standards and quality control samples

On each analytical run, stock solutions of oxaliplatin and Pt(DACH)Cl₂ were prepared in Milliq water and 0.9% saline, respectively. Calibration standards, at 50, 75, 100, 150, 200, 300, 400 and 500 nM and quality control samples, at 50, 200 and 500 nM, were prepared with the blank MDP in duplicate and were stored on ice until analysis. Calibration curves were generated by plotting internal standard-corrected platinum peak areas versus nominal concentrations of calibration standards.

Method validation

According to the criteria defined in the US Food and Drug Administration Guidance for Industry Bioanalytical Method Validation,²¹ several parameters including sensitivity, accuracy, precision, recovery and stability were determined and optimized for the assay. The limit of quantitation (LOQ), defined as the lowest concentration that was measurable with an accuracy and precision of less than 20%, was examined at 50 nM oxaliplatin in MDP. Intra- or inter-assay accuracy and precision were determined by analyzing the MDP samples containing oxaliplatin at concentrations of 50, 200 and 500 nM within a single run or over three different runs. The recovery of oxaliplatin was determined as the ratio of measured concentration to the nominal concentration in plasma containing 50, 200 and 500 nM oxaliplatin. For all the above assays, five replicates of samples were analyzed at each concentration. The aliquots of stability samples of 200 nM oxaliplatin in MDP were analyzed in triplicate after storage on ice for 2 and 4 h, and at −80 °C for 1, 4, 9 and 90 days, respectively.

In vitro incubations

Two μM oxaliplatin was incubated at 37 °C in a 0.9% physiological saline solution. Aliquots of 500 μL were taken at 5 min, 12, 24, 36, 48 and 64 h for analysis.

Patients and sampling

After approval by the local Human Ethics Committee, three subjects aged 65, 64 and 47 years, with metastatic colorectal cancer and adequate organ function, gave informed consent. Oxaliplatin diluted in 500 ml 5% glucose was infused over 2 h at 130 mg m⁻². Blood samples were collected into heparinized vacuum tubes immediately prior to the commencement of the infusion and at 1, 2, 2.16, 2.3, 2.5, 3 and 4 h thereafter. MDP samples were prepared as described above, at the bedside, immediately after acquiring patient blood, stored at −80 °C and were analyzed within 3 weeks.

For total platinum analysis, use was made of a previous ICP-MS method.²² Briefly, patient samples, calibration standards prepared in blank MDP (30 to 300 nM) and quality controls (50, 150, 300 nM) were added with 10 volumes of Milliq water containing 0.5 nM thallium as internal standard. Samples were taken up by an ASX-100 autosampler (CETAC Technologies, Nebraska, USA) at 0.1 ml min⁻¹ to the abovementioned HP4500 ICP-MS for measuring platinum isotopes with m/z 194/195 and thallium with m/z 205, with dwelling time of 1 s in triplicate. The ICP-MS assay was validated for determining total platinum in MDP with acceptable calibration curves (R²≥ 0.9924), accuracy (96–108%) and precision (c.v. %, 0.6–5.8%).

Data analysis

Statistical and regression analyses were performed using Prism 5.01 (GraphPad Software, San Diego, CA, USA). Pharmacokinetic parameters were determined by a non-compartmental model using WonNonLin 3.1 (Pharsight Co. Mountainview, CA, USA).

Results and discussion

Method development and validation

Previously, El-Khateeb et al had reported HPLC conditions for separating cisplatin and its active metabolites, using mobile
phase components with low chemical reactivity to platinum complexes.\(^{21}\) Subsequently, we showed that these HPLC conditions were suitable for use on-line to ICP-MS for the quantitative detection of cisplatin and monohydrated cisplatin.\(^{\text{17}}\) Using the same conditions, oxaliplatin eluted as a single platinum peak at \(~15\) min and Pt(DACH)Cl\(_2\) eluted at \(~12\) min (Fig. 1B).

When oxaliplatin was incubated in a physiological solution of NaCl (0.9\% w/v) at 37 °C, the chromatographic peak corresponding to oxaliplatin (retention time \(~15\) min) decreased with time (\(t_{1/2}\) 10 h), and the chromatographic peak corresponding to Pt(DACH)Cl\(_2\) (retention time \(~12\) min) appeared and increased with time (Fig. 2A and B). This demonstrated the specificity of the assay for oxaliplatin and Pt(DACH)Cl\(_2\), and was consistent with chemical transformation of oxaliplatin to Pt(DACH)Cl\(_2\) in this solution with chloride concentration similar to human extracellular fluid.\(^{24}\)

Prior to analysis, human plasma was processed by methanol deproteinization then stored at \(-80\) °C. Injection of blank MDP onto the HPLC column produced no interfering peaks or changes in ICP-MS background counts (Fig. 1A). Calibration curves for quantitation of intact oxaliplatin in MDP samples were linear from 50 to 500 nM with a mean correlation coefficient of 0.9974, and the accuracy of back-calculated calibration standards ranged from 98 to 102\%. The assay showed acceptable intra- and inter-assay accuracy (101–107\%), precision (3.30–7.12\%, \(n = 5\)) and recovery (95–108\%) at the low, medium and high concentrations (ESI Tables S1 and S2†), and LLOQ (50 nM) and LOD (5 nM or 2 ppb). For comparison, the LLOQ and LOD were higher than that of reported HPLC-ICP-MS assays for other platinum-based anticancer drugs,\(^{15,16,18}\) but adequate for detecting pharmacologically-significant levels of oxaliplatin and Pt(DACH)Cl\(_2\). Quantitative analysis confirmed that there was no significant loss of oxaliplatin, or formation of degradation products such as Pt(DACH)Cl\(_2\), in MDP during storage for up to 4 h on ice and 90 days at \(-80\) °C (ESI Table S3 and Fig. S1†).

### Clinical application

These validated sample processing and bioanalytical techniques were then applied to the quantitation of intact oxaliplatin in the plasma of colorectal cancer patients receiving oxaliplatin-based combination chemotherapy. Intact oxaliplatin was the only platinum-containing species detected on HPLC-ICP-MS chromatograms of MDP samples from three patients during and for up to 1 h after the end of the oxaliplatin infusion (ESI Fig. S2B†). Averaged values for peak plasma concentration (\(C_{\text{max}}\)) and area under the plasma concentration \(versus\) time curve (AUC\(_{0-4\ h}\)) for intact oxaliplatin were 9.2 \mu M and 13.9 \mu M h, respectively (Table 1). Intact oxaliplatin accounted for 63\% and 56\% of \(C_{\text{max}}\) and AUC\(_{0-4\ h}\) values for platinum in MDP, respectively. At the end of the drug infusion, intact oxaliplatin in MDP decreased rapidly with averaged elimination half-life of 0.25 h (ESI Fig. S2A†). In contrast to these findings, previous studies attempting to quantify intact oxaliplatin in cancer patients using other sample processing and bioanalytical techniques indicated that oxaliplatin was undetectable,\(^{11}\) accounted for only 40\% of free platinum levels,\(^{11}\) or did not relate oxaliplatin values to total platinum concentrations.\(^{8}\)

Pt(DACH)Cl\(_2\) was undetectable in plasma of patients treated with oxaliplatin in the current study. No significant platinum peaks corresponding to Pt(DACH)Cl\(_2\) (retention time \(~12\) min)

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**Table 1**  Pharmacokinetic data in cancer patients undergoing a 2 h infusion of oxaliplatin at 130 mg m\(^{-2}\)

<table>
<thead>
<tr>
<th>Concentration (\mu M) in MDP at the indicated time (h) after the commencement of infusion</th>
<th>(C_{\text{max}}/\mu M)</th>
<th>AUC(_{0-4\ h}/\mu M\ h)</th>
<th>(t_{1/2})/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxaliplatin</td>
<td>7.6 ± 0.5(^a)</td>
<td>8.2 ± 3.1</td>
<td>3.9 ± 0.8</td>
</tr>
<tr>
<td>Total platinum</td>
<td>13.1 ± 1.0</td>
<td>13.0 ± 4.6</td>
<td>8.8 ± 2.9</td>
</tr>
<tr>
<td>Percentage %</td>
<td>58 (^b)</td>
<td>63</td>
<td>44</td>
</tr>
</tbody>
</table>

\(^a\) Data values are expressed as mean ± standard deviation of \(n = 3\). \(^b\) Percentage of the mean measured values of oxaliplatin to total platinum.
Pt(DACH)Cl₂ is known to form as a degradation product of oxaliplatin during processing and bioanalytical techniques reported here are specific, reliable and well-validated for the quantitative determination of oxaliplatin in human plasma and have adequate sensitivity for detecting pharmacologically-significant levels of oxaliplatin and Pt(DACH)Cl₂ in clinical samples.

In conclusion, compared to other approaches, the sample processing and bioanalytical techniques reported here are specific, reliable and well-validated for the quantitative determination of oxaliplatin in human plasma and have adequate sensitivity for detecting pharmacologically-significant levels of oxaliplatin and Pt(DACH)Cl₂ in clinical samples.

Acknowledgements

We thank the patients, their families and nursing staff, and the Cancer Society of New Zealand for grant support.

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